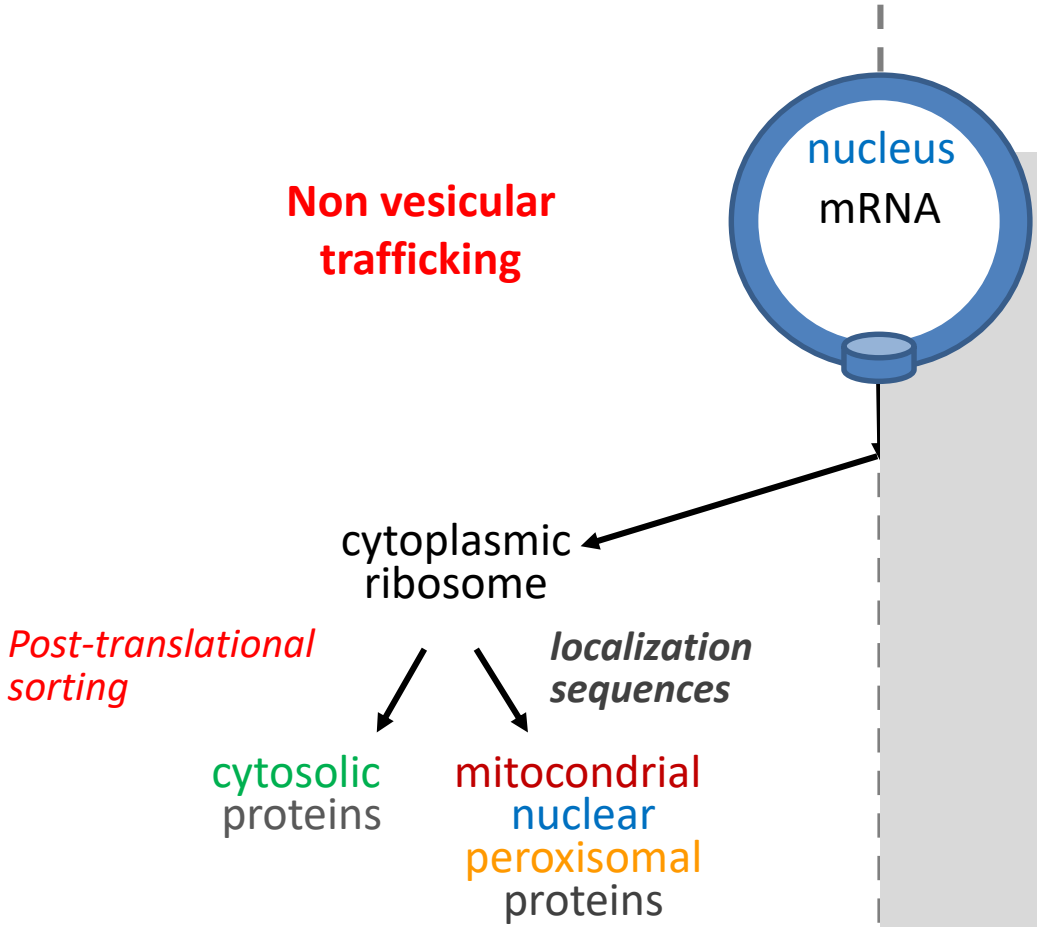
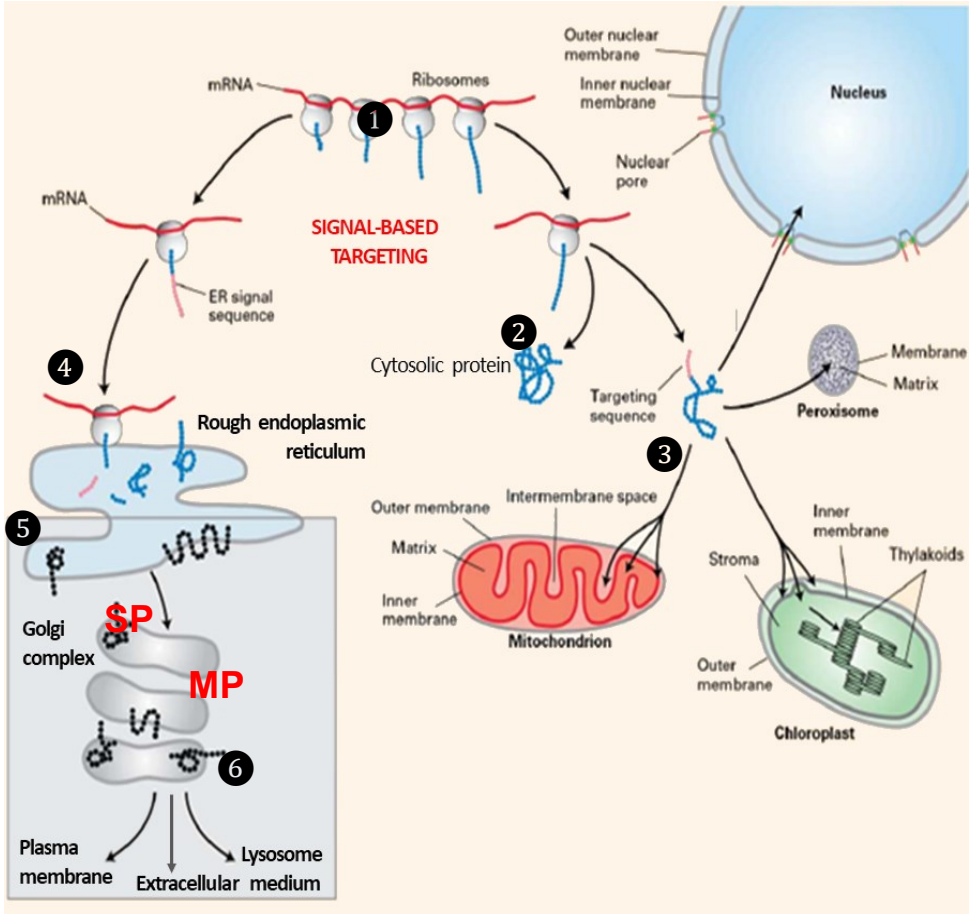
Modulo A4-3

NON VESICULAR SORTING PATWAYS



Major protein-sorting pathways in eukaryotic cells



- In the previous part of Module 4 we examined vesicular traffic through the ER and Golgi apparatus to the cytoplasmic membrane, endosomes and lysosome (❹❺)
- Some proteins synthesized on **cytosolic ribosomes** ❷ are sorted to **mitochondria/ chloroplasts,** the **nucleus,** and **peroxisomes**.
- This is a **post-translational event** and requires appropriate **targeting sequences**

VESCICULAR vs NON-VESICULAR TARGETING

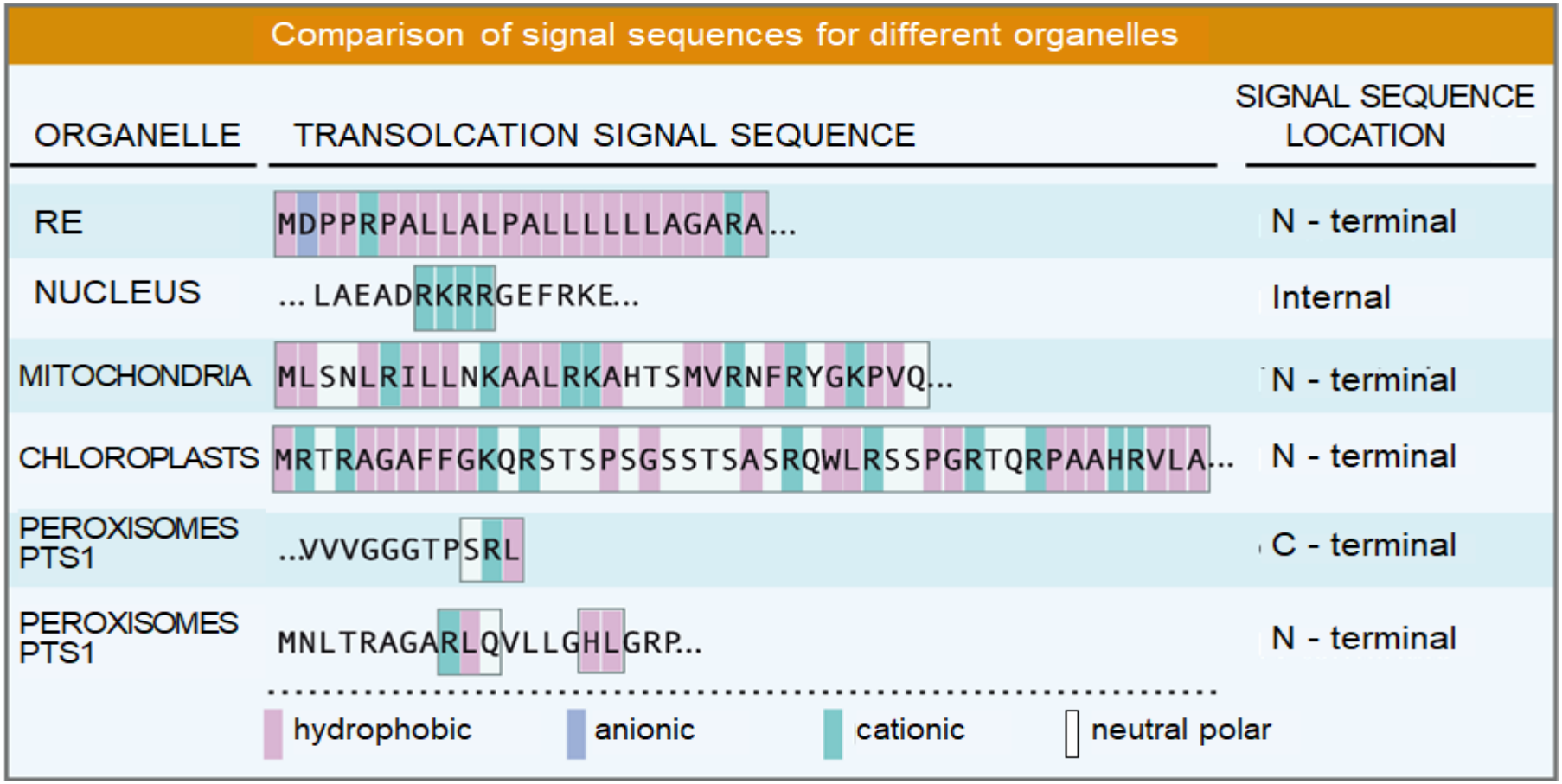
Uptake-Targeting Sequences That Direct Proteins from the Cytosol to Organelles*

	TARGET ORGANELLE	LOCATION OF SEQUENCE WITHIN PROTEIN	SEQUENCE REMOVAL	NATURE OF SEQUENCE
V	Endoplasmic reticulum (lumen)	N-terminus	Yes	Core of 6–12 hydrophobic amino acids, often preceded by one or more basic amino acids (Arg, Lys)
NV	Mitochondrion (matrix)	N-terminus	Yes	Amphipathic helix, 20–50 residues in length, with Arg and Lys residues on one side and hydrophobic residues on the other
NV	Chloroplast (stroma)	N-terminus	Yes	No common motifs; generally rich in Ser, Thr, and small hydrophobic residues and poor in Glu and Asp
NV	Peroxisome (matrix)	C-terminus (most proteins); N-terminus (few proteins)	No	PTS1 signal (Ser-Lys-Leu) at extreme C-terminus; PTS2 signal at N-terminus
NV	Nucleus (nucleoplasma)	Varies	No	Multiple different kinds; a common motif includes a short segment rich in Lys and Arg residues
NV	Organelle membranes & Subcompartments	Varies	No	Different or additional sequences

TARGETING IS MEDIATED BY SPECIFIC SEQUENCES

- **N-** or **C-terminal extensions** or **internal sequences** of proteins determine cellular localization

- **Signal Peptides** (**SP**): target to the RER
- **Transit Peptides** (**TP**): target endosymbiotic organelles (chloroplasts and mitochondria)
- **Peroxisomal Targeting Signals** (**PTS1 & 2**): target peroxisomes
- **Nuclear Localization Signal** sequence (**NLS**): target the nucleus



NON VESICULAR SORTING TO MITOCHONDRIA

- Endosymbiont hypothesis for the evolutionary origin of mitochondria

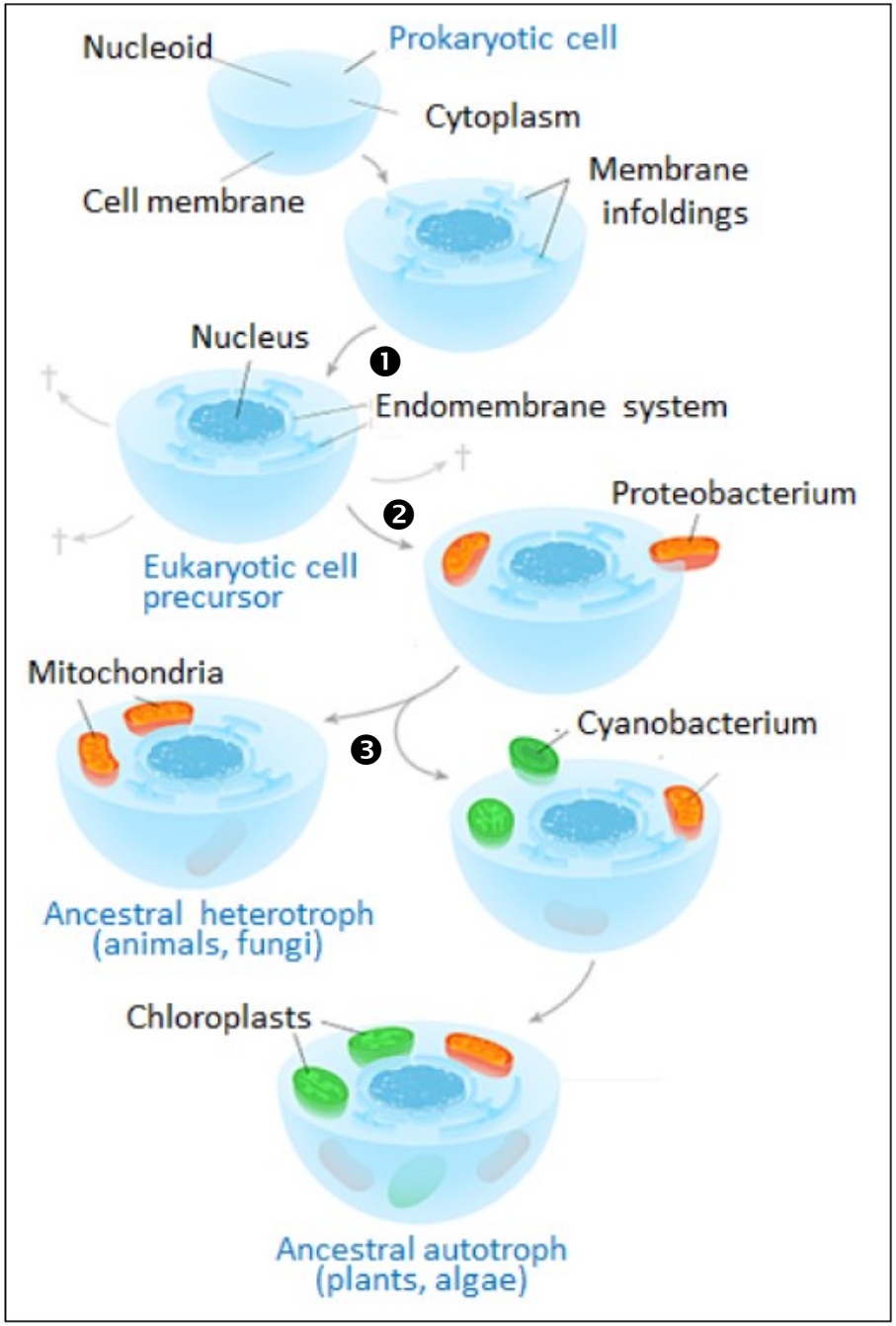
  - most likely arose from a bacterial symbiote (symbiont) whose closest contemporary relatives are in the *Rickettsiaceae* group [Gram(-) obligate intracellular parasites].

❶ The eukaryotic cell precursor developed a nucleus and endomembrane system

❷ Endocytosis of a bacterium generated organelles with two membranes: the outer membrane derived from the eukaryotic plasma membrane, the inner membrane from the bacterial cytoplasmic cell

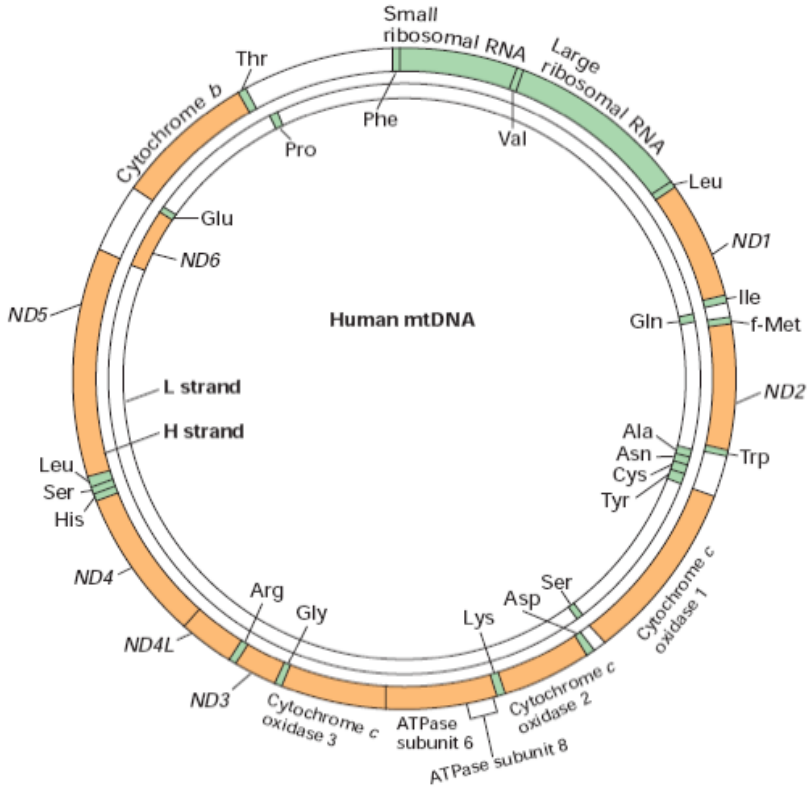
❸ Mitochondria and chloroplasts most likely arose from two separate bacterial symbiosis events

- Ancestral bacterial membrane proteins **retained their orientation**
  (cytoplasm → matrix/stroma)



MITOCHONDRIAL GENOME

- Human mitochondrial DNA (mtDNA) consists of 16.569 bp, as opposed to yeast which has ~ 70.000 bp
  - Encodes for **37 genes:** 2 rRNAs, 22 tRNAs, 13 ORFs for protein subunits involved in e^- transport and ATP synthesis
  - **MitoCarta3.0** (2020) however lists **1136 genes** for mitochondrial proteins
  - 99% of mitochondrial proteins are therfore encoded by nuclear genes, and of these,15% are involved in energy metabolism (75% of protein mass).



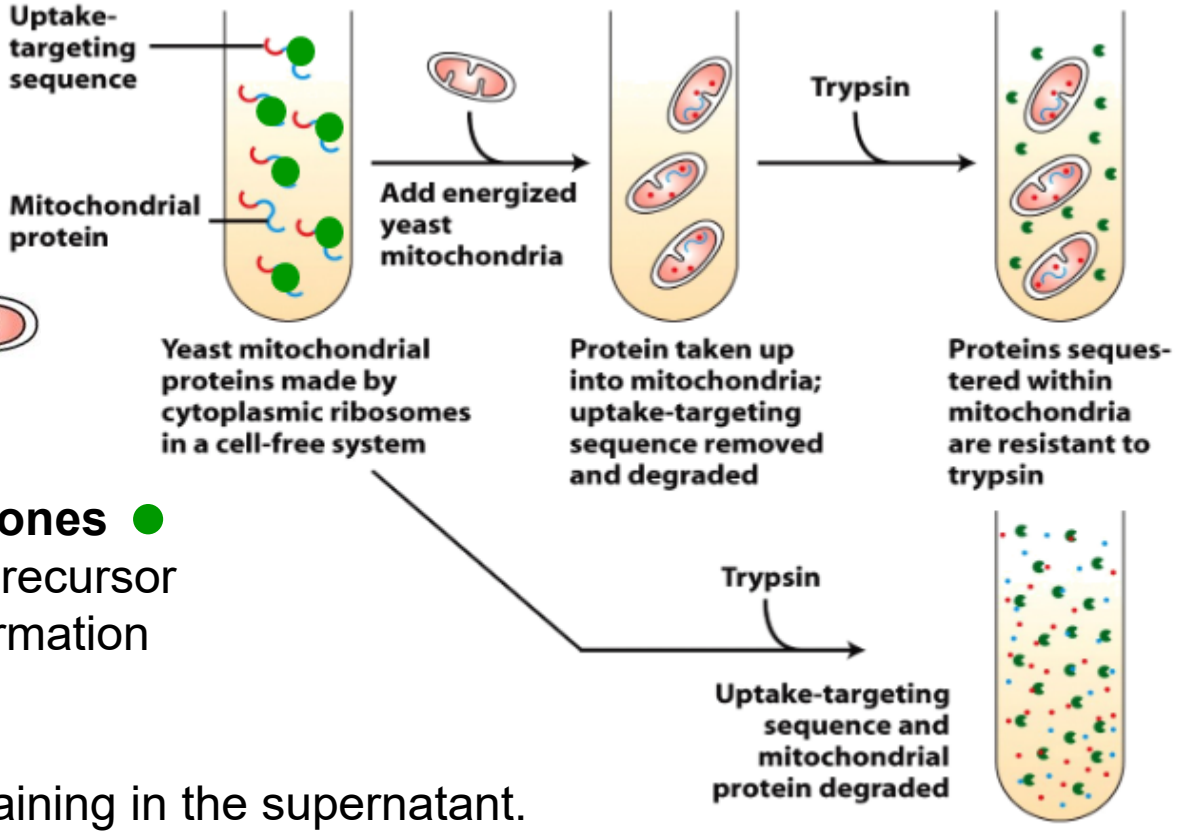
~ 25% of the mitochondrial proteome is required to regulate and express the mitochondrial genome that codes for only ~1% of mitochondrial proteins

- Most mitochondrial proteins are **synthesized in the cytoplasm**
- They must be **translocated** into the matrix or mitochondrial membranes

EXPERIMENTAL VERIFICATION OF MITOCHONDRIAL IMPORT

- Import of mitochondrial precursor proteins assayed in a **cell-free system**

- **Requirements for uptake**:
  - **cell-free translation** of proteins with **appropriate signal sequences**
  - **respiring mitochondria** (active proton-motive force)
  - **ATP**
  - Cytosolic extract with **chaperones** that can keep mitochondrial precursor proteins in an unfolded conformation



- **Trypsin is then added**
  - It degrades any proteins remaining in the supernatant.
  - any intact proteins must have **entered into the organelle** and are protected
  - this implies that there must be the presence of **OM receptors** and **translocation machinery** (translocons) in both Outer and Inner Mt membranes.
  - proteins must be **unfolded** (protected by chaperones) and have an **appropriate signal sequence**

Protein import pathways of mitochondria

❶ **α-helical precursor** → OM

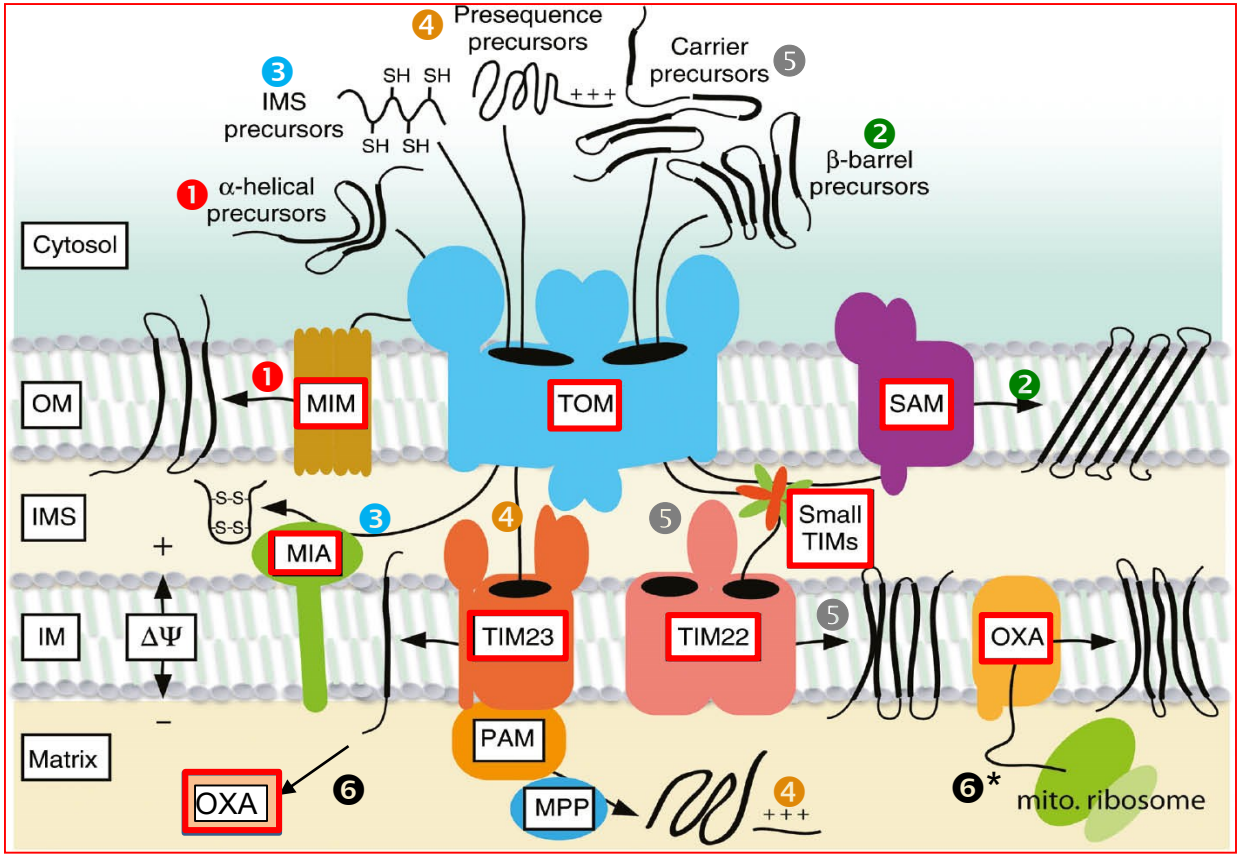
❷ **β-barrel precursor** → OM

❸ **IMS precursor** → **I**nter**MS**pace

❹ **Presequence precursor** (Mt matrix protein)

❺ **Carrier precursor** (IM carrier proteins)

❻ **IM proteins (Mt encoded)**



❶- ❻ **TOM** = **T**ranslocase **OM**

❷ **SAM** = **S**orting **A**ssembly **M**achinery (exo→IMS→OM)

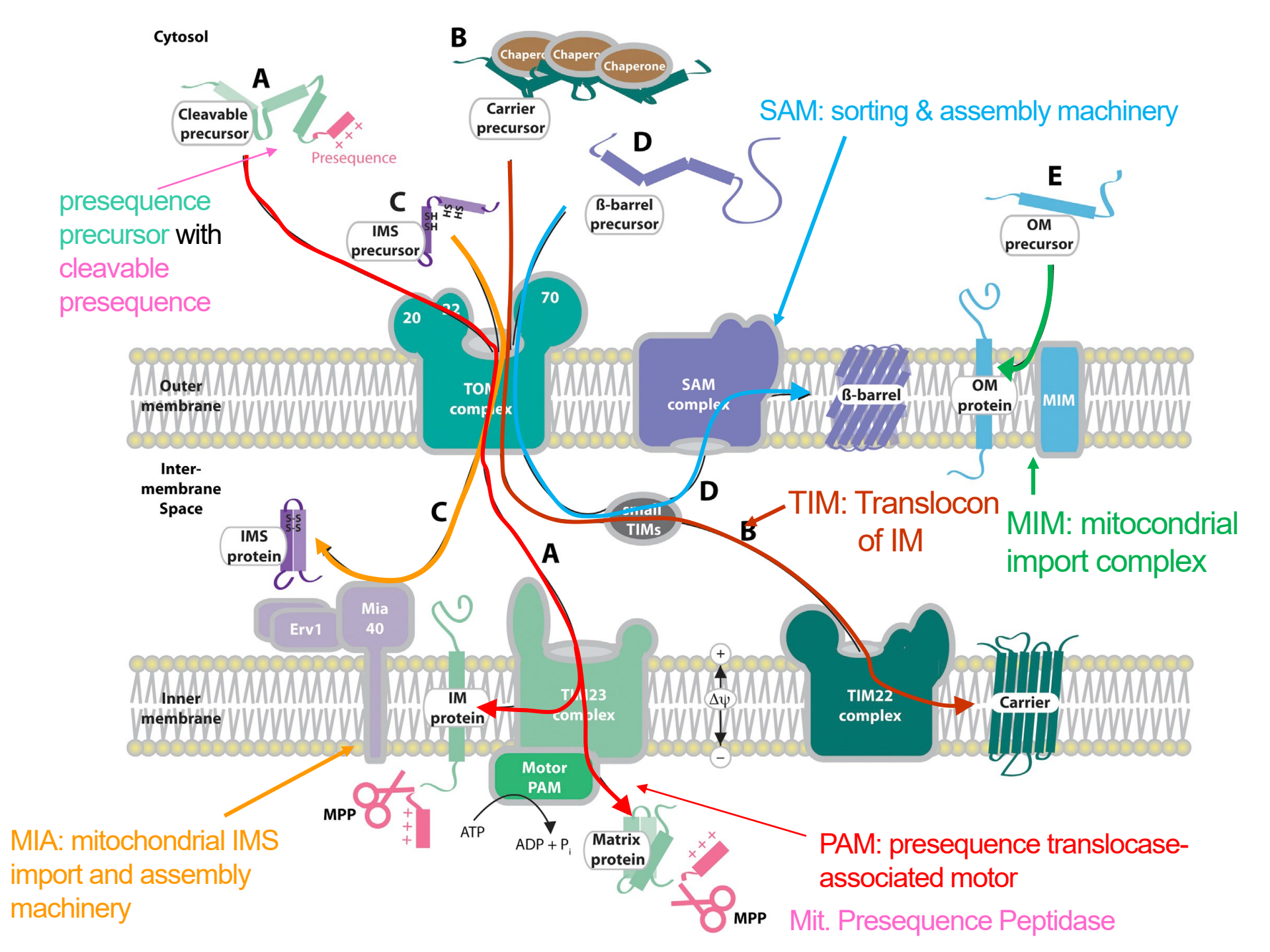
❶ **MIM** = **M**itochondrial **IM**port complex (exo → OM) (TOM dependent or independent)

❹ & ❺ **TIM** = **T**ranslocase **IM: TIM23** = **Preseq. Translocase,** assisted by ATP driven **PAM** (**P**T **A**ssoc. **M**otor). Preseq. cleaved by Mt Presq.Protease (**MPP**); **TIM22 = Carrier Translocase**

❷ & ❺ **Small TIMs** (8 -13) transfer hydrophobic chains to SAM or TIM22 for OM or IM delivery

❸ **MIA** = **M**t IMS **I**mport and **A**ssembly – uptake & ox folding of Cys-rich IMS proteins

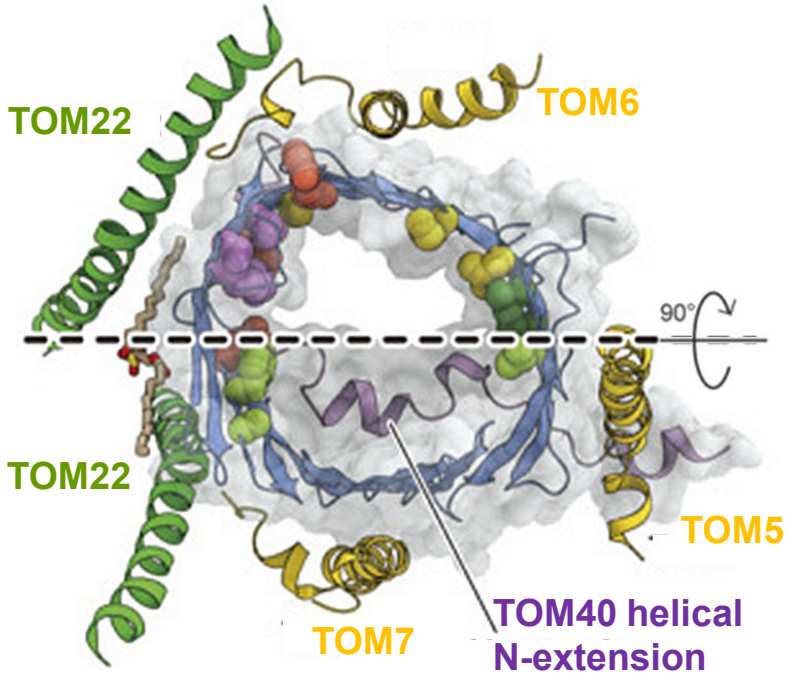
❻* **OXA1** = **OX**idase **A**ssembly: co-translational insertion of Mt encoded proteins into IM (*)

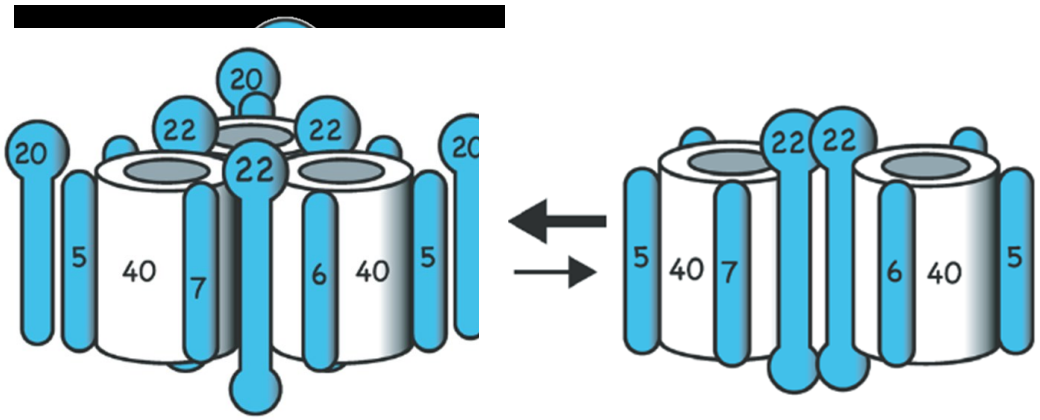

Cytosol
A
Cleavable precursor
Presequence
B
Chaperone
Carrier precursor
C
IMS precursor
D
ß-barrel precursor
E
OM precursor
SAM: sorting & assembly machinery
presequence precursor with cleavable presequence
Outer membrane
Inter-membrane Space
Inner membrane
TOM complex
SAM complex
ß-barrel
OM protein
MIM
small TIMs
TIM: Translocon of IM
MIM: mitocondrial import complex
IMS protein
Erv1
Mia 40
IM protein
TIM23 complex
TIM22 complex
Carrier
Motor PAM
MPP
ATP
ADP + P$_i$
Matrix protein
MIA: mitochondrial IMS import and assembly machinery
PAM: presequence translocase-associated motor
Mit. Presequence Peptidase

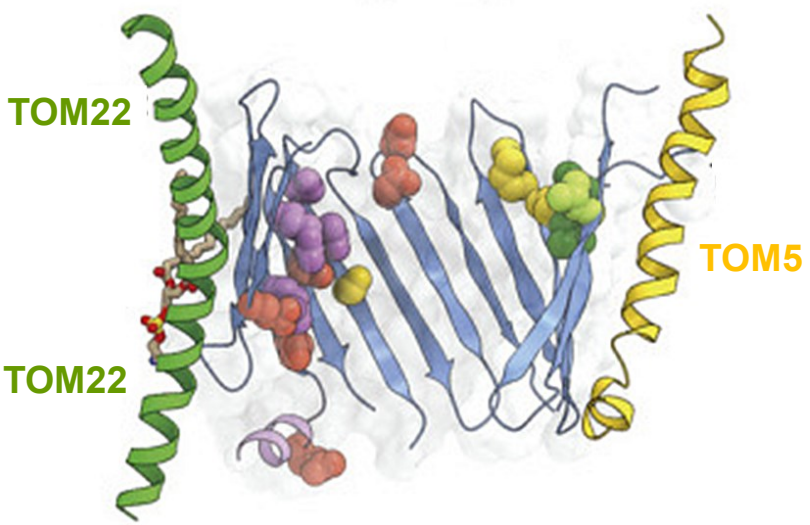
Protein import into the mitochondrion: the TOM40 complex

- The TOM complex is a **major entry gate** for most Mt proteins (**general import pore**)
  - **TOM40** consists of a β-barrel with 19 β-strands
  - **TOM 20** & **22** function as **receptors for TS**
  - **TOM 5,6 & 7** are the **small subunits.**

    TOM 5 – 22 all have single α-helical TM domains
- The **major form** of the TOM complex is trimeric, with three of each subunit
- It can be assisted by **Tom70** (**receptor for internal TM domains**)
- A minor form is dimeric and lacks TOM20.
- They are in dynamic exchange







Four major mitochondrial import pathways via TOM complex

- **Major entry gate** for most Mt proteins, which then branch into different sorting pathways

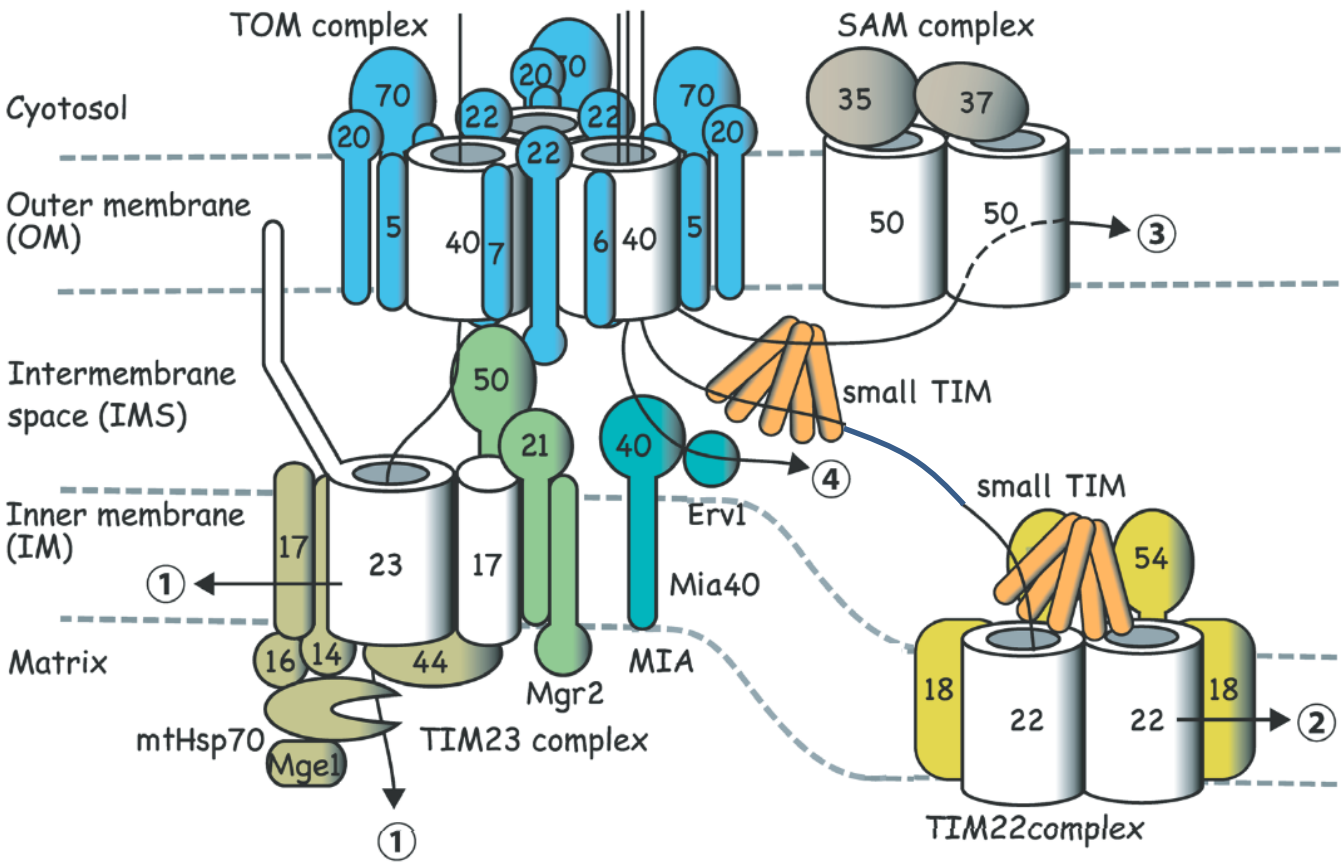
① **Proteins with presequence (TS)** are sorted through the TIM23 complex **into the matrix**

② **Polytopic IM proteins** (e.g. Carrier proteins) bind to IMS chaperones (small TIM) and are delivered to TIM22 for **integration into the IM**

③ **β-barrel OM proteins** (e.g. porins,TOM, SAM,) are delivered by sTIM to the SAM complex for **integration into OM**

④ **Soluble, Cys-rich IMS proteins** are **transferred to IMS**, **where** MIA (TIM40) and Erv1 oxidatively fold them

- The different pathways chosen depend on the **oligomerisation state** of **TOM** (trimer ←→dimer)



Protein import into the mitochondrial matrix / IM

❶ Precursor proteins are kept in a partially folded state by **cytosolic chaperones**

❷ **TS** binds to **import receptor TOM20/22**

❸ It transfers protein to **General import pore** (**TOM40**) at rare OM/IM **contact sites**

❹ Protein moves through TOM into the IMS and on to adjacent IM channel complex (**TIM23/17**)

❺ It then moves through TIM into matrix

❻ **ATP hydrolysis** by PAM and matrix chaperones drives translocation; **MMP** cleaves**TS**

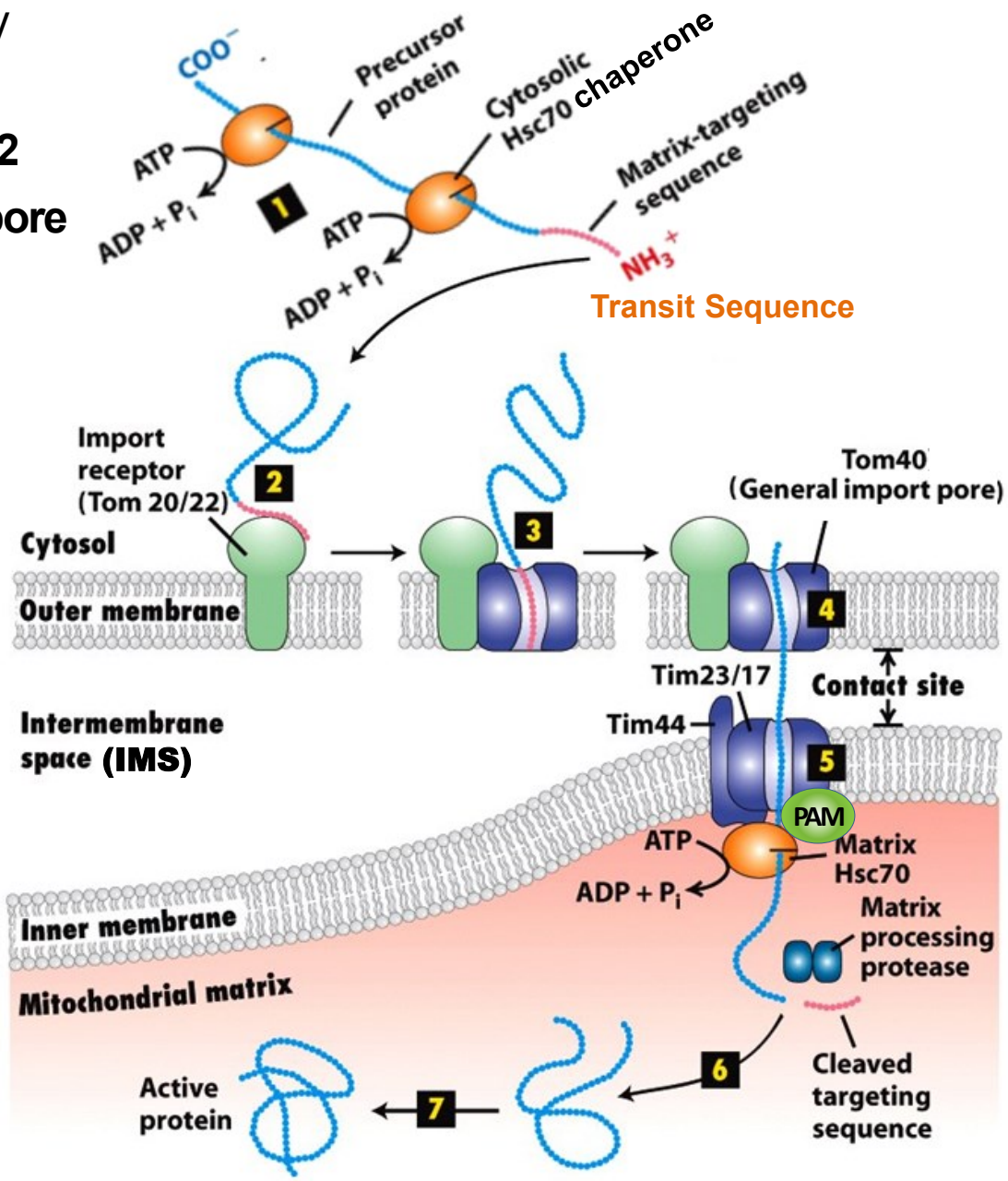
❼ Protein then folds with assistance of matrix chaperones

Three **energy inputs** are required for translocation:

1) ATP hydrolysis by $Hsc70^{cyt}$

2) ATP hydrolysis by $Hsc70^{mit}$ & PAM

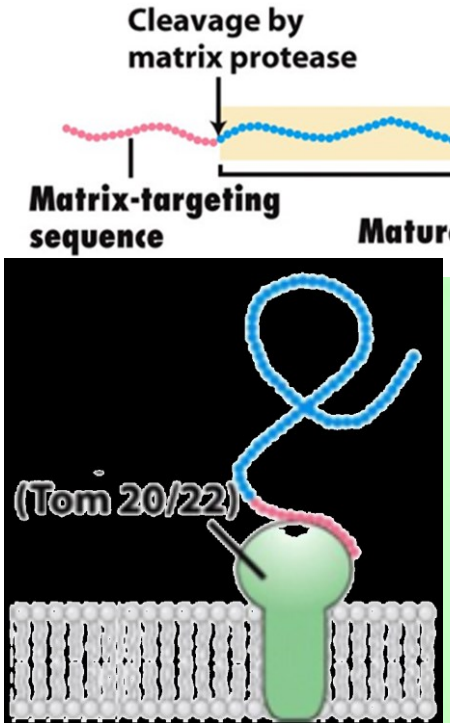
3) Proton-motive force of IM

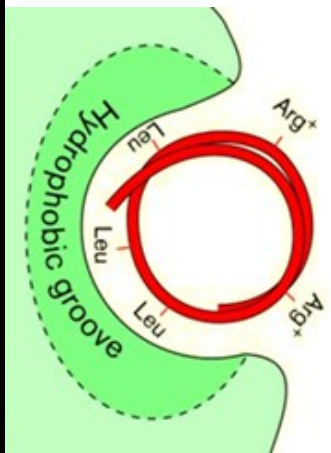


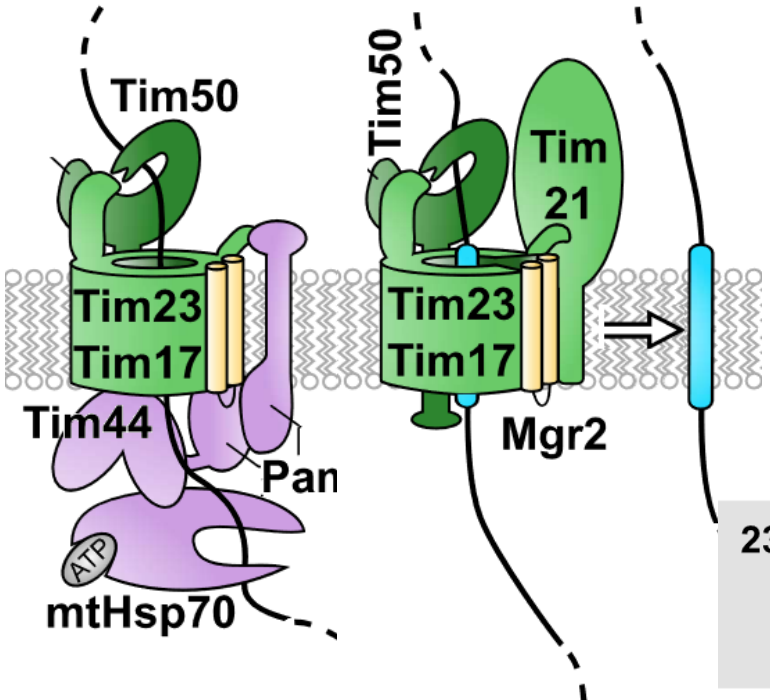
Protein import into the mitochondrial matrix & IM

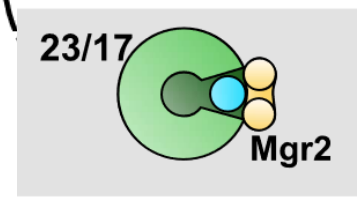
- ~ 60% of mitochondrial proteins have an **N-terminal targeting presequence**

1) The presequence has cationic and hydrophobic residues which form **amphipathic α-helices**, which allow binding to **Import Receptor** (**TOM20/22**)
2) Mt proteins then pass through TOM in an **unfolded state** (requires ^{C}Hsc70)
3) Tim50 then delivers it to the **TIM** complex (TIM17,23,44) for sorting:
   - **into the matrix** (assisted by PAM and ^{M}Hsc70)
   - **into the IM** if a **stop-transfer sequence** is present (assisted by Mgr2 & TIM21)
4) The cleavable **presequence is removed** by *Matrix Presequence Proteases* (*MPP*)









Experimental verification of mitochodrial import

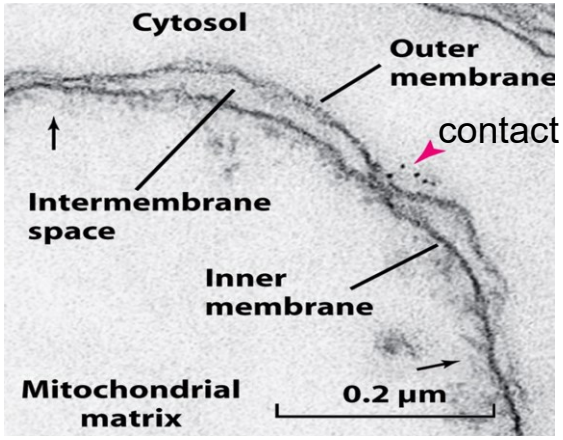
(1) A matrix-targeting sequence (TS) and a long unstructured spacer sequence were added to the cytoplasmic enzyme *DHFR* and the chimeric construct translated in a cell-free system.

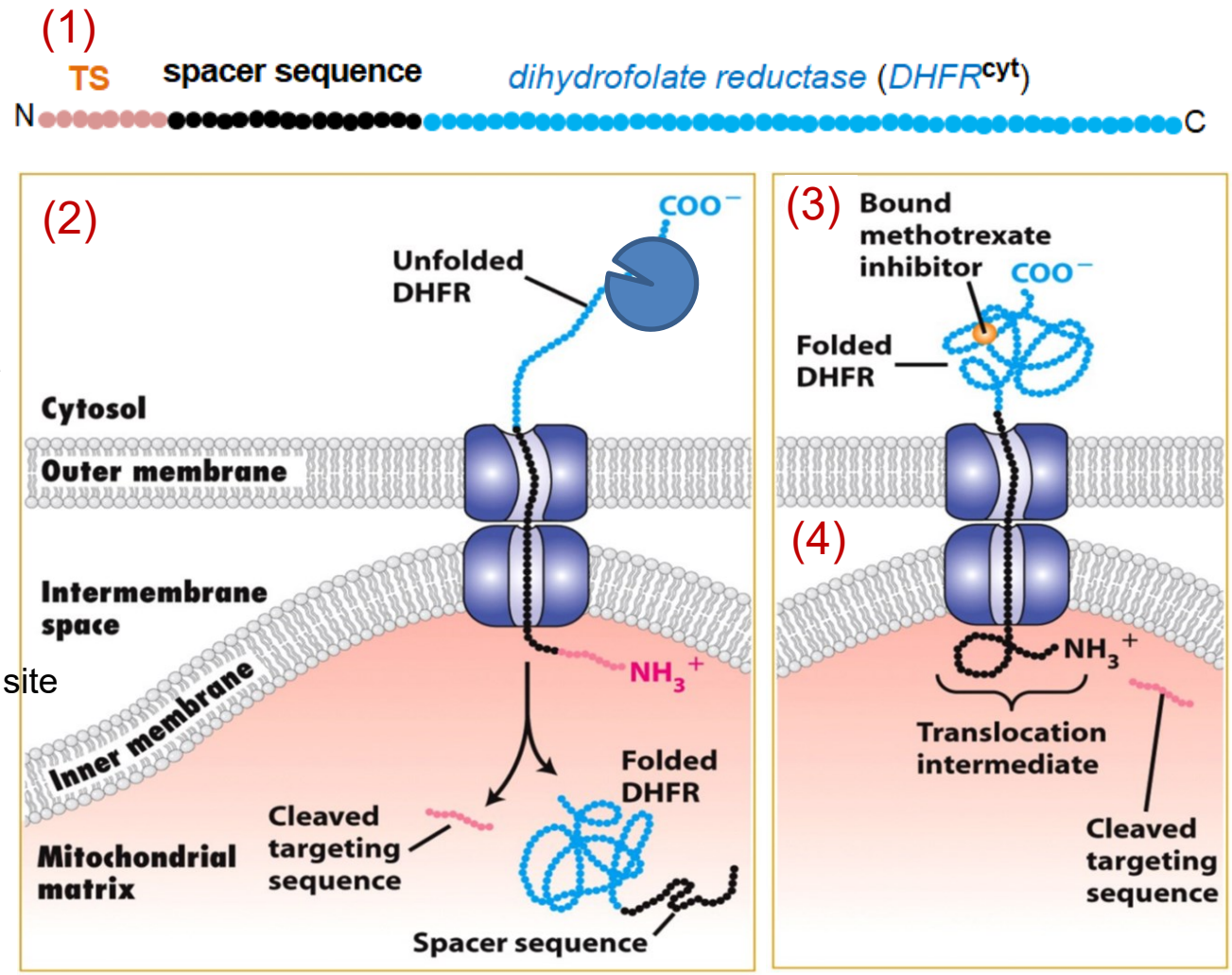
(2) If chaperones are present, it stays unfolded and translocates to the Mt matrix (TS is removed)

(3) Methothrexate strongly stabilizes the structure so the construct can't translocate completely

(4) The unfolded spacer sequence however allows the TR to translocate, locking the enzyme onto TOM40; TS is cleaved

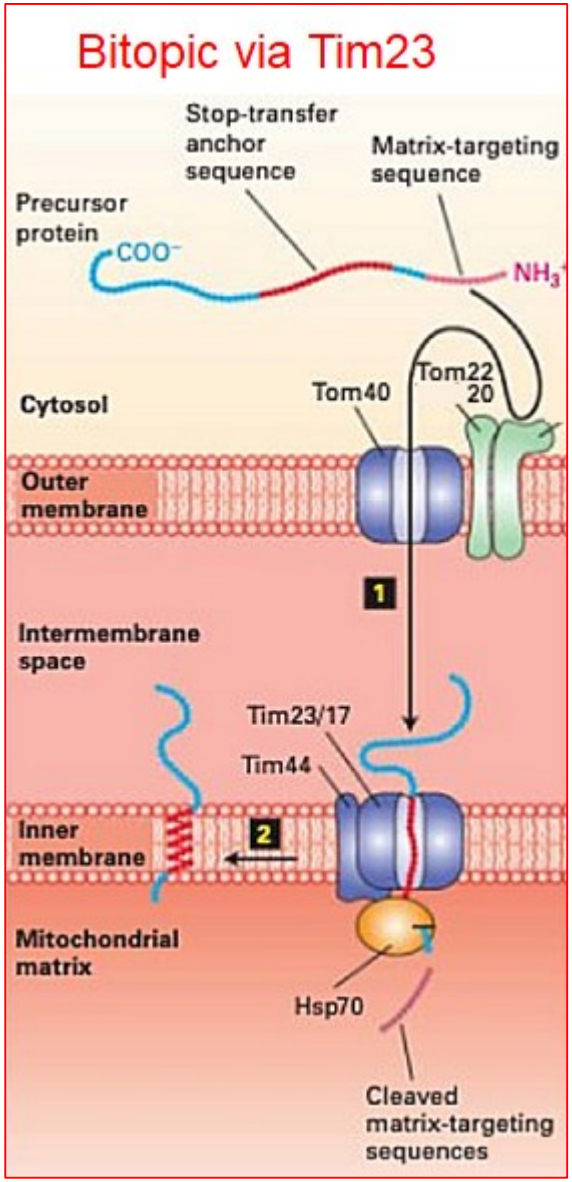
(5) Its localization on TOM at rare mitochondrial contact sites can be verified using *DHFR*-specific antibodies

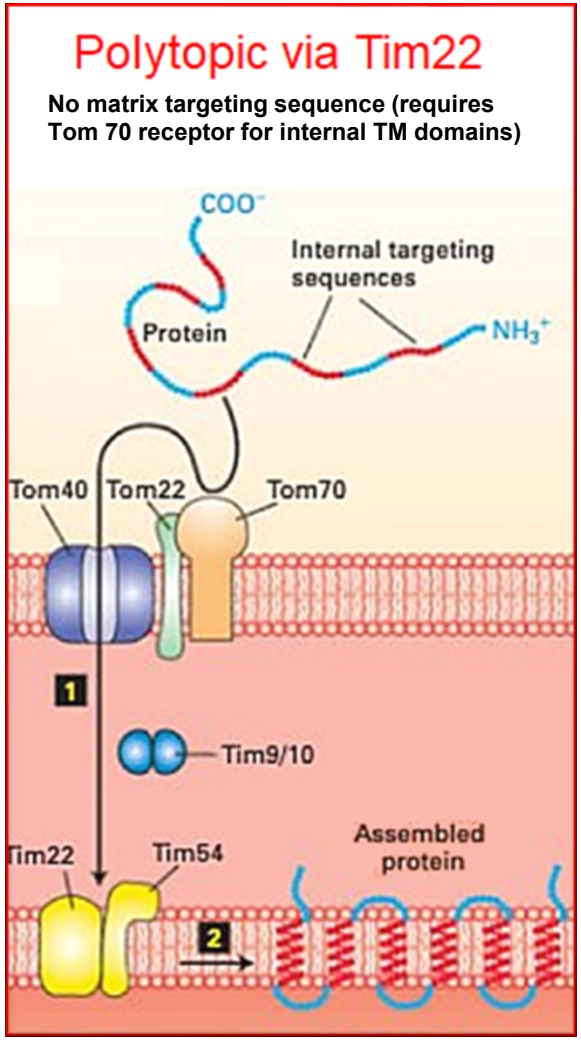




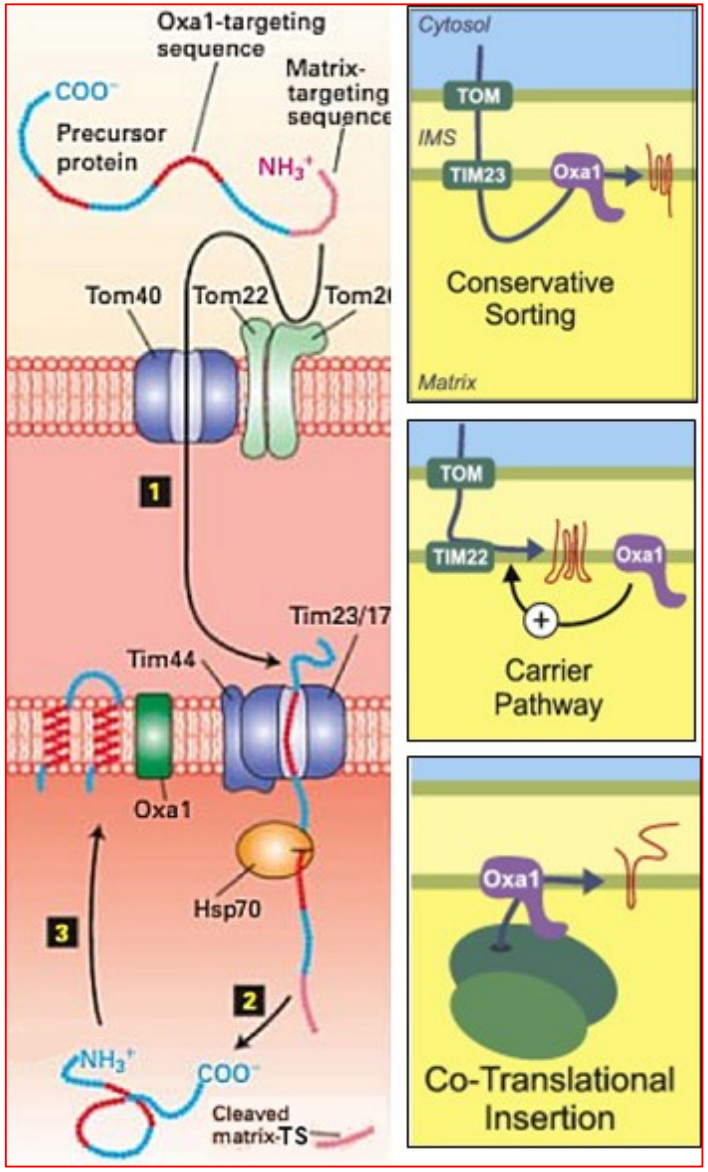
Different import pathways for mitochondrial IM proteins

- As with incorporation into ER membrane, this **requires multiple sequence signals**
  - can use different pathways depending on the type of protein (bitopic, polytopic etc.)



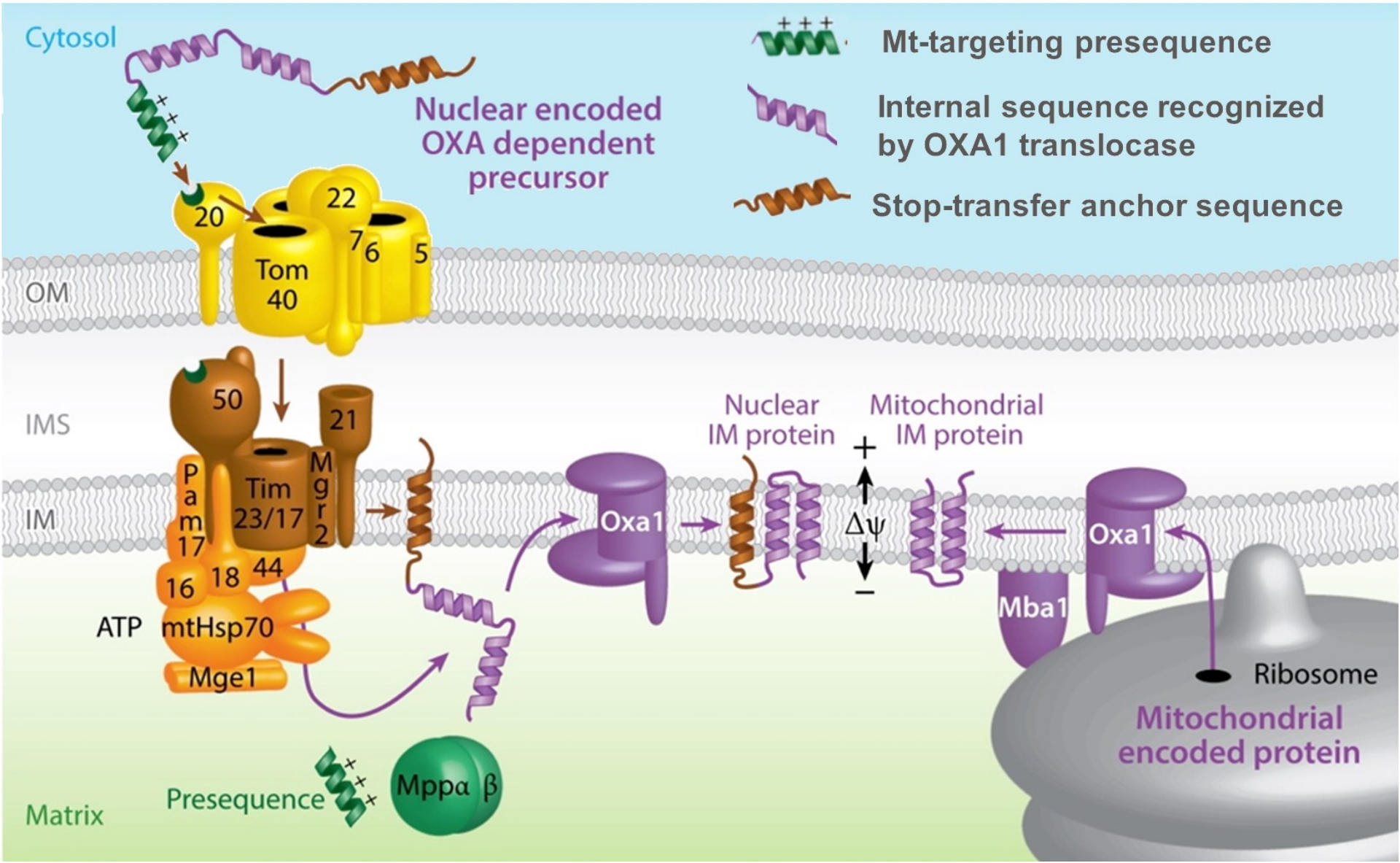


Polytopic via Oxa →



Import into mitochondrial IM

- Presequence-containing precursor proteins with **internal sequences recognised by Oxa1**



Insertion of proteins into intramembrane space (IMS)

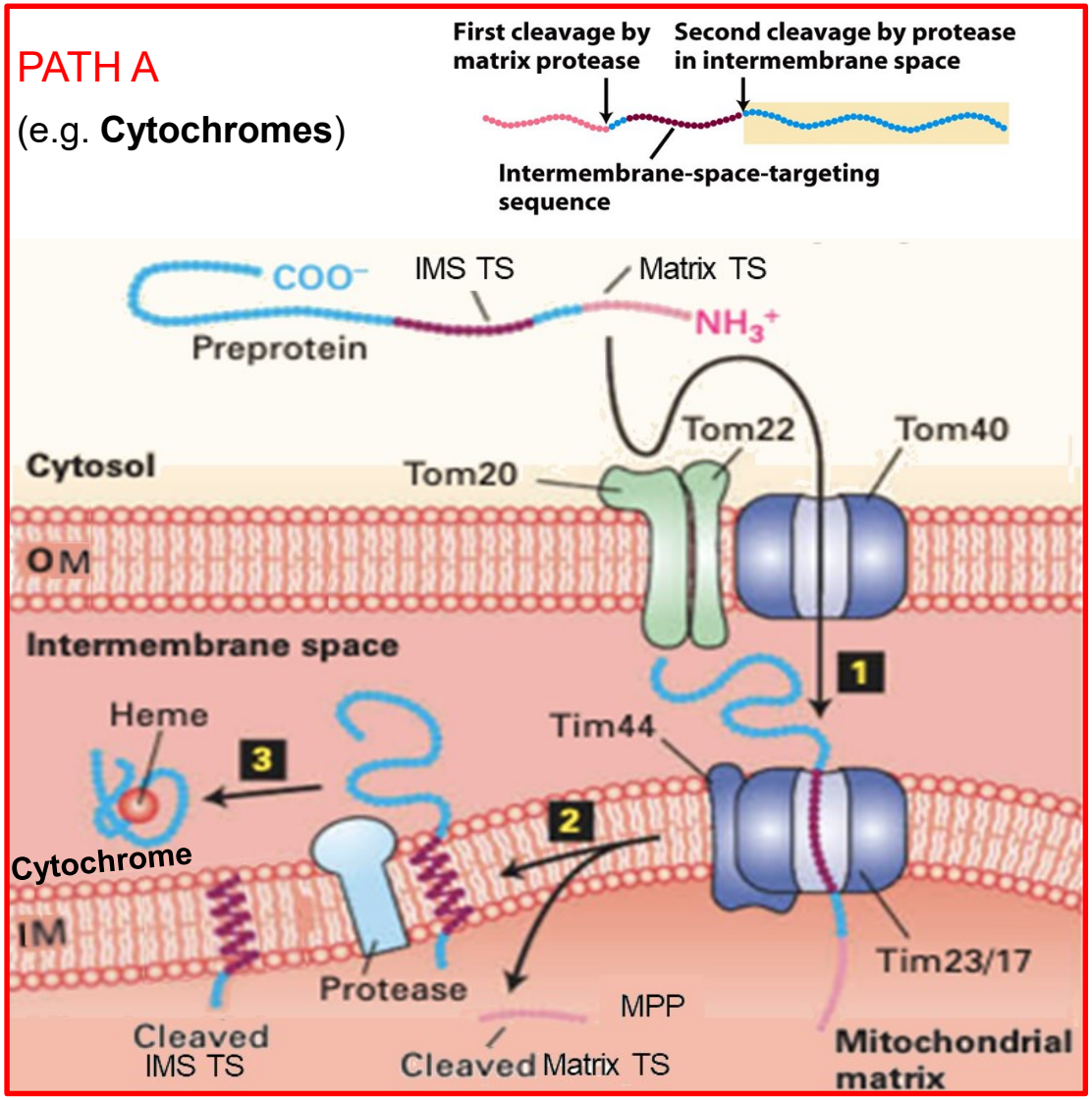
- As with other Mt proteins, these **first pass through TOM**, and can then follow **two paths:**

Path A (main): **cleavable TS** & **IM targeting sequences** (similar to bitopic IM proteins)

Path B (small Tim): **internal targeting sequence** for **general import pore**

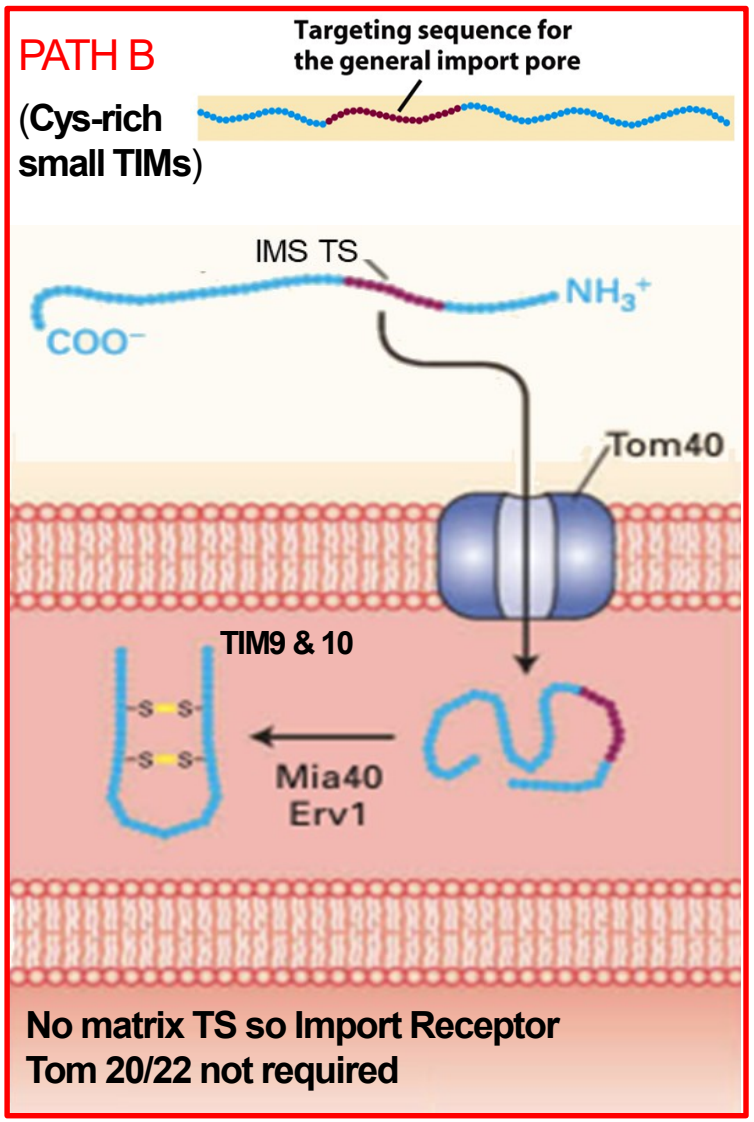
PATH A

(e.g. **Cytochromes**)



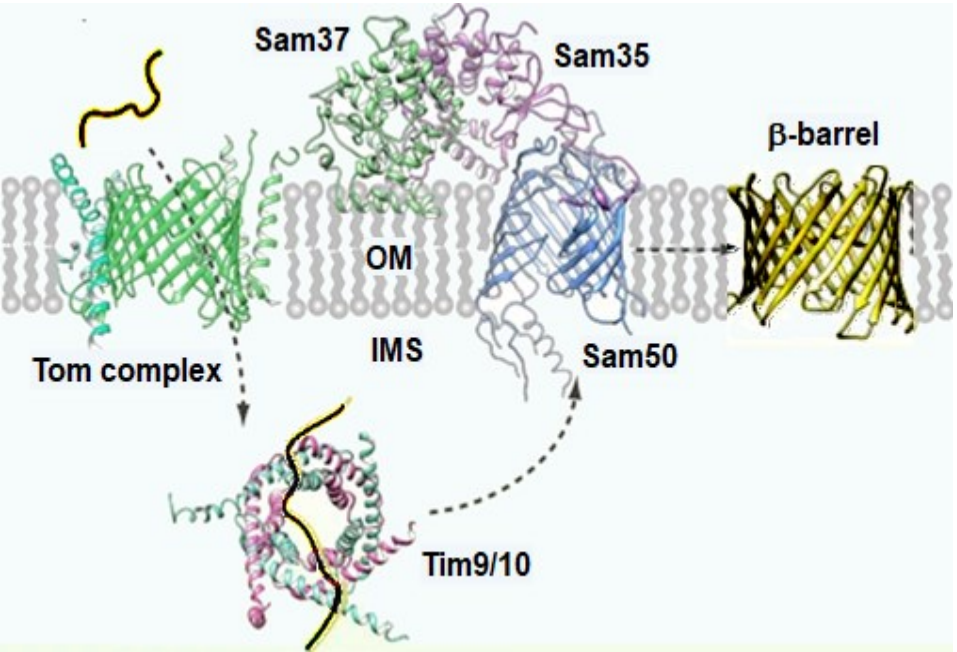
PATH B

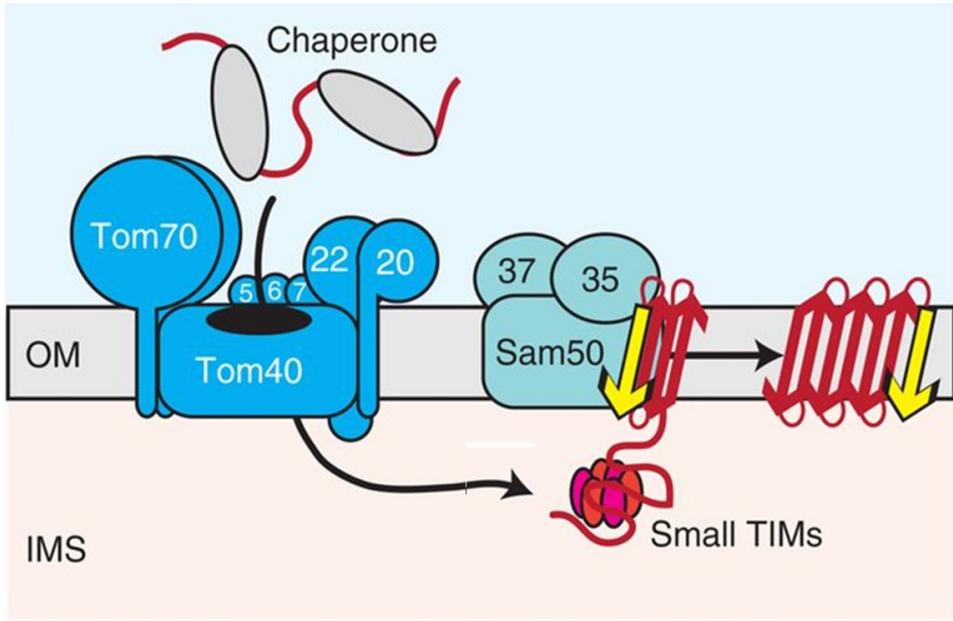
(**Cys-rich small TIMs**)



Insertion of mitochondrial OM proteins: β-barrels

- The OM contains two **major classes of MP** ; β-barrel (include **Tom** and **Sam**)
  α-helical TM domain (bitopic or polytopic)
- **β–barrel proteins** contain a specific import signal (**β-signal**) in the **last β-strand**
  - conserved **XGYZ** motif (**X** = bulky polar, **G** invariant, **YZ** = large hydrophobic)
- The **Tom70** subunit of Tom has a relevant role (**recognition of internal signal seq.**)
- Insertion via **SAM complex** with at least **3 core subunits**:
  - Sam37 forms super-complex with Tom; Sam50 imports chain; Sam 35 binds β-signal
- Small Tims (9/10) accompany the protein in the IMS from TOM to SAM





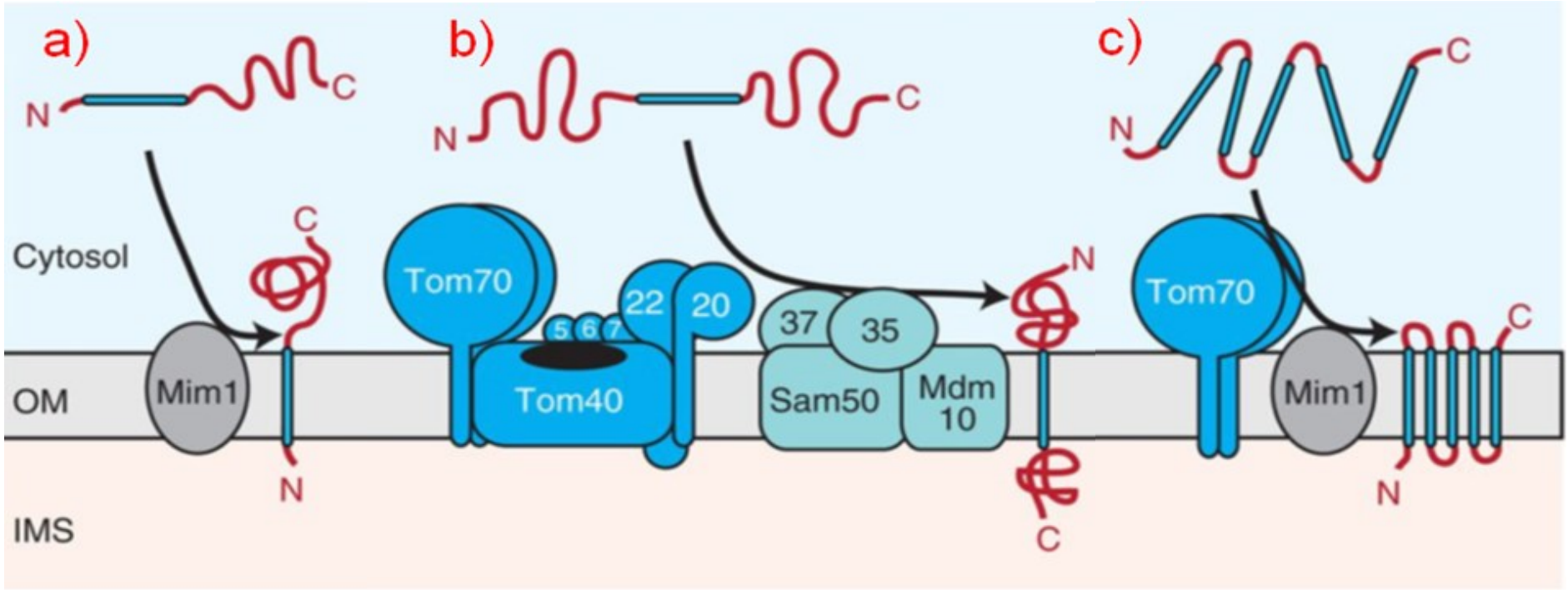
Insertion of OM proteins with α-helical TM domains

- Multiple pathways mediate insertion of α-helical proteins into Mt OM.

a) **Bitopic**, TMh located at the N-ter (N-anchored) — **Mim1 integration**, facilitated by Tom

b) **Bitopic,** TMh central or C-ter — **Tom integration,** facilitated by Tom70 & Sam, assisted by Mdm10

a) **Polytopic**, multiple TMh — **Mim1 integration** facilitated by Tom70 acting alone



Peroxixomes

- Peroxisome are **degradative intracellular organelles** in eukaryotic cells
  - contain *oxidases* in their matrix for **chemical oxidation** of **toxins**, **AA** & **FA**
  - participate in **lipid metabolism**
  - processing of **reactive oxygen species**.
  - catabolism of *D*-AA, polyamines, bile acids.

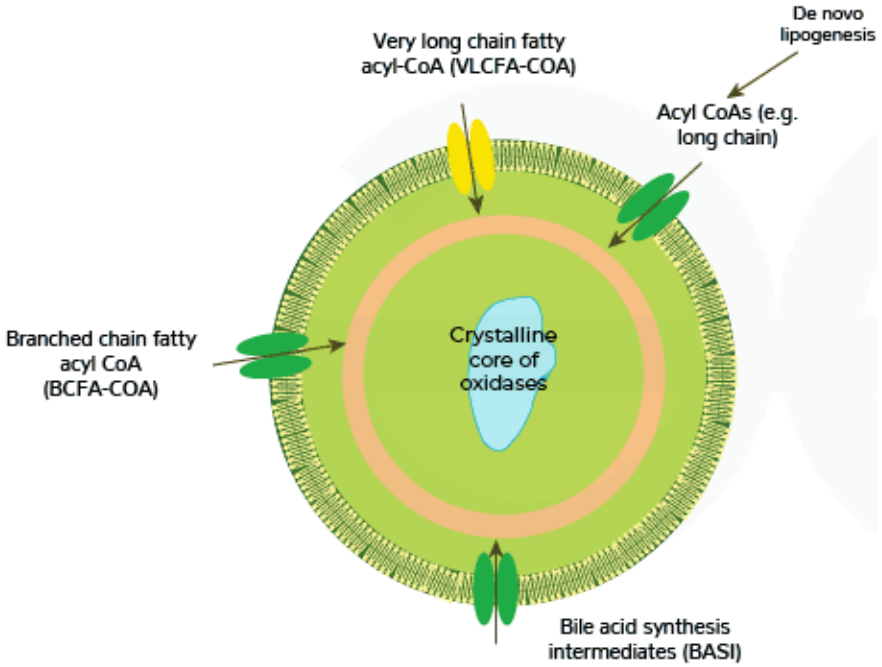
Oxidation of FA with very long or branched chains

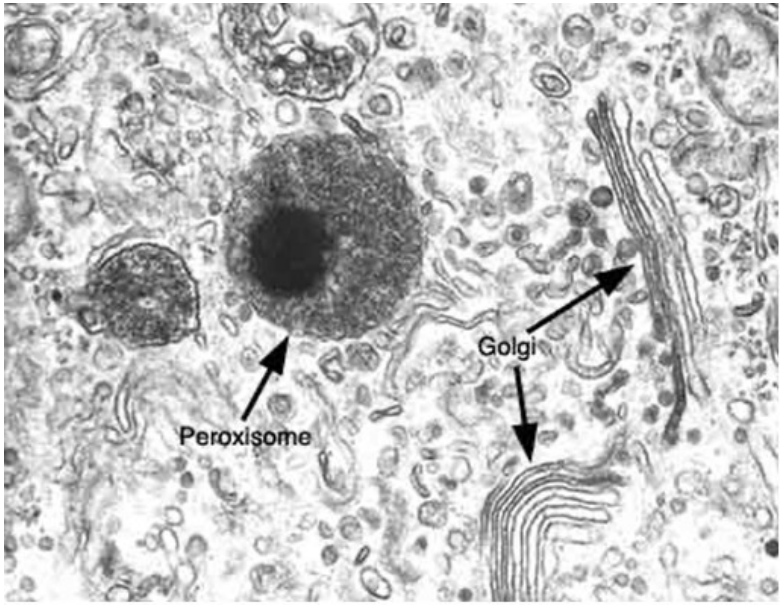
$$RH_2 + O_2 \rightarrow R + H_2O_2$$

$$H_2O_2 + R'H_2 \rightarrow R' + 2H_2O$$

Detoxification of H_2O_2

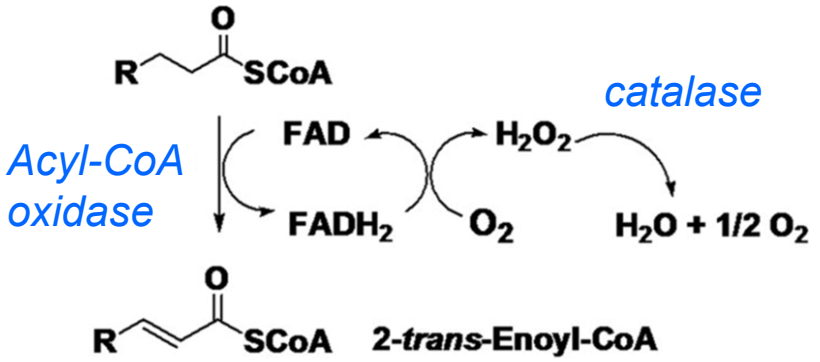
$$2H_2O_2 \xrightarrow{catalase} 2H_2O + O_2$$



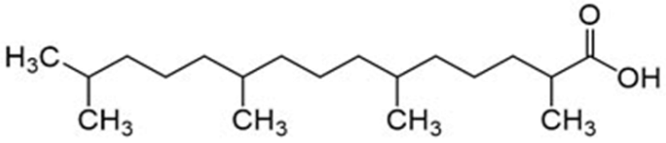


Functions of peroxisomes

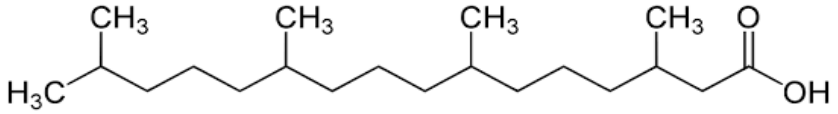
- Oxidation of **very long-chain fatty acids (VLCFA)**



- Oxidation of **branched-chain fatty acids (BCFA)**



Pristanic acid C19 (2,6,10,14-tetramethylpentadecanoic acid)

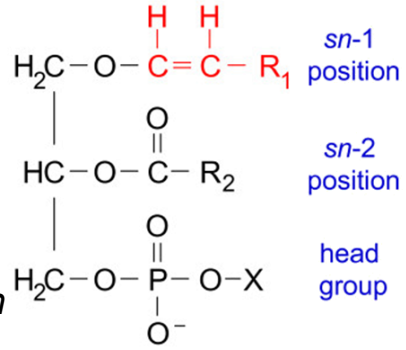


Phytanic acid C20 (3,7,11,15-tetramethyl hexadecanoic acid)

- Detoxification of **hydrogen peroxide (H_2O_2)** by the enzyme ***catalase***
  - this is a potentially dangerous product of fatty-acid oxidation
- Oxidation of **uric acid** (purine catabolism, not in humans)
- Participate in lipid synthesis (**plasmalogens**) (myelin)

Not fully proven:

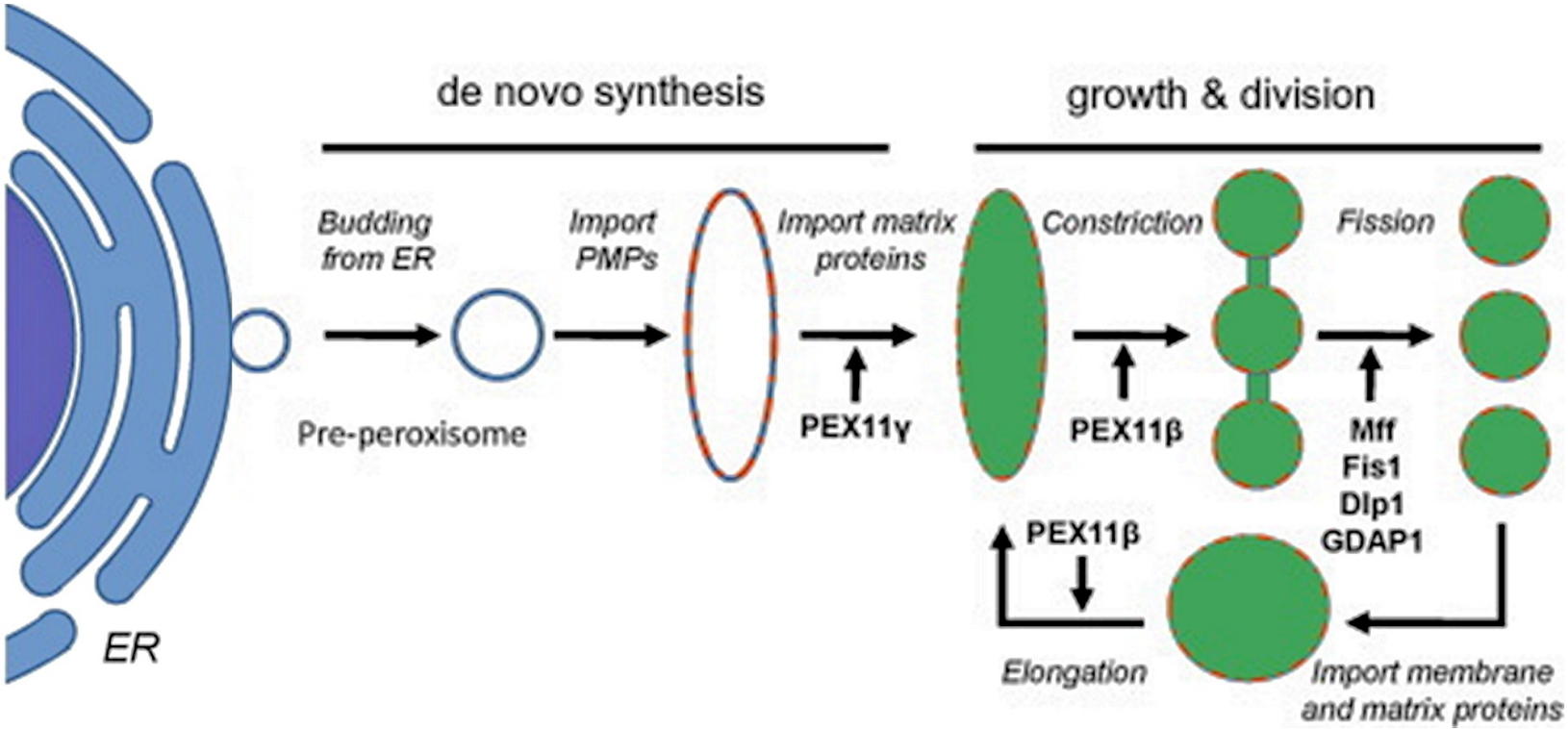
- Participate in the synthesis of cholesterol
- Participate in the synthesis of bile acids



glycerophospholipid with a plasmenyl chain linked by ether bond

Model of peroxisome biogenesis and division

- Peroxisomes may arise predominantly from either:
  - **pre-peroxisomal vesicles** originating from specialized ER compartments
  - **growth and division** of pre-existing peroxisomes (slide 26)
- **Vary in size and content** in different cells, but have same oxidative functions
- Mature, metabolically active peroxisomes **require import** of peroxisomal membrane proteins (**PxMP**s) and matrix proteins (**PxMxP**s).
- 3 sequential steps; **elongation**, **membrane constriction**, **fission**



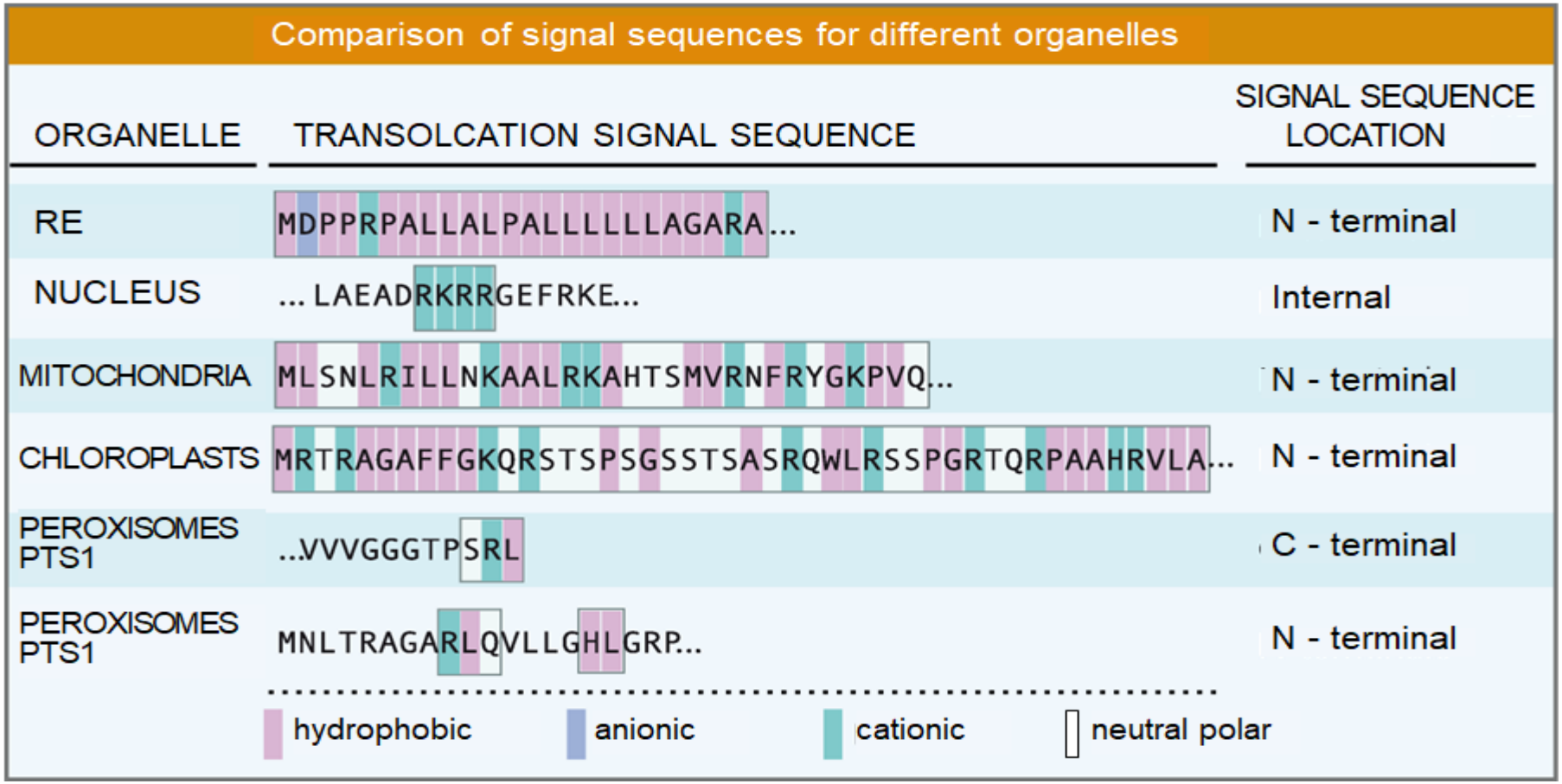
Import of peroxisomal proteins

- **P**eroxisome **M**atri**x** **P**roteins possess **peroxisomal targeting** sequences (**PTS**):

  - **PTS1** at the **C-terminus** consisting of the tripeptide SK/RL

  - **PTS2** near **N-terminus** with consensus sequence (R/K)(L/V/I) X_5 (Q/H)(L/A)

    \+ h p h



PTS1-directed import of peroxisomal matrix proteins

- Peroxisomal proteins are encoded by nuclear genes and synthesized in the cytosol
  - PTS are **recognized by peroxisome-specific receptors**
  - delivered to matrix or membrane by an **energy-dependent process**
  - proteins transported in **folded form**

❶ Most matrix proteins have **PTS1**, which interacts with cytosolic **Pex5 receptor**

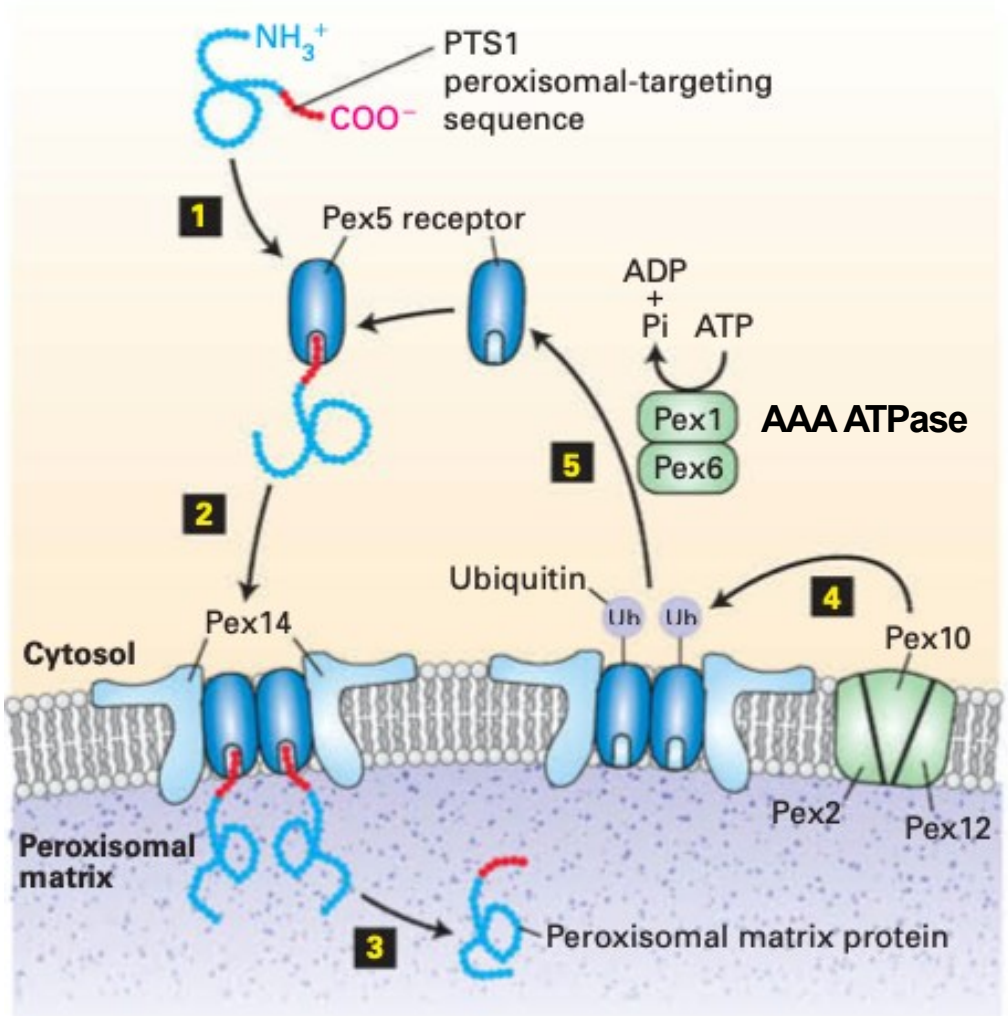
❷ A Pex5 dimer forms a multimeric complex with the **Pex14 receptor** in the peroxisomal membrane

❸ Pex5 releases its cargo in the matrix

❹ Peroxisomal membrane proteins Pex2, 10 &12 then **ubiquitinate Pex5**

❺ This allows the ATP driven release from Pex14 into cytosol; a process catalized by *ATPases* **Pex1** & **6** anchored to the peroxisomal membrane

- PTS1 signal is NOT cleaved



AAA or **triple-A** ATPases: (**A**TPase **A**ssociated with cellular **A**ctivities)

Peroxisomal protein import receptor cycle

(1) Cargo recognition in the cytosol

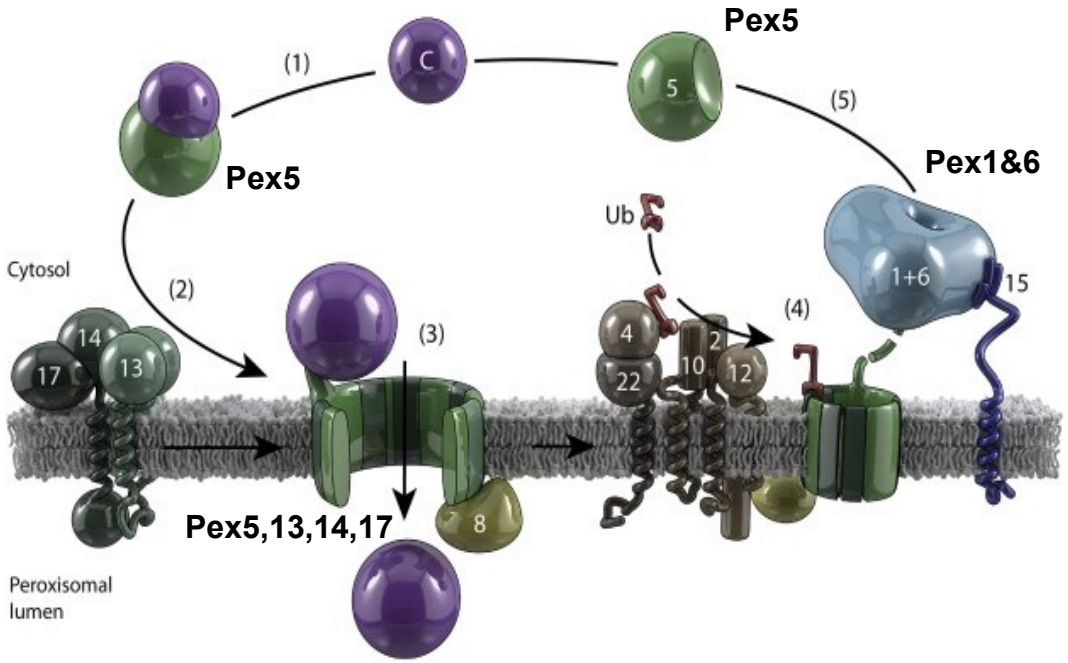
(2) Docking of Pex5–cargo Px membrane

(3) Pore assembly and protein translocation

(4) Receptor ubiquitination

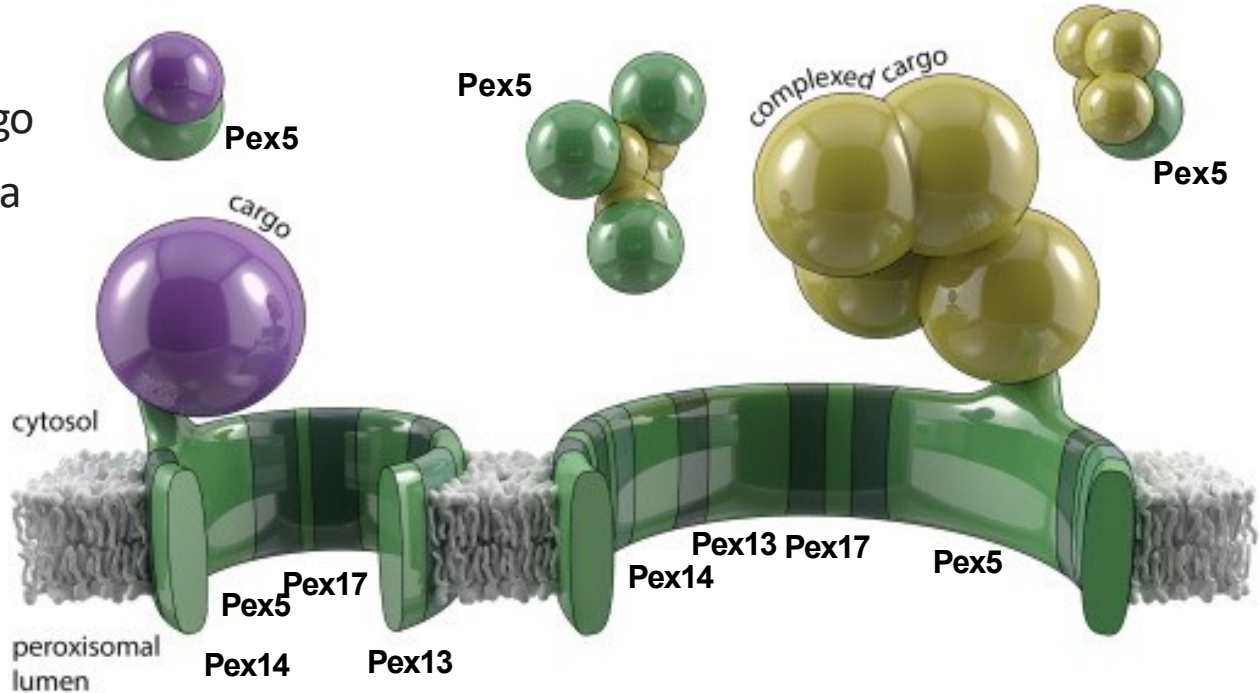
(5) Receptor export and recycling.

- Pex5 can exist as a soluble cytosolic receptor or as a PxMP, as part of the translocation pore (translocon)



Importing large folded cargo

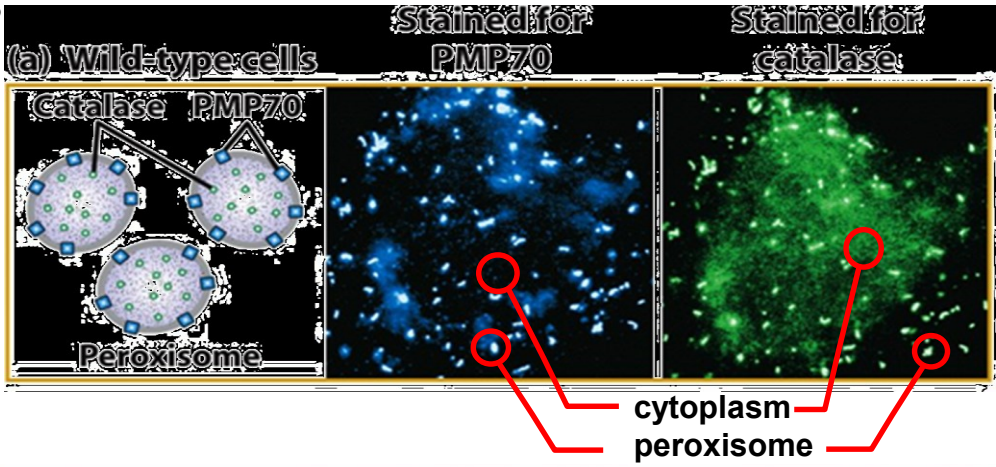
- The translocon expands to accommodate differently sized cargo
- LEFT translocon wide enough for a monomeric folded cargo protein
- RIGHT translocon when activated with an oligomeric cargo complex.



Different pathways for incorporation of PxPM and PxMxP

a) *Wt* Cells stained with fluorescent antibodies specific for PMP70 (a **PxMP)** and for *catalase* (a **PxMxP)**

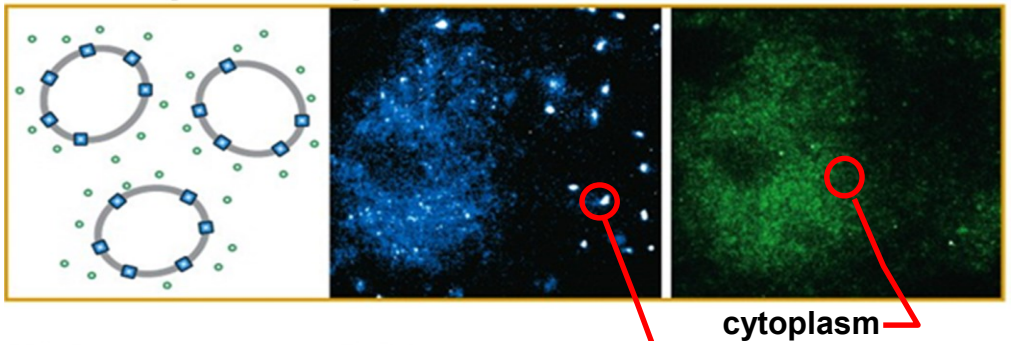
In *W-t* cells, both proteins are concentrated in Px and only difusely visible in cytoplasm



b) Pex-12 deficient patient (required for matrix incorporation)

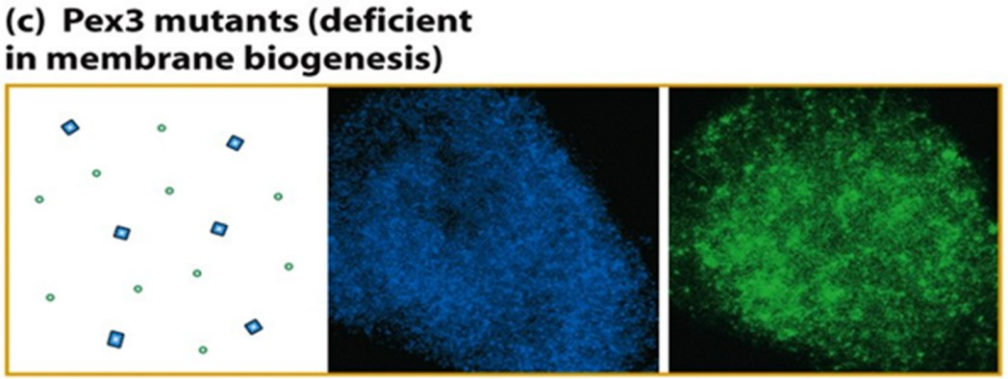
PxMP are incoporated into Px

PxMxP remain in cytoplasm



c) Pex3 deficient patient (required for membrane assembly)

peroxisomes do not form, both PxMP & PxMxP remain difuse in cytoplasm



Formation / division of peroxisomes

- **Model of peroxisomal biogenesis and division:**

❶ **Precursor membrane** derives from ER bringing some PxMP with it, including Pex proteins

❷ **1st stage** of **incorporation of PxPM** – Pex19 is the receptor for proteins with membrane-targeting sequences (e.g. PMP70). The Pex3/16 complex is required for membrane insertion

❸ The "**peroxisomal ghost**" forms, now capable of importing PxMxP

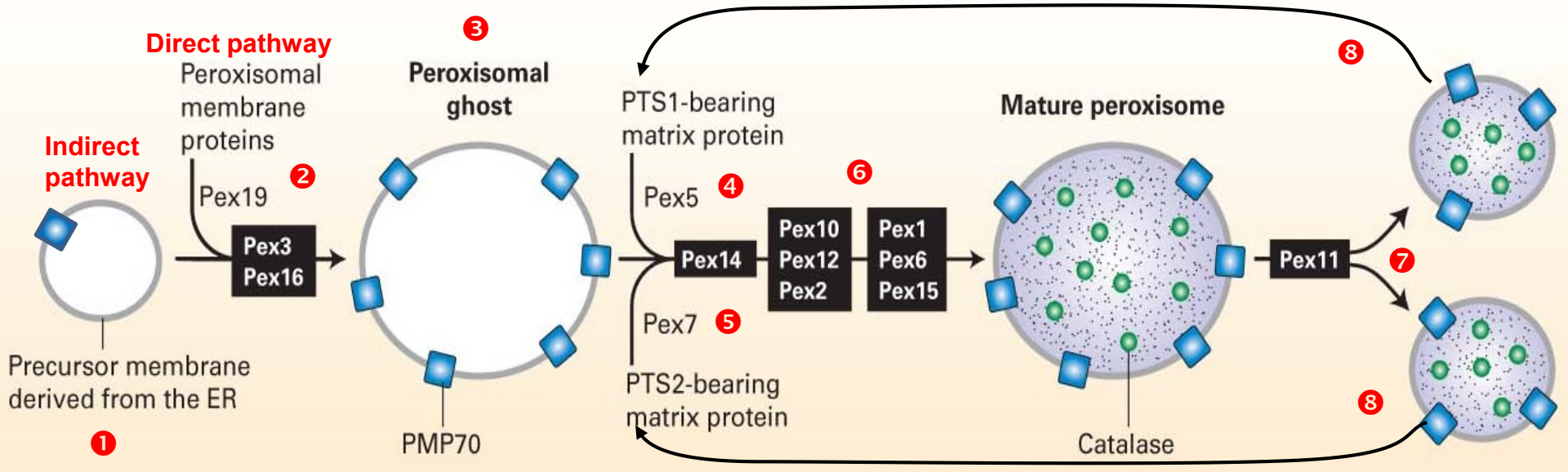
❹ Pex5 acts as receptor for proteins with PTS1 sequences

❺ Pex7 acts as receptor for proteins with PTS2 sequences

❻ Several other Pex proteins are required to assist PxMxP incorporation into the mature Px

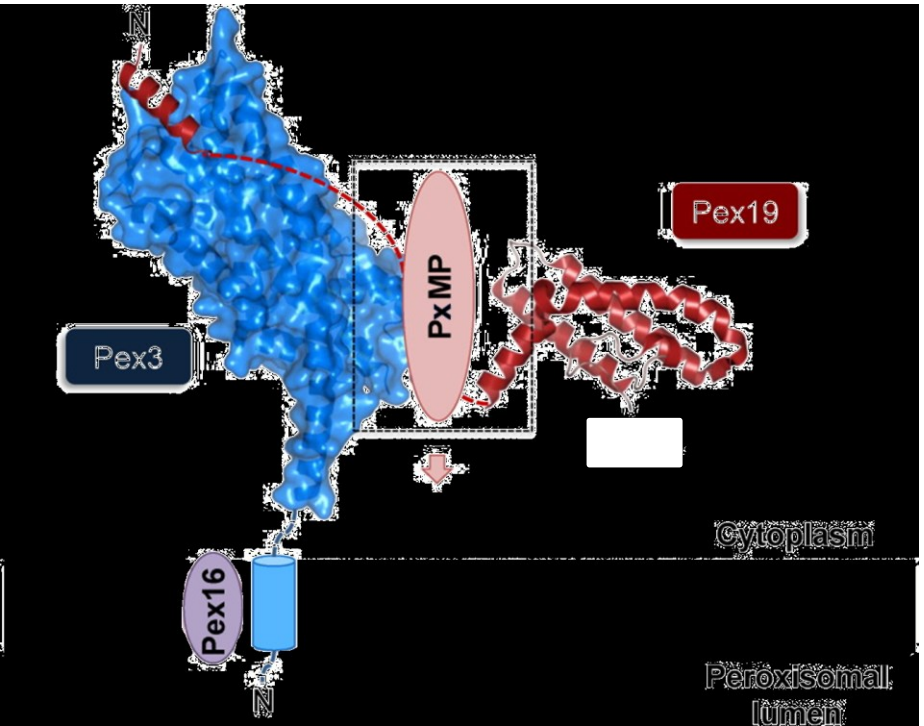
❼ Proliferation of peroxisomes in the cell then requires Pex11

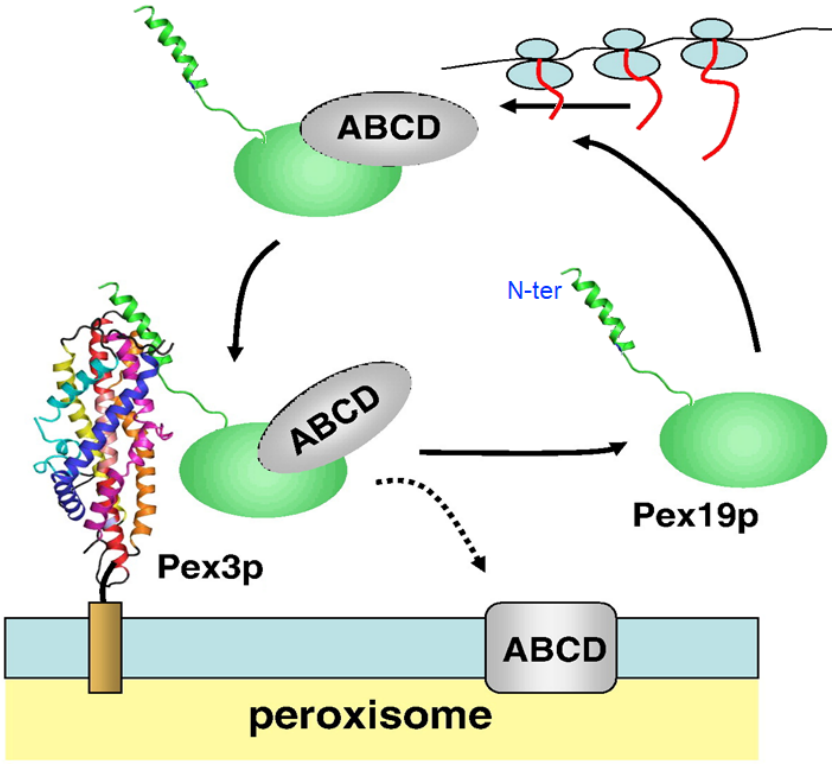
❽ Small Px enlarge by incorporation of more PxMP and PxMxP



Role di Pex19 & Pex3 in PxMP insertion

- This is illustrated by the targeting of the **ABCD1 transporter** to the peroxisomal membrane.
  - ABCD1 is translated on free ribosomes and selectively captured by Pex19p in the cytosol
  - Pex19p acts both as a cytosolic receptor and chaperone, interacting with hydrophobic TMD of ABCD1, keeping it stable and with a proper conformation in the cytosol
  - The ABCD1/Pex19p complex binds to Pex3p via a hydrophobic, helical Nt binding motif
  - ABCD1 is inserted into the peroxisomal membrane and Pex19p shuttles back to the cytosol
  - The insertion mechanism is as yet unclear





Peroxisome division by fission

- **Model of peroxisomal growth and division in mammalian cells**

❶ **activation of Pex11β** initiates **membrane remodelling and tubular membrane extension**

❷ **amphipathic α-helix** nature of Pex11β causes membrane bending (AMP-like behaviour)

❸ the membrane extension **acquires more PxMP** (e.g. Pex11β, Fis1**)**

❹ It then constricts and **acquires newly synthesized PxMxP**

❺ Pex11pβ and Mff complex concentrate at **constriction sites** and **DLP1 is recruited**

❻ The DLP1/Mff complex forms a ring structure that hydrolyses GTP and **severs the membrane**

- Mff = mitochondrial fission factor, and with DLP1, Fis1 act also in mitochondrial fission