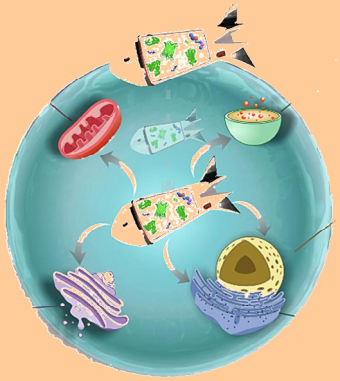
Cellular Biochemistry [918SM]



Module 4

Protein Sorting Pathways I

4_1 Vesicular traffick

4_2 Exocytosis & endocytosis

4_3 Nonvesicular traffick

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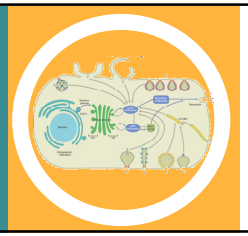
AA 2026-2027

Cellular Biochemistry [918SM]



Modul4_1

Vescicular trafficking

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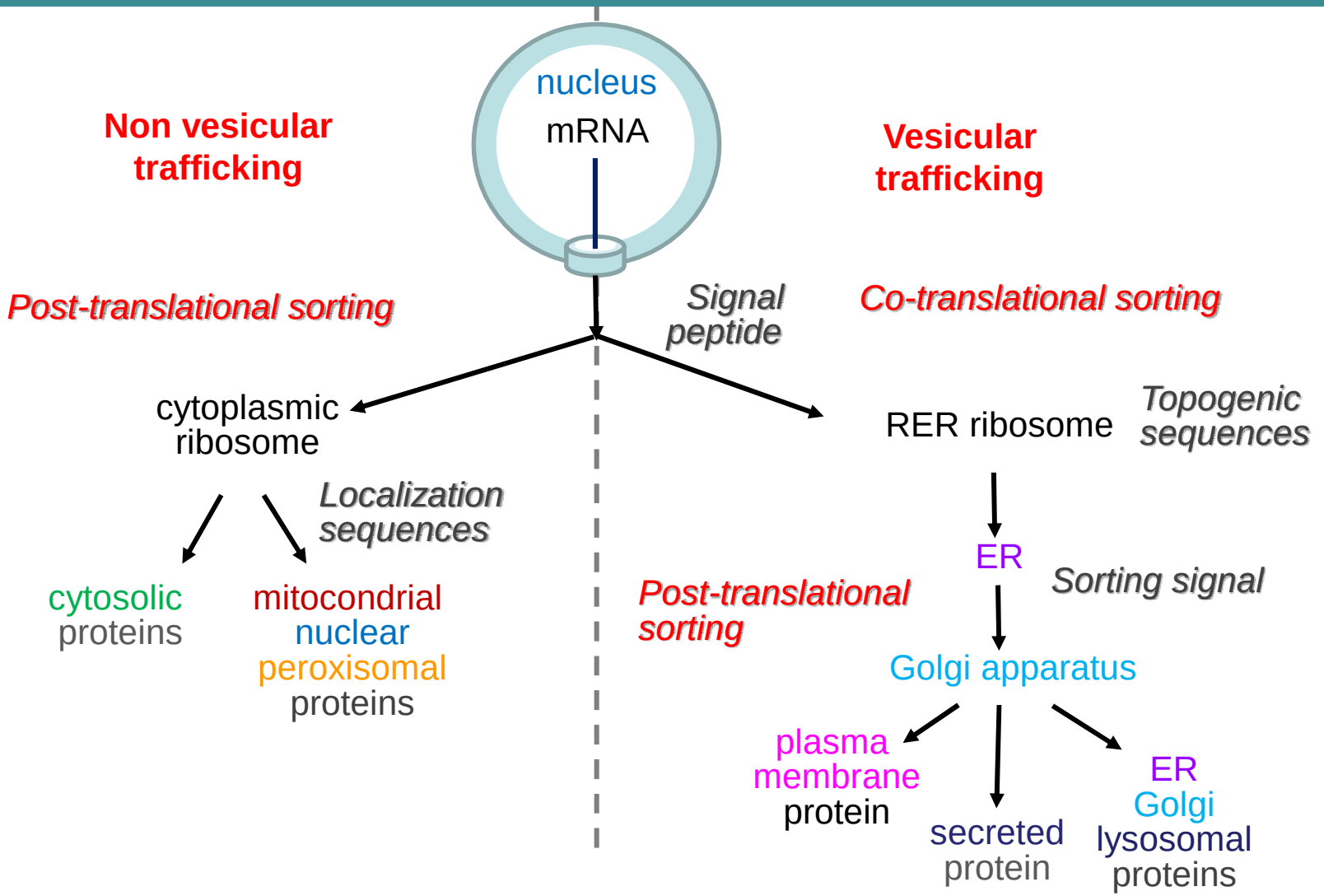
AA 2026-2027

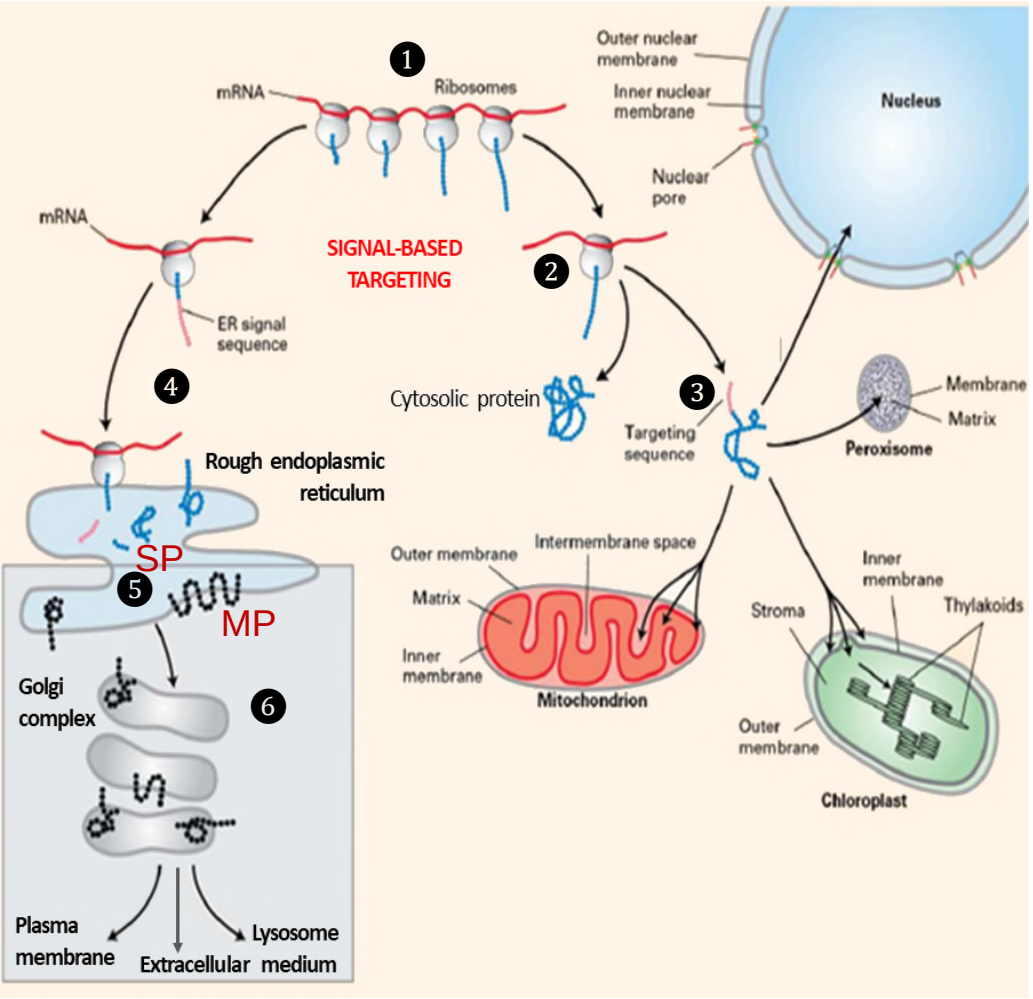
PROTEIN TRAFFICKING



Signal peptide:	N-terminal AA sequence interacting with signal recognition particle
Localization sequences:	AA signal sequences in proteins
Topogenic sequences:	AA signal sequences in proteins
Sorting signals:	AA signal sequences in proteins or PTMs (glycosylation)

SORTING PATHWAYS

Major protein-sorting* pathways in eukaryotic cells -



❶ mRNA; nucleus → cytosolic ribosome

❷ No signal peptide:

protein translation → cytosol

❸ Specific localization sequences:

proteins → mitochondria/nucleus etc.

❹ Signal peptide for ER :

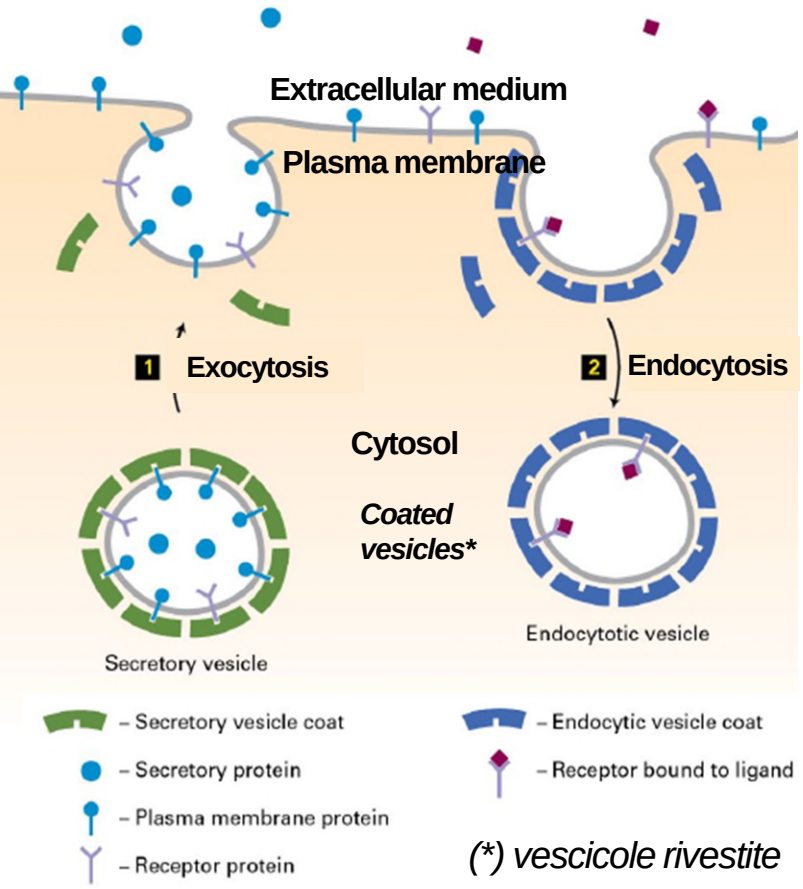
Ribosome + SRP(†) → RER

❺ protein → ER lumen (soluble proteins SP)
ER membrane (membrane proteins – MP)

❻ Vescicular trafficking of proteins:
ER → Golgi complex → plasma membrane
→ lysosome (LS)
→ LS membrane
→ secretion

sorting = smistamento

Basic principles of vesicular trafficking



(*) *vescicole rivestite*

Trafficking is required for:

1 Delivery of membrane proteins (MP) to plasma /organelle membranes and soluble proteins (SP) to organelle lumens or to the extracellular medium (secretion) from cells (Exocytosis)

- It makes use of coated vescicles*. The coat proteins are *i)* necessary for vescicles formation, *ii)* determine the type of protein cargo and *iii)* destination
- Cargo proteins (SP & MP) preserve their topology; secretory and organelle-directed SP never enter the cytosol. They bind to cargo receptors. For MP the domains facing *exo* will face the vesicle interior and then the extracellular medium or organelle lumen.

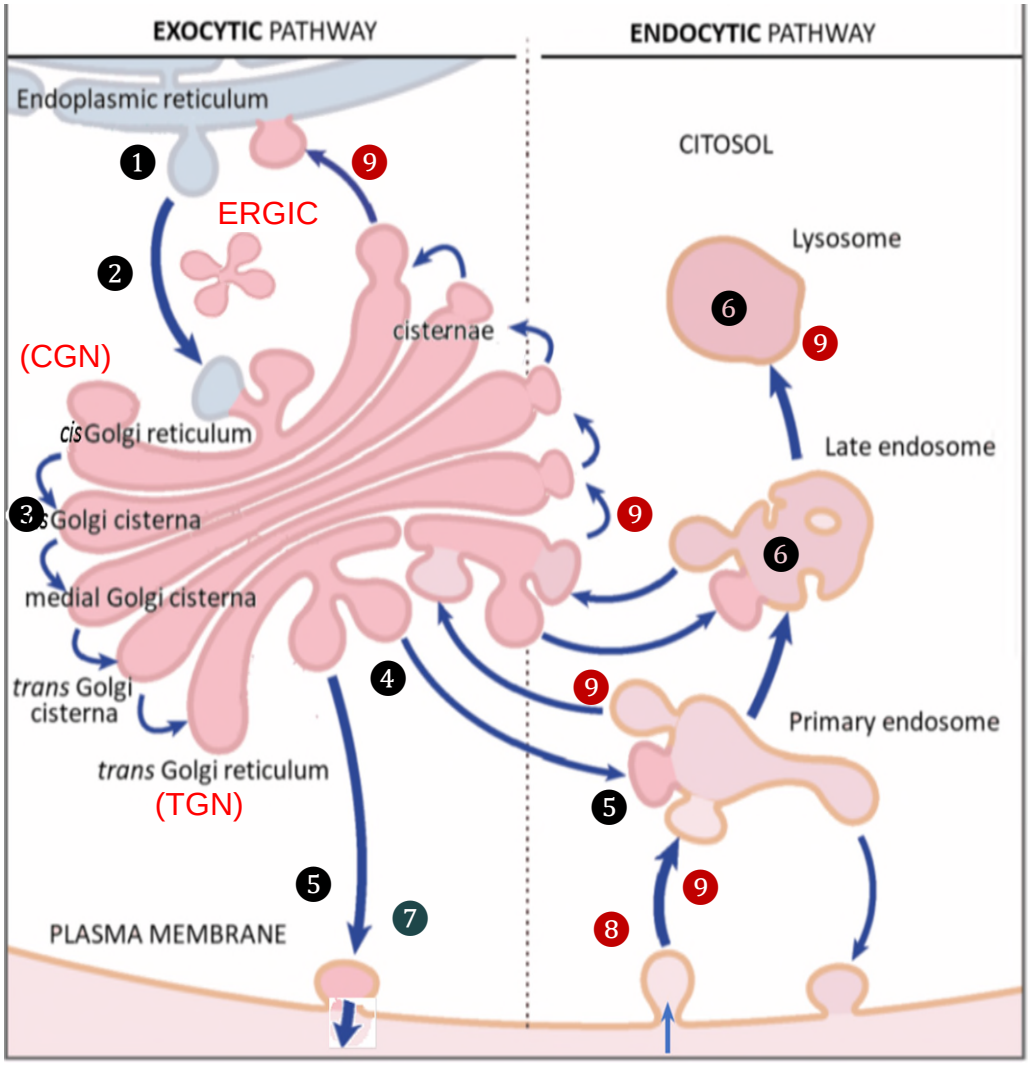
2 Protein internalization into cells (Endocytosis) uses similar mechanisms and serves for:

- internalization of activated receptors allowing to modulate receptor activity
- internalization of large nutrient molecules or of lipoproteins (e.g. LDL)

Secretory (protein export) and Endocytic (protein import) pathways are correlated

VESICULAR TRAFFICKING (CONT.)

Major routes in the secretory and endocytic pathways



❶ Vesicles bud* from ER, carrying soluble proteins (SP) or membrane proteins (MP)

❷ In animal cells, they fuse to form the ER-Golgi intermediate compartment (ERGIC) which matures into the cis-Golgi network (*CGN*).

❸ Golgi cisternae progress *cis* → *trans* to form the *trans*-Golgi network (*TGN*)

❹ Vesicles bud from *TGN* and ❺ fuse with the plasma membrane (PM) or endosomes

❻ Early endosomes (EE) (*primari*) mature to late endosome (LE) (*tardivi*) and then to the Lysosome (LS)

SP/MP exit cells by ❼ Exocytosis and enter by ❽ Endocytosis.

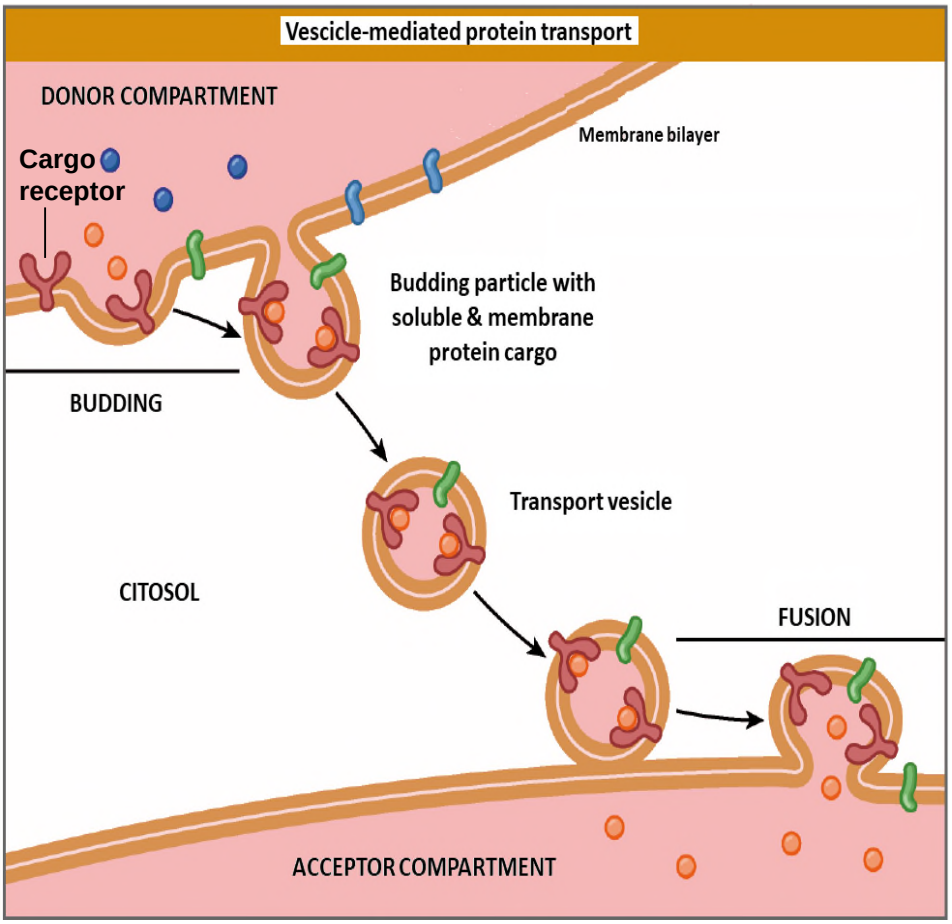
** to bud = germinare*

❶-❻ Anterograde Vesicles move SP/MP from ER → Golgi → PM/LS

❾ Retrograde Vesicles move SP/MP from PM → Early endosome → Lysosome or Golgi → ER

VESICULAR TRANSPORT

Unifying principle for protein trafficking in secretory and endocytic pathways



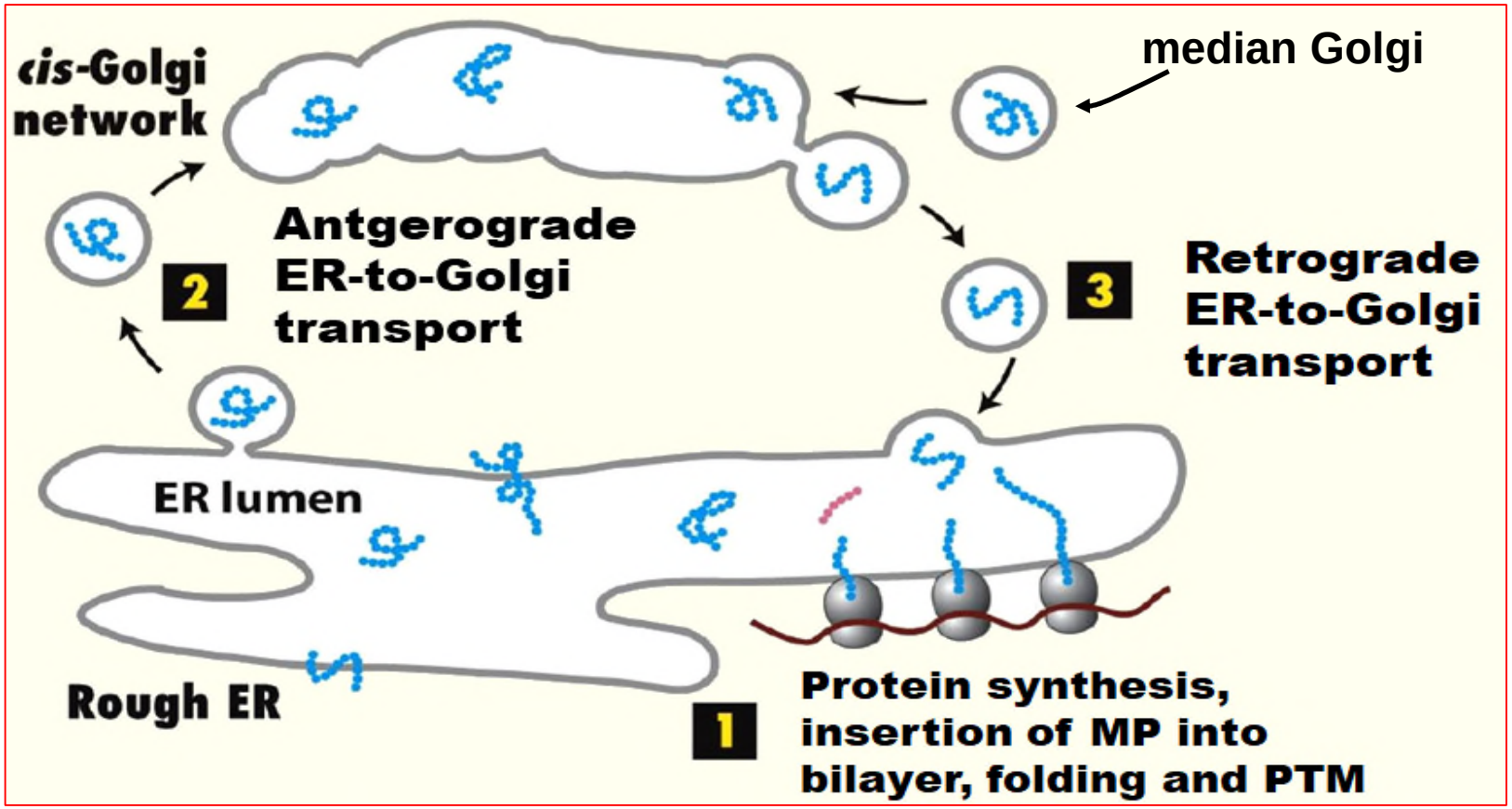
* germinazione

- SP/MP transport between compartments is mediated by transport vesicles
- Budding*: *"Cargo" proteins* collect in buds that form on the membrane of the donor compartment and fuse with membrane of the acceptor compartment to deliver their cargo (MP and SP on cargo receptors)
- Each step in the secretory and endocytic pathways uses a different type of vesicle
- The different vesicular transport steps are simply a variation on a common theme:

i) coating & budding from donor compartment
ii) uncoating of vesicle
iii) transport along cytoskeleton tracks (actin filaments or microtubules)
iv) fusion with acceptor compartment

TOPOLOGICAL RESTRAINT:
During vesicular traffic the same membrane face is always oriented toward the cytosol (*cyto / exo* orientation is always maintained)

ANTEROGRADE AND RETROGRADE TRANSPORT



❶ Protein synthesis in ER-bound ribosomes:

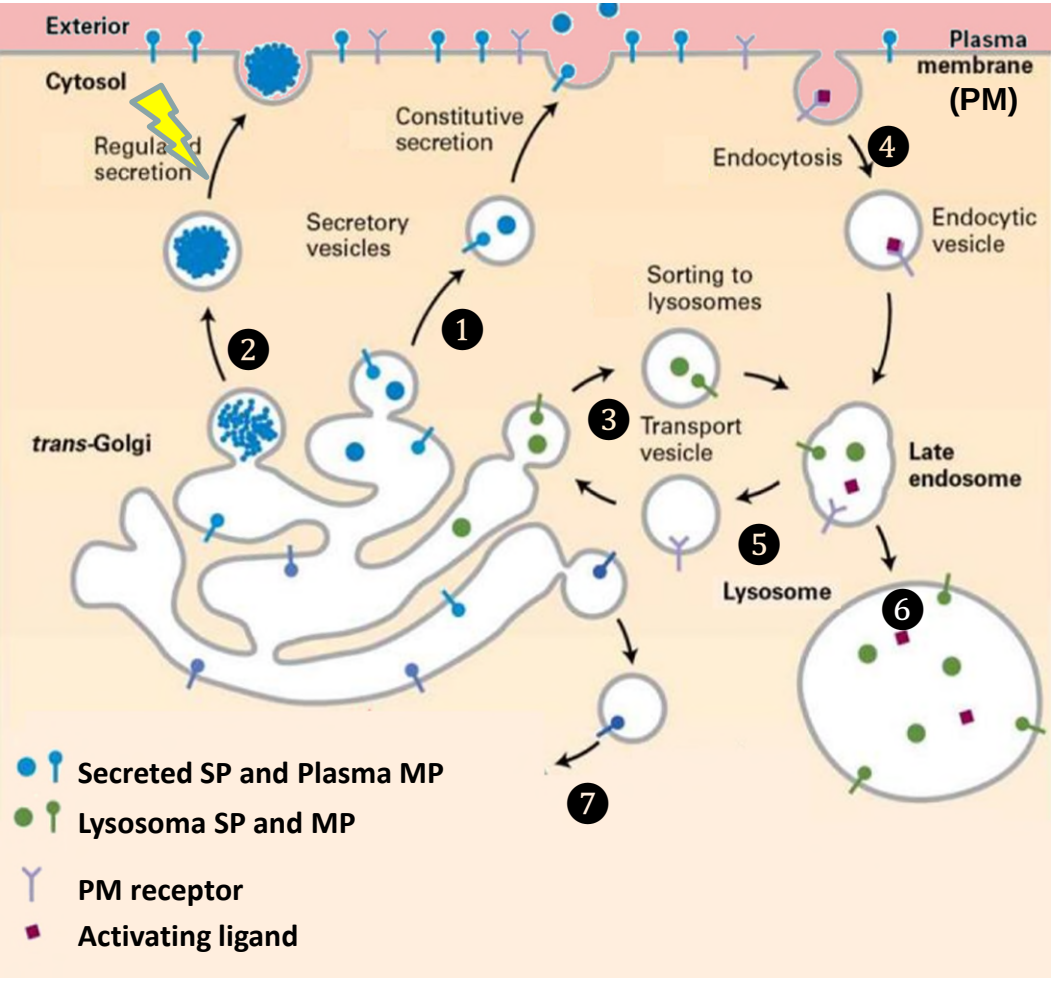
SP → folding/PTM MP → co-translational insertion in ER membrane/folding/PTM

❷ Anterograde vesicle bud from ER → fusion to ERGIC → formation of *cis*-Golgi reticulum

❸ Retrograde vesicles bud from *cis*-Golgi → fusion to ER

These vesicles carry ER-resident SP incorrectly sorted to golgi or ER MP for reuse (e.g. SP cargo receptors)

EXOCYTOSIS AND ENDOCYTOSIS



Exocytosis

Vesicles transport SP/MP cargo from *trans*-Golgi → PM after which:

1. Some SP are ***secreted continuously*** (Constitutive secretion)
2. In some cells SP are stored in ***secretory vesicles*** that require neuronal or hormonal signals for exocytosis (Regulated secretion)
3. Anterograde vesicles can also transport SP/MP cargo to lysosomes

trans-Golgi → late endosomes → lysosome

Endocytosis

4. Plasma MP (eg. activated receptors) taken up by endocytic vesicles budding from PM: PM → endosomes

Endocytosed material in the endosome can either:

5 1. be recycled to the plasma membrane (early E → recycling E → PM) e.g. receptor recycling
6. be degraded in the lysosome (early E → late E → lysosome → dismantled) cargo, e.g. LDL
7. enter retrograde transport (Endosome → TGN →→→ CGN → ER) rarer e.g. some toxins

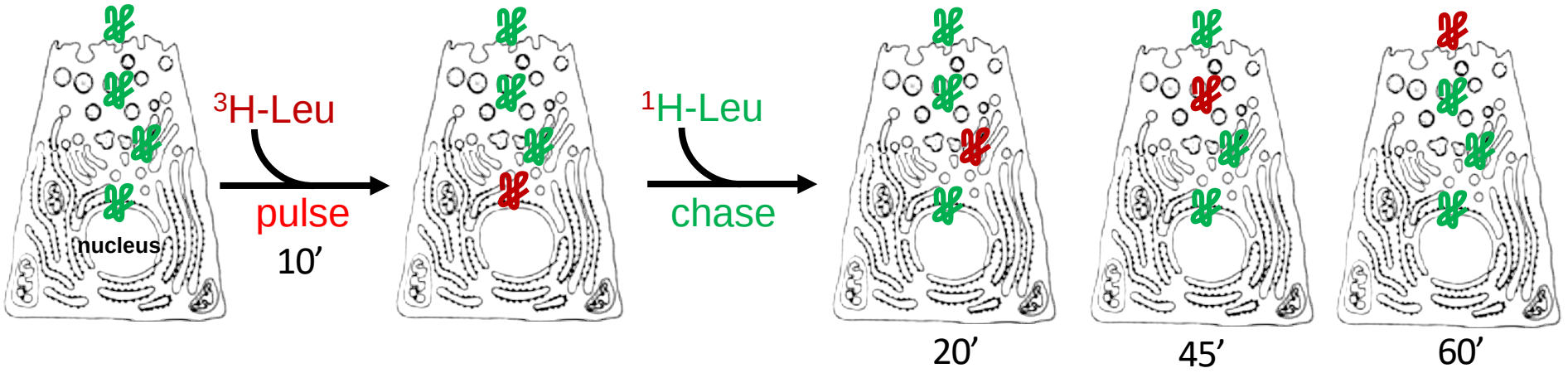
VESICULAR TRAFFICKING – EXPERIMENTAL VERIFICATION

Classic methods used to establish the different steps of vesicular protein transport

- The components required for formation and fusion of transport vesicles were identified in the 2nd half of 20th century by a remarkable convergence of classic biochemical and genetic approaches
- These studies were carried out by G. Palade (1960s, Nobel) on *live cells* to establish:

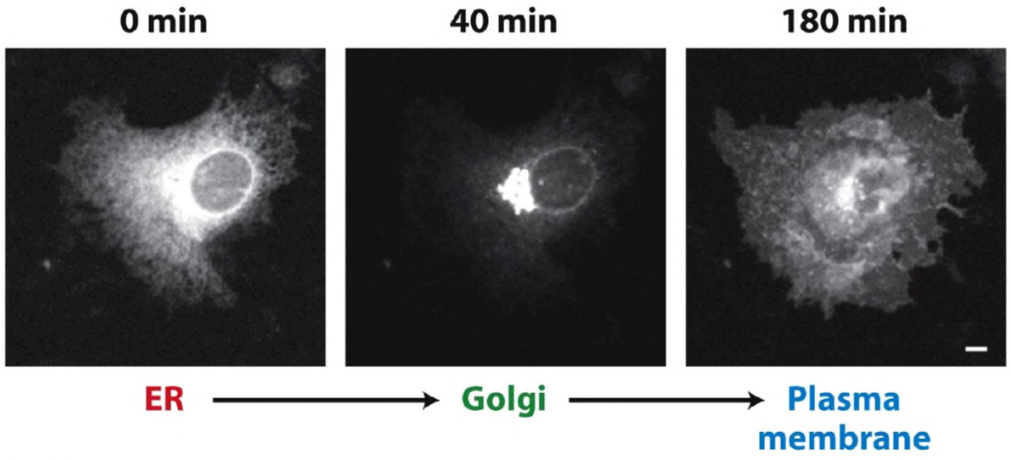
1) the order in which proteins move from organelle to organelle in the secretory pathway.
2) that secretory proteins were ***never*** released into the cytosol (*cyto /exo* conservation)

- They involved pulse-chase techniques. Radioactive amino acid (e.g. ^{3}H-Leu) were injected for a *brief period* (pulse) into experimental animal tissue (e.g. pancreas). Newly synthesized proteins (e.g. the secretory protein *Insulin*) incorporate ^{3}H-AA and are radioactively marked. Incorporation was then stopped by injecting cold ^{1}H-Leu (chase).
- At different times after injection, pancreatic cells were extracted, immediately fixed and the location of radioactively labelled protein (e.g. ^{3}H-Insulin) determined by autoradiography.
- Later, proteins could be safely & efficiently labelled using genetic methods (e.g. GFP fusion).

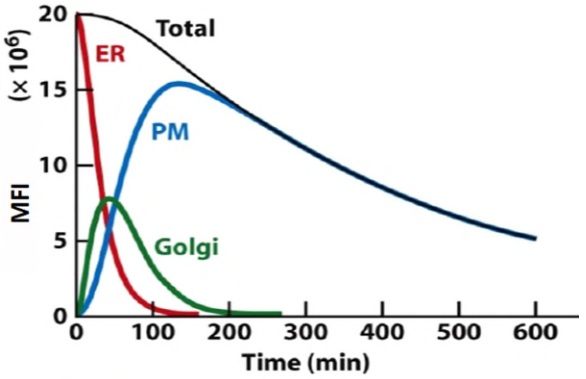


Later methods used to establish the different steps of vesicular transport

- Fluorescence microscopy: procedure for observing intracellular trafficking of a secretory protein in almost any type of cell, using the VSVG-GFP fusion protein (> 1970s)
- A gene encoding Vesicular Stomatitis Virus membrane glycoprotein *G* (VSVG) was fused to GFP (VSVG-GFP) and transfected into cultured mammalian cells.
- $VSVG^{ts}$, a temperature-sensitive mutant was used to form $VSVG^{ts}$-GFP, which folds at 32º C (*permissive temperature,* T^{p}) but denatures at 40º C (*non-permissive temperature,* T^{np})
- Protein movement can be blocked at any time by increasing the temperature:
  - *i)* at T^{p} (32˚ C) $VSVG^{ts}$-GFP folds and transport is allowed
  - *ii)* at T^{np} (40˚ C) $VSVG^{ts}$-GFP misfolds (partly denatures) and transport is turned off
  - *iii)* reversible process; returning to 32º C $VSVG^{ts}$-GFP refolds and transport resumes
- Allows to establish transit kinetics



Mean fluorescence intensity vs time



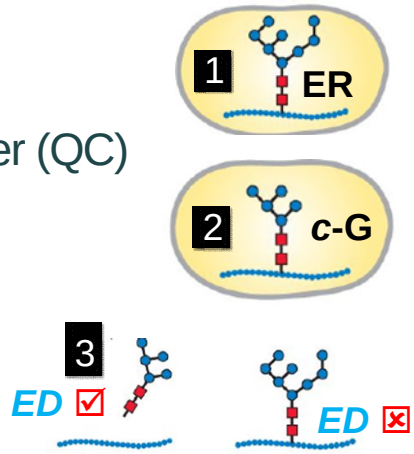
Monitoring PTM – mannose trimming of secreted proteins

– Other procedures for observing the progress of VSVG is to follow its PTM

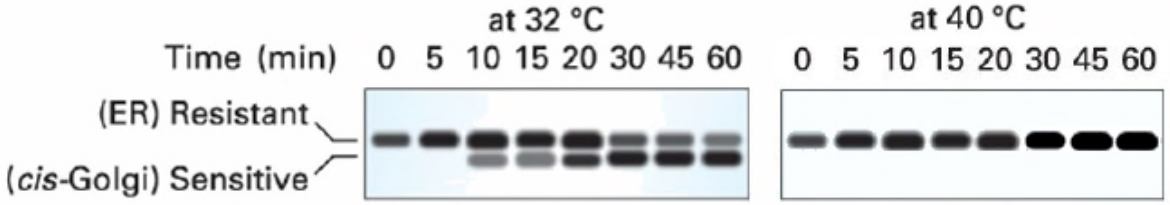
1 VSVG N-linked glycosylation in ER → $Man_8(GlcNAc)_2$ before Golgi transfer (QC)

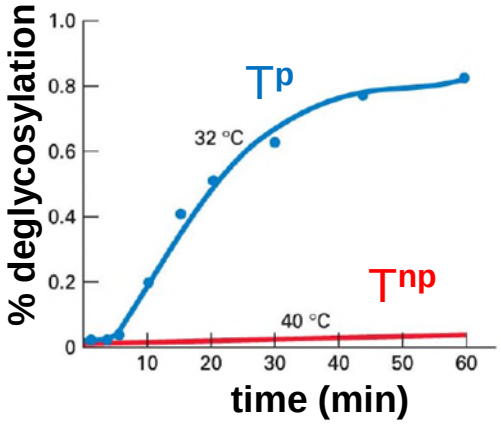
2 3 Man residues trimmed* in *cis*-Golgi by *Glycosidases* to $Man_5(GlcNAc)_2$

3 *Endoglycosidase D* can selectively cleave $Man_5(GlcNAc)_2$ making the protein lighter in gel electrophoresis but cannot cleave $Man_8(GlcNAc)_2$



– By extracting VSVG at different times and at different temperatures, its progress from ER to the Golgi can be followed.





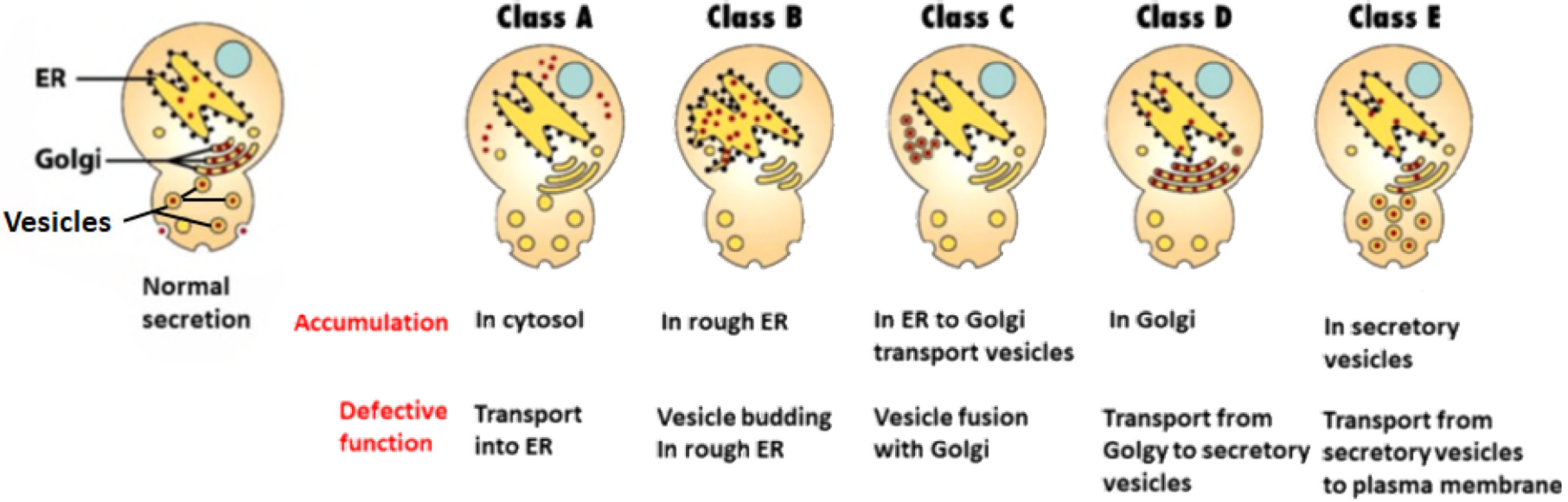
– This experiment allows to monitor the transit time from ER to *cis*-Golgi

– Other assays use specific later carbohydrate modifications that occur in Golgi compartments as they mature (*see slide 28*) to monitor the progression of VSVG through the Golgi complex.

* rimossi

Sec mutants in yeast cells (haploid yeast cells)

- Temperature-sensitive yeast *sec*^{ts} mutants used to identify the stages in the secretory pathway
- Mutated genes code for proteins that are essential components of the secretory pathway
- Permissive temperature (T^p): mutant proteins allow transport and secretion when folded were
- Non-permissive temperature (T^{np}): mutant proteins unstable and don't function, transport stops
- 5 classes of *sec* proteins identified (A–E) → protein accumulation in different compartments.

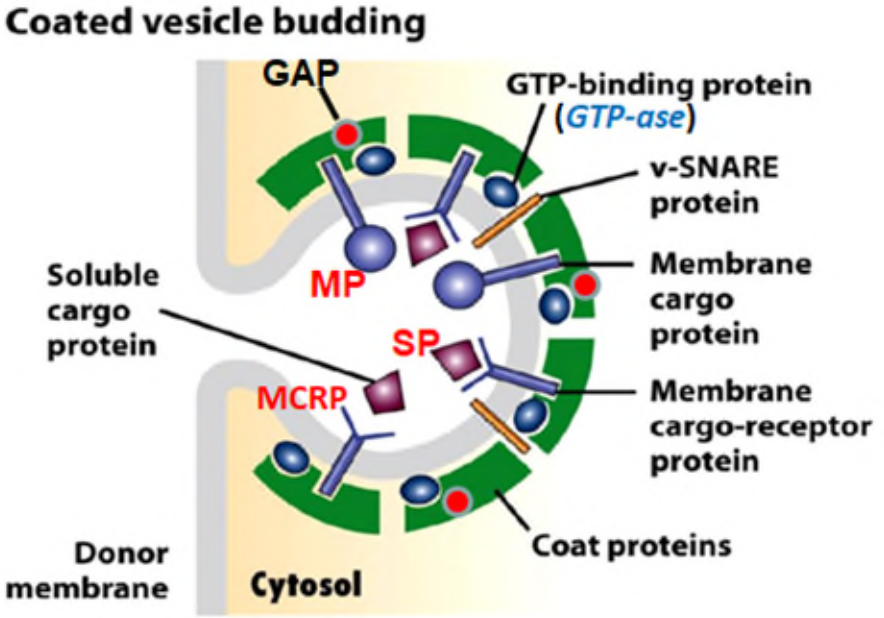


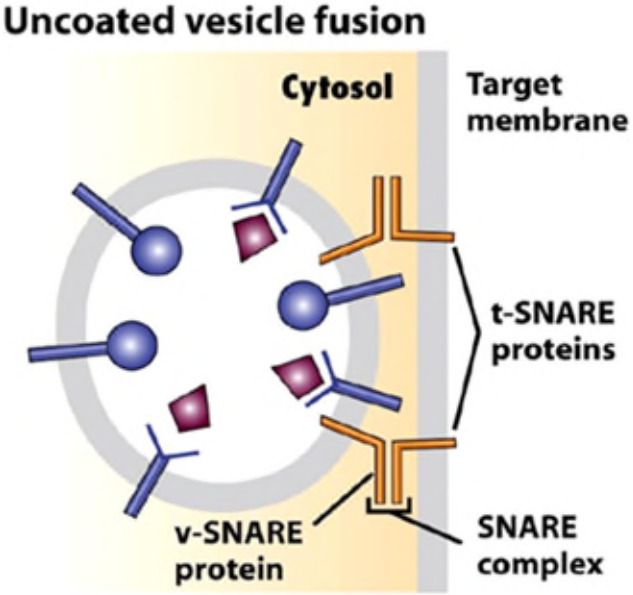
- When T^p → T^{np}, protein transport is blocked and identifies the particular point where the mutant pathway component is normally functioning.
- Analysis of haploid double *sec* mutants determined the order of the steps in the pathway.

VESICULAR BUDDING & FUSION

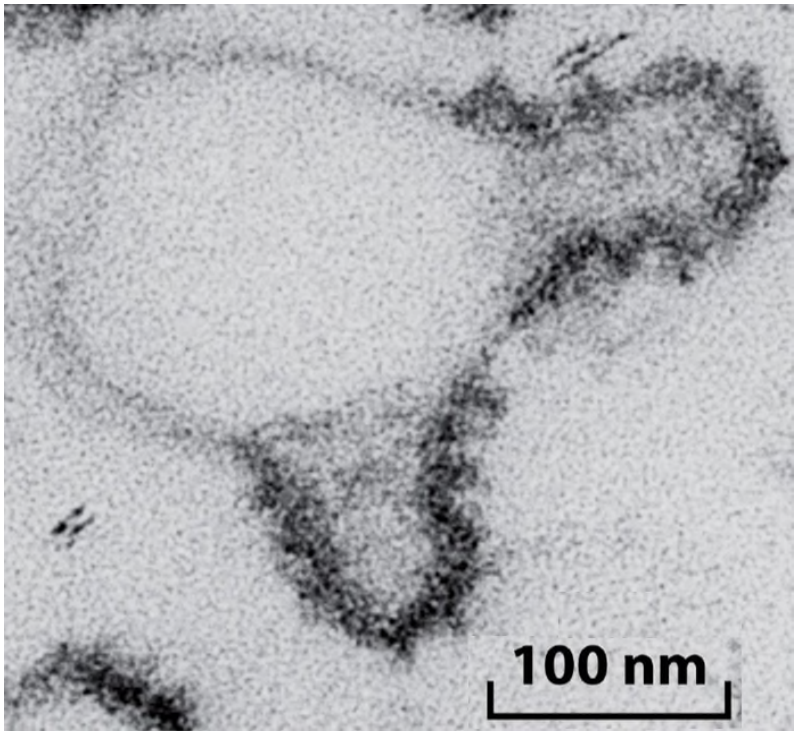
Basic molecular mechanisms of vesicle budding and fusion – 1

- Vesicle budding is driven by polymerization of coatamer proteins (COP) on the cytoplasmic side of the donor membrane, forming a proteinaceous coat around the vesicle
- Polymerization & budding requires a GTP-binding protein belonging to the *GTP-ase* superfamily, cycling from GDP-bound to GTP-bound forms. Curvature activates a GAP activity in a COP.
- Coatamer proteins (COP): *i)* when polymerized impose curvature to the budding vesicle
  i) recruit specific cargo MP and SP (via Membrane Cargo-Receptor Proteins - MCRP).
  iii) recruit v-SNARE proteins required for subsequent fusion of vesicle with acceptor membrane.
- After budding the coat is lost – due to GTP hydrolysis in *GTP-ase* mediated by the GAP activity (*GTPase activating proteins*), allowing fusion with acceptor membrane via SNARE interactions.





VESICULAR BUDDING & FUSION (cont.)



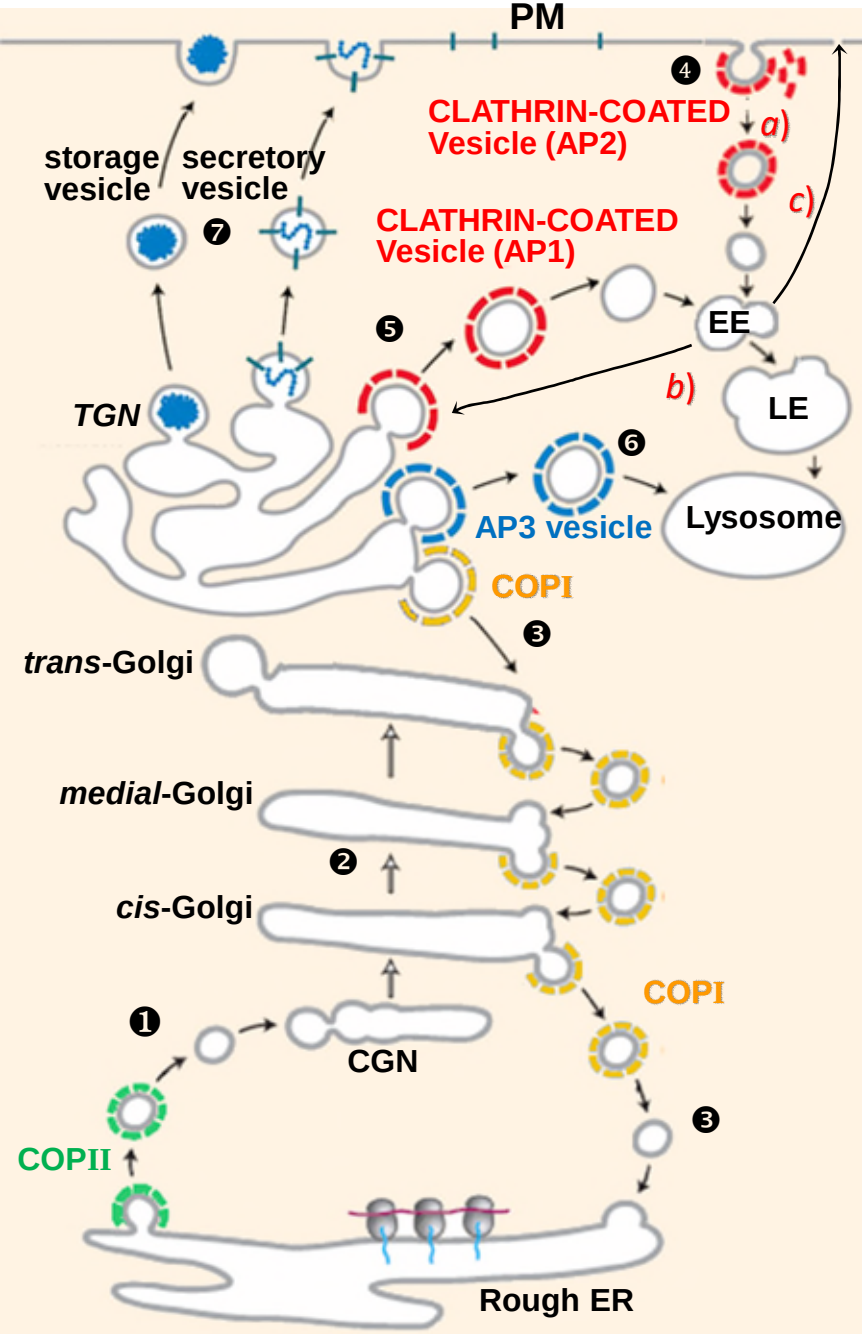
Basic molecular mechanisms – 1

- COPs are complexes with multiple subunits
- Budding can be studied *in vitro* using isolated or artificial membranes and purified COP.
- COP polymerize on the cytosolic face of the donor membrane forming a curved lattice*
- The lattice imposes high curvature typical of transport vesicles (∅ ~50 nm), and drives vesicle formation.
- 3 different types of vesicles are known that use different COPs and different G-proteins.

* reticolo

Vesicle Type	Donor M → Acceptor M	Coat protein subunits	*GTPase*
COPII	ER → *cis*-Golgi network	Sec23/Sec24; Sec13/Sec31; Sec16	*Sar1*
COPI	- *cis*-GN → ER - Retrograde transport (Golgi)	seven different Sec subunits	*ARF*
Clathrin coated	- *trans*-GN → endosome	Clathrin + AP1 or Clathrin + GGA	*ARF*
	- PM → endosome	Clathrin + AP2 (endocytic)	*ARF*
	- *t*-GN → lysosome	AP3	*ARF*

MAJOR TYPES OF COP VESICLES



COPII vesicles - anterograde

❶ transport proteins rough ER → *cis*-Golgi.

– move along microtubulin 'highways'

– loose COP, fuse to ERGIC → *cis*-Golgi reticulum

❷ cargo progression *cis*- → *medial*- → *trans*-Golgi (Golgi cisternal maturation)(not vesicular)

COPI vesicles - retrograde

❸ *trans*- → *medial*- → *cis*-Golgi → rough ER.

AP2 Clathrin-coated vesicles

❹ *a)* PM → EE → LE → lysosomes (degradation)

b) PM → EE → TGN (retrograde transport)

c) PM →EE →PM (recycling)

AP1 Clathrin-coated vesicles

❺ TGN → EE → LE → Lysosome (lysosomal cargo)

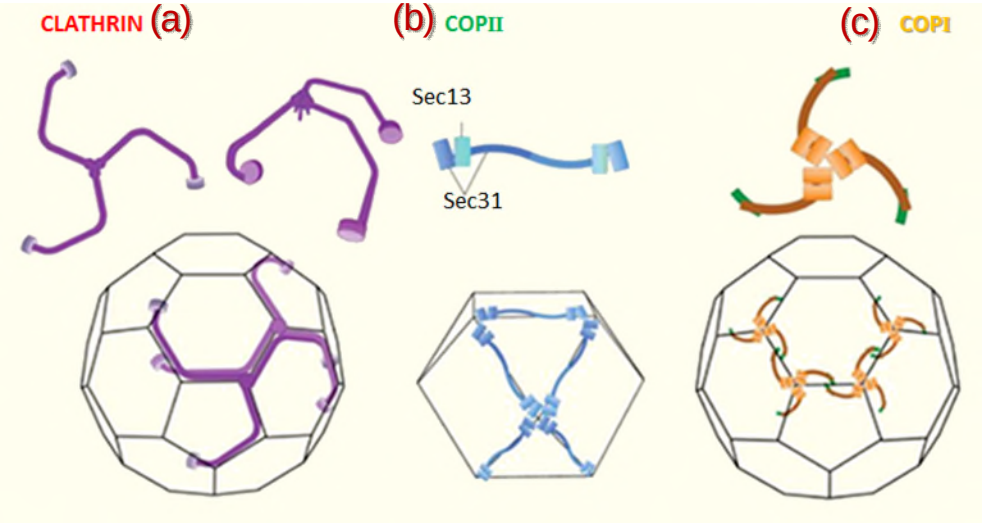
AP3-Coated vesicles

❻ TGN → Lysosome (lysosomal cargo) *(clathrin?)*

Secretory/storage vesicles

❼ COP uncharacterized (Clathrin, AP1/AP2 ?)

VESICLE COAT (COP) ARCHITECTURE

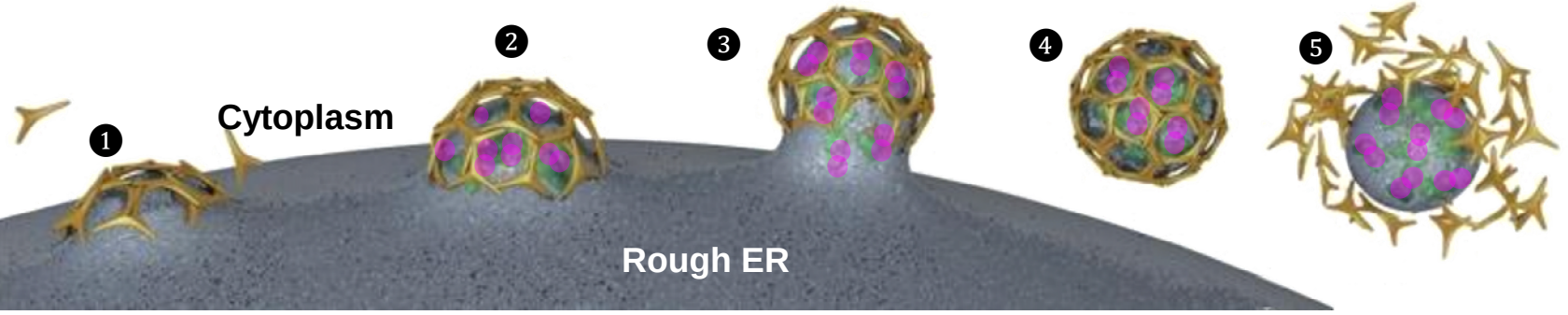


Structure

- (a) Clathrin triskelion forms an icosahedral coat in clathrin-coated vesicles ∅ 90 nm.
- (b) Sec31/Sec13 forms a tetramer and then a cuboctahedral coat (d) in COPII vesicles
- (c) Sec27 + other CoP triskelion forms an icosahedral lattice in COPI.

Assembly

1. When COP assemble at the membrane surface they force the lipid bilayer to curve.
2. As they assemble a polymeric lattice, they start packaging selected protein cargo.
3. As more COP are recruited, the coat shapes the enclosed membrane into a spheroid
4. The coated vesicle then pinches off* the membrane. Membrane curvature triggers GAPs
5. GTP hydrolysis triggers uncoating. Some proteins remain and mediate tethering to target membrane



* si stacca

GENERAL ARCHITECTURE OF TRANSPORT VESICLES

Membrane

Coatamer protein

Scaffold (Adaptor) Protein (AP)

Cargo MP

Cargo receptor

Cargo SP

ARF Sar1

GTPase/GTP

GAP

GEF

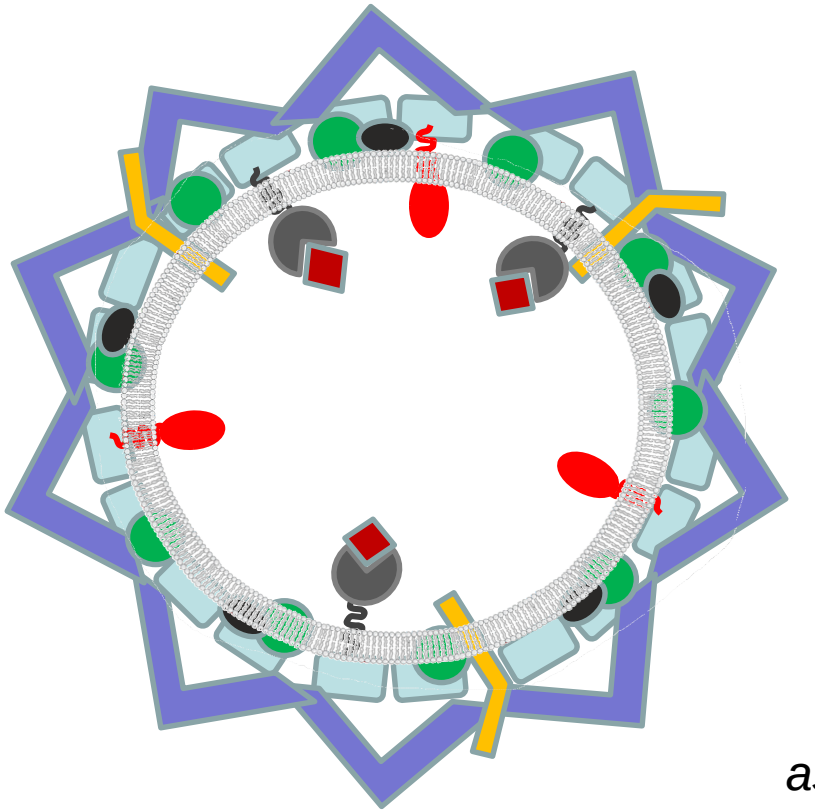
GTPase/GDP

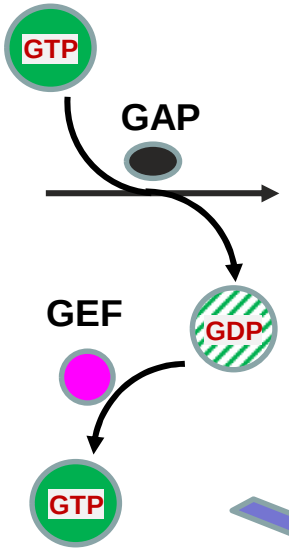
vSNARE

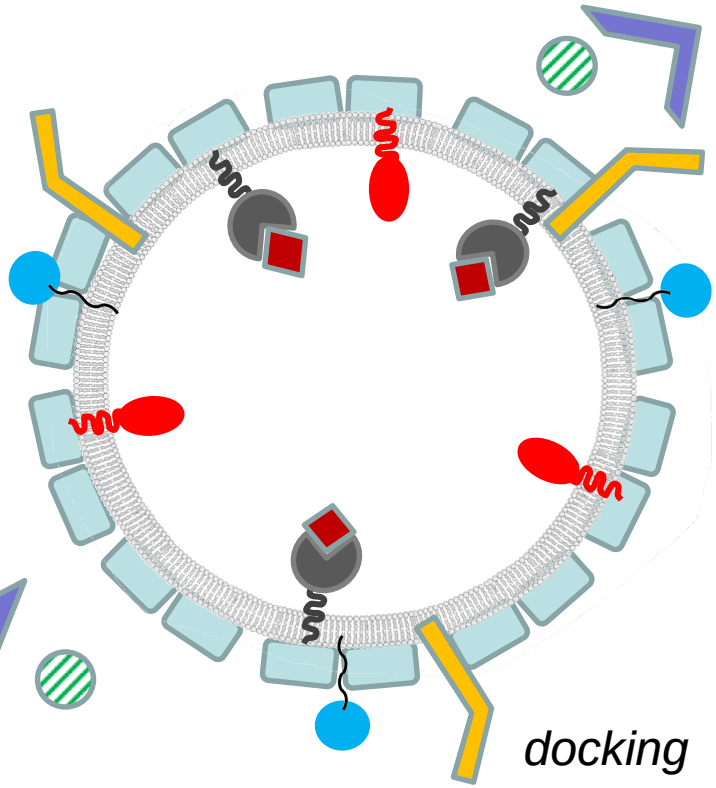
Rab GTPase

Coated vesicle (from Donor Membrane)

Uncoated vesicle (to Acceptor Mewmbrane)





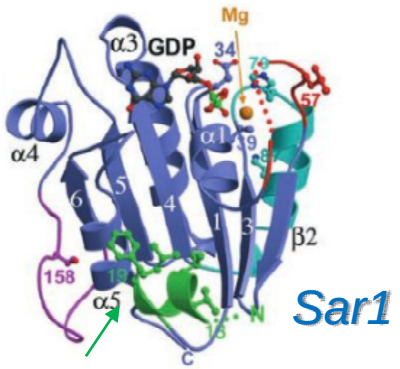


assembly/budding

docking

MOLECULAR MECHANISM OF VESICLE BUDDING

The coats of all three vesicle types contain GTP-binding proteins:

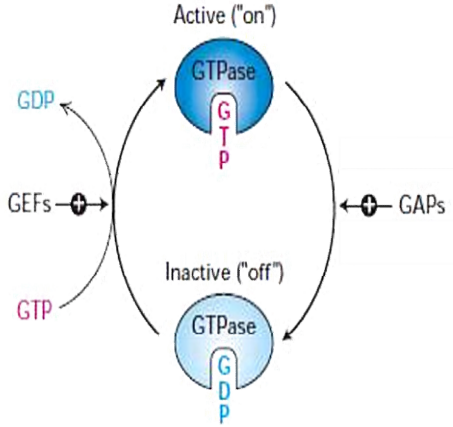


membrane anchor sequence

- these act as regulatory subunits for coat assembly/disassembly and belong to the *GTPase* superfamily - COPII → *Sar1*
  COPI → *ARF*
  clathrin vesicles → *ARF*
- They are small & monomeric and act as switch proteins: GDP → GTP exchange (activation) GTP hydrolysis (inactivation).
- similar in structure to the cytosolic *Ras G-proteins* (signalling) but not part of this family, and distinct also from *Rab GTPases* (see slides 23,24)
- GDP/GTP recruitment and exchange:

  activation is promoted by Guanine nucleotide-exchange factors (GEFs)

  inactivation is promoted by GTPase activating protein (GAP)

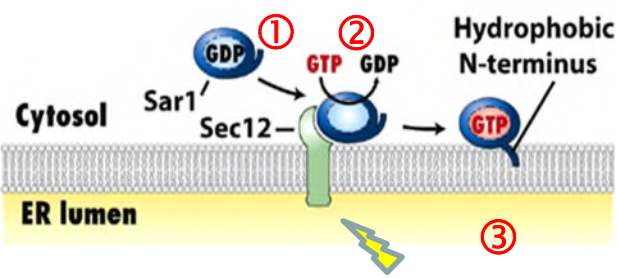


COP II activation

① ER membrane protein Sec12 is activated by the presence of protein cargo and recruits *Sar1*/GDP

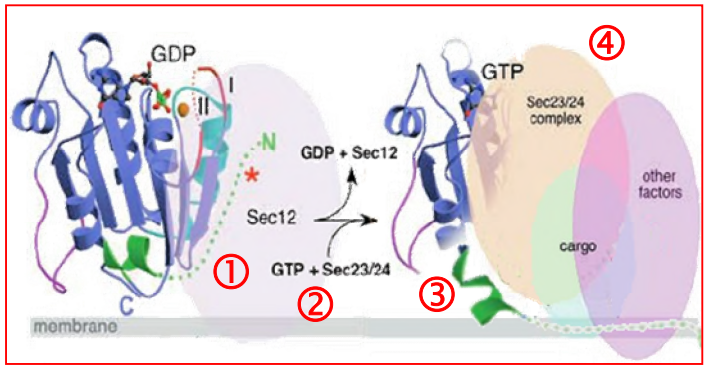
② Sec12 acts as GEF for *Sar1* promoting GDP/GTP exchange

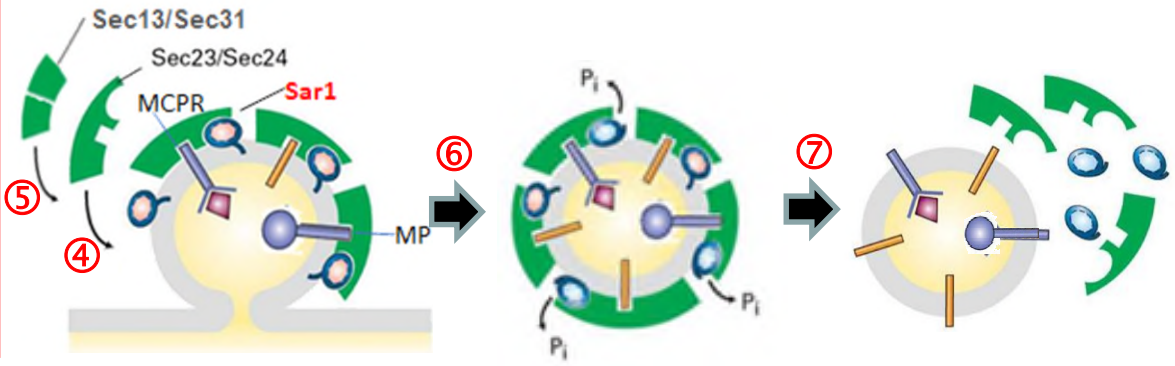
③ This causes a conformational change in *Sar1*, which exposes a hydrophobic helical sequence, allowing it to anchor to the ER membrane.



MOLECULAR MECHANISM OF VESICLE BUDDING (Cont.)

COP II activation (cont.)





– *Sar1* couples a cycle of GTP binding/hydrolysis to formation and dissociation of the COPII coat

④ *Sar1*-GTP recruits the COP complex Sec23/Sec24 and this complex recruits SP receptors (membrane cargo receptor protein MCRP) and cargo MP from the luminal side, recognising *specific sorting signal sequences*.

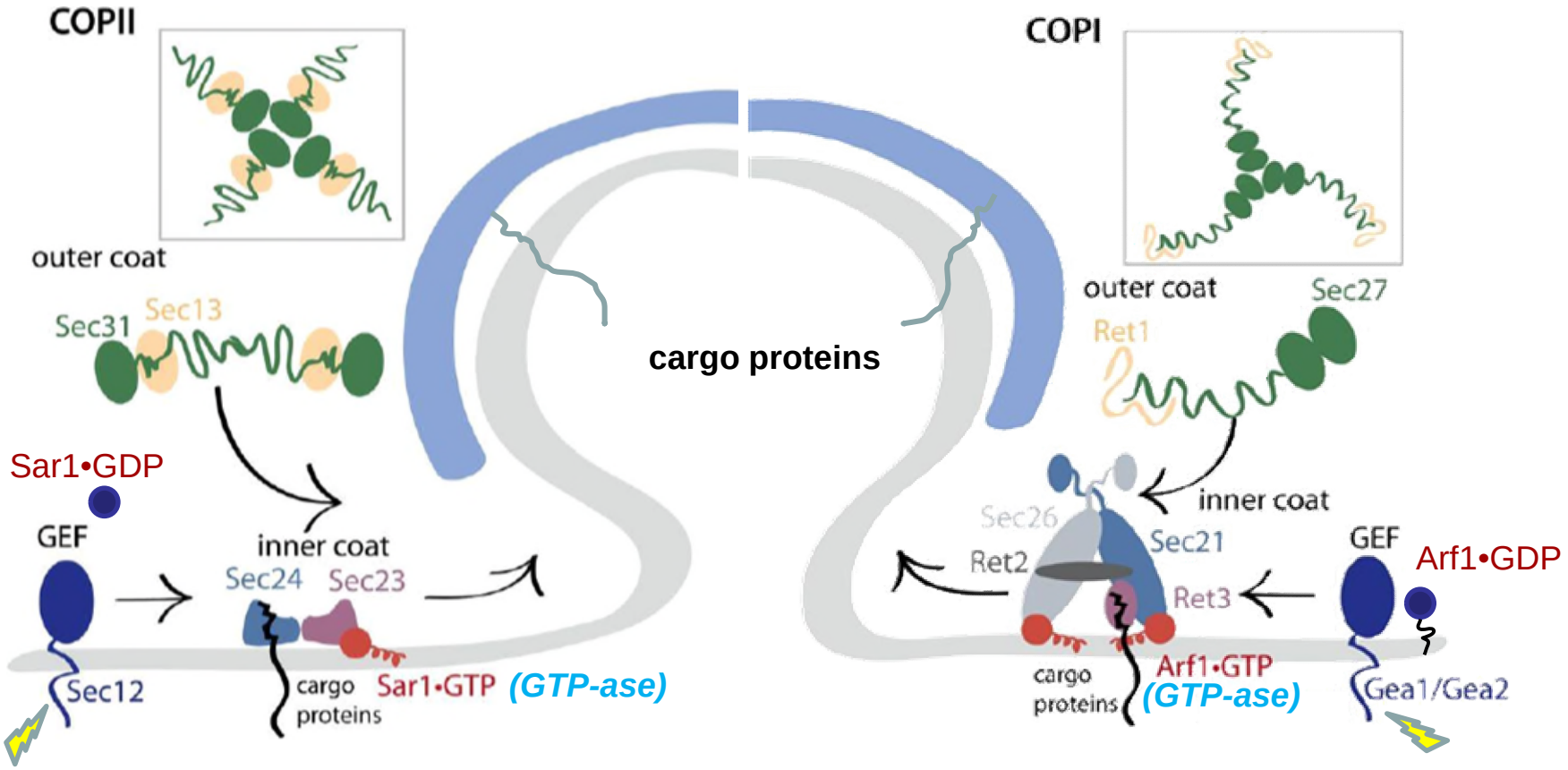
⑤ The coat is completed by recruitment of Sec13/Sec31 which forms the coatamer. *Sar1*-GTP drives polymerization and formation of the spheroid coat lattice.

⑥ *Pinching-off* requires *Sar1*-GTP, acting with an as yet unclear mechanism.

⑦ Sec23 is a GAP, activated by vesicle formation to promote hydrolysis to *Sar1*-GDP. This results in coat disassembly and vesicles can now fuse with target membranes

- *ARF* has a similar role in COPI and Clathrin-coated vesicles.
- This process is disrupted in *sec* mutants

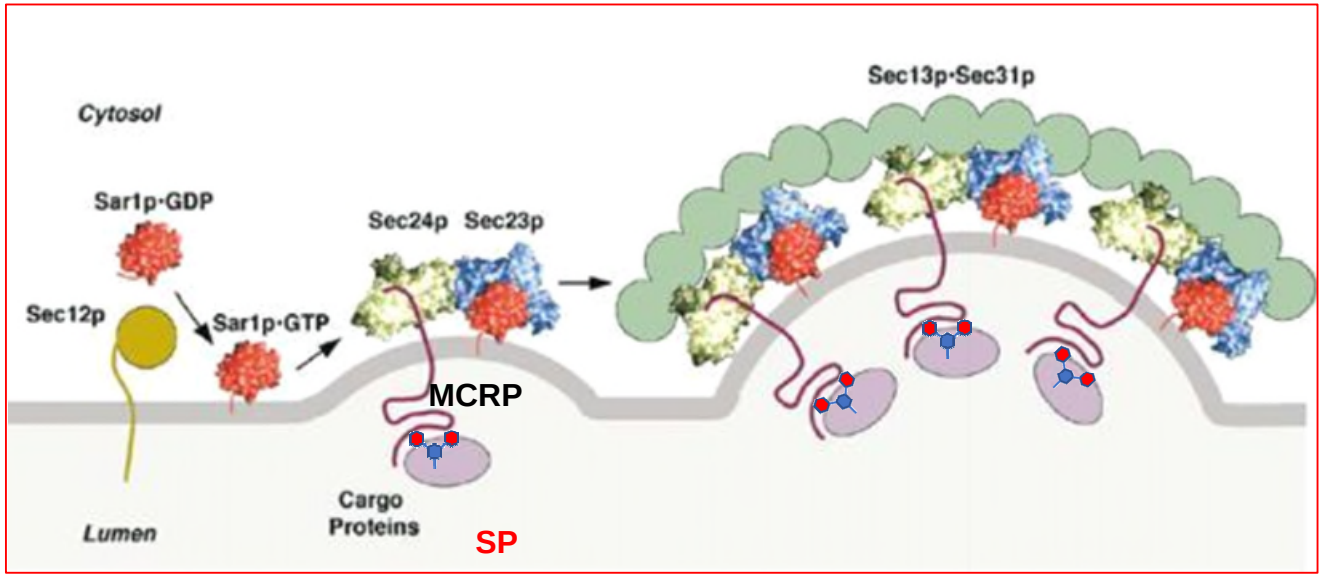
Molecular mechanism of vesicle budding – COPII vs COPI

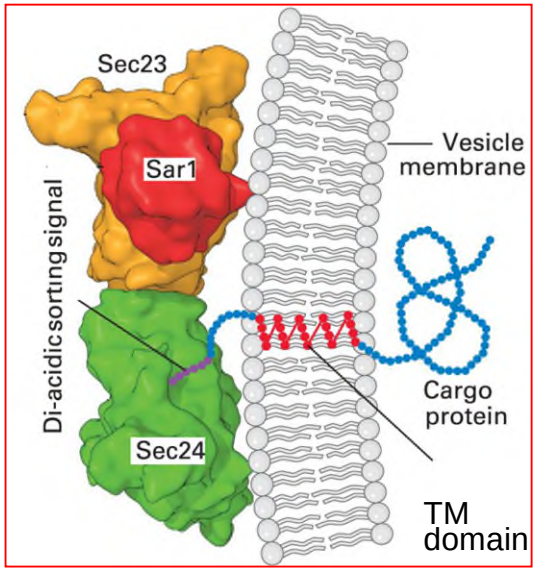


- COPII vesicles (left): Sec12 recruits *Sar1*-GTP to the membrane; *Sar1*-GTP recruits Sec23/Sec24 and then Sec13/Sec31 complexes to form the coat; Sec24 recruits cargo membrane proteins.
- COPI vesicles (right); Gea1/Gea2 complex recruits *ARF1*-GTP and this recruits Sec21/Ret3/ Sec26/Ret2 and then Sec27/Ret1 complexes to form coat; Ret3 recruits cargo membrane proteins.
- Sec13/Sec31 heterotetramers and Ret1/Sec27 triskelions polymerize to form the outer coats

MOLECULAR MECHANISM OF VESICLE BUDDING (Cont.)

Recruitment of cargo proteins into COPII and other vesicles





- Budding vesicle discriminate different MP & SP cargo to be transported to specific locations.
- MP specifically recruited into COPII have cytosolic segments with a diacidic sorting signal (DXE) towards the C-terminus which binds to a site in the **Sec24** subunit

- Glicosylated SP  bind to a **M**embrane **C**argo-**R**eceptor **P**rotein (MCRP) associated with the COPII for anterograde transport towards *cis*-Golgi.
- ER-resident SP have the sorting signal sequence KDEL and bind to the luminal domain of a MCRP (KDEL receptor) associated with **COPI** for retrograde transport

- MP that must return to the ER (e.g. the MCRP themselves) contain the KKXX sorting sequences and bind to **COPI** vesicle coat proteins for retrograde transport

MOLECULAR MECHANISM OF VESICLE BUDDING (Cont.)

Known sorting signals that direct proteins to specific transport vesicles

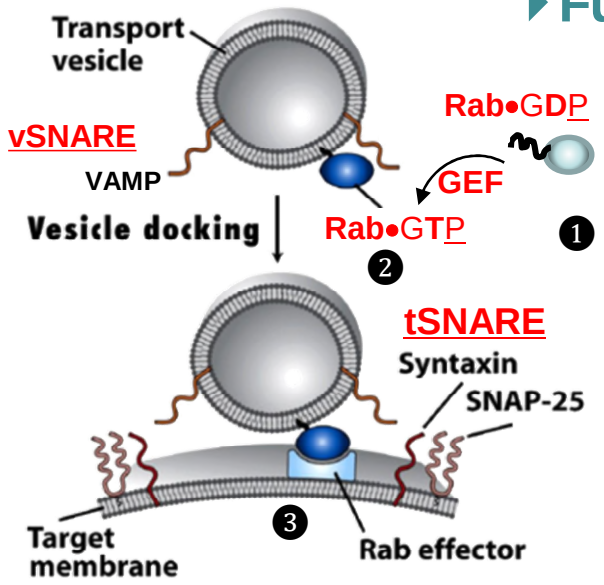
SORTED PROTEIN	SIGNAL	SIGNAL RECEPT.	VESICLE	TRAFFICK
ER proteins (luminal)	-KDEL-COOH	**KDEL receptor** (*c*Golgi membrane)	COPI	CGN→ER
ER proteins (membrane)	-KKXX-COOH	**COP (I) subunits**	COPI	CGN→ER
MCPR (cytosolic domain)	DXEXX-COOH	**COP (II) subunits** (Sec24)	COPII	ER→CGN→
Lysosomal SP (Golgi PTM)	Man-6-P	**Man-6-P receptor** (*t*Golgi membrane)	Clathrin/AP1	TGN→Lysosome
Lysosomal MP	-YXXF(ϕ)-	**AP1 subunit**	Clathrin/AP1	TGN→Lysosome
(Endocytosis) **M protein** (cytosolic domain)	-YXXF(ϕ)-	**AP2 subunit**	Clathrin/AP2	PM →Endosome
M protein (cytosolic domain)	-DXXXLL-	**AP2 complex**	Clathrin/AP2	PM →Endosome
DL recept. (cytosolic domain)	-NPXY-	**AP2 complex**	Clathrin/AP2	PM → Lysosome

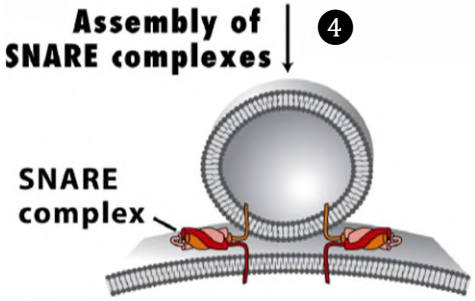
See later Slides

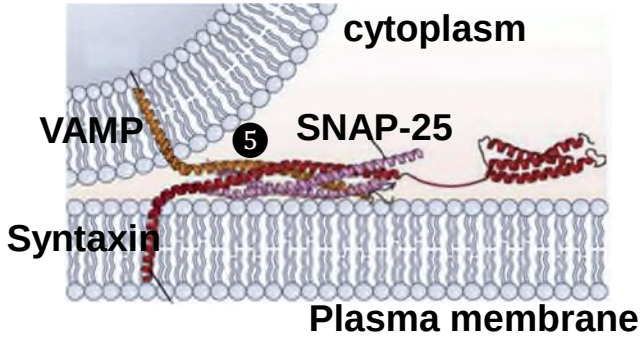
X = any AA, Φ = hydrophobic AA

MOLECULAR MECHANISM OF VESICLE FUSION

Fusion of uncoated vesicles with acceptor membranes







Process requires another set of *G-proteins* called *Rab*

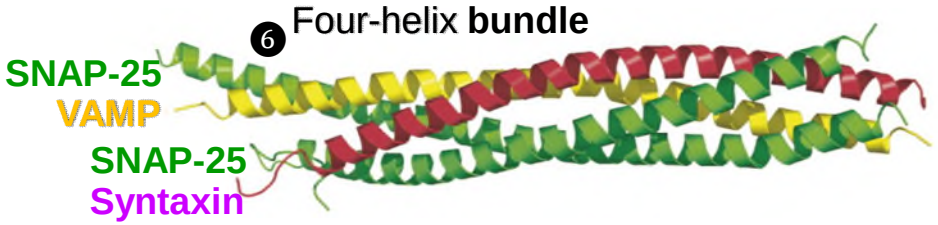
1. *Rab*-GDP are cytosolic proteins with an isoprenoid anchor
   - They belong to the *GTP-ase switch protein* superfamily
   - Specific *Rabs* are recognised by specific vesicle membrane proteins and anchor on the cytosolic side
   - *Rab*-GDP is kept cytosolic by GDP Dissociation Inhibitor (GDI) that prevents nucleotide exchange and hides anchor.

A **GEF** promotes exchange to *Rab*-GTP, which allows 2 lipid anchor exposure and binding to vescicle and 3 *Rab*-GTP to dock to Rab effectors in the acceptor membrane.

4 Cognate vSNAREs and tSNARES interact, allowing membrane fusion.

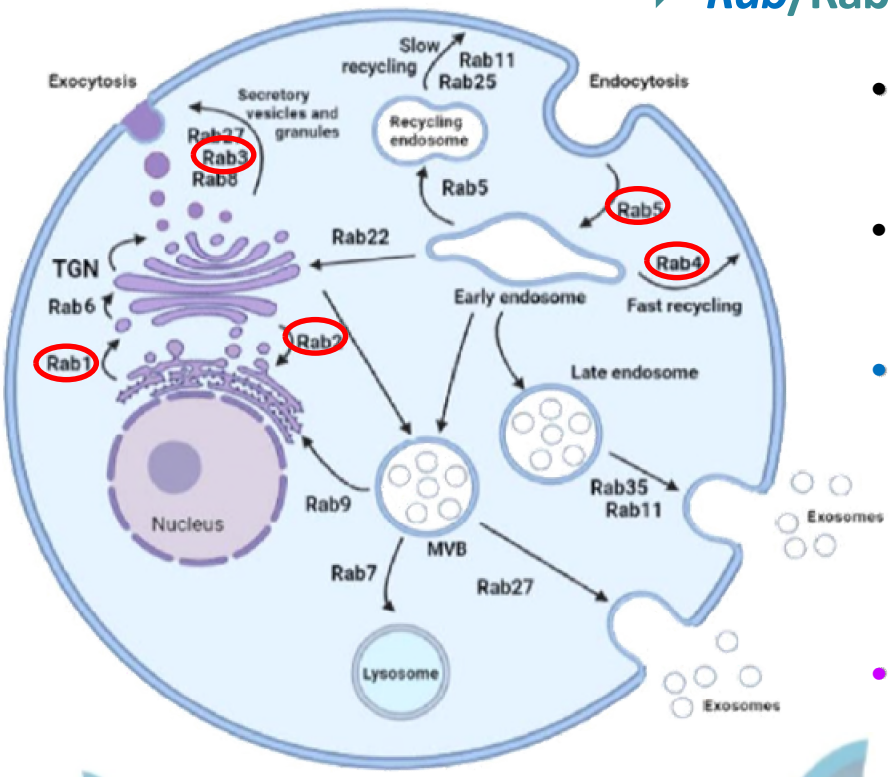
5 The best-understood example of vesicle fusion is during exocytosis of secreted proteins, where cognate SNAREs are VAMP (vSNARE) and the Syntaxin/SNAP-25 complex (tSNARE)

6 SNAREs contain repeating heptad sequences that allow formation of a very stable 4-helix bundle

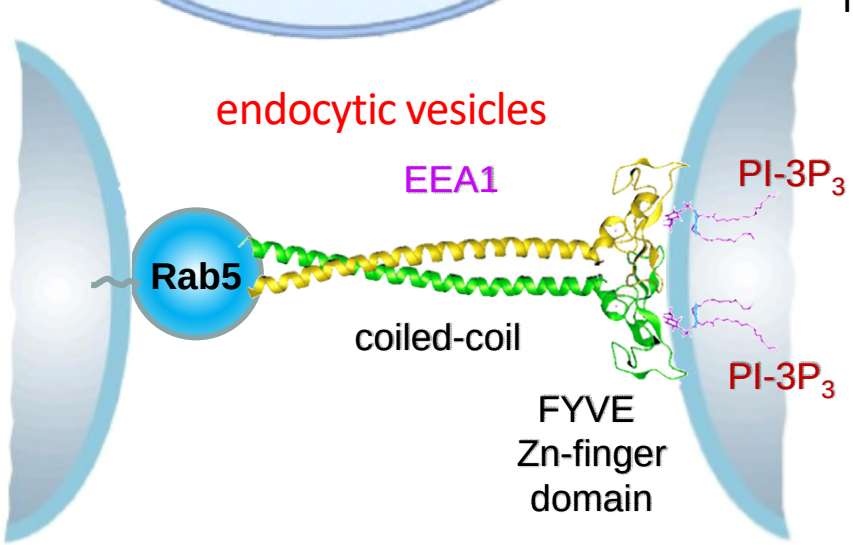


MOLECULAR MECHANISM OF VESICLE FUSION (cont.)

Rab/Rab effector combinations and trafficking specificity

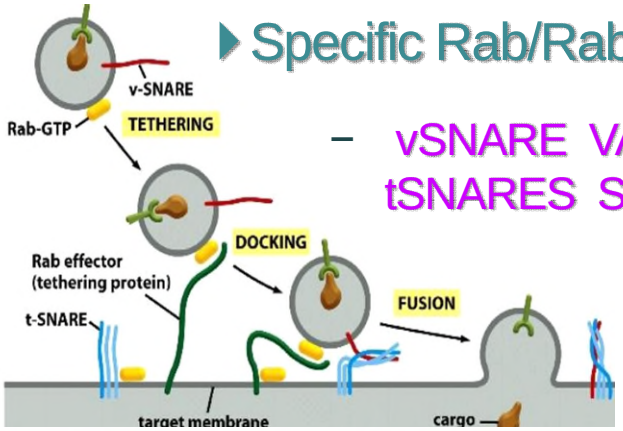


- Each vesicle and organelle type has at least one *Rab* protein on its cytosolic surface
- In yeast cells with mutant *Rab4* (Sec4), vesicles cannot dock to plasma membranes (Class E, *see slide 12*).
- *Rab* proteins determine the specificity of vesicle trafficking. E.g. *Rab5* (required for fusion of endocytic vesicles to form the Early Endosome) binds to *Early Endosome Antigen 1* (EEA1), a long protein that acts as Rab5 effector.
- EEA1 docks onto phosphatidylinositol-3-P in the membrane. When it binds to *Rab* it becomes flexible



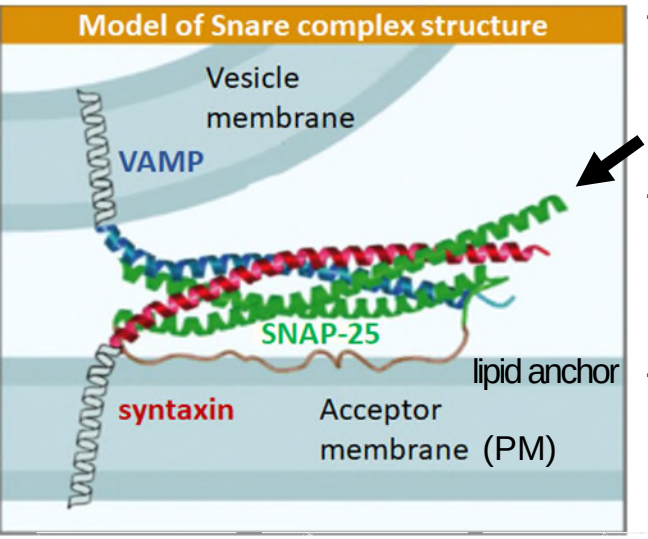
Rab	VT step	Vesicle	Context
Rab1	ER → *c*G	COPII	Anterograde transp.
Rab2	Intra-GN	COPI	Retrograde transp.
Rab3	*t*G →PM	Sec V	Secretion
Rab4	*t*G→PM	Rec V	Fast recycling
Rab5	PM→EE	Rec V	Endocytic fusion

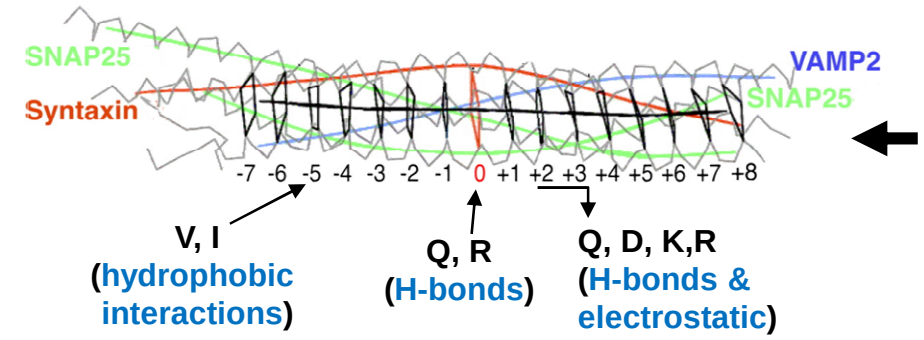
MOLECULAR MECHANISM OF VESICLE FUSION (cont.)

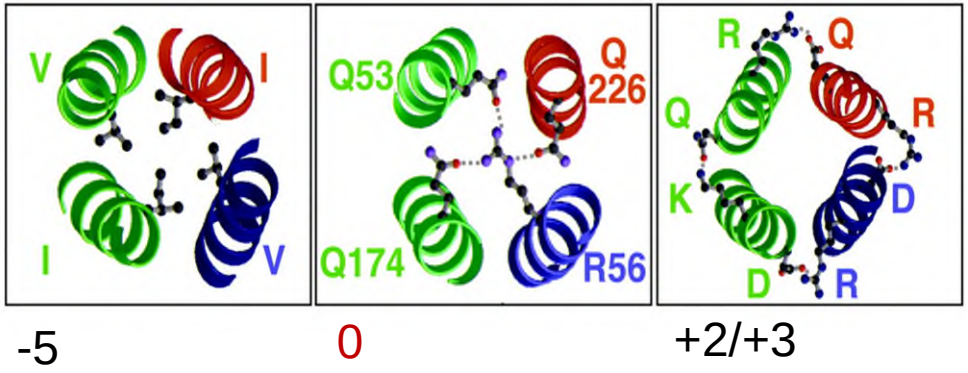


Specific Rab/Rab effector interactions and v/t SNAREs required for docking

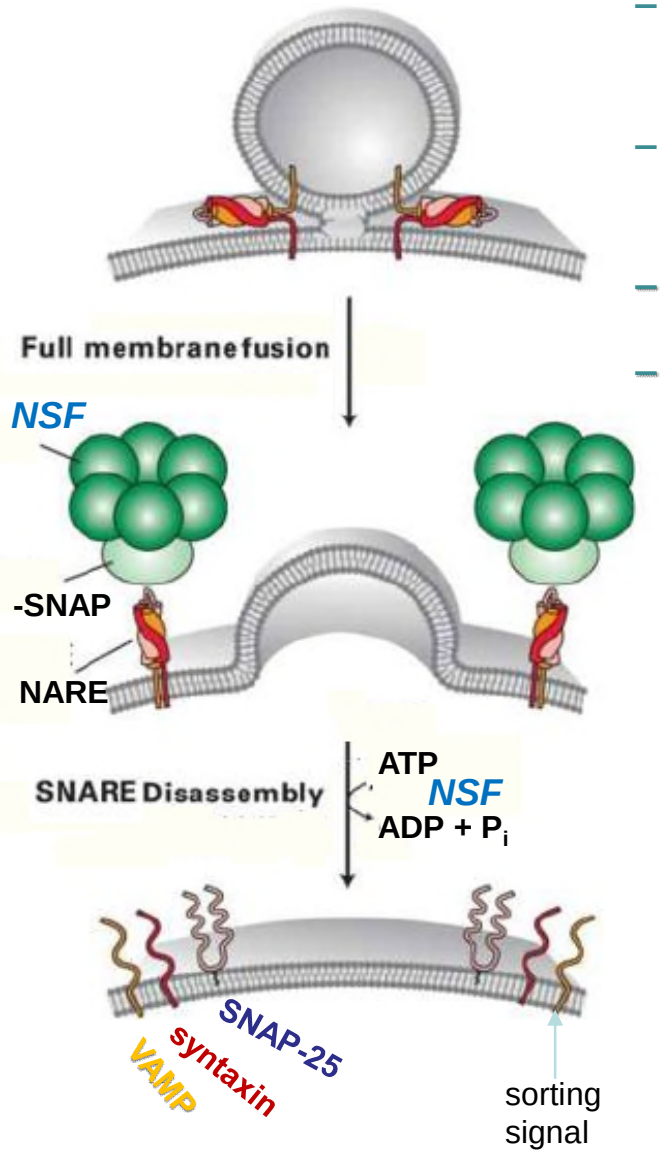
- vSNARE VAMP (*Vesicle-Associated MP*) of secretory vesicles binds to the tSNARES Syntaxin (integral PM protein) & SNAP-25 (lipid anchored to the PM)
- If liposomes with VAMP meet liposomes with synapsin/SNAP-25 they fuse slowly, so SNARE formation is sufficient and essential for vesicle fusion - *Rab*/Rab effector makes it faster.
- For exocytic vesicles, the SNARE complex helix bundle involves 1 helical heptad repeat from VAMP + 1 repeat from syntaxin + 2 repeats from SNAP-25
- Different SNARE components act for other vesicles (20 different vSNARE and tSNARE proteins known in eukaryotes) arranged in different ways, but they always form a four-helix bundle
- activation requires Ca^{++} ions and involves reversible interactions



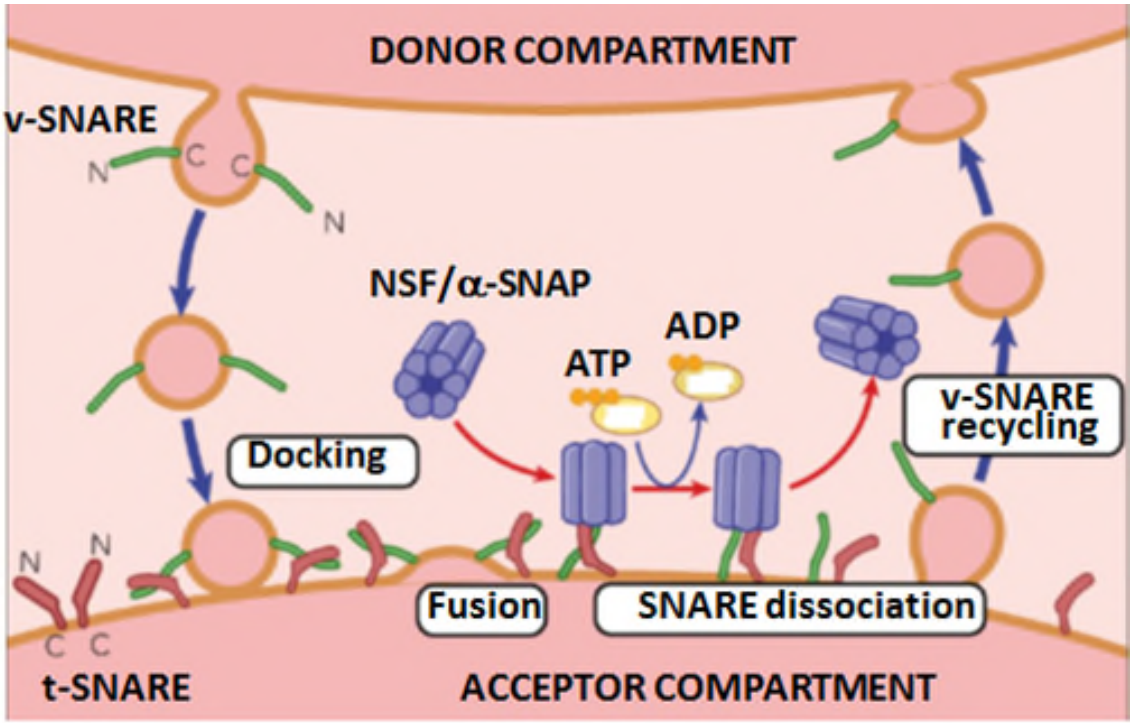




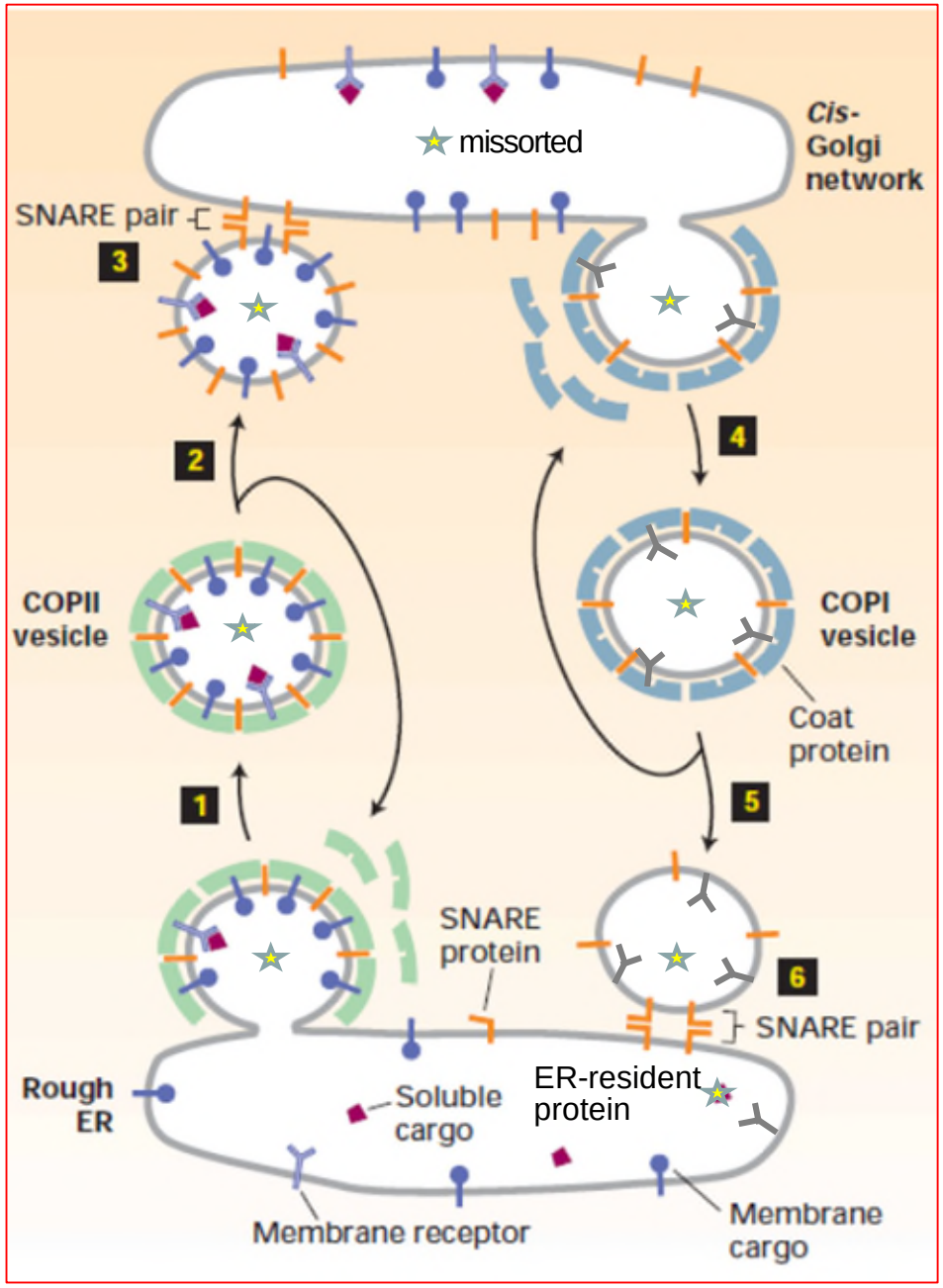
Dissociation of SNARE complexes is driven by ATP Hydrolysis



- SNARE complexes must be recycled for membrane fusion to continue, but they are very stable and require energy to dissociate
- It requires ATP hydrolysis by *NSF* (*N-ethylmaleimide sensitive factor*) complexed with α-SNAP proteins
- v-SNARE are recycled back to *trans*-Golgi (using sorting signal)
- Class C sec mutants (see slide 12) have defective *NSF*/ α-SNAP so there is no endocytosis



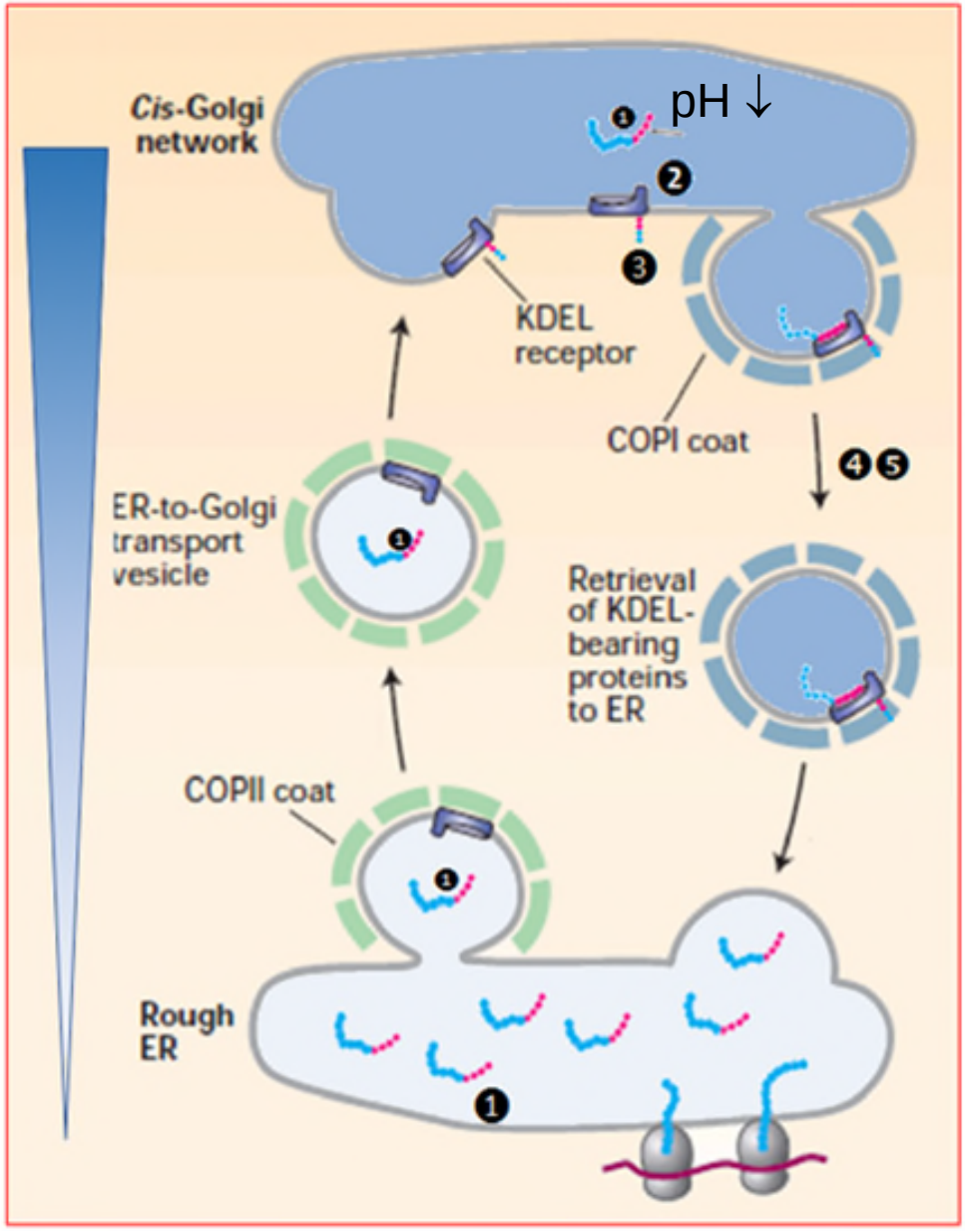
RETROGRADE TRANSPORT



- COPI vesicles mediate retrograde transport within the Golgi and from the Golgi to the ER
- They recycle proteins necessary for COPII vesicle anterograde function
- Yeast cells with COPI protein mutations are class B *sec mutants* *(Slide 12)*
- COPI retrograde transport is required for:
- Recycling Sec proteins: Class B mutants cannot recycle key MPs back to the rough ER, which is gradually depleted of proteins like v-SNAREs or MCPR. Vesicle fusion cannot occur and vesicle formation from the rough ER eventually halts
- Recycling of vesicle membranes: returning phospholipids to the rough ER
- Retrieval of missorted* ER proteins: ER-resident proteins (e.g. chaperones, disulfide isomerases) loaded by mistake into COPII vesicles (sorting errors).

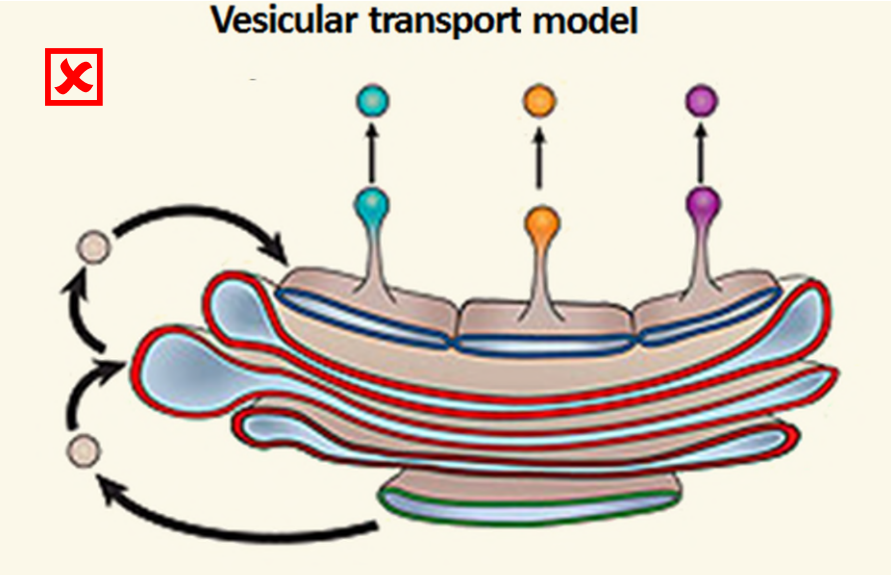
* Erroneamente smistate

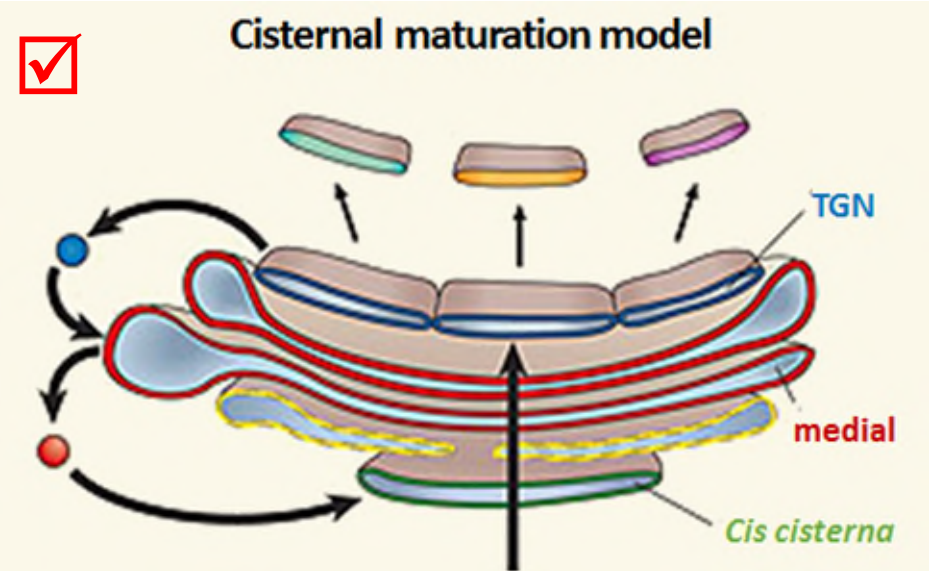
RETROGRADE TRANSPORT (cont.)



- ER-resident proteins carry the KDEL sorting signal at their C-terminus ❶ *(see slide 21)*
- This is recognized by the KDEL receptor ❷ in the membranes of transport vesicles and the *cis*-Golgi reticulum, binding at lower pH.
- The KDEL sorting signal is both *necessary and sufficient* to incorporate proteins into COPI vesicles for retrograde transport.
- ❸ KDEL receptor and other ER-resident membrane proteins have the Lys-Lys-Xaa-Xaa (KKXX) sequence at the C-terminus (in their cytosolic domain)
- ❹ They are incorporated into COPI and cycle from cis-Golgi → COPI → rough ER.
- ❺ Apart from the KDEL receptor, other ER MP (e.g. ER v-SNARE proteins) also have the KKXX signal.
- Binding/release of KDEL/KDELR exploits pH differences between the Golgi and ER lumens.

ANTEROGRADE TRANSPORT – 2 POSSIBLE MODELS





Anterograde vesicular transport model

- Each type of cisterna (cG, mG & *t*G) **remains in its place with unchanging enzymes**
- Cargo proteins move forward through the stack using anterograde vesicles moving from *cis* to median to *trans* cisternae.
- Evidence:

PRO – *cis* to *trans* movement of cargo proteins

CON – vesicles are too small to transport some cargo protein (e.g. tropocollagen fibrils)

Cisternal Maturation model

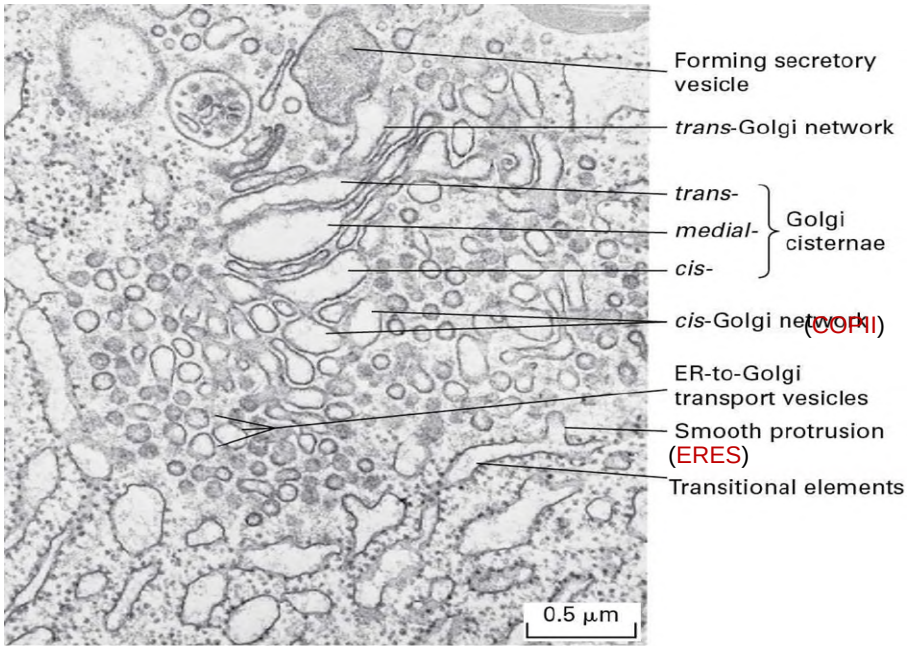
- A new *cis* cisterna is formed from ERGIC in turn formed by COPII vesicles coming from the RER.
- It then **moves through the Golgi stack***, maturing by accumulating medial, then *trans* enzymes (*pila)
- This requires the return of enzymes from later cisternae, through retrograde vesicles

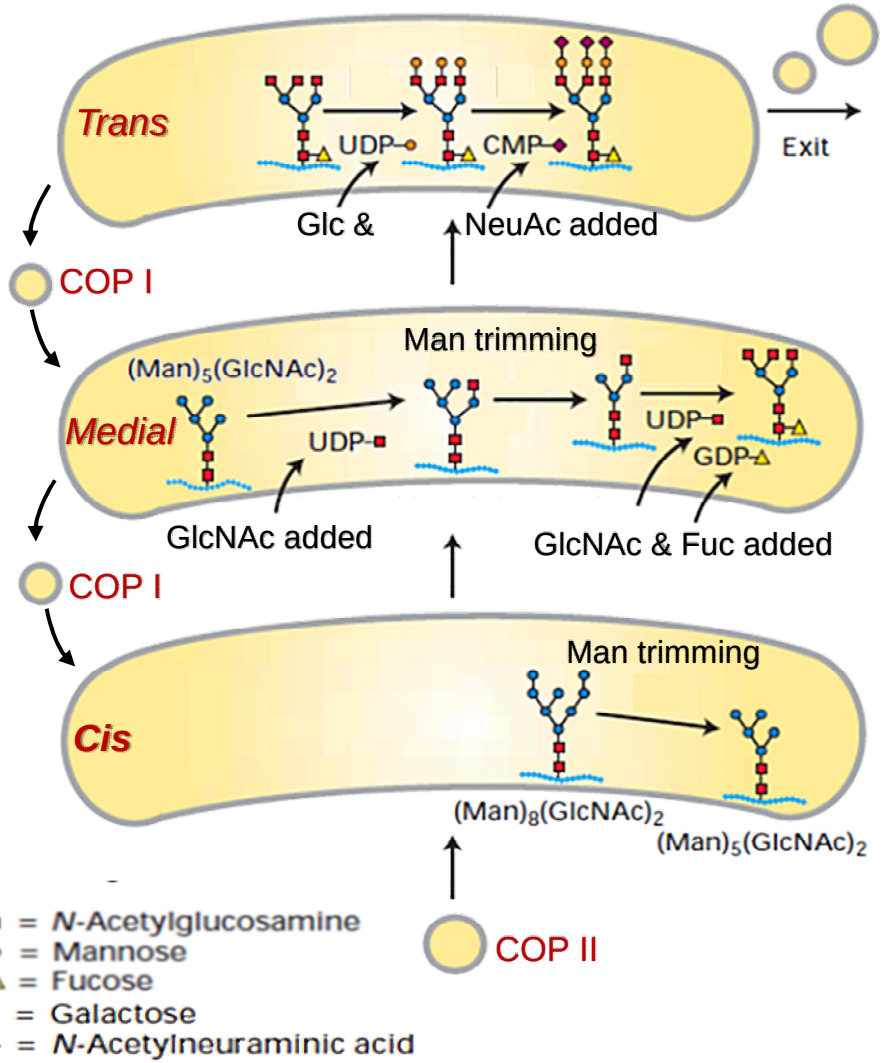
Evidence:

1) Compatible with efficient transit of larger proteins
2) Retrograde transport of Golgi enzymes has been verified

ANTEROGRADE TRANSPORT

- COPII vesicles released from ER exit sites (ERES) into the cytosol; in animal cells they fuse to form the ER-to-Golgi Intermediate Compartment (ERGIC) and reach the *cis*-Golgi network
- Anterograde movement through the Golgi then occurs by Cisternal Maturation (Progression)
- The Golgi complex is organized into 3 compartment types, arranged as flattened sacs or *cisternae*.
- Cisternae differ in their enzymes (*Glycosidases* & *Glycosyltransferases* for PTM)
- The Golgi complex acts as an assembly line that modifies N-& O-linked carbohydrates on transiting secretory proteins.

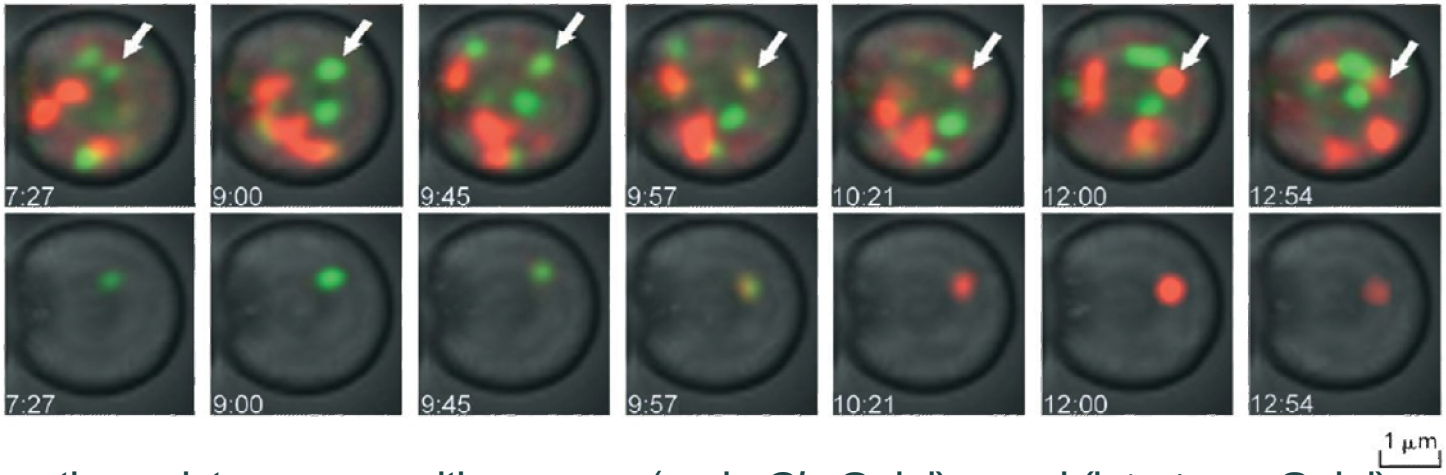




ANTEROGRADE TRANSPORT (cont.)

Anterograde movement in the Golgi network

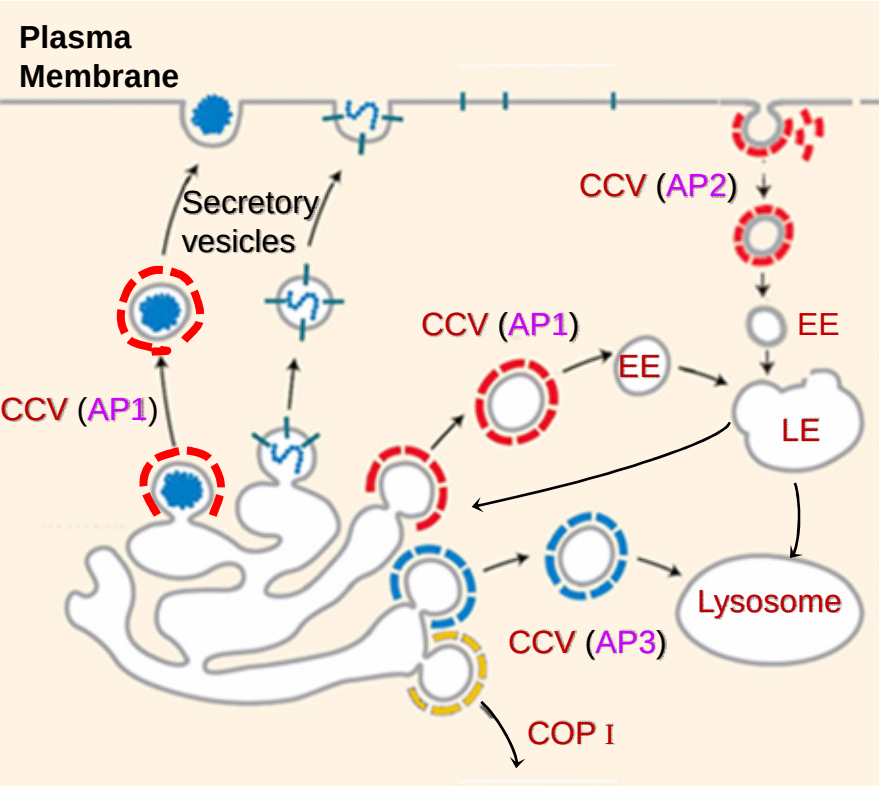
- Cargo proteins are not transported from one Golgi cisterna to the next, but rather the cisterna itself '*matures*' (progresses) from *cis* to *medial* to *trans.*
- *Cis*-cisternae become *medial* and *medial* become *trans*. This is known as *cisternal progression*.
- Retrograde transport by COPI vesicles is needed to transfer enzymes back from late cisternae (where they are no longer needed) to earlier cisternae (where they are required).
- Progression allows transit of large cargo proteins (e.g. tropocollagen fibrils in fibroblasts)
- Experimentally verified by GFP-labelling early Golgi enzymes and RFP-labelling late Golgi enzymes



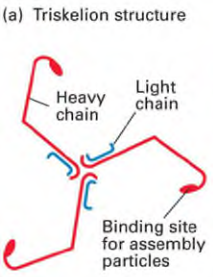
- At any given time, cisternae are either green (early *Cis*-Golgi) or red (late *trans*-Golgi).
- Focusing on only one cisterna (indicated by arrow) this starts as green (*cis* with early Golgi enzyme), but in time acquires the later Golgi red enzyme and looses the early green enzyme, to become completely red

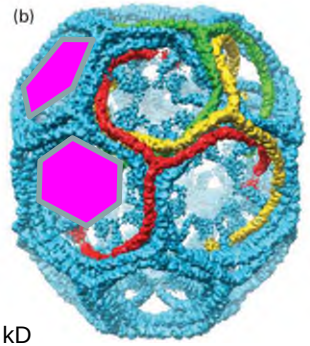
ANTEROGRADE TRANSPORT – LATE SECRETORY STAGES

Transport from *trans*-Golgi to cytoplasmic membrane & lysosomes

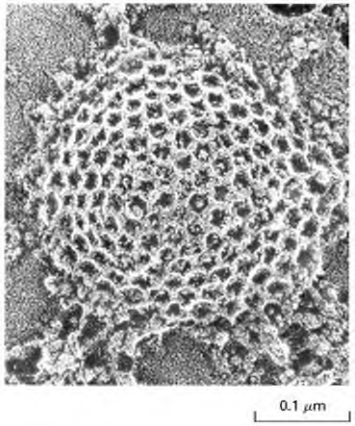


- Cargo proteins → *τ*-GN → Vesicle → Destination.
- Vesicles budding from *τ*-GN have outer layer of the fibrous protein Clathrin and inner layer of adaptor proteins (AP) (Clathrin-coated vesicle – CCV)
- 3 types of AP: AP1, AP2 and AP3, each with 4 different subunits).
- Clathrin light & heavy chains form triskelions (*slide 17*) that assemble with the AP.
- A polyhedral cage forms (icosahedron with pentagonal and hexagonal faces) which confers intrinsic curvature to CCVs.



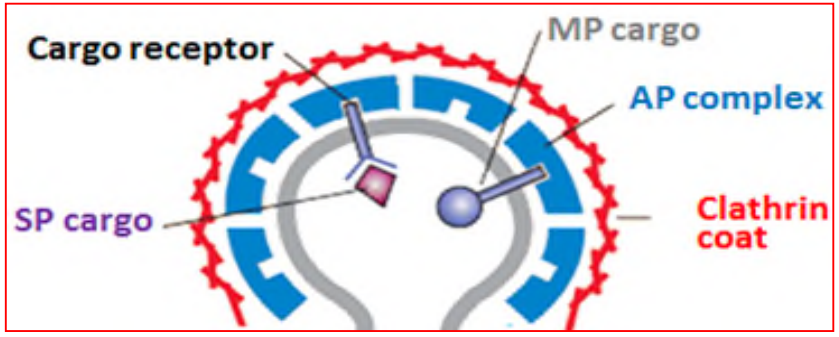


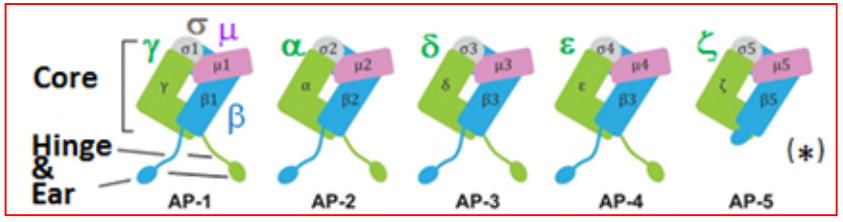
Light chain: ~38 kD
Heavy chain: ~180 kD
AP tetramer; ~340 kD

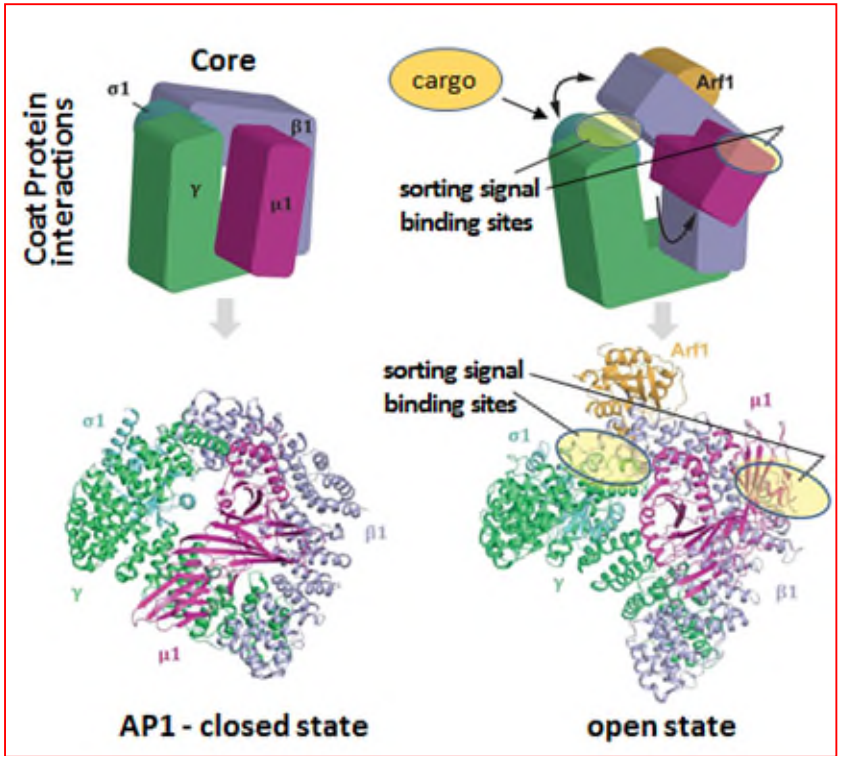


- Unlike COP, Clathrin/AP assemble spontaneously *in vitro* to form empty lattice cages - GTP hydrolysis or presence of membrane are NOT REQUIRED
- CCVs mediate several different transport steps. Cargo is selected by different AP subunits acting from the cytosolic side of the membrane

ADAPTOR COMPLEXES (AP)





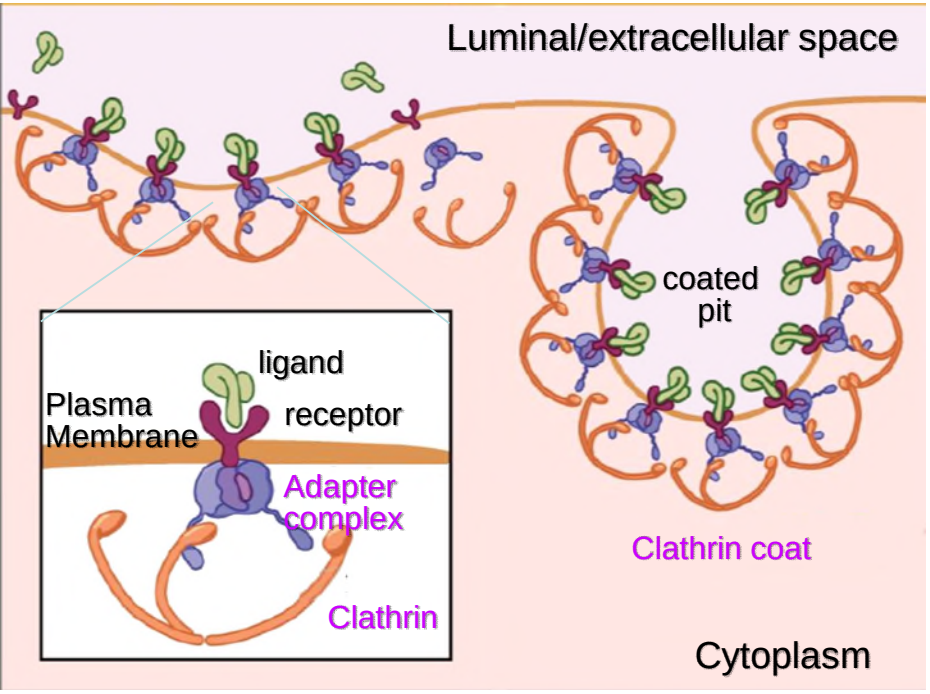


Clathrin/AP vesicles

- Clathrin polymerization occurs on donor membrane only in association with AP complexes, which assemble between the clathrin lattice and the membrane.
- Different AP determine which cargo proteins are specifically included in (or excluded from) a budding transport vesicle.
- AP complexes are hetero-tetramers each with 1 copy of 4 different subunits (σ, β, μ + α, γ, δ, ε or ζ)
- AP pass from CLOSED to OPEN STATES in the presence of *Arf1*/GTP (activated switch)
- The conformational change to the Open State exposes the binding sites for cargo proteins (recognise specific sorting signals).
- E.g. Cargo for Clathrin/AP-1 complex are proteins containing a Tyr-XX-Φ sequence (Φ = bulky hydrophobic AA, e.g. Phe) (see *slide 22*)

core = nucleo; hinge = cardine; ear (appendage) = appendice

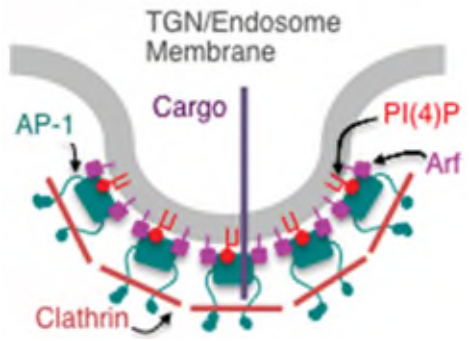
ADAPTOR COMPLEXES (cont.)

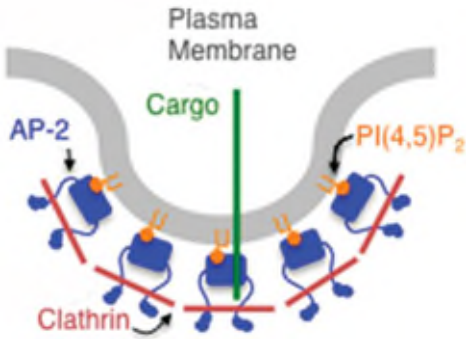


Adapter	Pathway	Subunits	Clathrin	Arf	PIP
AP-1	TGN→EE	4	✓	✓	PI(4)P
AP-2	PM→EE	4	✓	?	$PI(4,5)P_2$
AP-3	TGN→LS	4	?	✓	PI(3)P
GGA	TGN→LE	1	✓	✓	PI(4)P

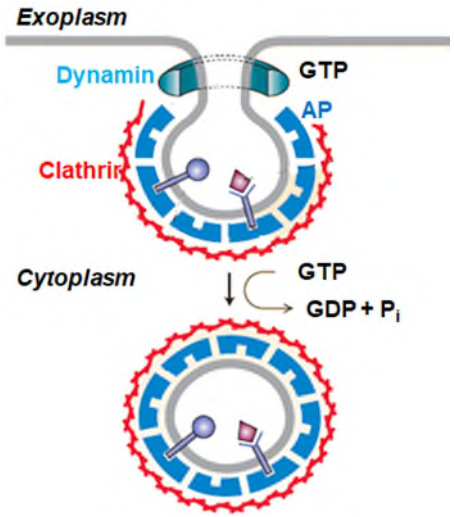
AP select the cargo proteins

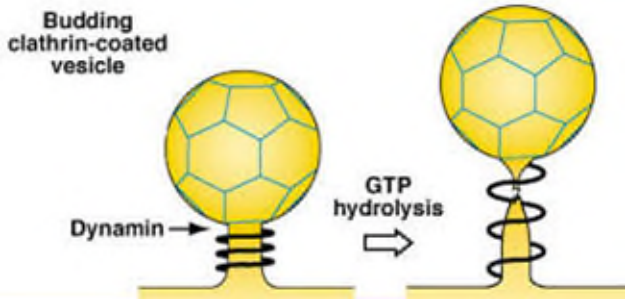
- Clathrin binds to a *clathrin-binding box* sequence on the β subunit of the AP complex (cytosolic).
- AP C bind MP or luminal MCPR and are central to vesicle formation/clathrin-mediated trafficking.
  - AP1 C: cargo from *T*GN → EE → LE → LS
  - AP2 C: cargo from PM → EE (→ TGN/LE)
  - AP3 C: cargo from TGN → Lysosome
  - GGA C: cargo from TGN → LE
- AP bind specific membrane phospholipids [PI(n)P]
- GGA has similar Clathrin-binding & cargo-binding elements (but monomeric $ different sorting seq.)
- *ARF GTPase* and different PIPs are required for AP recruitment and CCV assembly.

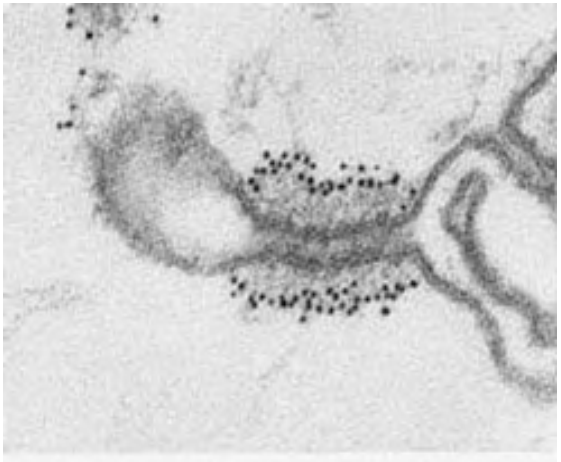




CLATHRYN COATED VESCICLES







Role of Dynamin in vesicle detachment (pinching off)

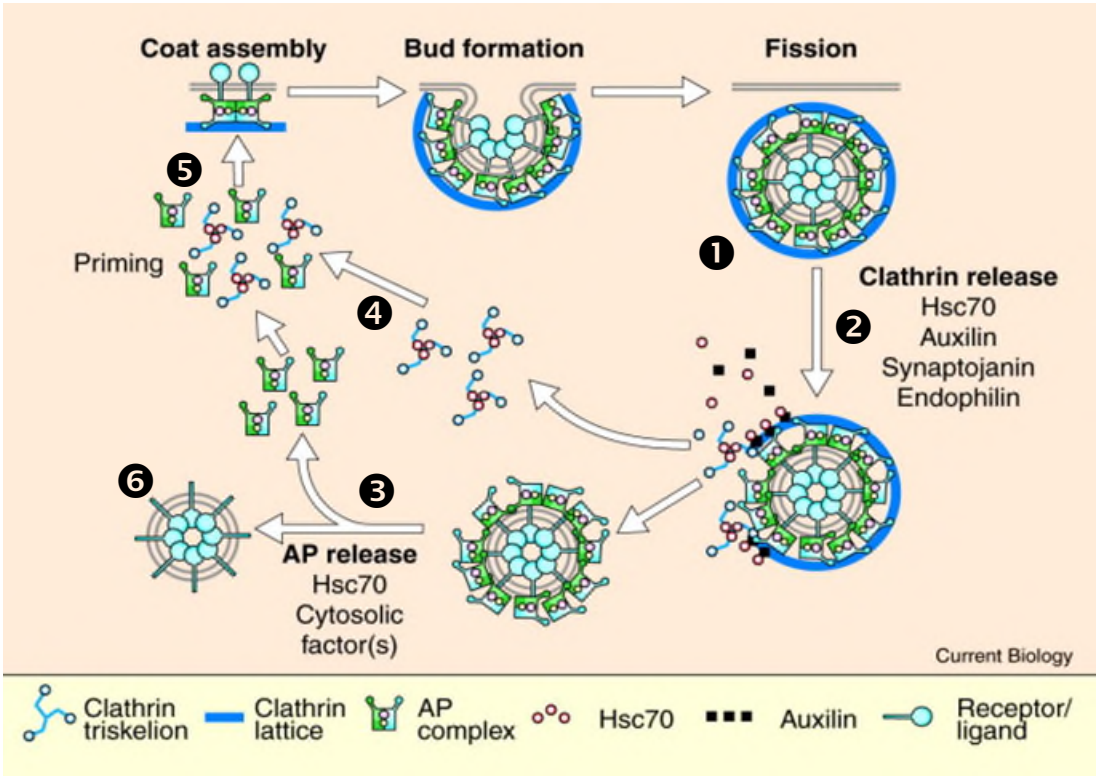
- A cytosolic protein, *Dynamin*, polymerizes around the neck portion of a budding CCV forming a spiral
- This requires binding of *Dynamin* to GTP
- *Dynamin* has a *GTPase* activity and when polymerization is completed it hydrolyses GTP→GDP
- GTP hydrolysis drives "contraction" of the polymerized dynamin, allowing the vesicle to "pinch off" the PM
- Non-hydrolyzable GTP analogues result in CCV buds with long necks. Polymeric dynamin spirals cannot pinch off.
- N.B. COPI & COPII vesicles do NOT require a separate dynamin-like *GTPase* to pinch-off. *ARF/SAR1* may mediate this process in conjunction with other proteins.

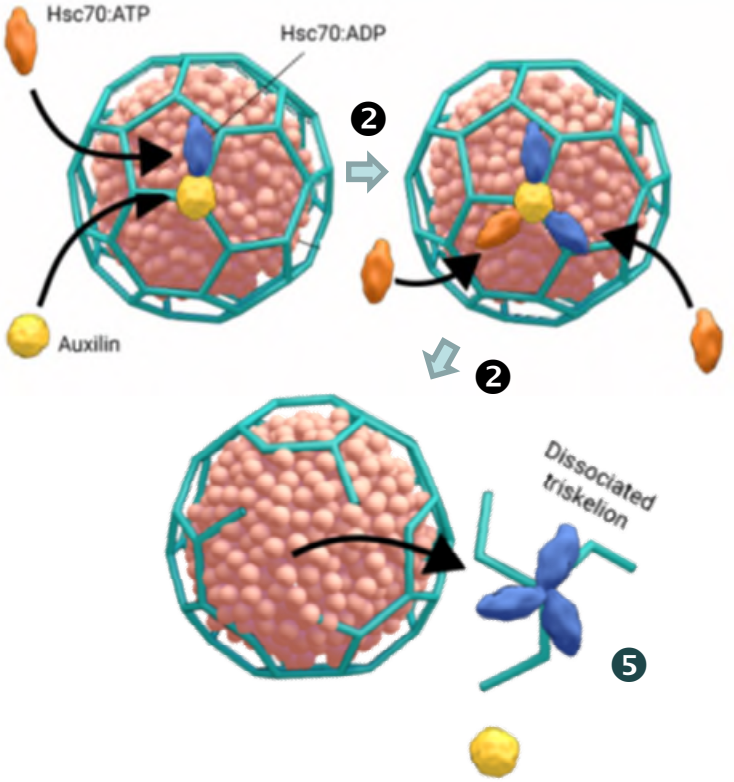
Uncoating and AP/clathrin recycling

– CCVs lose their coat soon after formation in a process assisted by *chaperonin ATPases*

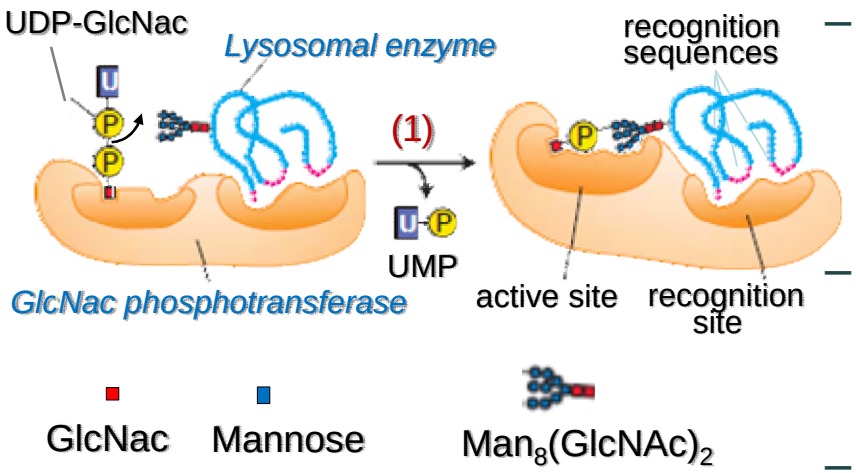
❶ uncoating is timed by *ARF*-ATP hydrolysis; ❷ 3 *Hsc70* chaperone bind in succession to a auxilin subunits and hydrolize in succession to mediate Clathrin release in an ATP powered process;

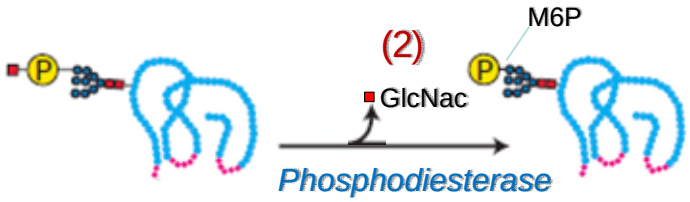
❸ *Hsc70* then also mediates the release of underlying AP subunit; ❹ AP and Clathrin are then recycled to the membrane. ❺ *Hsc70*-ADP protects Clathrin from premature polymerization and primes it for coat assembly; ❻ Uncoating exposes v-SNAREs for assembly with tSNARES and membrane fusion





Role of M-6-P in targeting of luminal lysosomal enzymes





- The sorting signal that directs soluble lysosomal *enzymes* from the *trans*-Golgi network to the late endosome is a carbohydrate residue: mannose 6-phosphate (M6P)
- Unlike in secreted proteins, mannose residues (Man) in lysosomal proteins are NOT TRIMMED-OFF, but one is phosphorylated
- In the *cis*-Golgi, the N-linked $Man_8(GlcNAc)_2$ oligosaccharide present on most lysosomal enzymes undergoes a two-step modification:

(1) *GlcNAc phosphotransferase* transfers a UDP-activated GlcNAc group to the C6 position of one or more Man.

(2) A *Phosphodiesterase* then removes the GlcNAc group, leaving Man-6-P on the lysosomal enzyme.

- M6P blocks further processing reactions of lysosomal enzymes (no trimming occurs)

SORTING TO LYSOSOMES (cont.)

Mannose-6-P receptor

– Clathrin/AP1 vesicles have an M6P receptor (for lysosomal *enzymes*)

① they bud from the *trans*-Golgi, and lose their coat proteins

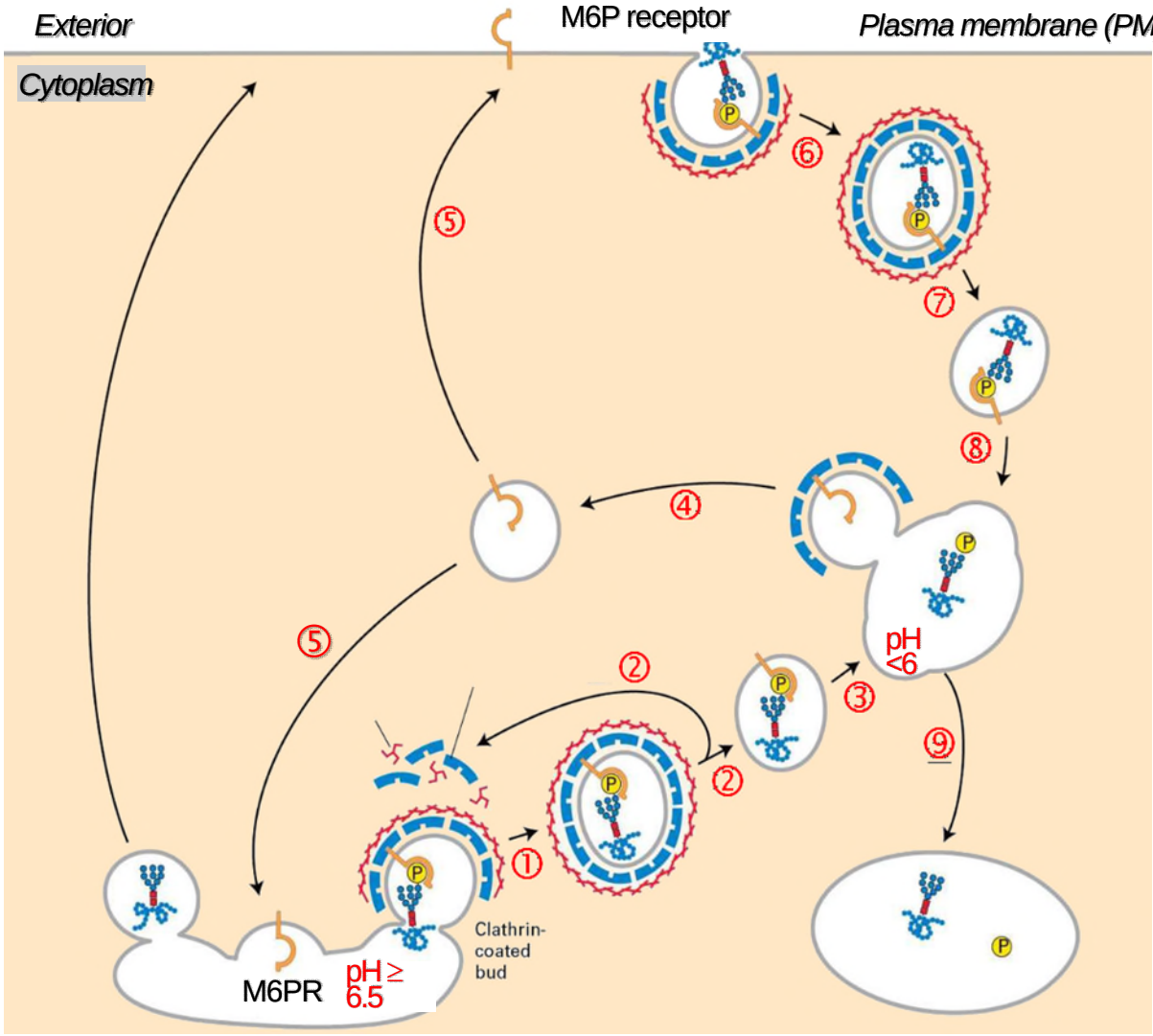
② Coat proteins recycle back to *t*-G

③ Uncoated vesicles fuse with late endosome, where the lower pH causes M6P to dissociate from the receptor

④ The receptor is incorporated into vesicles for recycling.

⑤ These can return to *t*-G or fuse with the plasma membrane

⑥ missorted M6P-labelled proteins sent to PM are recovered by PM M6P-Receptor



⑦ They are delivered to the late endosome by receptor-mediated endocytosis and ⑧ eventually reach the lysosome.

⑨ In the lysosome, mannoses are dephosphorylated by *Acid phosphatases*