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Mitochondrial drug-delivery systems for macromolecule and their therapeutic application to mitochondrial diseases

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Key words: Mitochondria, mitochondrial drug delivery, mitochondrial macromolecule delivery, MITO-Porter, membrane fusion, multifunctional envelope-type nano device (MEND), mitochondrial protein therapy, mitochondrial gene therapy, mitochondrial protein import machinery, mitochondrial DNA.

Abstract

Mitochondrial dysfunction has been implicated in a variety of human disorders—the so-called mitochondrial diseases. Therefore, the organelle is a promising therapeutic drug target. In this review, we describe the key role of mitochondria in living cells, a number of mitochondrial drug delivery systems and mitochondria-targeted therapeutic strategies. In particular, we discuss mitochondrial delivery of macromolecules, such as proteins and nucleic acids. The discussion of protein delivery is limited primarily to the mitochondrial import machinery. In the section on mitochondrial gene delivery and therapy, we discuss mitochondrial diseases caused by mutations in mitochondrial DNA, several gene delivery strategies and approaches to mitochondrial gene therapy. This review also summarizes our current efforts regarding liposome-based delivery system including use of a multifunctional envelope-type nano device (MEND) and mitochondrial liposome-based delivery as anti-cancer therapies. Furthermore, we introduce the novel MITO-Porter—a liposome-based mitochondrial delivery system that functions using a membrane-fusion mechanism.

Abbreviations

AAC, ATP/ADP carrier protein; ACE, angiotensin-converting enzyme; ACE-Cat, catalase conjugated with biotinylated anti-ACE antibody; ACE-SOD, CuZnSOD conjugated with biotinylated anti-ACE antibody; AIF, apoptosis-inducing factor; ANT, adenine nucleotide translocase; anti-PECAM/SOD, superoxide dismutases conjugated with anti-PECAM; Apaf-1, apoptotic protease activating factor-1; bp, base pair; Bmpr1a, bone morphogenetic protein receptor type 1 A; CM, cardiomyopathy; COS7 cells, cultured monkey kidney cells; CH, chaperone proteins; CPEO, chronic progressive external ophthalmoplegia; C/P molar ratio, condenser/protein molar ratio; CuZnSOD, copper-zinc-containing superoxide dismutases; cyt c, cytochrome c; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphatidyl ethanolamine; DOPE-LP, liposome composed of DOPE; DQAplexes, DQAsomes-DNA complexes; EPC, egg yolk phosphatidyl choline; EPC-LP, liposome composed of EPC; ETC, electron transport chain; Exo III, exonuclease III protein; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; GPx, glutathione peroxidase; IM, inner membrane; IMS, intermembrane space; INT-MTS, internal MTS; KSS, Kearns-Sayre syndrome; LHON, Leber's hereditary optic neuropathy; mtDNA, mitochondrial DNA; MELAS, myopathy, encephalopathy, lactic acidosis and stroke-like episodes; MEND, multifunctional envelope-type nano device; MEND (GFP), GFP-encapsulated in MEND; MERRF, myoclonic epilepsy with ragged-red fibers; MnSOD, manganese-containing superoxide dismutase; MP, mastoparan; MPP, mitochondrial processing peptidase; MTS, mitochondrial targeting signal peptide; MTS-ODN, MTS-conjugated ODN; NARP, neurogenic muscle weakness, ataxia, and retinitis pigmentosa; NLS, nuclear localization

signal peptide; N-MTS, amino-terminal MTS; 3NP, 3-nitropropionic acid; ODN, oligodeoxynucleotides; OM, outer membrane; PAM, presequence translocase-associated motor; PECAM, platelet-endothelial cell adhesion molecule; PEG, polyethylene glycol; PLL, poly-L-lysine; PNA, peptide nucleic acid; PPD, PEG-Peptide-DOPE conjugate; PT, permeability transition; PTD, protein transduction domain; PTP, mitochondrial permeability transition pore; R8, octaarginine peptide; R8-MEND, MEND modified with high-density R8 peptide; R8-MEND (ODN), ODN-encapsulated in R8-MEND; R8-MEND (GFP), GFP-encapsulated in R8-MEND; R8-MEND (Protein), protein-encapsulated R8-MEND; RES, reticuloendothelial system; Rho, sulforhodamine B; ROS, reactive oxygen species; SA, stearylamine; SAM, sorting and assembly machinery; SS-peptides, Szeto-Schiller-peptides; STR-R8, stearyl R8; TAT, trans-activating transcriptional activator; TAT-mMDH-GFP, TAT fusion protein that consisted of an MTS derived from mitochondrial malate dehydrogenase and green fluorescent protein; tBHP, t-butylhydroperoxide; Tf, transferrin; Tf-GALA-LP, Tf-LP equipped with GALA; Tf-LP, transferrin-modified liposomes; TIM, translocator of the mitochondrial inner membrane; TCA cycle, tricarboxylic acid cycle; TOM, translocator of the mitochondrial outer membrane; TPP, triphenylphosphonium cations; VDAC, voltage-dependent anion channel.

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1. Introduction

Recently, it has become increasingly evident that mitochondrial dysfunction causes a variety of human disorders, including neurodegenerative and neuromuscular diseases, obesity and diabetes, ischemia-reperfusion injury, cancer and inherited mitochondrial diseases [1-3]. Progress in genetics and molecular biology has revealed that mitochondria play a role in the homeostasis of vital physiological functions, including electron transfer, apoptosis, and calcium storage (Fig. 1) [4]. For example, diabetes, cancer, and inherited mitochondrial diseases are caused by dysfunctions of the electron transfer system, apoptosis regulation, and mutations in mitochondrial DNA (mtDNA), respectively [5-7]. Therefore, mitochondria are promising organelles for drug targeting.

The mitochondrion possesses both an outer and inner membrane that encloses the intermembrane space and the matrix, respectively (Fig. 1). The double-membrane structure most likely evolved to assist in the preservation of essential organelle functions. For example, for ATP synthesis to occur, the inner mitochondrial space must be acidic and there must be an electrochemical potential across the inner membrane. Thus, it is essential that the organelle selectively control the permeability of the mitochondrial membrane to chemicals. Therapeutic drugs must overcome this barrier to effectively deliver drugs to mitochondria. To date, numerous investigators have attempted to construct mitochondria-targeted delivery systems; however, it is difficult to efficiently deliver therapeutic drugs to mitochondria [8-10]. Therefore, the development of systems for the delivery of various drugs, including therapeutic chemicals, proteins, DNA, to mitochondria is desired.

In this review, we will describe unique features of mitochondria, such as electron

transfer system, induction of reactive oxygen species and apoptosis. In addition, we will review various lines of investigation concerning mitochondrial drug delivery. In particular, strategies for mitochondrial delivery of macromolecules, such as proteins and nucleic acids will be discussed. The discussion of protein delivery focuses primarily mitochondrial protein import machinery. In the section on mitochondrial gene delivery and therapy, we will describe the relationships between mitochondrial diseases and mutated and/or defective mtDNA and discuss in detail mitochondrial delivery of linear and circular DNA. This review will also summarize our current efforts regarding liposomal delivery system. Multifunctional envelope-type nano device (MEND), which is a novel non-viral vector that was designed based on a new packaging concept, "Programmed Packaging", will be described [11, 12]. Mitochondria-based treatments for cancer that use intracellular trafficking regulation systems also will be discussed [13, 14]. Furthermore, we will describe the novel MITO-Porter—a liposome-based mitochondrial delivery system that operates via membrane fusion—as a MEND for mitochondrial delivery [15, 16].

2. Mitochondrion possesses important roles in living cells

2. 1. Structural features of the mitochondrion and various functions

Mitochondria are composed of a double membrane. The inner-most mitochondrial space is the matrix, while the intermembrane space is located between the membranes (Fig. 1). It has been hypothesized that mitochondria originated by endosymbiosis. Research on mitochondrial DNA (mtDNA) supports the similarities between mitochondria and α-proteobacteria [17]. Like all membranes, mitochondrial membranes are composed of a phospholipid bilayer with embedded proteins. The outer membrane, like the plasma

membrane, has a lipid to protein ratio of 1:1. The outer membrane does not offer a barrier to small molecules (< 5 kDa), which can simply diffuse through pores in the membrane formed by a membrane-spanning protein called porin. Large molecules, such as proteins, cross the outer membrane via a unique protein-import apparatus. Mitochondrial targeting signal peptide (MTS) delivers many proteins to mitochondria via protein import machinery. ATP synthesis occurs in the matrix, so it is the inner membrane that provides most of the mitochondrial barrier function. The composition of the inner membrane differs from that of the outer membrane in that it is more proteinacious and contains an unusual phospholipid—cardiolipin. Specific compounds reach the matrix space via a number of transport proteins in the inner membrane—each responsible for the transport of a specific ligand. For example, the ATP/ADP carrier (AAC) allows ADP to cross the inner membrane, while simultaneously transferring ATP out of the matrix space. In addition to metabolite transporters, the inner membrane also has the respiratory chain complex and ATP synthase. Within the human mitochondrial matrix space is the small mitochondrial genome of 16,569 bp that encodes 13 hydrophobic proteins, all of which are involved in electron transfer system, along with 22 transfer RNAs and two ribosomal RNAs [18, 19]. The matrix space is also the site for major metabolic pathways, including the tricarboxylic acid cycle (TCA cycle), the urea cycle and fatty acid oxidation (β oxidation).

2. 2. The electron transfer system and ATP synthesis

In major mammalian tissues, 80 to 90% of ATP is generated by oxidative phosphorylation in the mitochondria. The mitochondrial respiratory chain, located in the

inner mitochondrial membrane (Fig. 1), is composed of enzymes and coenzymes that transport reducing equivalents—hydrogen atoms or electrons—from respiratory substrates to molecular oxygen in accordance with the redox potential of the components of the respiratory chain.

The electron transport chain (complexes I, III, IV, and complex II, if one includes the oxidation of succinate) makes use of the considerable free energy released as a result of the oxidation of NADH (Fig. 2) [20]. This hydrogen/electron current forms three cascades in which the redox energy is adequate for the extrusion of protons from the mitochondrial matrix to the intermembrane space. The electrochemical proton gradient thus formed, also designated as the proton motive force ($\Delta\mu_H$), is the driving force for the back flow of protons through the ATP synthase complex (Fig. 2) [21]. This gradient is composed of the electric component ($\Delta\ \psi^2$) and the proton concentration gradient. Thus, mitochondria are unique cellular organelles that build up a transmembrane electric potential of up to 200 mV, have a negative integral charge, and an internal pH of approximately 8.

Perhaps the most important mitochondrial function is the syntheses of ATP from ADP and phosphate. Therefore, dysfunction of ATP-production is associated with large number of mitochondrial diseases. For example, mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS), myoclonic epilepsy with ragged-red fibers (MERRF) and chronic progressive external ophthalmoplegia (CPEO) have been attributed to defects in the respiratory chain [22]. As described above, electron transfer system requires various small molecules such as coenzyme Q_{10}, in addition to proteins [20]. Therefore, mitochondrial delivery of these small molecules and proteins has the probability of mitochondrial diseases therapy.

2. 3. Mitochondria and reactive oxygen species

Mitochondria are a major source of superoxide anion ($O_2^{\cdot-}$) and other reactive oxygen species (ROS) that may result from $O_2^{\cdot-}$ (Fig. 3) [23]. $O_2^{\cdot-}$ is produced by the electron transport chain [24-26]. Although the end product of the respiratory chain is water generated by the four-electron reduction of molecular oxygen by cytochrome oxidase (complex IV) (Fig. 2), a minor fraction of O_2 is involved in one-electron reduction processes, generating ROS, as follows: $O_2^{\cdot-}$, hydrogen peroxide (H_2O_2) and the extremely reactive hydroxyl radical ($HO^{\cdot}$). Generation of ROS occurs mainly at complex III due to proton cycling between ubiquinone, cytochromes *b* and c_1, and iron-sulfur proteins [27]; although complex I may also contribute to this process (Fig. 2). In the presence of mitochondrial superoxide dismutase, $O_2^{\cdot-}$ can be converted to H_2O_2, which can then diffuse out of mitochondria into the cytoplasm (Fig. 3). In the presence of high iron concentrations, H_2O_2 can form the highly reactive hydroxyl radical ($OH^{\cdot}$), via the Fenton reaction. $O_2^{\cdot-}$ can also react with nitric oxide to form the highly reactive peroxynitrite anion ($ONOO^-$).

Normally, ROS are decomposed or their peroxidation products are neutralized by natural defense systems, consisting primarily of mitochondrial (manganese-containing) and cytosolic (copper-zinc-containing) superoxide dismutases (MnSOD and CuZnSOD, respectively), glutathione peroxidase, and phospholipid hydroperoxide glutathione peroxidase (Fig. 3) [23, 28-31]. However, under conditions of increased ROS generation, e.g., ischemia-reperfusion, exposure to some xenobiotics, inflammation, aging, and ultraviolet or ionizing irradiation, or of an impaired antioxidant defense system, ROS may

accumulate, causing mtDNA mutations [32], lipid peroxidation [33], protein oxidation [34], damaging the cell and whole organism [25, 26, 35-37].

This ROS-induced damage alters the function of many metabolic enzymes in the mitochondrial matrix, as well as those comprising the electron transport chain. A particularly relevant protein that loses function upon oxidation is superoxide dismutase. Loss of superoxide dismutase activity further compromises antioxidant capacity, leading to additional oxidative stress. mtDNA mutations are associated with many degenerative diseases, including Parkinson's disease, Alzheimer's disease, Huntington's disease and amyotrophic lateral sclerosis [38, 39]. Antioxidant supplements and drugs have been used to scavenge free radicals that are produced in mitochondria. Drugs inhibit mitochondria, thereby killing cancer cells; they protect cells from oxidative damage; and, they repair defects. Because of the complex nature of the mitochondrion, different strategies may be required for mitochondrial uptake of different pharmacotherapeutic agents.

2. 4. Mitochondria and apoptosis

Mitochondria are involved in apoptotic programmed cell death. In general, two semi-interdependent routes may lead to apoptosis [40]. One of them is initiated by ligand binding to the so-called death receptors at the cell surface [41], whereas the route involves mitochondria [5]. In the latter case, an early trigger of the apoptotic pathway is an increase in the permeability of the outer membrane, causing release of apotogenic factors, such as cytochrome c (cyt c) [42] (Fig. 4). Along with another mitochondrial protein, a cascade of events in the cytosol that activates intracellular proteases of the caspase family [43] and, eventually, results in partial self-digestion of the cell (Fig. 4). The mechanism by which cyt

c is liberated from mitochondria to the cytosol is debatable. Various mechanisms, such as Bax/Bak channels, permeability transition pores (PTP) and membrane damage, may mediate apoptogenic factor-release [42]. In contrast, Bcl-2 and Bcl-XL inhibit apoptosis [44-47]. These proteins belong to the Bcl-2 family, which also contains pro-apoptosis proteins, such as Bax and Bid.

Opening of PTP has been proposed as a mechanism for cyt c release from mitochondria to the cytosol [42]. Although the mitochondrial permeability transition (PT) was first observed in the 1950s, this phenomenon remains incompletely understood. Because of the intense interest in the pathways involved in apoptosis and exocytotoxicity, many studies concerning of the PT have been reported to date [48, 49]. The PT is defined as sudden loss of the membrane potential, $\Delta\Psi$. Typically, PT is detected by loss of fluorescent dyes, for which mitochondrial uptake and concentration is dependent on $\Delta\Psi$. In vitro experiments have detected a PTP that permits solutes smaller than 1,500 Da to cross the mitochondrial inner membrane. The PTP consists of a voltage-dependent anion channel (VDAC) in the mitochondrial outer membrane and an adenine translocator (ANT) in the mitochondrial inner membrane (Fig. 1). VDAC is a highly conserved protein with homology to bacterial porin that forms an outer membrane pore [50]. VDAC plays a major role in mitochondria associated-apoptosis; however, there are many conflicting theories about the nature of its role. The pro-apoptotic protein, Bax, co-immunoprecipitates with VDAC, suggesting an interaction between the two proteins [51]. Other investigators have shown that Bax promotes the high conductance state of VDAC that allows cyt c to be released from the intermembrane space [52]. Rostovtseva et al. found that the pro-apoptotic protein, tBid, but not Bax, bound VDAC and prevented the exchange of ATP/ADP between

the cytosol and mitochondria, leading to mitochondrial swelling and rupture of the mitochondrial membrane. Subsequently, cyt c was released into the cytoplasm, leading to cell death [53].

ROS are generally regarded as toxic metabolites and, as such, are decomposed by specialized enzymes: catalase, peroxidases, and superoxide dismutases. Nevertheless, the fraction of ROS that escape catalytic removal may cause significant additional oxidative stress in mitochondria. ROS play a key role in the promotion of cyt c release from mitochondria [54]. Under normal conditions, cyt c is bound to the inner membrane by association with cardiolipin [55]. Peroxidation of cardiolipin leads to dissociation and release of cyt c through the outer membrane into the cytosol [56]. The mechanism by which cyt c is released through the outer membrane is not clear. In the cytosol, cyt c causes activation of caspase 9, which in turn, activates the caspase cascade (Fig. 4). We will describe mitochondria-based therapies for cancer that involve apoptosis in section 6. 3.

3. Mitochondrial small-molecule drug delivery systems as therapeutic strategies

A variety of small-molecule drugs have been investigated as potential therapeutic agents for mitochondrial diseases [9]. In this section, we discuss the potential role of small compounds targeted to the mitochondria as therapeutic drugs. In addition, we describe effective antioxidant drug delivery to the mitochondria.

3. 1. Delivery of small-molecule drugs via passive targeting

Oral administration of exogenous coenzymes of the respiratory chain, such as vitamins B_1 or B_2, succinic acid and cytochrome c (cyt c), and of ATP itself has been

investigated as way to compensate for dysfunctional mitochondria. Although these strategies have limited positive therapeutic effects, they do not allow for complete recovery of lost function [57-60]. Without a drug carrier, the tissue distribution, cellular uptake and intracellular trafficking of therapeutic agents into mitochondria cannot be actively controlled and, therefore, are insufficient for therapeutic activity.

In contrast, Koga et al reported that oral administration of L-arginine without a carrier was an effective mitochondrial disease therapy. L-arginine is suitable for oral administration because it is hydrophilic. The therapeutic effects of L-arginine were investigated in patients with mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) [61]. During the acute phase of MELAS, L-arginine concentrations were significantly lower in patients than in control subjects. L-arginine infusion improved all symptoms, suggesting that stroke occurs during the acute phase of MELAS. Oral administration remarkably decreased the frequency and severity of stroke-like episodes. Flow-mediated dilation decreased to a greater degree in patients than in controls. Moreover, two years of oral supplementation with L-arginine improved endothelial function to the level of controls and normalized plasma L-arginine concentrations in patients. Thus, L-arginine therapy appears to be a promising treatment for stroke-like episodes in MELAS patients.

3. 2. Antioxidant delivery using lipophilic triphenylphosphonium cations

Selective targeting of antioxidants to mitochondria in intact cells was recently reported by Murphy and coworkers [62-66]. First, selective delivery of an effective antioxidant, vitamin E, to mitochondria was attempted in an in vitro cell culture system

using vitamin E covalently coupled to lipophilic triphenylphosphonium cations (TPP) (Fig. 5A) [62]. Such positively charged compounds, can accumulate in mitochondria up to a thousand-fold, driven by a transmembrane electric potential of approximately 200 mV (negative inside) in fully energized mitochondria. The accumulation of positively charged compounds can be further increased by the electric potential of 30 to 60 mV at the plasma membrane. Thus, the calculated ratio of intra- to extra-mitochondrial concentrations may reach 10000:1. In the in vitro cell culture system, TPP-modified vitamin E crossed the lipid bilayers and accumulated within the mitochondrial matrix more effectively than native vitamin E.

Moreover, MitoQ, which is ubiquinone conjugated with TPP, selectively accumulates in mitochondria [63, 64, 66]. In mitochondria, MitoQ is reduced by the respiratory chain to its active ubiquinol form. The ubiquinol derivative thus formed is an effective antioxidant that prevents lipid peroxidation and protects mitochondria from oxidative damage. After detoxifying reactive oxygen species (ROS), the ubiquinol moiety is regenerated by the respiratory chain enabling its antioxidant activity to be recycled. MitoQ protects mitochondria against oxidative damage both in vitro and in vivo, following oral administration.

3. 3. Antioxidant therapy using Szeto-Schiller (SS)-peptides

Recently, Zhao and coworkers reported that mitochondrial antioxidant delivery using a cell-permeable peptide reduced ROS within cells [67, 68]. They developed a novel approach to targeted delivery of antioxidants to the inner mitochondrial membrane using Szeto-Schiller (SS) peptide antioxidants (Fig. 5B). The structural motif of these SS peptides

centers on alternating aromatic residues and basic amino acids (aromatic-cationic peptides). SS peptides scavenge hydrogen peroxide and peroxynitrite and inhibit lipid peroxidation. The antioxidant action of these compounds can be attributed to the tyrosine and dimethyltyrosine residues. These peptide antioxidants are cell-permeable and are concentrated up to 1000-fold in the mitochondrial inner membrane. These peptide antioxidants significantly reduced intracellular ROS and cell death caused by t-butylhydroperoxide (tBHP) in neuronal N_2A cells (EC_{50} in the nM range). SS peptide antioxidants also decreased mitochondrial ROS production inhibited the permeability transition (PT) and swelling, and prevented cyt c release induced by Ca^{2+} in isolated mitochondria. In addition, SS peptides inhibited 3-nitropropionic acid (3NP)-induced PT in isolated mitochondria and prevented mitochondrial depolarization in cells treated with 3NP. Because ROS and PT have been implicated in myocardial stunning associated with reperfusion in ischemic cardiac muscle, Zhao et al. concluded that these peptide antioxidants significantly improved contractile force in an ex vivo heart model. The authors also speculated that these mitochondrial inner membrane-targeted antioxidants might be very effective treatments for aging and diseases associated with oxidative stress.

3. 4. Delivery of selective electron scavengers

Wipf and coworkers recently reported a novel type of mitochondria-targeted electron and ROS scavenger, XJB-5-131 (Fig. 5C) [69-71]. This scavenger consists of coupling a payload portion with electron- and ROS-scavenging activity and a targeting portion, which promotes selective accumulation in mitochondria. The payload portion of XJB-5-131 consists of a stable nitroxide radical. By accepting one electron, nitroxide

radicals are converted to hydroxylamines. Hydroxylamines are effective ROS scavengers and in the process are converted back into nitroxides [72]. Furthermore, nitroxide radicals have superoxide dismutase mimetic activity [73, 74], which helps prevent the reaction of $O_2^{\cdot-}$ with nitric oxide, thereby inhibiting formation of the highly toxic species, $ONOO^-$. The targeting portion of XJB-5-131 consists of the Leu-D-Phe-Pro-Val-Orn fragment of gramicidin S, which is a membrane-active cyclopeptide antibiotic [75]. Because antibiotics of this type have a high affinity for bacterial membranes [76], and because of the close relationship between bacteria and mitochondria [77], this fragment effectively targets XJB-5-131 to the mitochondria.

Wipf et al. validated the ability of XJB-5-131 to prevent ileal mucosal barrier dysfunction in rats subjected to lethal hemorrhagic shock [71]. XJB-5-131 ameliorated peroxidation of mitochondrial phospholipid, cardiolipin in ileal mucosal samples from these rats. In addition, XJB-5-131 ameliorated lethal hemorrhagic shock-induced activation of the pro-apoptotic enzymes, caspases 3 and 7, in ileal mucosa. Furthermore, intravenous treatment with XJB-5-131 (2 μmol/kg) significantly prolonged survival of rats subjected to profound blood loss (33.5 mL/kg), despite administration of only a minimal volume of crystalloid solution (2.8 mL/kg) without blood transfusion. Based on the results of these studies, it was concluded that mitochondrially targeted electron acceptors and superoxide dismutase mimics are potentially valuable therapeutics for the treatment of serious acute conditions, such as lethal hemorrhagic shock, which are associated with marked tissue ischemia.

4. Mitochondrial protein delivery and therapeutic strategies

In mitochondria, many proteins perform vital cell functions. In turn, dysfunctional proteins may cause disease, by inducing of cell death. Thus, replacement of defective proteins via mitochondrial protein delivery can be a useful treatment of disease. In this section, we provide an overview of mitochondrial protein import machinery, and discuss mitochondrial delivery using the mitochondrial targeting signal peptide (MTS) and related therapeutic strategies. We also describe the protein transduction domain (PTD), which is a novel mitochondrial targeting device.

4. 1. Mitochondrial protein delivery via the protein import machinery

Mitochondria contain ~1000 different proteins. Greater than 99% of all known mitochondrial proteins are synthesized on cytosolic ribosomes as precursor proteins that must be imported into the organelle. Import of these proteins into mitochondria is performed via protein translocator complexes in the outer and inner membranes. Proteins are sorted to one of four intra-mitochondrial locations—the outer membrane, inner membrane, intermembrane space, and matrix [78-81] (Fig. 6).

4. 1. 1. The mitochondrial targeting signal peptide and sorting site

Precursor proteins that possess an amino-terminal MTS (N-MTS) are destined for the intermembrane space, inner membrane, or matrix (Fig. 6A). Typically, an N-MTS consists of approximately 20–30 amino acids. Although there is no sequence identity shared among N-MTSs, they all form amphiphilic α-helices that are enriched in basic, hydroxylated, and hydrophobic residues [82-84]. The N-MTS is cleaved off by the matrix processing peptidase (MPP) in the matrix upon import [85]. In contrast, outer and polytipic

inner membrane proteins are mostly synthesized without an N-MTS, but contain an internal MTS (INT-MTS) within the mature region of the protein (Fig. 6A). In contrast to N-MTSs, these targeting signal sequences have not been well-characterized.

4. 1. 2. The TOM complex as a general point of entry for mitochondrial proteins

Cytosolic chaperones, which prevent protein aggregation and folding, import mitochondrial preproteins into mitochondria [86]. The initial entry gate for nearly all mitochondrial proteins is the translocator of mitochondrial outer membrane (TOM) complex (Fig. 6A). Once across the TOM complex, proteins are sorted to one of the four intra-mitochondrial locations. The TOM complex consists of the channel-forming β-barrel protein Tom40, import receptors, and small Tom proteins [85, 87]. Tom40 forms the channel across the membrane and contains N-MTS-binding sites that are spatially arranged from the cis to the trans side of the pore with a diameter of 2-2.5 nm [88-92]. Two primary import receptors, Tom20 and Tom70, are loosely associated with Tom40. Tom20 is the major import receptor that preferentially recognizes N-MTS containing proteins. Endo and coworkers examined the structure of the receptor domain of rat Tom20 in a complex with an N-MTS using NMR. They found that Tom20 has a hydrophobic groove on the surface, to which the hydrophobic side of the amphiphilic helix of the presequence binds [93]. In contrast, Tom70 preferentially recognizes INT-MTS that contain inner membrane proteins. Moreover, Tom70 functions as a docking site for the cytosolic chaperone Hsp70 [94]. Translocation of proteins with INT-MTS is not well-understood, but it appears that the protein spans Tom40 as a hairpin-like structure, thereby exposing the loop that connects the two transmembrane domains to the intermembrane space [95]. Both Tom20 and Tom70

transfer their substrate to Tom22, which functions as a central import receptor within the TOM complex [96, 97].

After translocation across the outer membrane, the sorting pathways diverge to either the outer membrane or intermembrane space, or to translocator complexes of the inner membrane complex (TIM), including the TIM22 and TIM23 complexes. The protein import machinery contains two main import pathways that are used by N-MTS or INT-MTS (Fig. 6A). Pathway A is used by N-MTS destined for the intermembrane space and the TIM23 complex, which sorts them to inner membrane or matrix (please see section *4. 1. 3.* regarding protein sorting via a TIM23 complex). Localization of proteins containing an N-MTS to the intermembrane space is illustrated by the import of cytochrome b_1 and cytochrome b_2 [98-101]. These proteins are targeted to the TIM23 complex, but are arrested in the pore by an uncharged "sorting signal" that immediately follows the N-MTS. In contrast, proteins of the intermembrane space that contain cysteine motifs are imported and oxidized by the mitochondrial intermembrane space assembly (MIA) system [102-105].

Pathway B is used by pre-proteins containing INT-MTS that are destined for the outer membrane and TIM 22 complex, which sorts them to the inner membrane (please see section *4. 1. 4.* regarding protein sorting via TIM23 complexes). Outer membrane β-barrel proteins are inserted into the membrane by the sorting and assembly machinery (SAM) complex [106-109]. β-barrel proteins are first translocated through the TOM complex into the intermembrane space. At the trans-side of the TOM complex, they are bound by the TIM chaperone complex, which consists of a hexameric assembly of Tim9 and Tim10. The TIM chaperone complex guides β-barrel preproteins to the SAM complex, thereby preventing aggregation of these particularly hydrophobic polypeptides in the aqueous environment of

the intermembrane space. In contrast, outer membrane proteins containing a single α-helical transmembrane domain are released directly to the membrane from the TOM complex; however, the mechanism by which this transfer occurs remains unclear [110].

4. 1. 3. Protein sorting by the TIM23 complex

The majority of mitochondrial preproteins are synthesized with positively charged, N-MTS, including preproteins destined for the intermembrane space, inner membrane and matrix. All of these preproteins require the TIM23 complex for import to their final location (Fig. 6B). The TIM23 complex consists of Tim17, Tim21, Tim23, and Tim50 [97, 111-114]. Tim23 forms a channel 1.3-1.4 nm in diameter across the inner membrane, which specifically responds to the addition of N-MTS [115].

First, proteins containing an N-MTS are imported into the TOM complex. Upon translocation through the Tom40 channel, Tim 50 interacts with the preprotein [112, 113, 116], and the N-MTS binds to the tail of Tom22, which is located in the intermembrane space [96, 97]. Subsequently, Tim21 binds to Tom22 and promotes release of N-MTS. The N-MTS-fused protein inserts into the Tim23 channel and then is sorted to the matrix or inner membrane. Matrix proteins are sorted via the TIM23 complex and the presequence translocase-associated motor (PAM). Tim17 and Tim21 are involved in switching between the TIM23 complex, which inserts proteins into the inner membrane, and the complex that imports proteins into the matrix [117]. The PAM complex is essential for preprotein transport into the matrix in an ATP-dependent manner [118]. Once in the matrix, the N-MTS of most precursor proteins is removed by mitochondrial processing peptidase (MPP) to form mature proteins [119]. Matrix proteins are folded and assembled into

complexes by the heat shock proteins [120].

Single-pass inner membrane proteins contain an N-MTS and are inserted into the membrane by one of two mechanisms [121]. Some proteins have a hydrophobic "stop-transfer" signal that arrests the protein in the TIM23 complex, followed by lateral release of the protein directly into the inner membrane [115, 122-124]. Other precursor proteins are imported into the matrix with the same sequence and requirements as matrix proteins. These proteins are then exported from the matrix to the inner membrane [125-127]. The export process involves the inner membrane protein, Oxa1 [128, 129], which is also required for insertion of mitochondrially-encoded proteins into the inner membrane [130, 131].

4. 1. 4. Protein insertion into the inner membrane by the TIM22 complex

Polytopic inner membrane proteins with INT-MTS, like the large family of mitochondrial metabolite carriers, are guided through the intermembrane space by the small TIM proteins—Tim8, Tim9, Tim10, Tim12, and Tim13 (Fig. 6C) [132, 133]. Two soluble 70-kDa complexes (Tim9/10 and Tim8/13) mediate transfer of these proteins to the TIM22 complex [134]. Most polytopic inner membrane proteins are transferred from the TOM complex to the TIM22 complex via Tim9/10 [135-140]. Other proteins cross the intermembrane space with the help of Tim8/13 [141]. For the mitochondrial ADP/ATP carrier (AAC), it has been demonstrated that Tim9/10 binds to and thereby protects hydrophobic α-helical sequences within the preproteins that later become transmembrane segments [95, 142, 143]. Tim9/Tim10 is required for release of carrier preproteins from the TOM complex and delivery to the TIM22 complex, which consists of Tim22, Tim54 and

Tim18. Tim9/Tim10 peripherally binds to the TIM 22 complex via the adaptor protein Tim12. Tim22 forms a voltage-activated channel that specifically responds to the addition of peptides that resemble INT-MTS [144]. Protein insertion at the twin-pore TIM22 complex is coordinated by $\Delta\Psi$ and the INT-MTS [133, 145].

4. 2. Mitochondrial protein delivery using the mitochondrial targeting signal peptide and related therapeutic strategies

The MTS has the potential to be a useful device for selective and effective delivery of therapeutic proteins to mitochondria in patients suffering from mitochondrial diseases. Candidate proteins for this strategy include superoxide dismutase, apoptosis-inducing proteins and anti-apoptosis proteins. Targeting of these candidate proteins to the mitochondria would protect mtDNA and nuclear DNA from reactive oxygen species (ROS) and from excessive apoptosis. Several studies concerning delivery of exogenous proteins to the mitochondria have been reported. In a basic study of mitochondrial delivery using MTS, pDNA encoding an MTS-fused protein was used to transfect cultured cells. The MTS directs the cargo protein inside the mitochondria, where it is then cleaved, allowing for localization and function of the fused protein [146-148] (Fig. 6).

Protein delivery via MTS has facilitated various lines of mitochondrial research. Zhang et al reported the photosensitization properties of mitochondrially localized green fluorescent protein (GFP) [148]. First, a mammalian expression vector was constructed using a thermostable variant, GFP5, fused at its N-terminus to the 16-amino acid mitochondrial targeting sequence of human 3-oxoacyl-CoA thiolase. The recombinant

pDNA was then transfected into cultured monkey kidney (COS7) cells under conditions suitable for transient expression. Confocal laser scanning microscopy revealed that the pattern GFP5 localization in the mitochondria was almost identical to that of MitoTracker Red, which selectively stains mitochondria. Photoirradiation at 390-570 nm to excite the GFP5 localized in mitochondria of transfected COS7 cells resulted in significant cell death. Thus, it was concluded that the combination of GFP5 and an MTS can be used in the development of mitochondria-specific photosensitizers.

Another useful application of MTS is mitochondrial gene therapy with restriction enzymes [146, 149, 150]. Tanaka et al. proposed gene therapy based on mitochondrial delivery of the restriction endonuclease, *Sma*I. *Sma*I has been used in the diagnosis of neurogenic muscle weakness, ataxia and retinitis pigmentosa disease (NARP) or Leigh's disease. This disease is caused by an Mt8993T→G mutation that results in Leu156Arg replacement, which blocks the proton translocation activity of subunit a of F_0F_1-ATPase, and selectively digests mutant mtDNA. The *Sma*I gene was transiently fused to MTS in hybrids carrying mutant mtDNA. Mutant mtDNA was specifically eliminated from mitochondria targeted by *Sma*I, followed by repopulation with wild-type mtDNA, resulting in restoration of both intracellular ATP levels and mitochondrial membrane potential. Moreover, in vivo electroporation of pDNA expressing mitochondrion-targeted *EcoR*I decreased cytochrome c oxidase activity in hamster skeletal muscle, but did cause degenerative changes in nuclei. These data indicate that this approach may be a novel form of gene therapy for unique mitochondrial diseases [146].

Although the MTS strategy appears promising, it may not be applicable in certain cases (Fig. 7). Mitochondrial delivery using MTS is severely limited by the size of the cargo

[100, 101, 151]. Therefore, MTS cannot deliver unfolded proteins or macromolecules, such as pDNA and mtDNA to mitochondria. Furthermore, MTS may not function in mitochondrial diseased cells. For example, MTS could not be used to correct defects in mitochondrial protein import (e.g., X-linked human deafness-dystonia syndrome [Mohr-Tranebjaerg syndrome]) [152] of MTS-conjugated cargo. Similarly, MTS may be of little benefit in the case of mtDNA-encoded-proteins, as these proteins are generally too hydrophobic to maintain the unfolded conformation that is required for MTS-mediated import [153]. Therefore, a novel protein-delivery system that is independent of the physical characteristics of the proteins is required to realize effective treatments for human diseases caused by mitochondrial dysfunction.

4. 3. Mitochondrial delivery using membrane-permeable peptides

Recent studies have revealed that the PTD is a promising device to improve delivery of various biologically active molecules. One PTD derived from the human immunodeficiency virus-1 TAT protein consists of 11 amino acids, including 6 arginine and 2 lysine residues. This PTD rapidly translocates cargo across the cell membrane both in vivo and in vitro. The TAT peptide has been employed to deliver oligonucleotides, peptides, full-length proteins, and even 200-nm liposomes [154-156]. Furthermore, TAT-fusion proteins can cross the nuclear membrane [157, 158].

In addition to translocation across the nuclear membrane, a recent study demonstrated the ability of TAT to deliver cargo to mitochondria. Asoh et al. showed that PTD fused to the FNK protein, which was constructed from Bcl-X_L by site-directed mutagenesis of three amino acids to improve its cytoprotective activity, protected some cell

types from both necrotic and apoptotic cell death [159-161]. These results suggest that the PTD-fused FNK protein translocates across both the plasma and mitochondrial membranes (Fig. 8A). Mitochondrial delivery that uses membrane-permeable peptides has advantages over the MTS, which depends on the protein import machinery.

Del Gaizo et al. constructed a TAT fusion protein consisting of MTS derived from mitochondrial malate dehydrogenase and GFP (TAT-mMDH-GFP) (Fig. 8B) [162, 163]. Incubation of isolated mitochondria and intact cells with TAT-mMDH-GFP resulted in accumulation of TAT fusion protein in mitochondria via an MTS-independent pathway. Assuming that TAT non-selectively penetrates the mitochondrial membrane, this protein may re-distribute to the cytosol after it accumulates in mitochondria. To avoid this situation, TAT may be fused with the N-terminus of full-length mMDH. In this system, MTS is cleaved specifically in the matrix, allowing GFP to separate from TAT. As a result, GFP is effectively trapped within the matrix.

Shokolenko et al. attempted mitochondrial delivery of exonuclease III protein (Exo III) via the combination of PTD and MTS (Fig. 8C) [164]. An MTS-Exo III-TAT-fusion protein was constructed by the fusion of MTS and TAT with Exo III at the N- and C-terminus, respectively. Overexpression of Exo III reportedly increases the sensitivity of cancer cells to oxidative stress, resulting in diminished cell survival [165]. The transduced protein was effectively targeted to the mitochondrial matrix, where it decreased repair of mtDNA, rendering the cells more susceptible to the lethal effects of oxidative stress. In this strategy, PTD functions as a cytoplasmic delivery device and the mitochondrial targeting activity of MTS may compensate for the non-specific protein delivery of PTD. As a result, efficient cytoplasmic and mitochondrial delivery is achieved.

Maiti et al. reported a novel class of guanidine-containing molecular transporters that use inositol dimers as the scaffold [166]. These transporters exhibited very high membrane-translocating capacity in various cell types. Their internalization–localization patterns appeared to be quite different from those observed using arginine-rich peptides such as TAT. They showed that these transporters exhibited high selectivity for mitochondrial localization. These results suggest that the guanidine group of the arginine residue in the TAT peptide plays a critical role in the enhancement of mitochondrial delivery. Currently, Maiti et al. are working to elucidate the structure–selectivity relationship of these guanidine-containing transporters.

5. Mitochondrial gene delivery and therapy

5. 1. Relationship between mitochondrial diseases and mutations and/or defects in mitochondrial DNA

Each mitochondrion contains approximately 1000 proteins, of which only 13 are mitochondrial genome-encoded. Human mitochondrial DNA (mtDNA) is a circular 16,569-base pair (bp) molecule that encodes 13 genes involved in oxidative phosphorylation. Two rRNAs and 22 tRNAs are necessary for expression of the mtDNA gene products (Fig. 9). The vast majority of diseases with mitochondrial origin result from incorporation of damaged or mutated proteins into complexes of the electron transport chain [167-169]. Any of 13 genes that are encoded by the mitochondrial genome or those encoded by a nuclear gene may be damaged, as the complexes are composed of subunits encoded by both mitochondrial and nuclear genes. Mitochondrial diseases that arise from defective nuclear-encoded proteins were recently reviewed by Mackenzie et al. [81]. The review

discusses pyruvate dehydrogenase deficiency, type 1 primary hyperoxaluria, severe alcoholic liver disease and human deafness dystonia syndrome. Several other mitochondrial diseases occur because of mutations in nuclear-encoded mitochondrial proteins, such as those involved in mtDNA maintenance/ replication, and mtDNA polymerase [170, 171].

Here, we focus on the relationships between mitochondrial diseases and mutations and/or defects in mtDNA. Because mtDNA is free of exons, mtDNA has a much higher information density than nuclear DNA. Therefore, mtDNA is more susceptible to mutation, than nuclear DNA (>20 fold), resulting in a high frequency of mitochondrial diseases [172, 173]. Genetic mutations that are responsible for mtDNA-associated diseases can be categorized into four types: missense, protein synthesis, insertion-deletion, and copy number (Fig. 9). Examples of diseases resulting from missense mutations include Leber's hereditary optic neuropathy (LHON) [174], neurogenic muscle weakness, ataxia, and retinitis pigmentosa (NARP) [175]. Protein synthesis mutations occur in the region encoding tRNA and include mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) [176], myoclonic epilepsy with ragged-red fibers (MERRF) [177], and maternally inherited cardiomyopathy (CM) [178]. Deletions in mtDNA have been found in the majority of chronic progressive external ophthalmoplegia (CPEO) and Kearns-Sayre syndrome (KSS) cases [179]. A marked decrease in the number of mtDNA molecules is associated with familial mitochondrial myopathy [180].

5. 2. Mitochondrial gene therapy

Ideally, all mtDNA would be mutation-free. Under these ideal conditions, "homoplasmy" results. However, in certain situations, cells have both mutant and wild-type

mtDNA. This phenomenon is termed, “heteroplasmy”. In the case of mitochondria-related diseases, when the percentage of mutant mtDNA exceeds a certain threshold, mitochondrial dysfunction becomes clinically apparent [172, 175]. Accordingly, delivery of a large quantity of wild-type mtDNA into the mitochondrial matrix of diseased cells would decrease the proportion of mutated mtDNA, thereby suppressing mitochondrial diseases (Fig. 10).

The loci of mtDNA mutations are known for many mitochondrial diseases. For example, the major point mutations that cause MERRF, MELAS, and CM are localized at bp 8344 (A to G; $tRNA^{Lys}$) [177], bp 3243 (A to G; $tRNA^{Lue\ (UUR)}$) [176] and bp 4269 (A to G; $tRNA^{Ile}$) [178], respectively (Fig. 9). Accordingly, precise site-specific correction of mtDNA mutations is a sophisticated strategy for the treatment of genetic disorders. Using in a cell-free DNA repair assay with RNA/DNA oligonucleotides, Chen et al. demonstrated that isolated rat liver mitochondria contain the machinery required for repair of genomic single-point mutations [181]. They also showed that the levels of gene conversion with mitochondrial extracts were similar to those observed with nuclear extracts. Based on the results of these experiments, it was concluded that RNA/DNA oligonucleotides might provide a novel approach to the treatment of certain mitochondrial-based diseases.

Taylor et al. proposed selective inhibition of mutant mtDNA replication, which would allow propagation of only wild-type DNA, as treatment for mitochondrial disease [182]. Taylor et al. validated this approach by synthesizing peptide nucleic acids (PNAs) complementary to human mtDNA templates containing a deletion breakpoint or single base mutation to cause disease. They showed that the antigenomic PNAs specifically inhibited replication of mutant, but not wild-type mtDNA templates using an in vitro replication

run-off assay. They concluded that their antigenomic PNA therapy could help patients with heteroplasmic mtDNA disorders. Because mutations in mtDNA cause disease as described above, mitochondrial gene delivery leading to the complementing normal mtDNA, repair of mutated mtDNA, inhibition of replication for mutated mtDNA, and/or degradation of mutated mtDNA would be a novel strategy for the cure of mitochondrial disease (Fig. 10).

5. 3. Mitochondrial delivery of oligodeoxynucleotides and peptide nucleic acids

As described above, the delivery of therapeutic oligodeoxynucleotides (ODN) and PNA to the mitochondrial matrix holds promise for the treatment of mitochondrial diseases that result from mtDNA mutations (Fig. 10). To date, DNA has been introduced into isolated mitochondria by covalently linking the mitochondria targeting signal peptide (MTS) to either ODN, double-stranded DNA, or PNA [183-185] (Fig. 11A). Seibel et al. showed that these conjugates are imported into mitochondria through the outer and inner membranes via the protein import machinery. Reportedly, DNA 17 bp to 322 bp in length can be used in this strategy [184]. A similar strategy has also been developed for the mitochondrial delivery of PNA (Fig. 11A). Using membrane permeability toxin as a device for cytoplasmic delivery, MTS-conjugated PNA can be imported into mitochondria. This method provides a viable strategy for modification of mtDNA in cultured cells, animals, and humans [183].

5. 4. Strategy for circular DNA delivery using mitochondriotropic lipoplexes

As described in section 4. 1., conjugation of MTS permits the delivery of exogenous protein to mitochondria. However, mitochondrial delivery is severely limited by

cargo size [151] (Fig. 7). Therefore, an alternative strategy for the delivery of macromolecules, such as mtDNA and pDNA, to mitochondria is needed for effective mitochondrial gene therapy. Weissig et al. attempted delivery of pDNA to mitochondria using DQAsomes, which are mitochondriotropic and cationic 'bola-lipid'-based vesicles [186, 187] (Fig. 11B). Previously, DQAsomes-DNA complexes (DQAplexes) were shown to selectively release pDNA upon contact with isolated mitochondria derived from rat liver [187]. In addition, DQAplexes apparently escape endosomes without losing their pDNA, and specifically release pDNA proximal to mitochondria [186]. More recently, Weissig et al. attempted delivery of pDNA to the mitochondrial matrix by means of improved DQAsomes [188]. In the future, effective delivery of circular DNA to the mitochondrial matrix using such novel approaches is expected to be used in mitochondrial gene therapy.

6. Application of mitochondrial liposome-based delivery to disease therapy

6. 1. Drug-carrier design for achievement of mitochondrial drug therapy

Research in the area of mitochondrial disease therapy has focused on the molecular mechanisms of mitochondrial diseases and on drug delivery systems that are targeted to mitochondria. Despite intensive research, viable mitochondrial drug delivery systems are rare. To achieve efficient mitochondrial drug delivery, methods of drug encapsulation into carriers that take the physical characteristics of the drugs into account must first be developed. Second, the carriers must be efficiently targeted and internalized by specific cell types. Finally, sophisticated regulation of intracellular trafficking is needed to release the drug carrier from the endosome to the cytosol and, thereafter, deliver it to the mitochondria [10].

Ultimately, it is necessary to program all devices that are required to target specific cell types and to regulate intracellular trafficking into one drug carrier, so as to trigger the function of each device at the appropriate intracellular location and time. [11]. We have developed a novel non-viral gene delivery system based on a new packaging concept, which we termed, “Programmed Packaging”. This novel gene delivery system—the multifunctional envelope-type nano device (MEND)—has been described previously [11, 12]. Programmed Packaging was proposed to develop rational non-viral gene delivery systems equipped with various functional devices that could overcome barriers nucleic acid-delivery to the nuclei of target cells [11]. Because there are multiple causes of mitochondrial disease, the carrier design should be flexible, so that it can be modified to target different tissues and to deliver various types of cargo, depending on the particular disease. We are optimistic that flexible design of MEND will meet the requirements of drug delivery systems for a variety of mitochondrial diseases.

6. 2. Efficient macromolecule packaging using a multifunctional envelope-type nano device (MEND)

The efficient packaging of therapeutic compounds and macromolecules into a drug carrier is an important step in the development of successful delivery systems. For example, in the case of doxorubicin (an anticancer drug), efficient packaging increases pharmacological activity [189]. Because of the wide variety of mitochondrial diseases with multiple etiologies the number of molecules that are candidate therapeutics is large. For example, gene-complement therapy requires delivery of mitochondrial DNA (mtDNA), gene-repair therapy depends on functional nucleic acid, maintenance of the electron transfer

system and apoptosis regulation require protein translocation to mitochondria. Although, these therapies are attractive, the lack of efficient macromolecule-packaging methods prevents gene- and protein-based therapies.

Recently, we developed a MEND that permits efficient and simple packaging of pDNA [11, 12]. This MEND consists of a condensed pDNA core and a lipid envelope equipped with various functional devices that mimic envelope-type viruses (Fig. 12). Condensation of pDNA into a compact core prior to its inclusion in a lipid envelope has several advantages as follows: protection of pDNA from DNase; size control; and, improved packaging efficiency because of electrostatic interactions between the condensed core and the lipid envelope. To date, we have efficiently packaged not only pDNA, but also functional nucleic acids, proteins, and other substances into MEND. The MEND is expected to deliver drugs to target locations in amounts sufficient to exert a pharmacological effect.

After pDNA condensation, complexes are incorporated into lipid envelopes, such that the pDNA core and lipid envelope exist as separate structures, rather than a disordered mixture, to control topology. Various functional devices are easily incorporated into the core particle and onto the MEND surface, as follows: cell-targeting activity by addition of ligands for specific receptors; endosomal escape capability via introduction of pH-sensitive fusogenic peptides; and, organelle-targeting activity by incorporation of organelle-targeting devices. Moreover, we have achieved nuclear and cytosolic delivery using MEND [16]. We believe that the MEND has significant potential as a mitochondrial-disease therapeutic when optimized for mitochondrial delivery.

6. 3. Mitochondrial liposome-based drug delivery for cancer therapy

Treatment of diverse mitochondrial diseases requires the selective delivery of drugs to target tissues, which vary depending on the particular disease. Cellular internalization of small-molecule drugs requires transporters, as does internalization of macromolecules. Therefore, both selective drug targeting and efficient cytoplasmic delivery are critical. To date, we have developed liposome-based drug delivery systems, such as octaarginine (R8)-modified liposomes and cell-targeting liposomes equipped with a pH-dependent fusogenic peptide [13, 157]. Modification of liposome-surfaces with ligand targeting devices was expected to result in liposome delivery to target organs. However, rapid elimination of liposomes from systemic circulation, due to the high rate of clearance by the reticuloendothelial system (RES) and other organs, prohibits liposomes and lipoplexes administered intravenously as anti-tumor treatments [190, 191]. It is generally accepted that half-life in systemic circulation can be increased by surface modification of carriers with PEG [191, 192]. Moreover, limiting the size of carriers to less than 200 nm allows for efficient accumulation in tumor tissue due to enhancement of the permeability and retention effect [193, 194]. The next section describes cancer therapy based on liposome delivery to mitochondria of cancer cells.

6. 3. 1. Efficient cytoplasmic delivery using transferrin-modified liposomes equipped with a pH-sensitive fusogenic peptide

Recently, we developed transferrin-modified liposomes (Tf-LP) targeted to tumor cells that up-regulate transferrin (Tf) receptor expression [13]. Tf is potent target ligand for cells that express Tf receptors, such as tumor cells, as it results in liposome uptake via receptor-mediated endocytosis. However, an encapsulated fluorescent marker

(sulforhodamine B, Rho) delivered in Tf-LP became trapped in the endosomal/lysosomal compartments (Fig. 13A). Thus, a pH-sensitive fusogenic peptide, GALA, was introduced in the preparation of Tf-LP to enhance endosomal escape. When the GALA peptide was co-encapsulated in the liposomes with Rho, the encapsulated marker remained in the endosome/lysosome fraction and was subsequently degraded. In contrast, when GALA was modified with cholesterol and displayed on the surface of the liposome (Tf-GALA-LP), encapsulated Rho was efficiently released from the endosome to the cytosol (Fig. 13B). Therefore, the topology of GALA is critical to its function [13].

6. 3. 2. Mitochondrial delivery of mastoparan using a Transferrin/GALA system for selective cancer therapy

To apply Tf-GALA-LP to the delivery of a functional peptide, mastoparan (MP) was encapsulated in the liposomes. MP is a 14-amino acid amphipathic peptide obtained from wasp venom (sequence: INLKALAALAKKIL-NH_2), which induces mitochondrial permeability transition (PT) (Please see section 2. 4. regarding PT) [195-197]. After induction of PT, the permeability of the mitochondrial inner membrane increases and any components with a molecular weight 1500 Da leak from the mitochondrial matrix. Next, mitochondrial swelling is induced and the structure of mitochondrial membrane changes drastically. As a result, cytochrome c (cyt c) is released to the cytosol from the mitochondrion, inducing apoptosis. Therefore, selective delivery of MP to tumor cells appears to be a useful strategy for cancer treatment (Fig. 13).

To examine the usefulness of encapsulation of MP into Tf-GALA-LP, K562 cells were treated with MP encapsulated in liposomes and free MP, and subsequently, induction

of PT was evaluated [14]. In the case of free MP, cyt c was detected in the culture medium and cytosol. This result indicates that MP entered the K562 cells through the cell membrane [198], and cyt c, after release from the mitochondria, leaked out of the cells through the disrupted cell membrane. Thus, encapsulation appears to be very important for prevention of non-specific MP activity. In contrast, MP encapsulated in Tf-GALA-LP was internalized to target tumor cells via Tf receptor-mediated endocytosis. Subsequently, MP was delivered to the cytosol, leaving the plasma membrane intact. In fact, cyt c was observed only in the cytosolic fraction. Thus, MP encapsulated in Tf-GALA-LP was successfully delivered to the cytosol. Non-specific MP activity was not detected and the encapsulate MP peptide did not leak from the liposomes to outside the cells. These results clearly demonstrated the utility of MP encapsulated in Tf-GALA-LP for cancer therapy.

6. 4. MITO-Porter: a nano-carrier system for delivery of macromolecules into mitochondria via membrane fusion

6. 4. 1. The novel MITO-Porter concept: mitochondrial delivery via membrane fusion

Mitochondrial delivery using mitochondrial targeting devices, such as the mitochondrial targeting signal peptide (MTS) or protein transduction domain (PTD), is an attractive strategy. As described above, several studies have demonstrated that conjugation of a mitochondrial-targeting device to either exogenous protein or oligodeoxynucleotides (ODN) allows effective mitochondrial delivery. However, there are many obstacles to clinical application of these strategies, such as generation of new in-frame fusion proteins, covalent chemical conjugation, and denaturation. Furthermore, mitochondrial delivery using

MTS is severely limited by the size of the cargo [151]. Therefore, delivery of bioactive macromolecules, such as pDNA and folded proteins, is difficult to achieve (Fig 7).

Recently, we have proposed the MITO-Porter as a MEND for mitochondrial delivery [15, 16]. The MITO-Porter is a liposome-based nano-carrier that delivers cargo to mitochondria via a membrane-fusion mechanism (Fig 14). Mitochondrial delivery using the MITO-Porter requires the following three steps: (1) the carrier must be delivered to the cytosol; (2) intracellular trafficking of the carrier, including mitochondrial targeting, must be regulated; and, (3) mitochondrial delivery via membrane fusion must be regulated. We are hopeful that MITO-Porter can be used to deliver any carrier-encapsulated molecule, regardless of its size or physicochemical properties, to the mitochondrion.

The first barrier to intracellular targeting is the plasma membrane. Therefore, the transduction activity of the MITO-Porter must be sufficient for mitochondrial delivery. Futaki et al. reported that R8 delivered exogenous protein into cells as effectively as TAT peptide, based on fluorescence microscopic observation of fluorescein-labeled peptides [199]. The peptides were internalized within 5 minutes via macropinocytosis, which is similar to clathrin-independent endocytosis [200]. We also showed that high density-R8-modified liposomes are internalized primarily via macropinocytosis and are delivered to the cytosol, while retaining an aqueous-phase marker [157]. Therefore, we chose R8 as a cytoplasmic delivery device for the MITO-Porter. We also expected that R8, which mimics TAT, might have mitochondrial targeting activity as described in section 4. 3.

6. 4. 2. Validation of binding between R8-modified liposomes and isolated mitochondria.

Liposomes with different lipid compositions were prepared by the hydration method to investigate their fusogenic activity with mitochondrial membrane. 1,2-Dioleoyl-sn-glycero-3-phosphatidyl ethanolamine (DOPE), a neutral, fusogenic, cone-type lipid, which is commonly used in drug delivery, was selected as the main lipid component (DOPE-LP). As a control, egg yolk phosphatidyl choline (EPC), a neutral, cylinder-type lipid was used in place of DOPE (EPC-LP). First, the mitochondrial binding activity of these liposomes was investigated (Fig. 15). Fluorescent-labeled liposomes were incubated with isolated rat liver mitochondria and the fluorescence intensity of the liposomes bound to mitochondria was measured. Binding activity was quantified as the percentage of mitochondria-bound liposomes out of the total amount applied.

Unmodified EPC liposomes had low binding activity, regardless of lipid composition (Fig. 15A). Because mitochondria maintain a high negative potential, it assumed that carrier access to mitochondria requires liposomes that carry a positive charge. In an attempt to increase liposome-binding activity, positively charged R8 peptides were attached to the surface of the liposomes. Liposome binding activity increased significantly after R8-surface modification, regardless of lipid composition (Fig. 15A). Similar results were obtained for DOPE-LP (Fig. 15B). These results show that addition of a cationic peptide, R8, to the liposome surface enhances binding to mitochondria, which have a high negative charge.

6. 4. 3. Identification of liposomes that fuse with the mitochondrial membrane

For our strategy to effectively deliver cargo to mitochondria, liposomes must fuse with the mitochondrial membrane after mitochondrial binding (Fig. 14). Therefore, we

screened liposomes with different lipid compositions for high fusion efficiency with isolated rat liver mitochondria using fluorescence resonance energy transfer (FRET) analysis [15]. Octaarginine modified liposomes (R8-LP) composed of DOPE showed higher fusogenic activity than liposomes comprised of EPC. In the absence of R8, fusogenic activity was inadequate. These results suggest that strong electrostatic binding between R8-LP and mitochondria stimulates liposomal fusogenic activity. Based on these results, we concluded that R8 and DOPE are essential to membrane fusion after mitochondrial membrane binding.

Two highly fusogenic lipid compositions that were identified during screening were used construct the MITO-Porter. We concluded that R8-LP composed of DOPE and containing either sphingomyelin (SM) or phosphatidic acid (PA) fused efficiently mitochondria. We termed these specially designed MEND, MITO-Porter [15]. Here, we discuss the effects of R8- and DOPE-modification on membrane fusion with mitochondria. The fusion activities of a series of liposomes composed of SM or PA are summarized in Fig. 16. In the case of EPC-LP composed of SM, no significant increase of fusion activity was detected after modification with R8. In contrast, fusion activity was increased 5.3-fold by replacing EPC with DOPE (Fig. 16A). Modification with R8 further increased the activity by 2.2-fold (Fig. 16A). In total, the fusion activity of R8-DOPE-LP was 12-fold higher than that of EPC-LP (Fig. 16A). In the case of liposomes composed of PA, the fusion activity of DOPE-LP was only 1.5 fold-higher than that of EPC-LP (Fig. 16B). However, modification of liposomes with R8 significantly enhanced the fusion activities of both EPC-LP and DOPE-LP by 4.3-fold and 3.7-fold, respectively (Fig. 16B). These results show that the increase in fusion activity of liposomes composed of SM can be explained by the substitution of DOPE for EPC. In contrast, the fusion activity of PA was increased by

R8-modification. The enhanced fusogenic activity observed for R8-LPs composed of PA, which is anionic lipid, may be attributed to increased binding to mitochondria, which possess a highly negative potential.

6. 4. 4. Mitochondrial macromolecule delivery using MITO-Porter

The mitochondrion possesses at double-membrane structure that consists of an outer membrane and an inner membrane, which form the intermembrane space and matrix (Fig. 1). We expected that MITO-Porter could deliver macromolecules to the intermembrane space by passing through the outer membrane, and not only binding to the outer membrane. Here, we summarize our study that evaluated macromolecule delivery using MITO-Porter [15]. Green fluorescent protein (GFP), a model macromolecule, was encapsulated into the MITO-Porter and incubated with isolated rat liver mitochondria. Next, the mitochondria were subfractionated into an outer membrane fraction and an intermembrane space fraction by digitonin treatment. Localization of GFP in each fraction was detected by Western blot analysis using antibodies specific for the N-terminal amino acid sequence of GFP (Fig. 17A).

As shown in Fig. 17B, in the case of MITO-Porter, GFP was detected in both the outer membrane and intermembrane space fractions (lanes 3-6); while in the case of R8-EPC-LP (low fusion activity), GFP was detected only in the outer membrane fraction (lanes 7-10). These results indicate that MITO-Porter delivered macromolecules to the mitochondria, while R8-EPC-LP could only bind to the outer membrane and failed to deliver GFP to the mitochondria. GFP was not detected in either the outer membrane or intermembrane space fractions when GFP alone was incubated with isolated rat liver

mitochondria (Fig. 17B, lanes 1, 2). Unexpectedly, the molecular weight of GFP detected in the intermembrane space fraction (28 kDa) was smaller than that of the full-length molecule (33 kDa). This result suggested the possibility that GFP was cleaved by a protease(s) in the intermembrane space. We tested this hypothesis, and confirmed that the truncated protein retains its ability to fluoresce [15]. These results support the assertion that the MITO-Porter delivered its GFP cargo to the intermembrane space by passing through the outer membrane.

6. 4. 5. Evaluation of mitochondrial targeting activity

We showed that MITO-Porter deliver macromolecules to mitochondria in the vitro experiment described above. However, to apply the MITO-Porter to living cells, intracellular trafficking of the carrier must be regulated. Mitochondrial targeting of MITO-Porter is critical to successful delivery of cargo to mitochondria in living cells. Accordingly, we evaluated mitochondrial targeting activity of the MITO-Porter to ascertain whether R8 facilitates interaction between the MITO-Porter and mitochondria in living cells.

Fluorescence-labeled liposomes were added to HeLa cell-homogenate and the mixture was incubated for 30 min at 25^{o}C. The resulting solution was centrifuged to isolate mitochondria, and then fluorescence intensities of the carriers bound to mitochondria were measured. Mitochondrial targeting activity (%) was calculated as the percentage of mitochondria-bound liposomes to the total amount applied. In this experiment, we used cell homogenate, which contained not only mitochondria, but also other organelles, such as, nucleus, Golgi apparatus, lysosome, peroxisome, as mitochondrial binding activity could be

better evaluated in model living cells than in isolated mitochondria.

Results of this experiment indicated that the efficiency of MITO-Porter-binding to mitochondria was approximately 10-fold greater than that of R8-unmodified liposomes. Mitochondrial targeting activity of MITO-Porter was approximately 20%, and the majority of the remaining carriers were detected in the cytosolic fraction. This result suggests that R8 can deliver large drug carriers, such as liposomes, to mitochondria even in a homogenate, which is a better model for living cells than isolated mitochondria, although delivery may not be efficient. Thus, addition of a positive charge to the carrier is important for mitochondrial binding, as mitochondria possess the highest negative potential known in living cells.

6. 4. 6. MITO-Porter system delivers cargo to mitochondria in living cells

MITO-Porters delivered GFP—a model macromolecule—to the mitochondria, as confirmed by confocal laser scanning microscopy of living cells (Fig. 19). HeLa cells were incubated with the carrier, stained with MitoFluor Red 589, and observed with confocal laser scanning microscopy to visualize red-stained mitochondria (Fig. 19A). In the case of MITO-Porter, GFP (green) co-localized with mitochondria (red); thus, colocalization of the signals appeared as a yellow signal in the merged images (Fig. 19B (a), (b)). In contrast, little co-localization was observed in the case of the R8-EPC-LP control carrier (Fig. 19B (c)) [15]. This result was consistent with that of the confocal laser scanning microscopy analyses, in which some, but not all, MITO-Porters accumulated in mitochondria (Fig. 18). Similar results were also found for the intracellular distribution of the carriers themselves [15]. These results demonstrate the principle of MITO-Porter-based mitochondrial delivery.

Mitochondria form a continuous, highly dynamic, reticulum or network, that is continually undergoing fusion and fission in living cells [201-204]. Therefore, this natural plasticity may effectively promote delivery by the MITO-Porter. We believe that the mitochondrial macromolecule delivery via the MITO-Porter has potential as a novel therapeutic strategy. Currently, we are studying therapeutic applications of the MITO-Porter.

6. 5. Perspective on use of liposome-based macromolecule delivery to mitochondria as therapy for mitochondrial diseases

In this review, we described various useful mitochondrial delivery systems. However, clinical application of mitochondrial drug delivery requires targeting of the carrier drug not only to mitochondria in living cells, but also to diseased cells. Moreover, mitochondrial delivery systems must deliver adequate amounts of the drug to effectively treat disease. Therefore, a drug carrier that meets all of these requirements is needed. Liposome-based delivery systems are one type of mitochondrial drug delivery system with potential clinical application.

We expect that the combination of an attractive mitochondrial delivery device and our MEND system would significantly improve mitochondrial delivery and provide effective mitochondrial disease therapy. A hypothetical combination of mitochondrial targeting device and MEND delivery is as follows: (i) drugs with an attractive mitochondrial targeting device, such as MTS and PTD, are condensed into nano-particles; (ii) the condensed particles are then efficiently encapsulated in a lipid bilayer using the MEND preparation technique; and, (iii) the MEND is equipped with the Transferrin/GALA cytoplasmic delivery system. Thus, construction of MEND with various delivery devices

has the potential to allow for selective and effective mitochondrial delivery of drugs.

Our MITO-Porter system will be tested in vivo. Drug carriers, such as cationic liposomes, are rapidly eliminated from systemic circulation, due to the high rate of clearance by the RES and other organs [190, 191]. It is generally accepted that the half-life in systemic circulation can be extended by carrier-surface modification with PEG [191, 192]. However, PEG negatively affects cellular uptake and intracellular trafficking [205]. Recently, we have reported the development of a PEG-Peptide-DOPE conjugate (PPD) for cancer gene therapy in vivo [206]. In this strategy, PEG is removed from the carriers via cleavage by a matrix metalloproteinase, which is uniquely expressed in tumor tissues. In vivo studies revealed that PPD effectively stabilized the carriers in systemic circulation and facilitated tumor accumulation. Moreover, intravenous administration of the PPD-modified gene carrier stimulated gene expression in tumor tissue. Future studies will attempt to develop mitochondrial delivery system for intravenous administration of drug carriers based on the PPD system.

7. Conclusion

Mitochondria were first recognized as the site of cellular energy production. Recently, the mitochondrion was recognized as the organelle that regulates apoptosis. Currently, the relationships between mitochondrial dysfunction and intractable disease are being investigated. As a result, the mitochondrion has attracted attention as a target organelle for the treatment of disease. This review summarized recent reports concerning the molecular pathology of mitochondrial diseases and mitochondrial drug delivery systems. In particular, we described strategies for mitochondrial delivery of macromolecules, such as

proteins and nucleic acids. We also described our liposome-based drug delivery systems, such as MEND, the Transferrin/GALA system and MITO-Porter. In the future, we hope to establish a clinically applicable nano-device for effective mitochondrial delivery of therapeutic compounds and also attempt to achieve mitochondrial gene therapy. It is our hope that these optimized delivery systems will provide effective therapies for the many patients who suffer from mitochondrial diseases.

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Figure legends

Figure 1 Various roles of mitochondria.

Mitochondrion possesses two membrane structure consisted of outer membrane (OM), where the main events related to apoptosis are triggered, and inner membrane (IM), which has proteins involved in electron transport chain, ATP synthase, and etc. The inner most is the matrix, which pools mitochondrial DNA (mtDNA) and also has major metabolic pathways, while the one between the membranes is called intermembrane space IMS. AAC, ATP/ADP carrier protein; AIF, apoptosis-inducing factor; ANT, adenine nucleotide translocase; cyt c, cytochrome c; MTS, mitochondrial targeting signal peptide; PTP, mitochondrial permeability transition pore; ROS, reactive oxygen species; TCA cycle, tricarboxylic acid cycle; VDAC, voltage-dependent anion channel.

Figure 2 Mitochondrial electron transfer system.

This figure shows electron transport chain with oxidative phosphorylation. The electron transport chain extrudes protons from the matrix to the intermembrane space (IMS) (1).

use of the considerable free energy released as a result of the oxidation of NADH (1). The electrochemical proton gradient thus formed (2). This gradient, which is composed of the electric component ($\Delta \psi^2$) and the proton concentration gradient, is the driving force for the back flow of protons through the ATP synthase complex (3). Q, ubiquinone (coenzyme Q); cyt c, cytochrome c; ROS, reactive oxygen species; TCA cycle, tricarboxylic acid cycle; IM, intermembrane space.

Figure 3 Reactive oxygen species (ROS) generated from mitochondria can damage cells.

Free radicals generated by the electron transport chain can result in oxidative damage to mitochondrial DNA, proteins and lipid peroxidation. CuZnSOD, cytosolic (copper-zinc-containing) superoxide dismutases; MnSOD, mitochondrial (manganese-containing) superoxide dismutases; GPx, glutathione peroxidase.

Figure 4 Mitochondria and apoptosis.

This figure shows apoptosis signaling associated with mitochondria. A large number of cell-death stimuli induce release of apoptogenic factors, such as cytochrome c (cyt c) and apoptosis-inducing factor (AIF), from mitochondria. Cyt c binds apoptotic protease activating factor-1 (Apaf-1) and then stimulates caspase-9. AIF degrades nuclear DNA, independent of caspase. Bax/Bak, PT pore, and membrane damage models have been proposed as mechanisms of apoptogenic factor-release. Cas, caspase; Procas, procaspase.

Figure 5 Mitochondrial delivery systems for small-molecule drugs.

A, Triphenylphosphonium (TPP) is lipophilic cation. Uptake into mitochondria through the lipid bilayer can be driven by the large negative potential across the mitochondrial membrane. The attachment of a lipophilic cation such as TPP leads to the uptake of an attached, small lipophilic molecule.

B, The SS (Szeto-Schiller) peptides are aromatic-cationic peptides and can scavenge reactive oxygen species (ROS). Moreover, these peptides are localized to the mitochondrial inner membrane, independent of the membrane potential.

C, XJB-5-131 consists of coupling a payload portion, a stable nitroxide radical with electron- and ROS-scavenging activities, and a targeting portion. The targeting portion is composed of Leu-D-Phe-Pro-Val-Orn fragment of gramicidin S, which promotes selective accumulation within mitochondria.

Figure 6 Mitochondrial protein delivery via the protein import machinery.

This figure shows the mitochondrial protein import pathway via the protein import machinery. N-MTS, amino-terminal mitochondrial targeting signal peptide; INT-MTS, internal mitochondrial targeting signal peptide; OM, outer membrane; IM, inner membrane; IMS, intermembrane space; TOM, translocator of the mitochondrial outer membrane; SAM, the sorting and assembly machinery; TIM, translocator of the mitochondrial inner membrane; PAM, presequence translocase-associated motor; MMP, mitochondrial processing peptidase; CH, chaperone proteins.

A, This figure shows the TOM complex as a general import pore and variations on the two main import pathways. Pathway A is used by preproteins with N-MTS destined for the IMS and TIM23 complex, which sorts them to IM or matrix. Pathway B is used by the preproteins with INT-MTS destined for SAM complex, which sorts them to OM and TIM 22 complex, which sorts them to IM via Tim9/10 complex.

B, This figure shows protein sorting via the TIM23 complex. First, preproteins with N-MTS are imported to the TOM complex. Upon translocation through the TOM complex, matrix proteins are sorted via the TIM23 complex. The PAM complex is essential for preprotein transport to the matrix in an ATP-dependent manner. Once the protein is delivered inside the mitochondria, the N-MTS is cleaved by an MMP, and the protein is then refolded into its

mature form. Single-pass inner membrane proteins contain an N-MTS and are inserted into the membrane by one of two protein import pathways: “stop-transfer” and the Oxa1 complex.

C, This figure shows protein insertion via the TIM22 complex. Most polytopic inner membrane proteins with INT-MTS are transferred from the TOM complex to the TIM22 complex via Tim9/10. The TIM22 complex then drives protein insertion into the IM.

Figure 7 Mitochondrial protein import machinery and limitations to mitochondrial delivery.

Mitochondrial targeting signal peptide (MTS) can deliver unfolded proteins (A) and small molecules (C). However, proteins derived from mitochondrial DNA (mtDNA) (B), unfolded proteins and macromolecule (D) cannot be delivered to mitochondria using MTS. Furthermore, MTS cannot deliver cargo to mitochondria when mitochondrial protein import is defective.

Figure 8 Mitochondrial protein delivery using membrane-permeable peptides.

A, A protein conjugated with protein transduction domain peptide (PTD) is delivered to mitochondria without passing through the protein import machinery. This strategy allows delivery of large macromolecules via an unknown mechanism.

B, A TAT fusion protein consisting of mitochondrial targeting signal peptide (MTS) and GFP (TAT-mMDH-GFP) was constructed. In this system, MTS is cleaved specifically in the matrix region, allowing GFP to separate from TAT. As a result, GFP is effectively trapped within the matrix. MPP, mitochondrial processing peptidase.

C, An MTS-Exo III-TAT-fusion protein was constructed by fusion of MTS and TAT with exonuclease III protein at the N- and C-terminus, respectively. In this strategy, PTD functions as a cytoplasmic delivery device and the mitochondrial targeting activity of MTS may compensate for the non-specific protein delivery of PTD.

Figure 9 Map of human mitochondrial DNA.

Human mitochondrial DNA (mtDNA) is 16,569 bp long. It contains 13 polypeptide-encoding genes, 2 ribosomal RNA (12S rRNA and 16S rRNA), and 22 transfer RNAs (tRNAs). The closed circles mark the positions of the tRNAs, which are indicated by the three letters of their cognate amino acids. ND, NADH dehydrogenase coding subunits; CO, cytochrome oxidase coding subunits; ATP, F_1F_0-ATP synthase coding subunits. Point mutations corresponding to the mitochondrial diseases, such as MELAS, CM, MERF, are also indicated (see text for details).

Figure 10 Mitochondrion in normal and diseased cells and therapeutic strategy.

When mitochondria have only one type of mutant or wild-type mitochondrial DNA (mtDNA) is denoted as homoplasmy. In contrast, the situation when mitochondria have both mutant and wild-type mtDNA is denoted as heteroplasmy. When the amount of mutant mtDNA is greater than a certain threshold level, mitochondrial diseases may result. Strategies for mitochondrial gene therapies are proposed to treat these diseases.

Figure 11 Strategy for mitochondrial gene delivery.

A, The attachment of a mitochondrial targeting signal peptide (MTS) to oligodeoxynucleotides (ODN) or peptide nucleic acid (PNA) allows mitochondrial delivery via the protein import machinery.

B, DQAsomes-DNA complexes selectively release DNA, when the carriers gain access to mitochondria. DNAs released proximal to mitochondria might be taken up by mitochondria.

Figure 12 Multifunctional envelope-type nano device (MEND).

The multifunctional envelope-type nano device (MEND) consists of condensed nano particles, coated with a lipid envelope modified with functional devices. Various macromolecules, such as mtDNA and proteins, can be efficiently encapsulated in MEND.

Figure 13 Mitochondrial delivery of mastoparan with transferrin and GALA system.

Transferrin (Tf)-modified liposomes equipped with GALA (Tf-GALA-LP) can be internalized by tumor cells via receptor-mediated endocytosis (1). In the endosomes, GALA enhances the fusion between liposomes and endosomes at lower pH and mastoparan (MP) is released into the cytosol (2). Mastoparan that escapes from the endosomes can attack the mitochondria, inducing permeability transition (PT), and cytochrome c (cyt c) is released from the mitochondria (3). In this figure, intracellular localization of Tf-LP (A) and Tf-GALA-LP (B) is shown. In this experiment, LP-encapsulating sulforhodamine B were incubated with K562 cells and then analyzed using confocal laser scanning microscopy.

Figure 14 MITO-Porter, a novel mitochondrial delivery system based on membrane

fusion.

This figure shows the concept of mitochondrial delivery using MITO-Porter. MITO-Porter is surface-modified with R8 to stimulate entry into cells and facilitate mitochondrial delivery. MITO-Porter can be internalized into cells via macropinocytosis (1). MITO-Porter in the cytosol can bind to mitochondria via R8 (2). Encapsulated compounds are delivered to the intra-mitochondrial compartment via membrane fusion (3). OM, outer membrane; IMS, intermembrane space; IM, inner membrane.

Figure 15 Mitochondrial-binding assay of liposomes with various lipid compositions.

Fluorescence-labeled liposomes were incubated with mitochondria for 30 min at 25ºC. Binding activities (%) of liposomes containing egg yolk phosphatidyl choline (EPC-LP) (A) and liposomes containing 1,2-dioleoyl-sn-glycero-3-phosphatidyl ethanolamine (DOPE-LP) (B) were calculated from the fluorescence intensity of the labeled liposomes bound to mitochondria. The black and white bars represent octaarginine-modified liposomes and unmodified liposomes, respectively. Chol, cholesterol; SM, sphingomyelin; CHEMS, Cholesteryl hemisuccinate (5-cholesten-3-ol 3-hemisuccinate); PA, phosphatidic acid; PS, phosphatidyl serine; PG, phosphatidyl glycerol; PI, phosphatidyl inositol; CL, cardiolipin. Data are means ± S.D. (n = 3-6). ** Significant differences between octaarginine-modified liposomes and unmodified liposomes ($p < 0.01$ by student's t-test).

Figure 16 Effects of modification with R8 and DOPE on membrane fusion with mitochondria.

Fusion activities (%) of a series of liposomes containing sphingomyelin (SM) or phosphatidic acid (PA)—highest mitochondrial fusogenic liposomes—were reevaluated: a, liposomes containing egg yolk phosphatidyl choline (EPC-LP); b, octaarginine modified EPC-LP; c, liposomes containing 1,2-dioleoyl-sn-glycero-3-phosphatidyl ethanolamine (DOPE-LP); d, octaarginine modified DOPE-LP. Data are means ± S.D. (n = 3). * Significant difference between EPC-LP and other liposomes ($p < 0.05$ by one-way ANOVA, followed by Bonferroni correction). ** Significant difference between EPC-LP and other liposomes ($p < 0.01$ by one-way ANOVA, followed by Bonferroni correction).

Figure 17 Localization of GFP-encapsulated MITO-Porter in mitochondria.

A, This figure illustrates the experimental protocol. Green fluorescent protein (GFP) or GFP-encapsulating carriers were incubated with mitochondria for 30 min at 25ºC (1). After incubation, mitochondria were subfractionated into the mitochondrial outer membrane fraction (OM) and the mitochondrial intermembrane space fraction (IMS) by digitonin treatment (2). A total of 2.5 μg of protein from each sample were subjected to Western blot analysis to detect GFP (3).

B, This figure shows the result of Western blotting analysis. Lanes 1, 2, GFP alone; lanes 3, 4, MITO-Porter containing sphingomyelin (SM); lanes 5, 6, MITO-Porter containing phosphatidic acid (PA); lanes 7, 8, octaarginine modified liposomes composed of egg yolk phosphatidyl choline (EPC) and SM; lanes 9, 10, octaarginine modified liposomes composed of EPC and PA. Bands at 33 kDa and 28 kDa represent full-length GFP (lanes 3, 5, 7, 9) and the GFP fragment (lanes 4, 6), respectively. OM (lanes 1, 3, 5, 7, 9) and IMS (lanes 2, 4, 6, 8, 10) indicate the outer membrane and intermembrane space fractions,

respectively.

Figure 18 Evaluation of mitochondrial targeting activity.

This figure shows mitochondrial targeting activity of MITO-Porter. Fluorescence-labeled carriers were added to HeLa cell-homogenate and then the mixture was incubated for 30 min at 25^{o}C. The resulting solution was centrifuged to isolate the mitochondria, and then fluorescence intensities of the carriers bound to mitochondria were measured. Mitochondrial targeting activity (%) was calculated as the percentage of mitochondria-bound liposomes to the total amount applied. Data are means ± S.D. (n = 4). ** Significant differences between MITO-Porter, which is modified octaarginine (R8), and unmodified liposomes ($p < 0.01$ by student's t-test).

Figure 19 MITO-Porter delivers macromolecules to mitochondria in living cells.

A, This figure illustrates the experimental protocol. Carriers encapsulating green fluorescent protein (GFP) were incubated with HeLa cells. Mitochondria were stained with Mito Fluor Red 589 prior to analysis by confocal laser scanning microscopy (CLSM).

B, This figure shows intracellular localization. a, MITO-Porter containing sphingomyelin (SM); b, MITO-Porter containing phosphatidic acid (PA); and c, octaarginine-modified liposomes composed of egg yolk phosphatidyl choline (R8-EPC-LP). The clusters present in the mitochondria and cytosol are indicated by the arrows, denoted as I or II, respectively.

Fig. 1

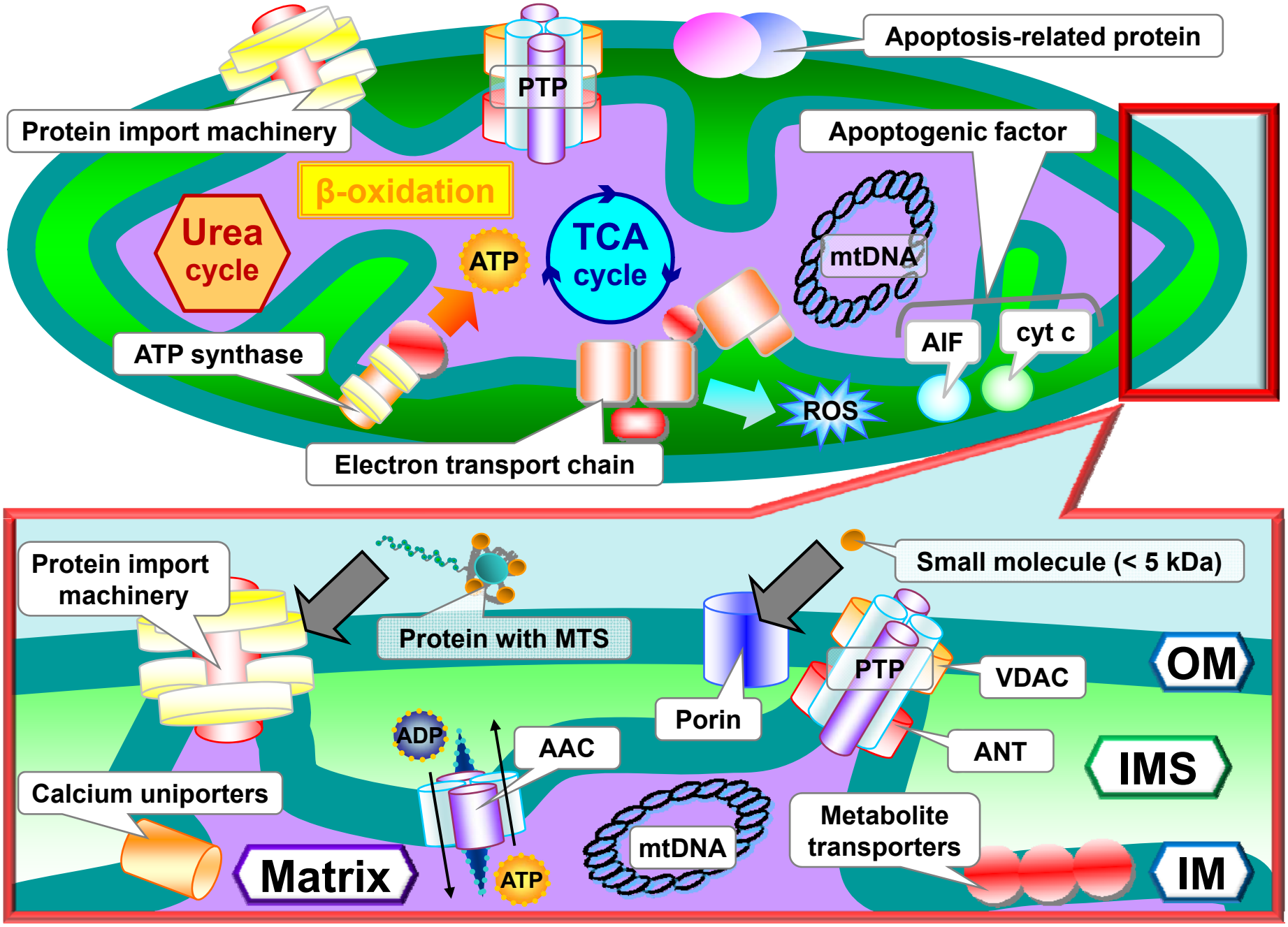

Apoptosis-related protein
PTP
Protein import machinery
Apoptogenic factor
β-oxidation
Urea cycle
TCA cycle
ATP
mtDNA
ATP synthase
AIF
cyt c
ROS
Electron transport chain
Protein import machinery
Protein with MTS
Small molecule (< 5 kDa)
PTP
VDAC
OM
Porin
ADP
AAC
ANT
IMS
Calcium uniporters
Matrix
mtDNA
Metabolite transporters
ATP
IM

Fig. 2

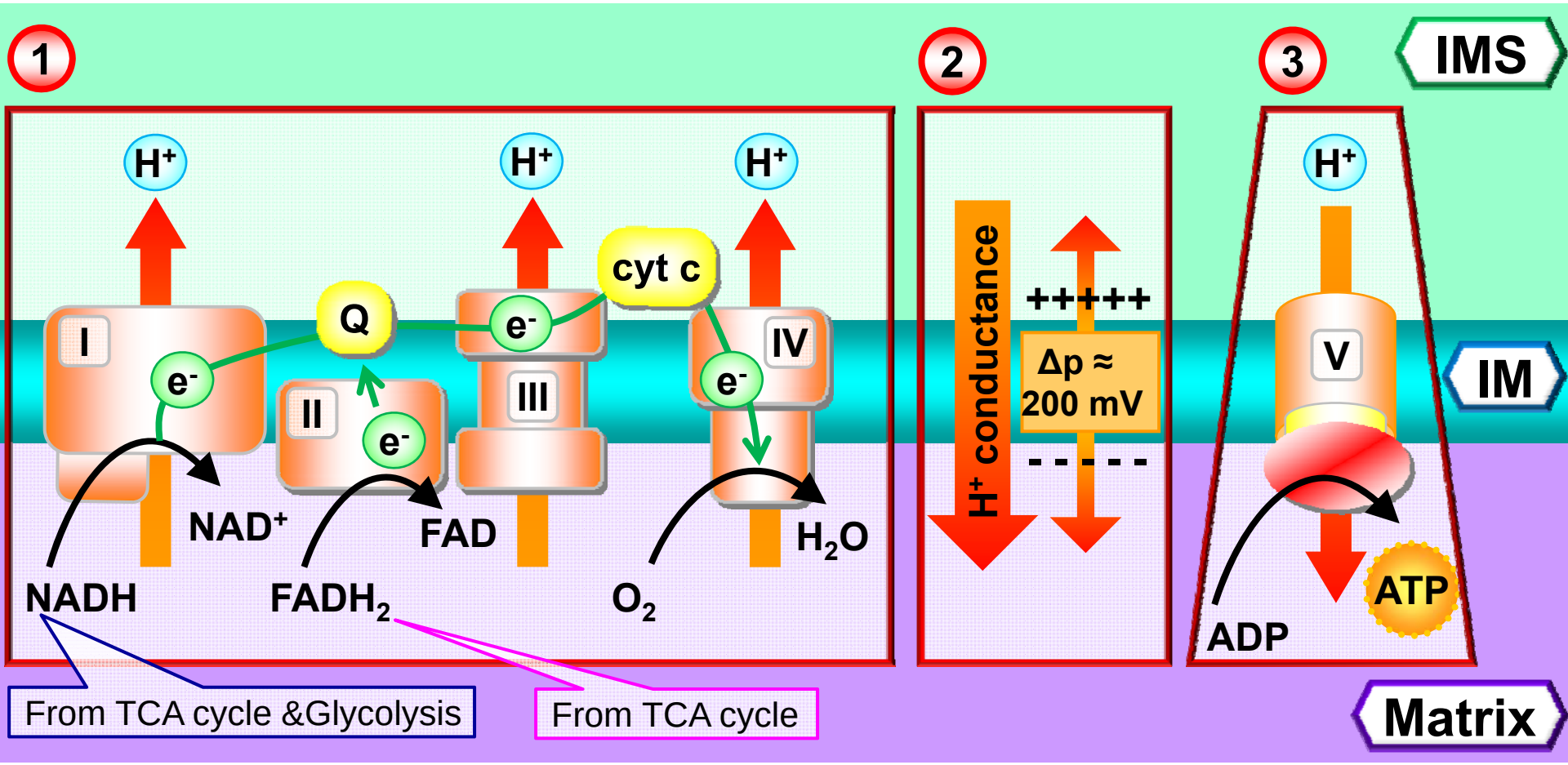

1
2
3
IMS
IM
Matrix
H+
H+
H+
H+
I
II
III
IV
V
Q
cyt c
e-
e-
e-
e-
NAD+
FAD
H2O
NADH
FADH2
O2
H+ conductance
+++++
Δp ≈
200 mV
- - - - -
ADP
ATP
From TCA cycle &Glycolysis
From TCA cycle

Fig. 3

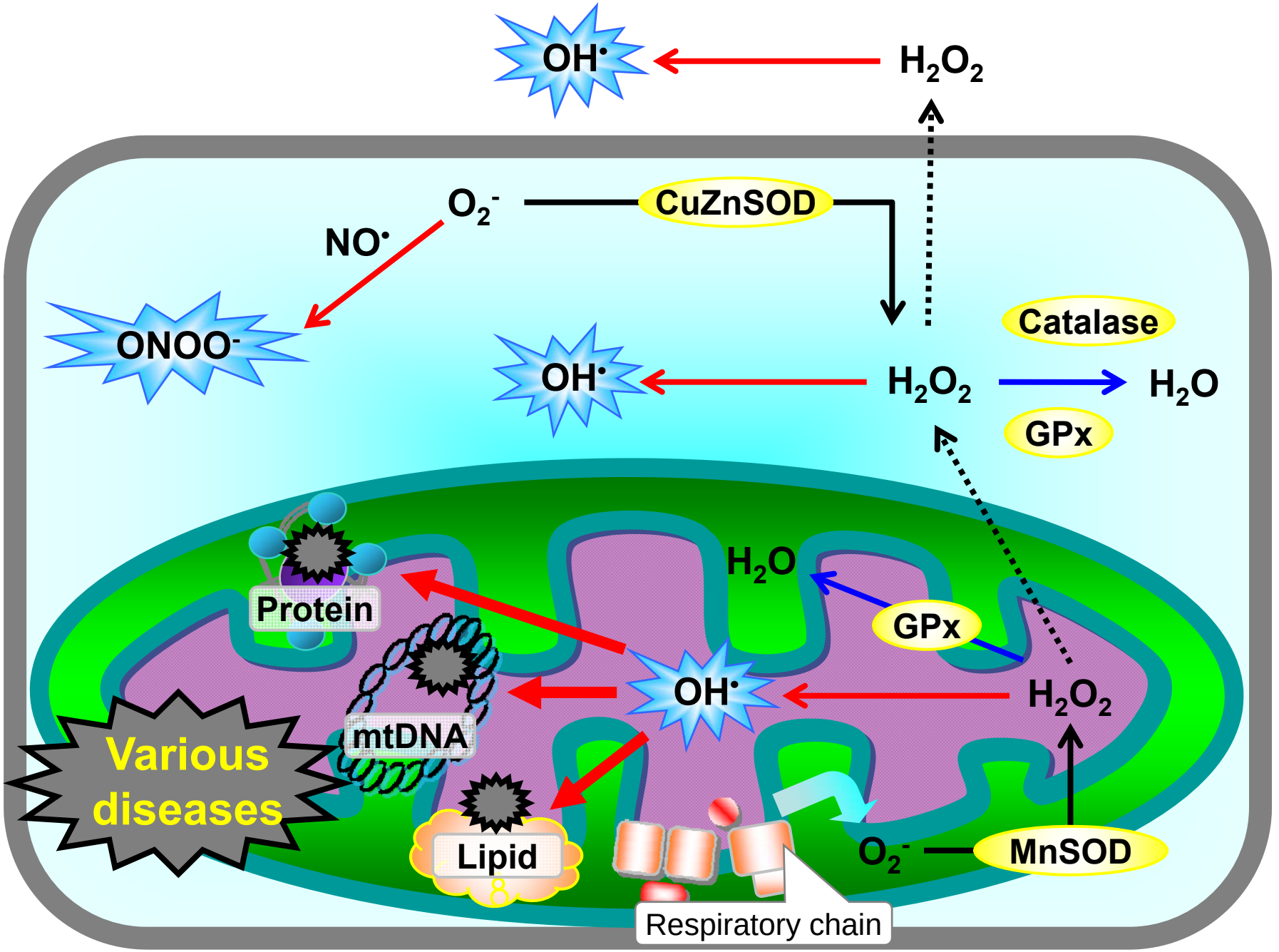

OH·
H2O2
O2-
CuZnSOD
NO·
ONOO-
Catalase
OH·
H2O2
H2O
GPx
Protein
H2O
GPx
OH·
H2O2
mtDNA
Various diseases
Lipid
O2-
MnSOD
Respiratory chain

Fig. 4

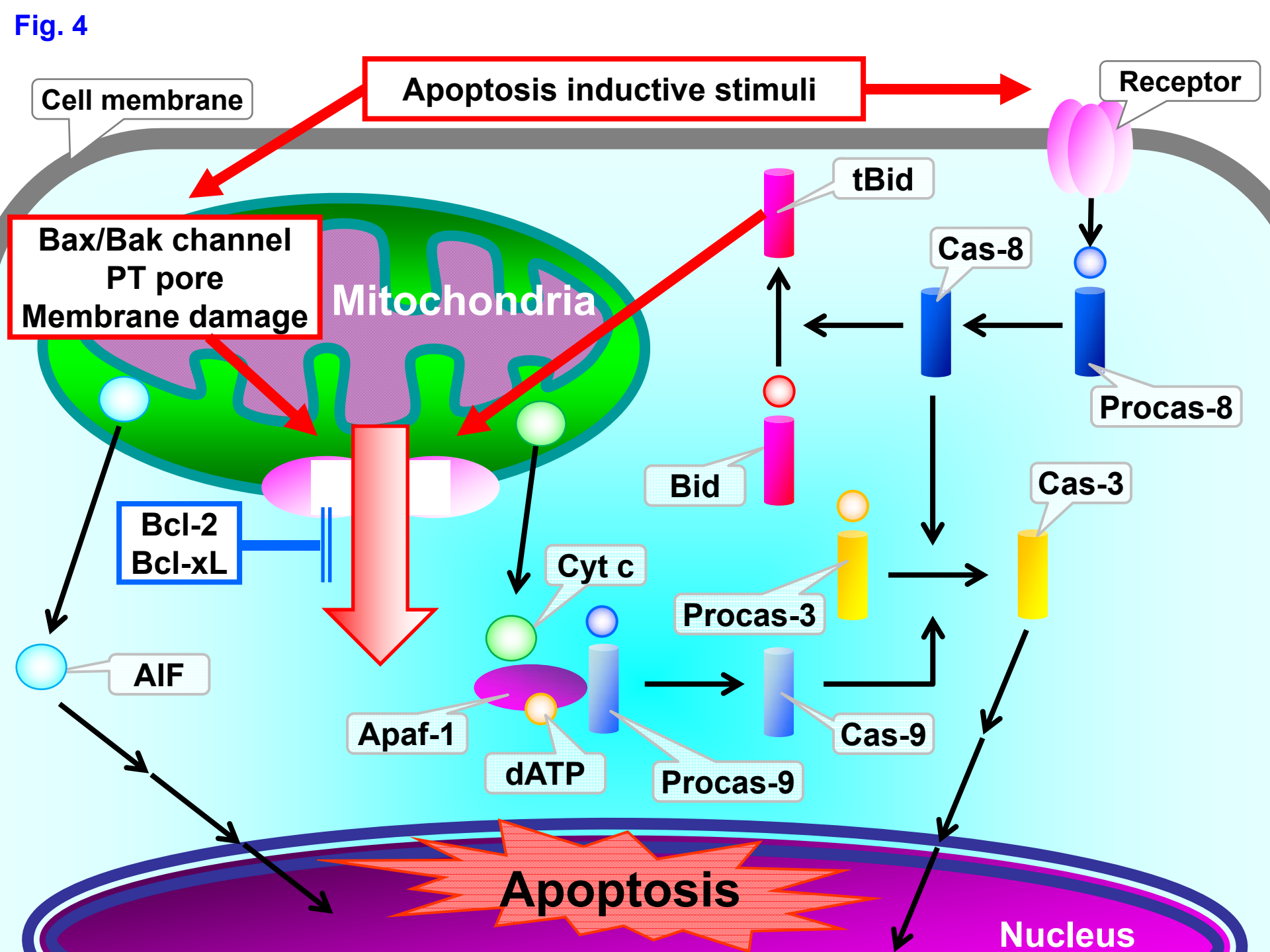

Cell membrane
Apoptosis inductive stimuli
Receptor
Bax/Bak channel
PT pore
Membrane damage
Mitochondria
tBid
Cas-8
Procas-8
Bid
Bcl-2
Bcl-xL
Cyt c
Cas-3
Procas-3
AIF
Apaf-1
dATP
Procas-9
Cas-9
Apoptosis
Nucleus

Fig. 5

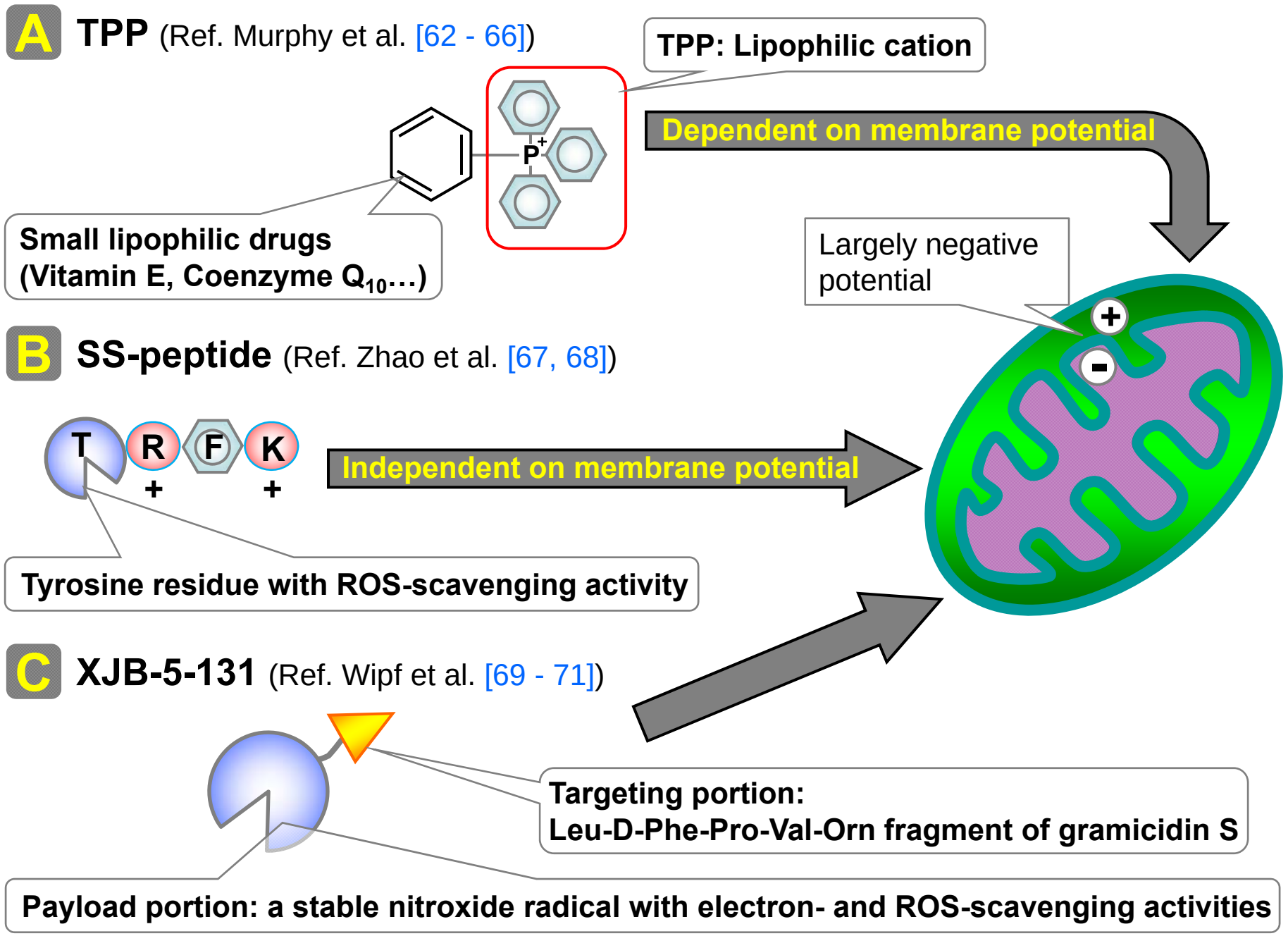

A TPP (Ref. Murphy et al. [62 - 66])
TPP: Lipophilic cation
Dependent on membrane potential
Small lipophilic drugs
(Vitamin E, Coenzyme Q10…)
Largely negative
potential
B SS-peptide (Ref. Zhao et al. [67, 68])
T R F K
+ +
Independent on membrane potential
Tyrosine residue with ROS-scavenging activity
C XJB-5-131 (Ref. Wipf et al. [69 - 71])
Targeting portion:
Leu-D-Phe-Pro-Val-Orn fragment of gramicidin S
Payload portion: a stable nitroxide radical with electron- and ROS-scavenging activities

Fig. 6A

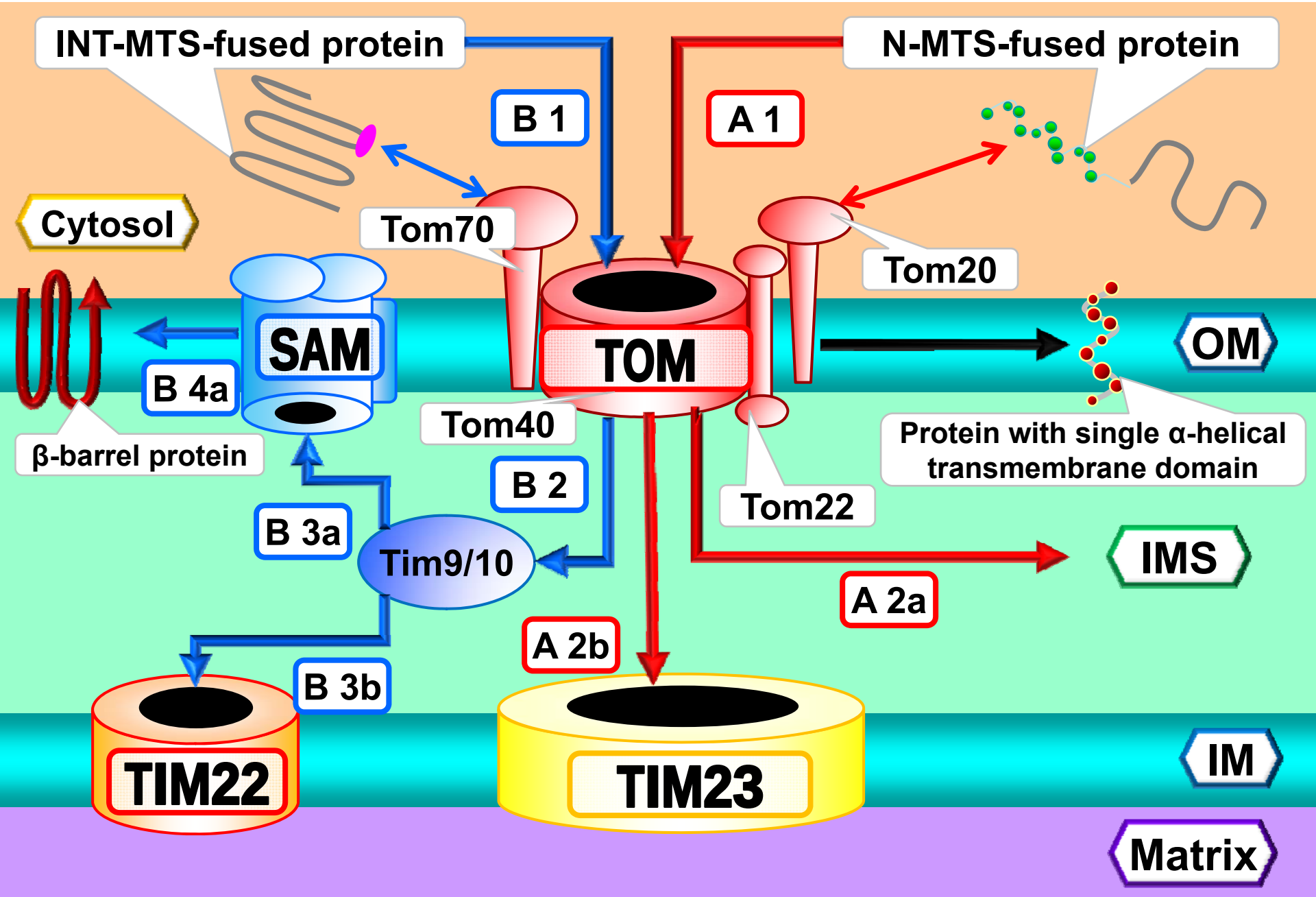

INT-MTS-fused protein
N-MTS-fused protein
B 1
A 1
Cytosol
Tom70
Tom20
SAM
TOM
OM
B 4a
β-barrel protein
Tom40
Protein with single α-helical transmembrane domain
B 2
Tom22
B 3a
Tim9/10
IMS
A 2a
A 2b
B 3b
TIM22
TIM23
IM
Matrix

Fig. 6B

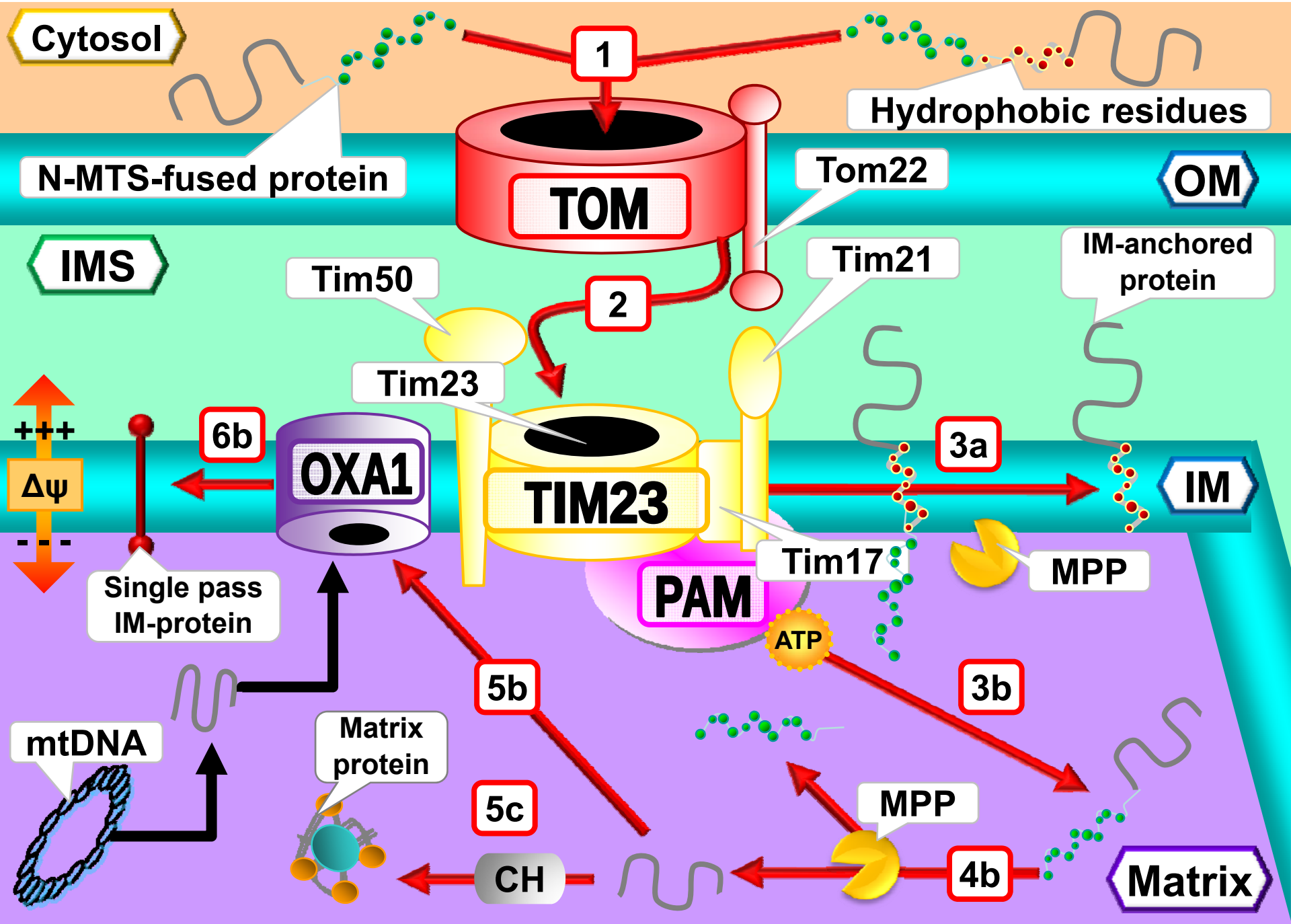

Cytosol
1
Hydrophobic residues
N-MTS-fused protein
TOM
Tom22
OM
IMS
Tim50
2
Tim21
IM-anchored protein
Tim23
+++
Δψ
- - -
6b
OXA1
TIM23
3a
IM
Single pass IM-protein
Tim17
MPP
PAM
ATP
5b
3b
mtDNA
Matrix protein
5c
MPP
CH
4b
Matrix

Fig. 6C

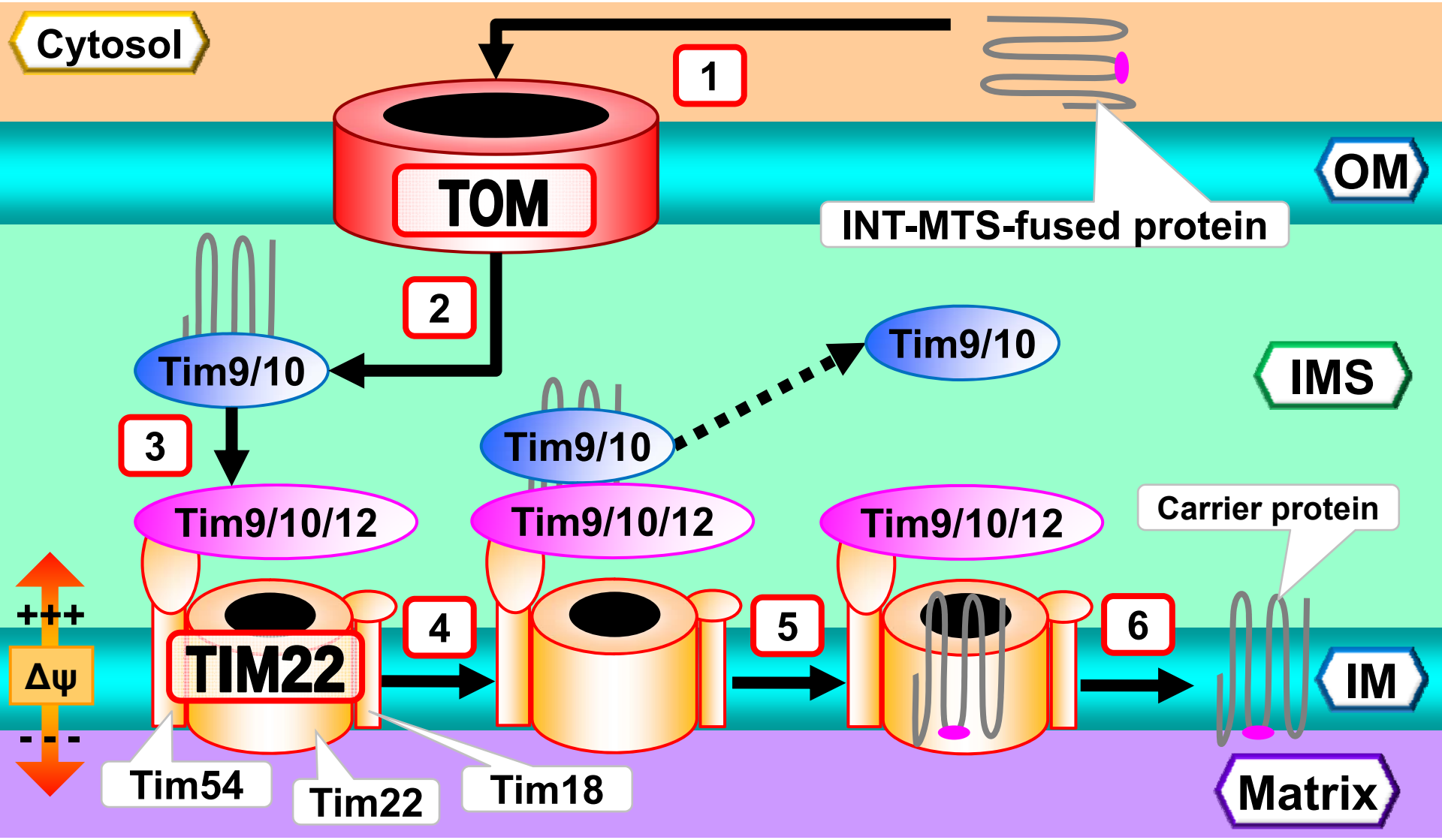

Cytosol
1
TOM
OM
INT-MTS-fused protein
2
Tim9/10
Tim9/10
IMS
3
Tim9/10
Tim9/10/12
Tim9/10/12
Tim9/10/12
Carrier protein
+++
Δψ
- - -
TIM22
4
5
6
IM
Tim54
Tim22
Tim18
Matrix

Fig. 7

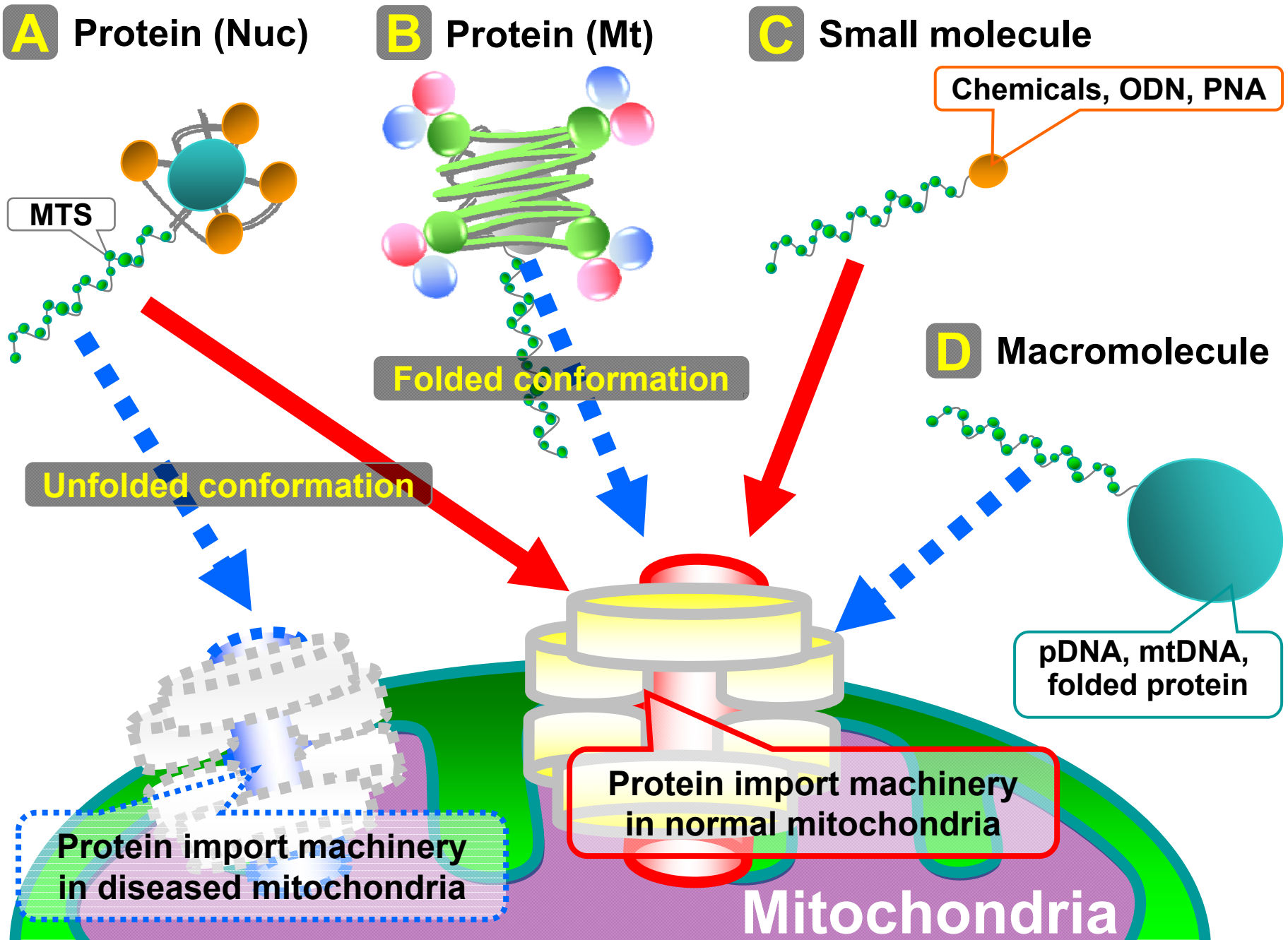

A Protein (Nuc)
B Protein (Mt)
C Small molecule
Chemicals, ODN, PNA
MTS
Folded conformation
D Macromolecule
Unfolded conformation
pDNA, mtDNA,
folded protein
Protein import machinery
in normal mitochondria
Protein import machinery
in diseased mitochondria
Mitochondria

Fig. 8

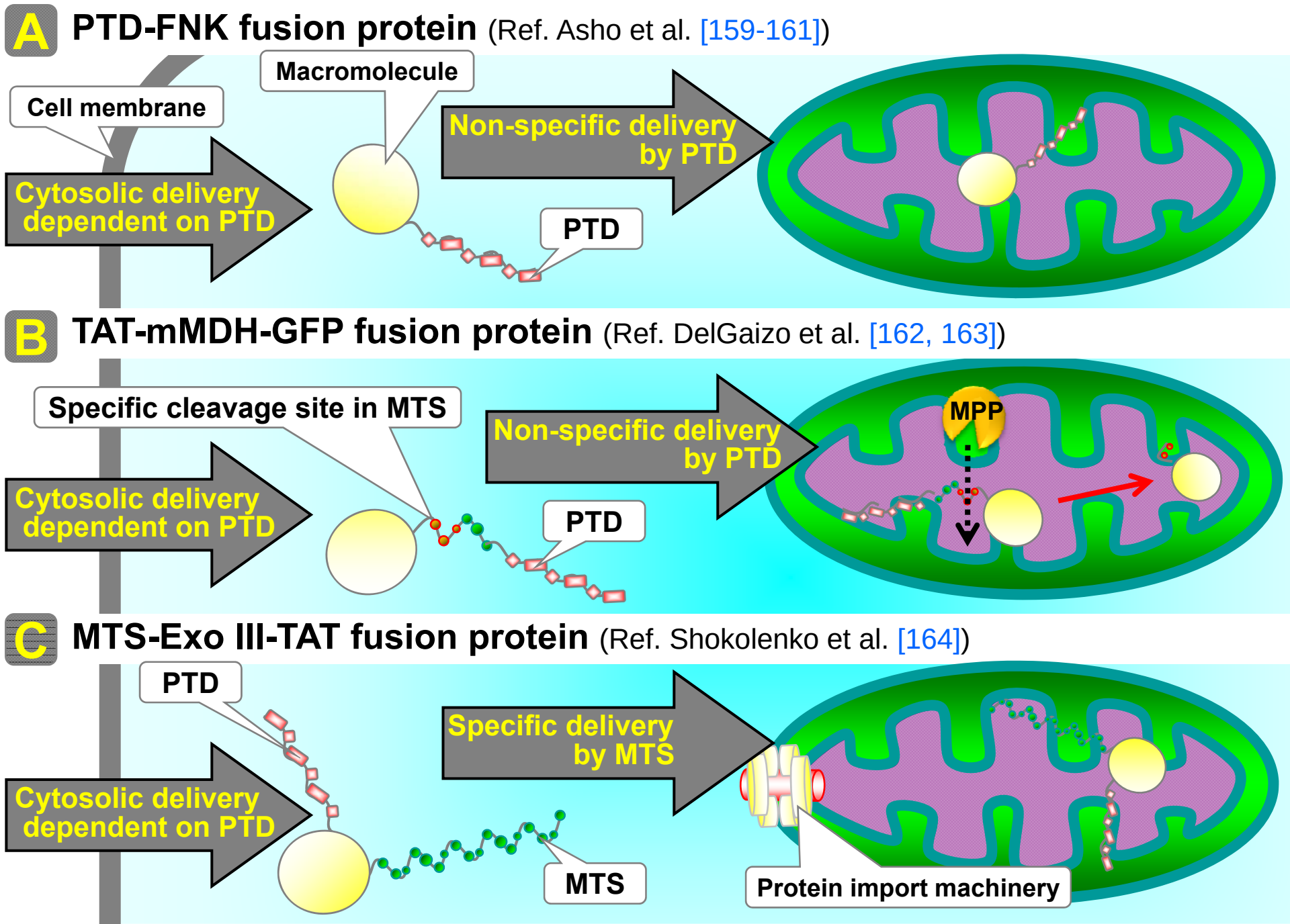

A
PTD-FNK fusion protein (Ref. Asho et al. [159-161])
Macromolecule
Cell membrane
Non-specific delivery by PTD
Cytosolic delivery dependent on PTD
PTD
B
TAT-mMDH-GFP fusion protein (Ref. DelGaizo et al. [162, 163])
Specific cleavage site in MTS
Non-specific delivery by PTD
MPP
Cytosolic delivery dependent on PTD
PTD
C
MTS-Exo III-TAT fusion protein (Ref. Shokolenko et al. [164])
PTD
Specific delivery by MTS
Cytosolic delivery dependent on PTD
MTS
Protein import machinery

Fig. 9

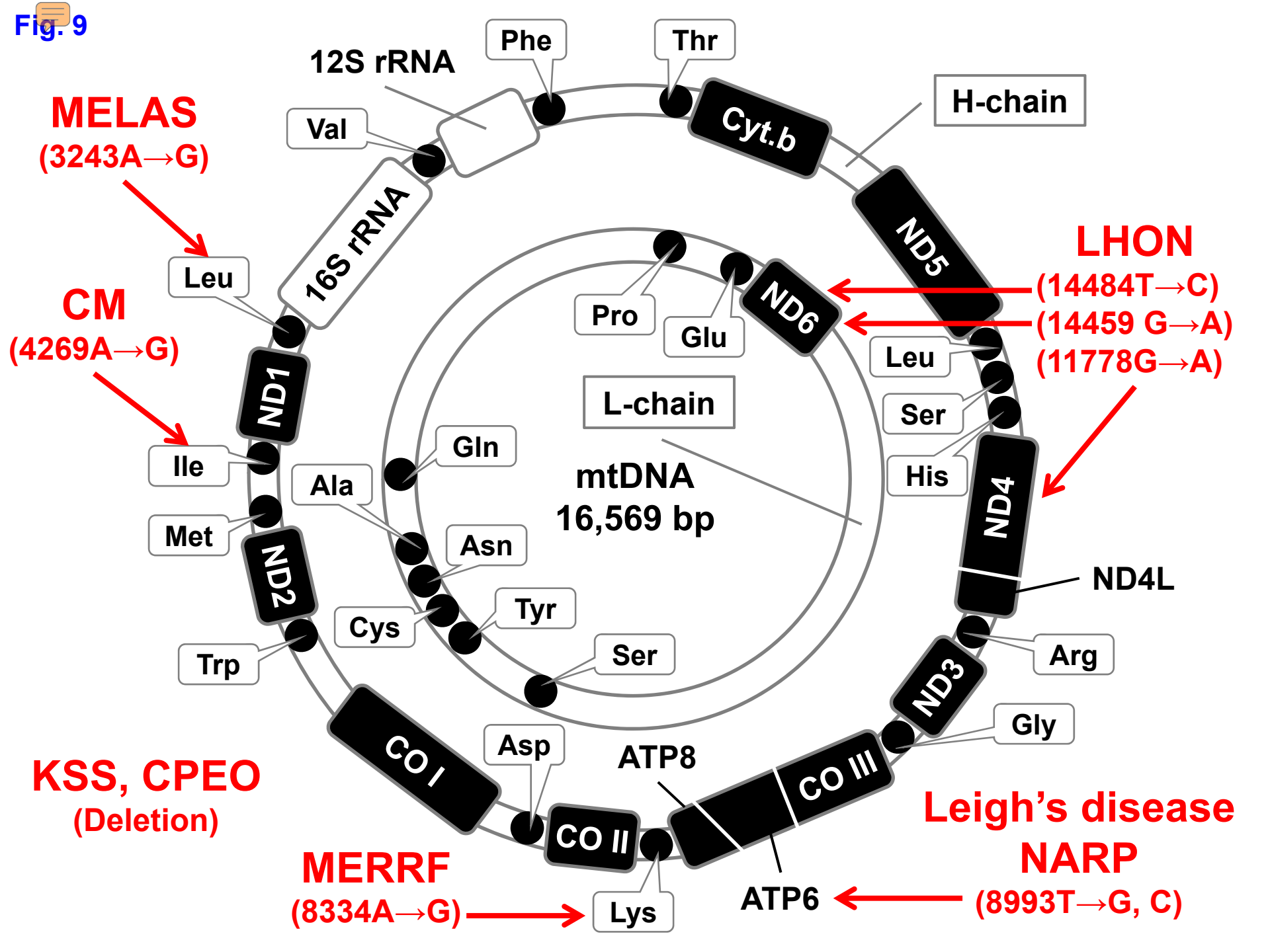

12S rRNA
Phe
Thr
Cyt.b
H-chain
MELAS
(3243A→G)
Val
16S rRNA
ND5
LHON
(14484T→C)
(14459 G→A)
(11778G→A)
Leu
CM
(4269A→G)
ND6
Pro
Glu
Leu
Ser
His
ND1
L-chain
Gln
Ala
mtDNA
16,569 bp
ND4
Ile
Met
ND2
Asn
ND4L
Tyr
Cys
Trp
Ser
Arg
ND3
Gly
CO I
Asp
ATP8
CO III
KSS, CPEO
(Deletion)
CO II
Leigh's disease
NARP
MERRF
(8334A→G)
Lys
ATP6
(8993T→G, C)

Fig. 10

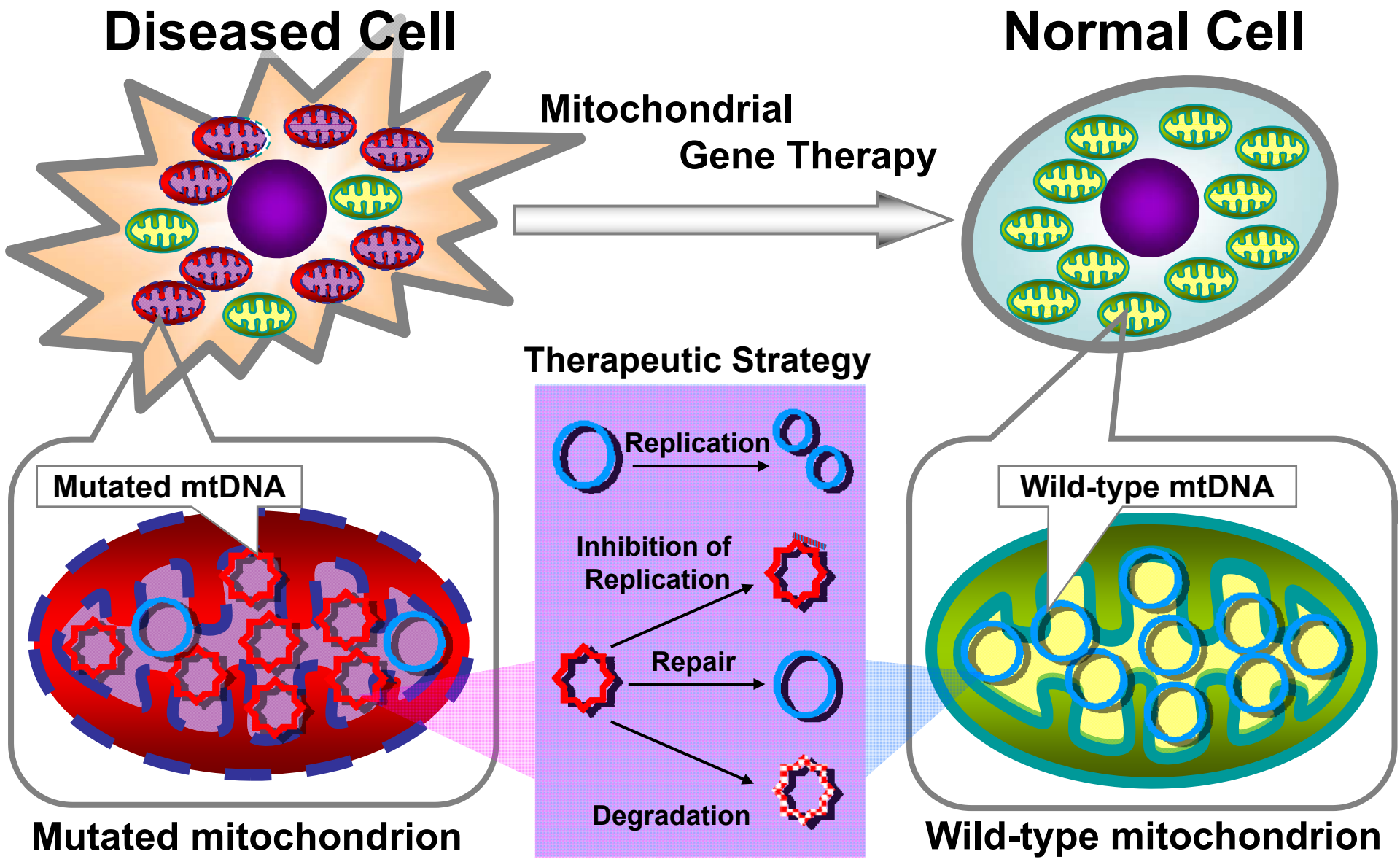

Diseased Cell
Normal Cell
Mitochondrial
Gene Therapy
Therapeutic Strategy
Mutated mtDNA
Wild-type mtDNA
Replication
Inhibition of
Replication
Repair
Degradation
Mutated mitochondrion
Wild-type mitochondrion

Fig. 11

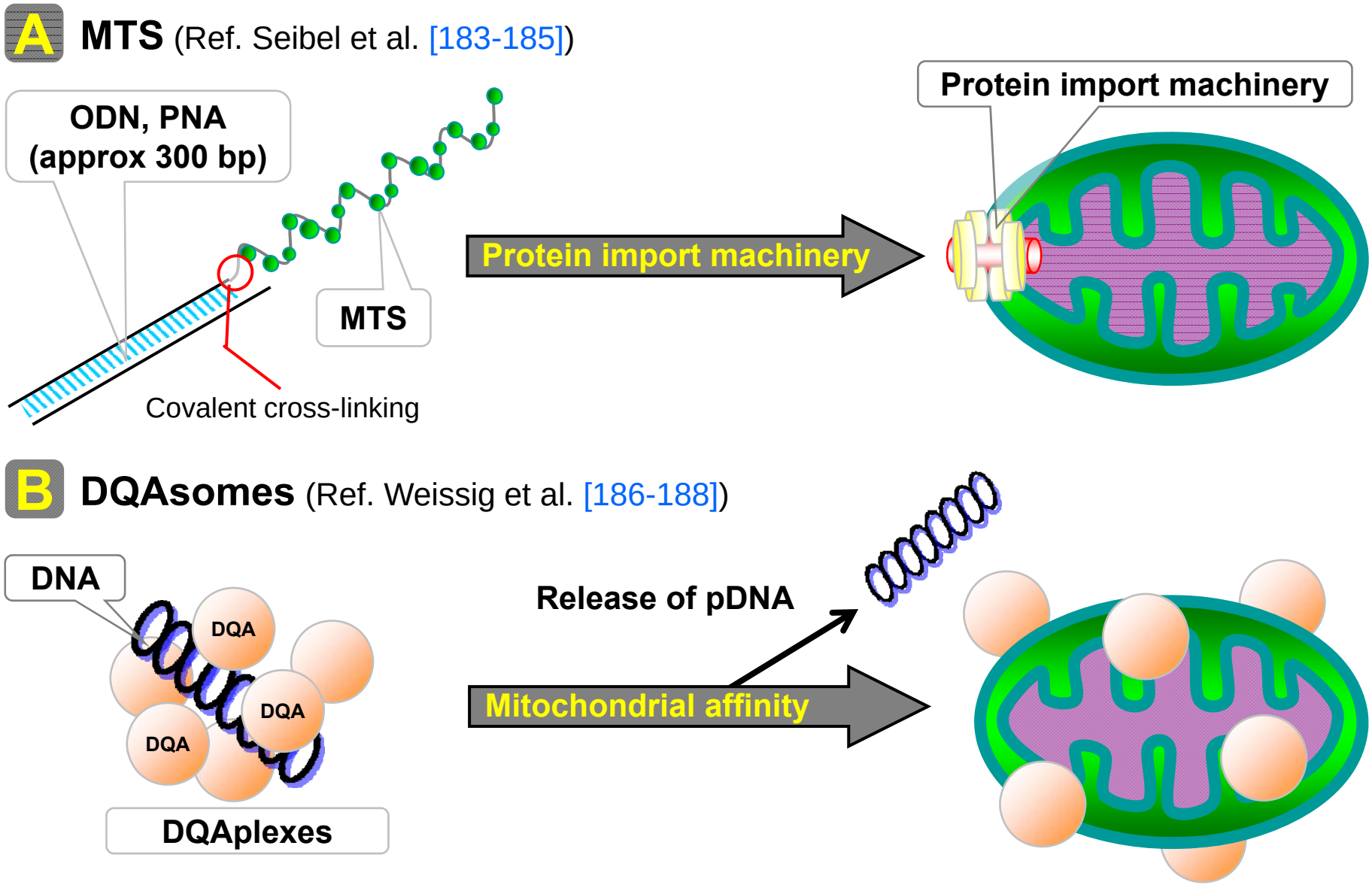

A
MTS (Ref. Seibel et al. [183-185])
ODN, PNA
(approx 300 bp)
MTS
Covalent cross-linking
Protein import machinery
Protein import machinery
B
DQAsomes (Ref. Weissig et al. [186-188])
DNA
DQA
DQA
DQA
DQAplexes
Release of pDNA
Mitochondrial affinity

Fig. 12

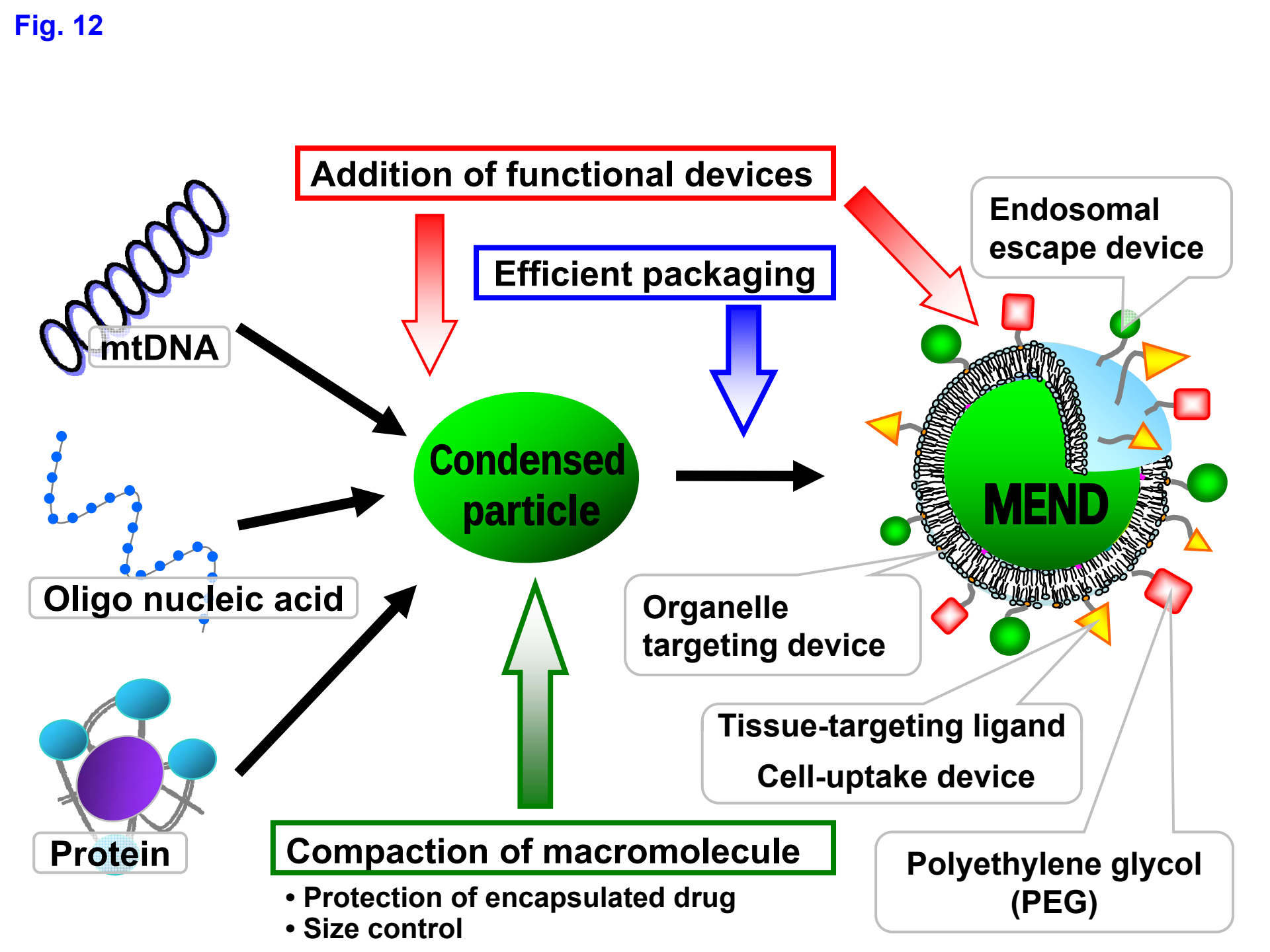

Addition of functional devices
Efficient packaging
Endosomal escape device
mtDNA
Condensed particle
MEND
Oligo nucleic acid
Organelle targeting device
Tissue-targeting ligand
Cell-uptake device
Protein
Compaction of macromolecule
• Protection of encapsulated drug
• Size control
Polyethylene glycol (PEG)

Fig. 13

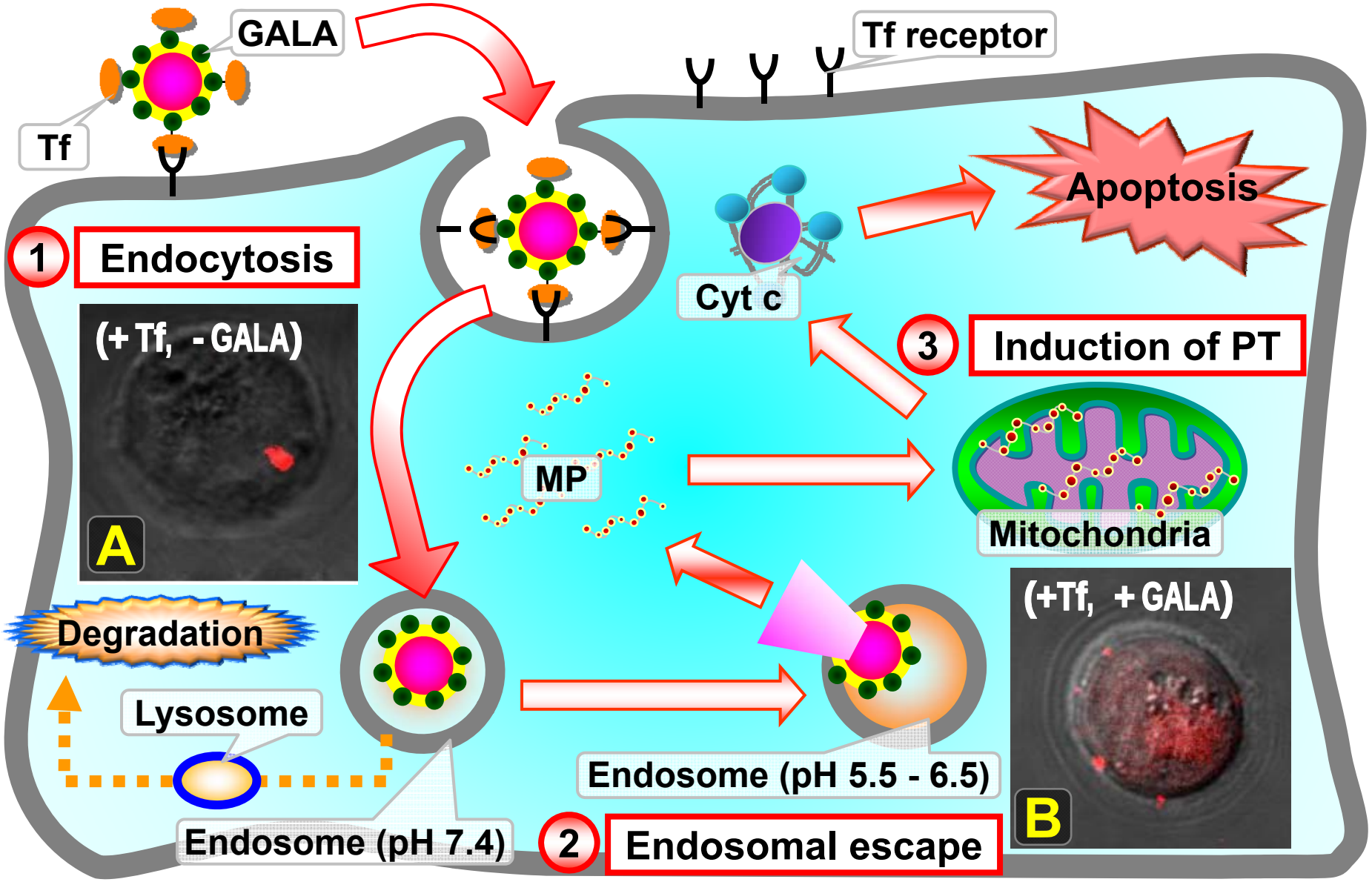

GALA
Tf
Tf receptor
1 Endocytosis
(+Tf, -GALA)
A
Degradation
Lysosome
Endosome (pH 7.4)
Cyt c
Apoptosis
3 Induction of PT
MP
Mitochondria
(+Tf, +GALA)
B
Endosome (pH 5.5 - 6.5)
2 Endosomal escape

Fig. 14

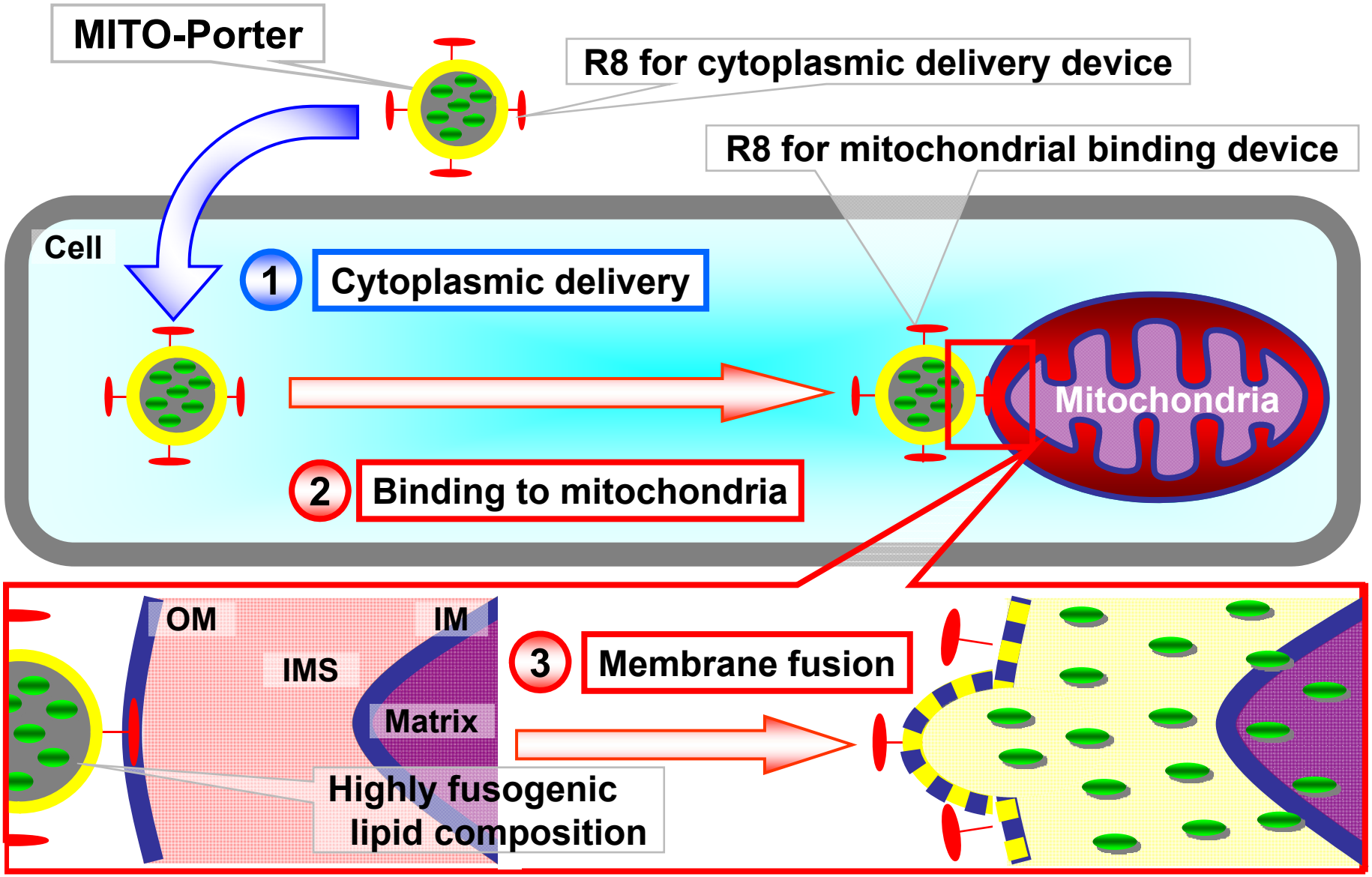

MITO-Porter
R8 for cytoplasmic delivery device
R8 for mitochondrial binding device
Cell
1
Cytoplasmic delivery
Mitochondria
2
Binding to mitochondria
OM
IM
IMS
Matrix
3
Membrane fusion
Highly fusogenic
lipid composition

Fig. 15

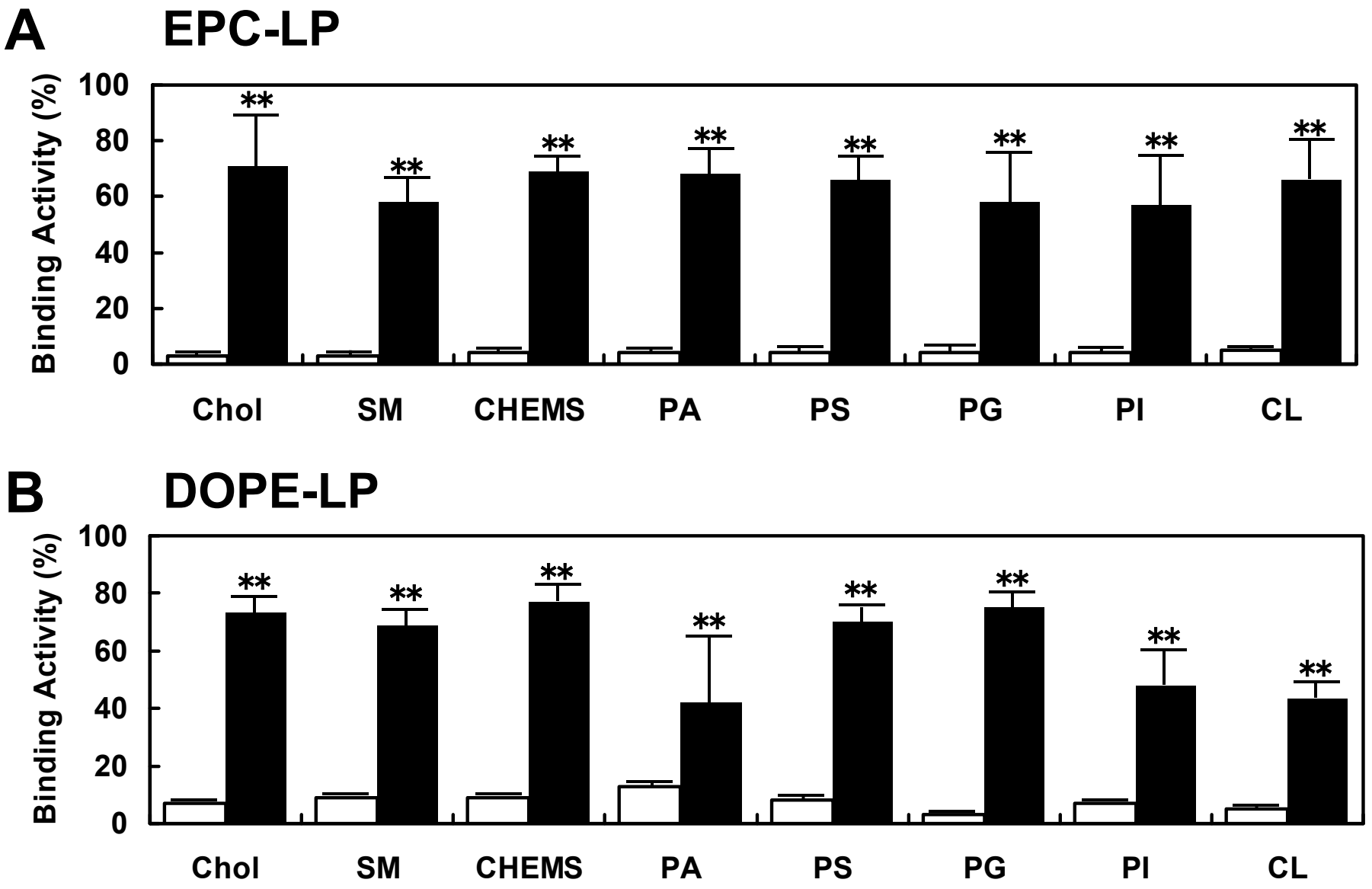
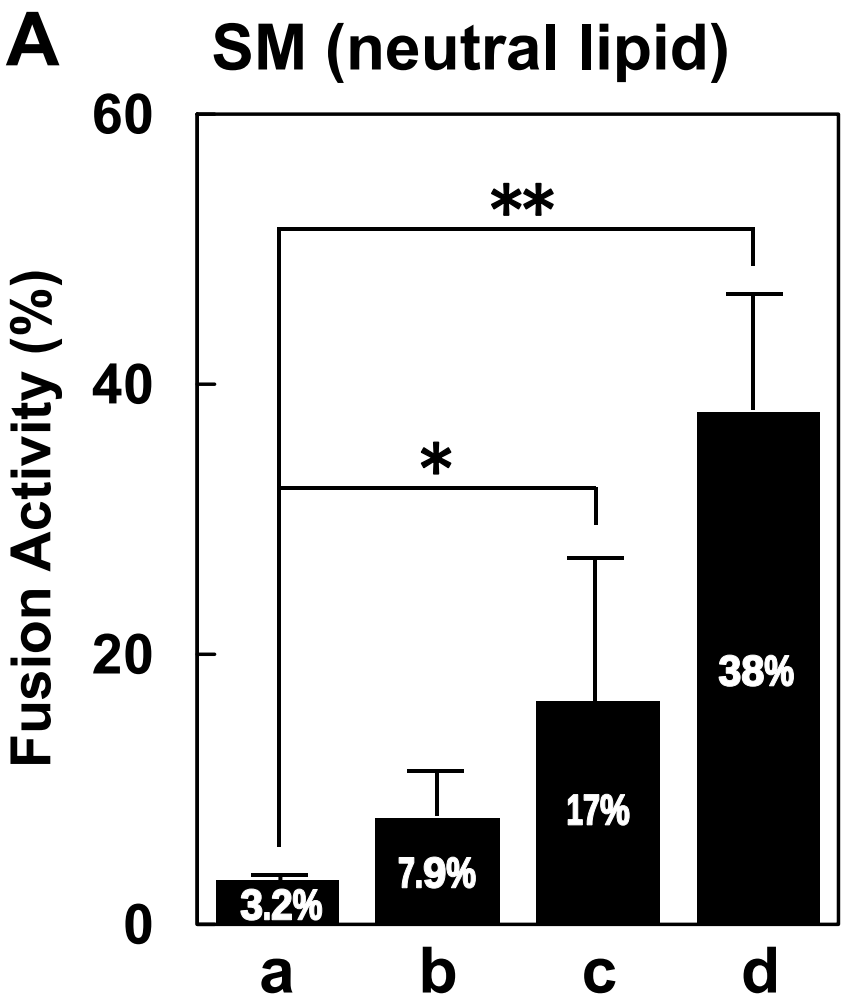
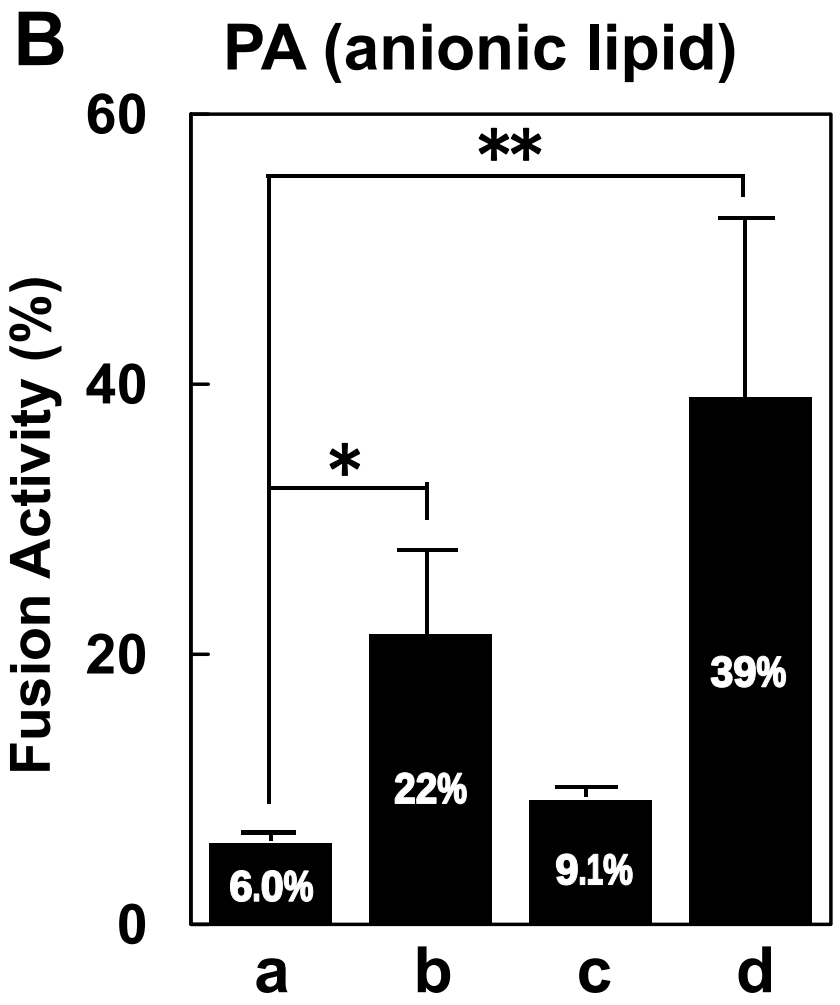

A
EPC-LP
Binding Activity (%)
100
80
60
40
20
0
**
Chol
SM
CHEMS
PA
PS
PG
PI
CL
B
DOPE-LP
Binding Activity (%)
100
80
60
40
20
0
**
Chol
SM
CHEMS
PA
PS
PG
PI
CL

Fig. 16



EPC	+	+	-	-
DOPE	-	-	+	+
R8	-	+	-	+



EPC	+	+	-	-
DOPE	-	-	+	+
R8	-	+	-	+

Fig. 17

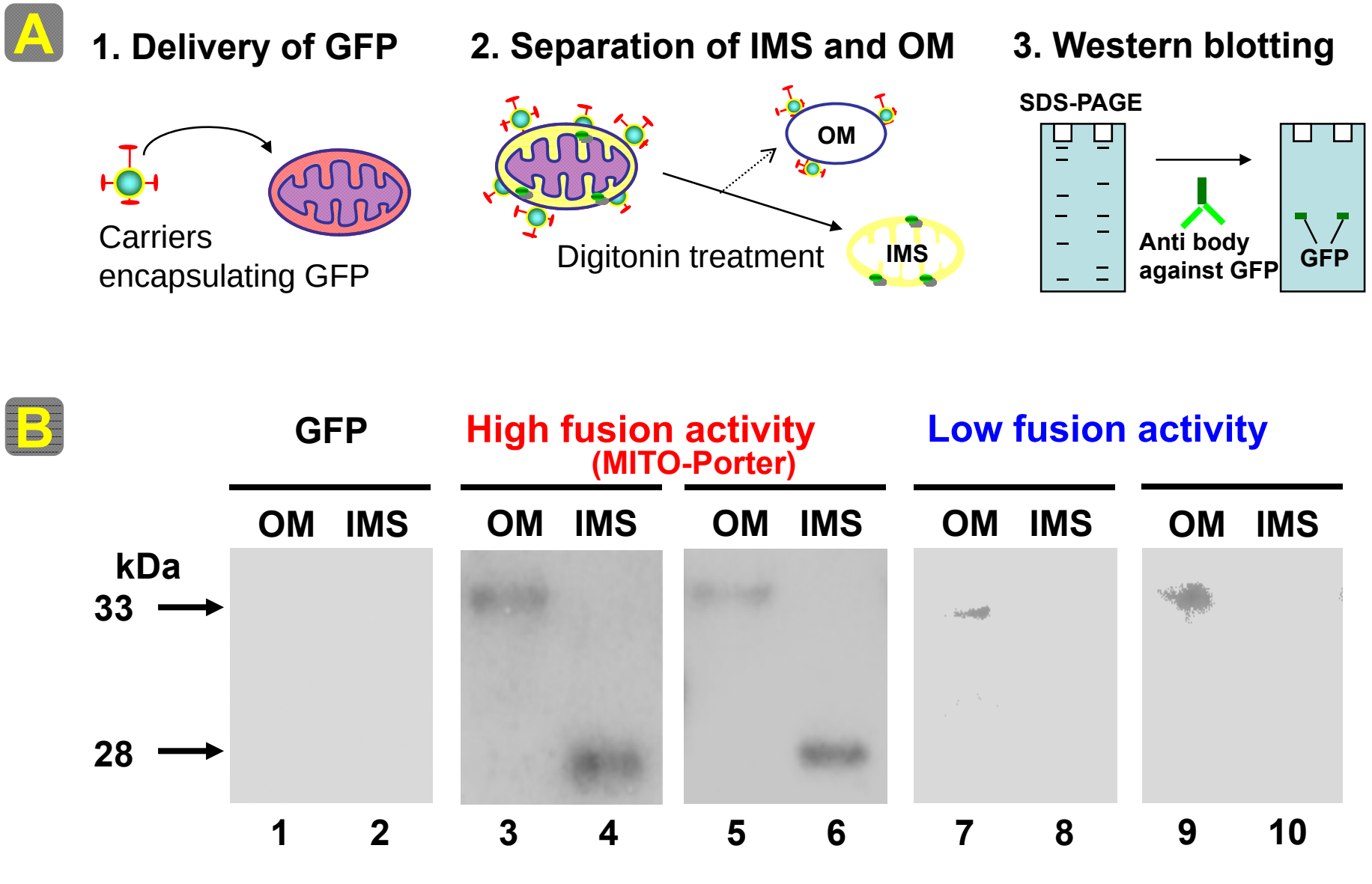

A
1. Delivery of GFP
Carriers
encapsulating GFP
2. Separation of IMS and OM
OM
Digitonin treatment
IMS
3. Western blotting
SDS-PAGE
Anti body
against GFP
GFP
B
GFP
High fusion activity
(MITO-Porter)
Low fusion activity
OM IMS
OM IMS
OM IMS
OM IMS
OM IMS
kDa
33
28
1 2 3 4 5 6 7 8 9 10

Fig. 18

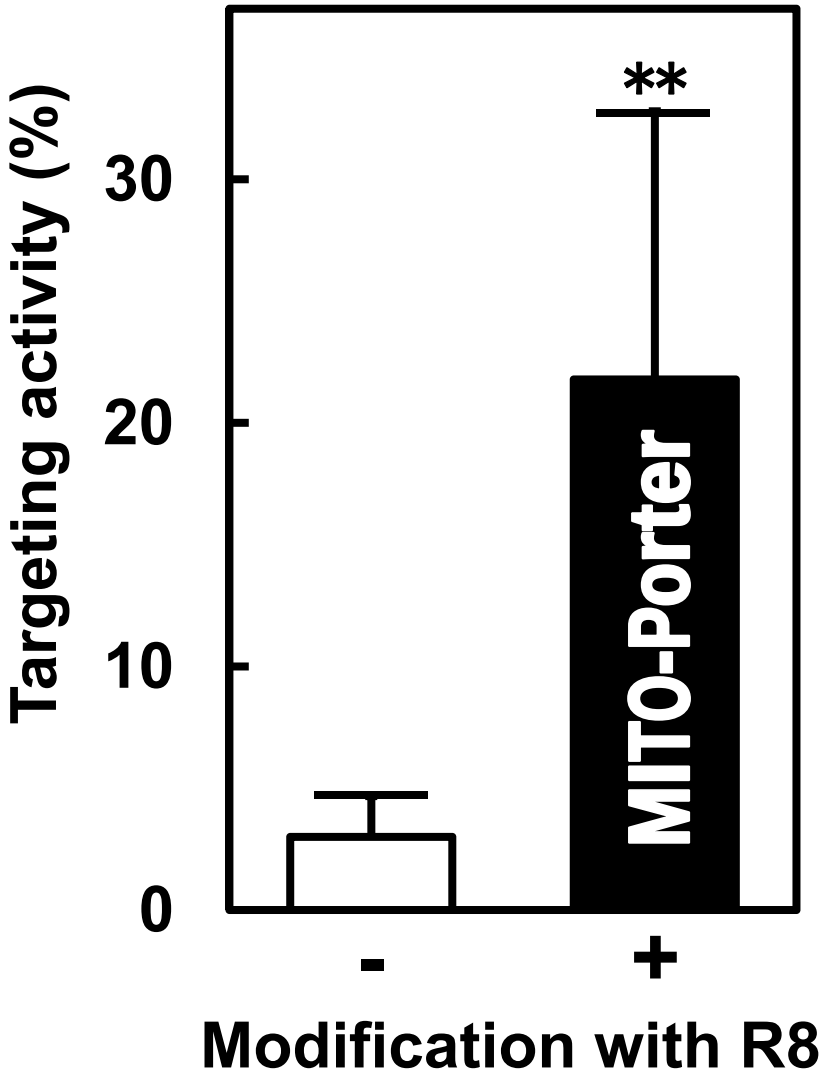
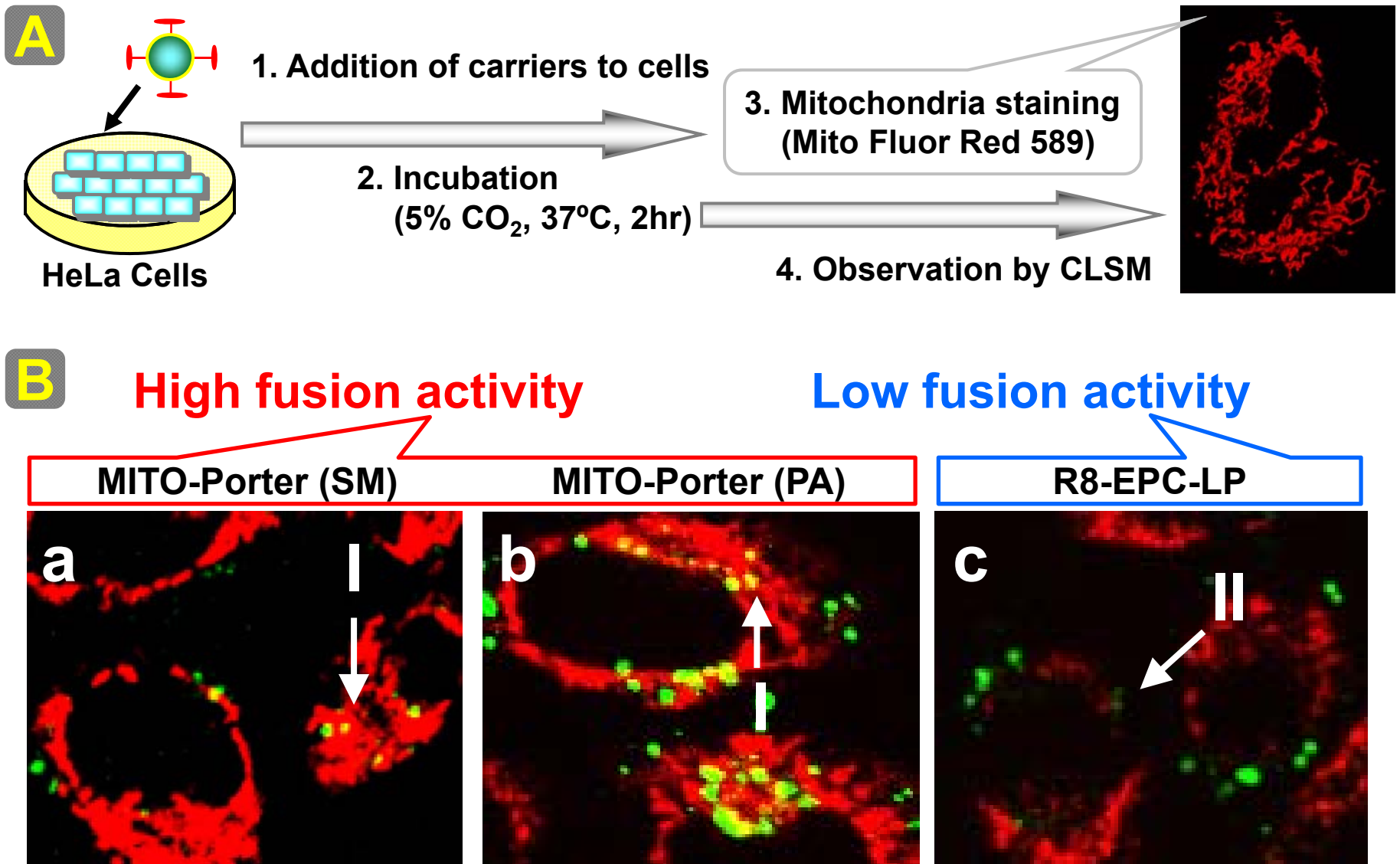

Targeting activity (%)
30
20
10
0
**
MITO-Porter
-
+
Modification with R8

Fig. 19


A
1. Addition of carriers to cells
2. Incubation
(5% CO_2, 37ºC, 2hr)
3. Mitochondria staining
(Mito Fluor Red 589)
4. Observation by CLSM
HeLa Cells
B
High fusion activity
Low fusion activity
MITO-Porter (SM)
MITO-Porter (PA)
R8-EPC-LP
a
b
c
I
I
II