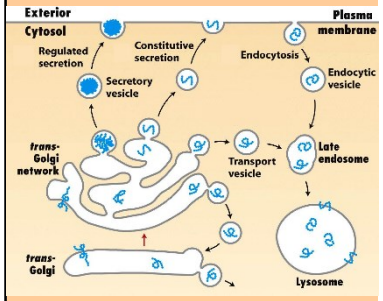
Cellular Biochemistry [918SM]



Module 4

Protein Sorting Pathways I

4_1 Vesicular traffick

4_2 Exocytosis & endocytosis

4_3 Nonvesicular traffick

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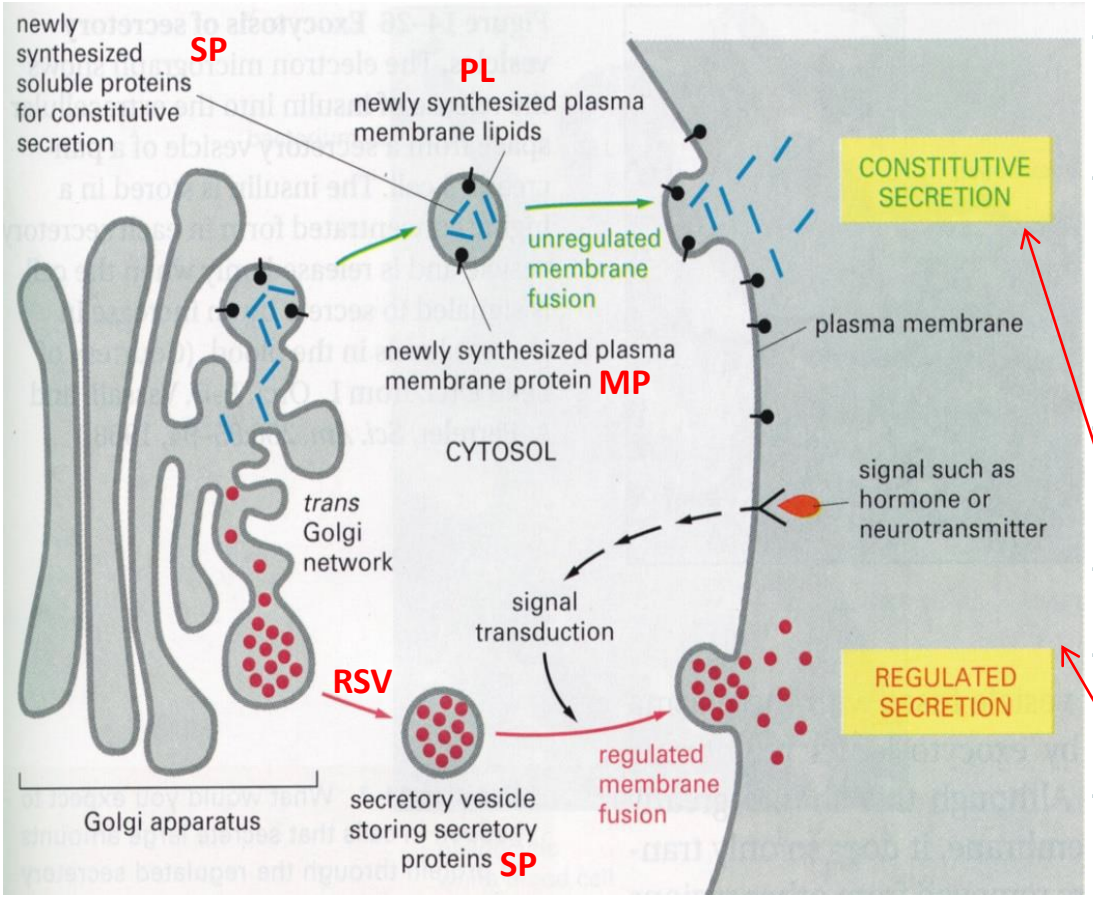
AA 2025-2026

PROTEIN SECRETION

Transport from the trans-golgi network to the cell exterior: exocytosis

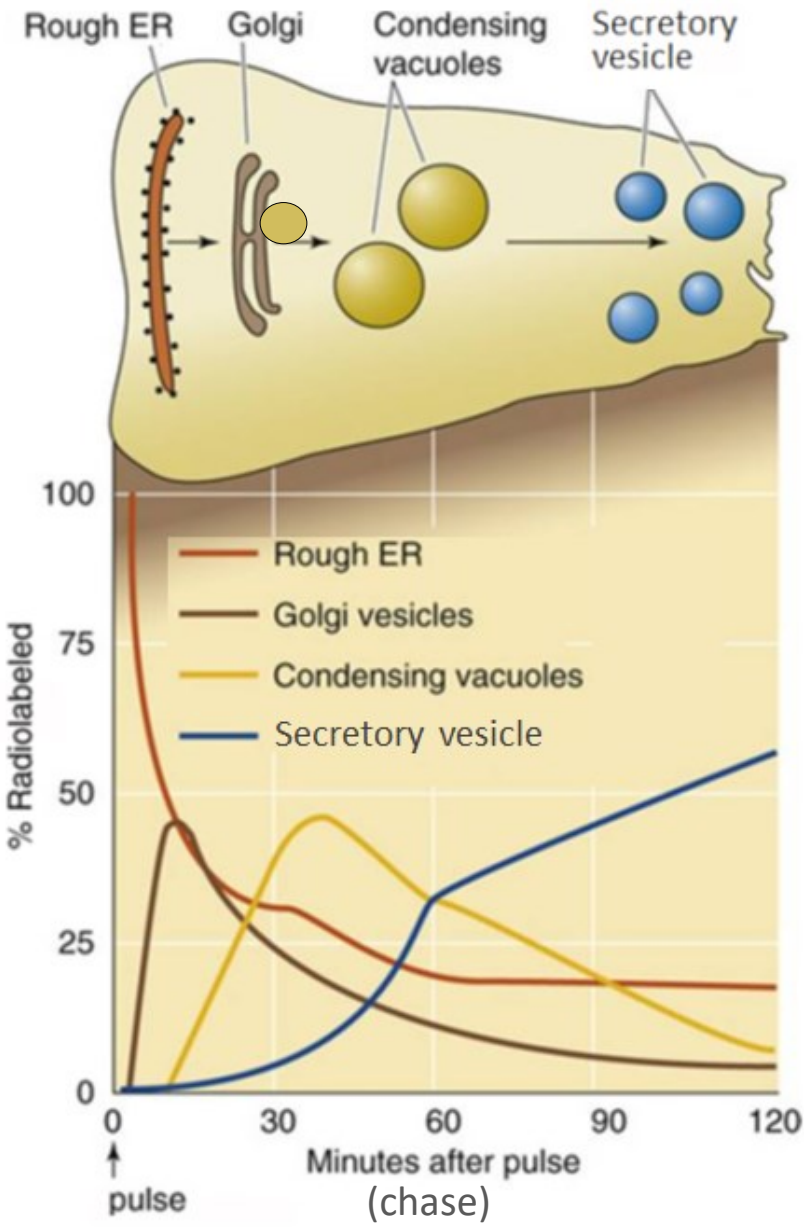


- Two types of vesicles move cargo from the *trans*-Golgi network to the cell surface:
- Unregulated transport vesicles carry soluble & membrane proteins (SP, MP) & phopspholipids (PL) which they secrete constitutively
- Regulated secretory vesicles (RSV) carry soluble secretory proteins (SP) in storage
- All eukaryotic cells → constitutive secretion
- Specialized secretory cells → regulated secretion (triggered by a specific stimulus)
- Common mechanisms govern regulated secretion by RSV

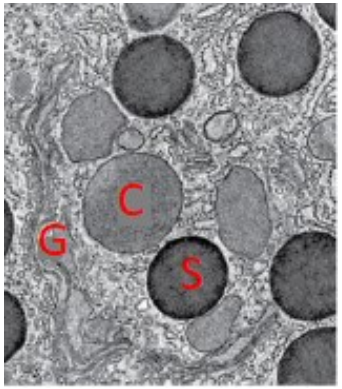
- In an experiment, recombinant insulin, trypsinogen and adrenocorticotropic hormone, expressed in a single cell, were seen to colocalise into RSV, even though they are normally produced by different cells and do not have a common sorting sequence.
- The key aspect of protein storage in RSV for regulated secretion of proteins seems to be aggregation.

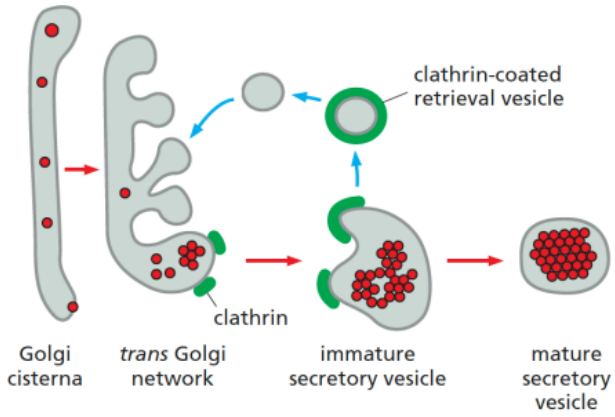
PROTEIN SECRETION (cont.)

Role of protein aggregation in *trans*-Golgi sorting to the regulated pathway



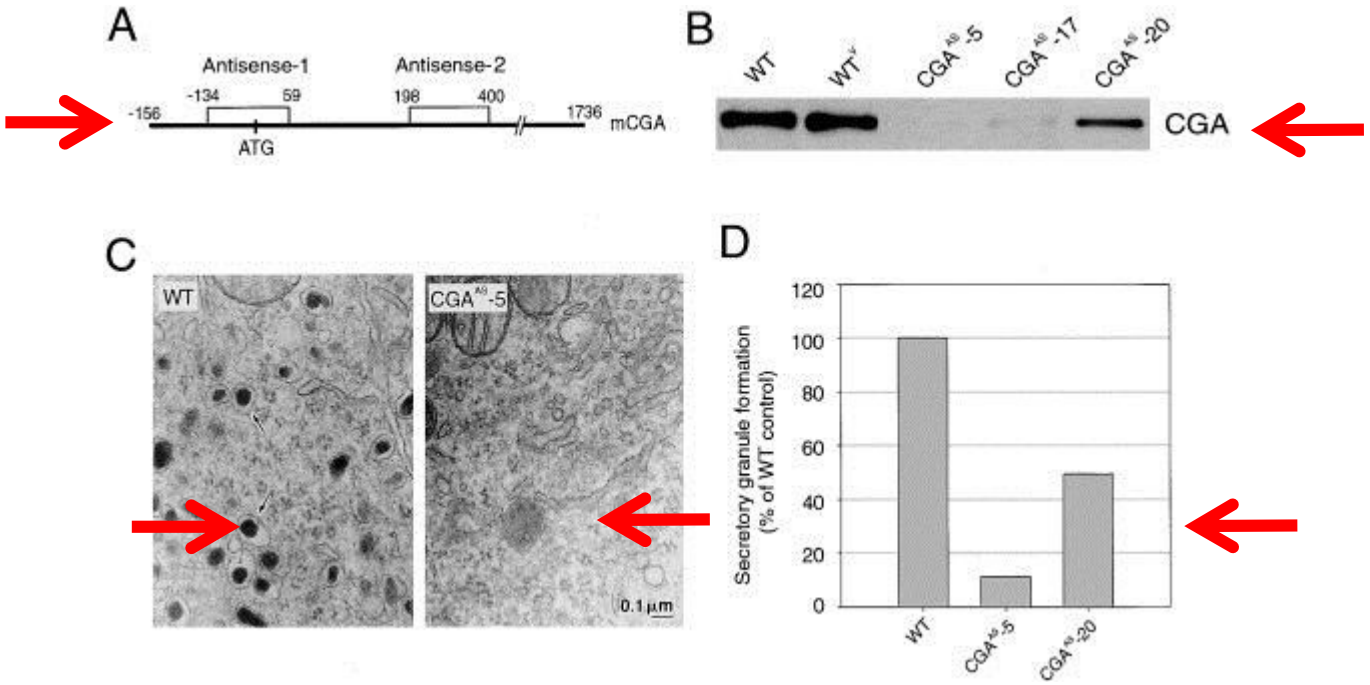
- Sorting into the regulated pathway occurs in stages and is controlled by selective protein aggregation.
- Budding vesicles, and immature vesicles that have just budded from the *trans-Golgi network* (G in micrograph) contain diffuse aggregates of secreted proteins (condensing vacuoles, C), indicating that proteins destined for RSV selectively aggregate before incorporation.
- Mature secretory vesicles (S) form with the aid of Clathrin, are smaller & denser, due to a higher level of aggregation.
- In mammalian cells, Chromogranins & Secretogranins are found RSVs, and form aggregates in the conditions of the *TGN* (pH ≈ 6.5, 1 mM Ca^{2+}) – they have a role in sorting proteins into RSV.





Role of chromatogranin A - Analysis of depleted PC12 cells

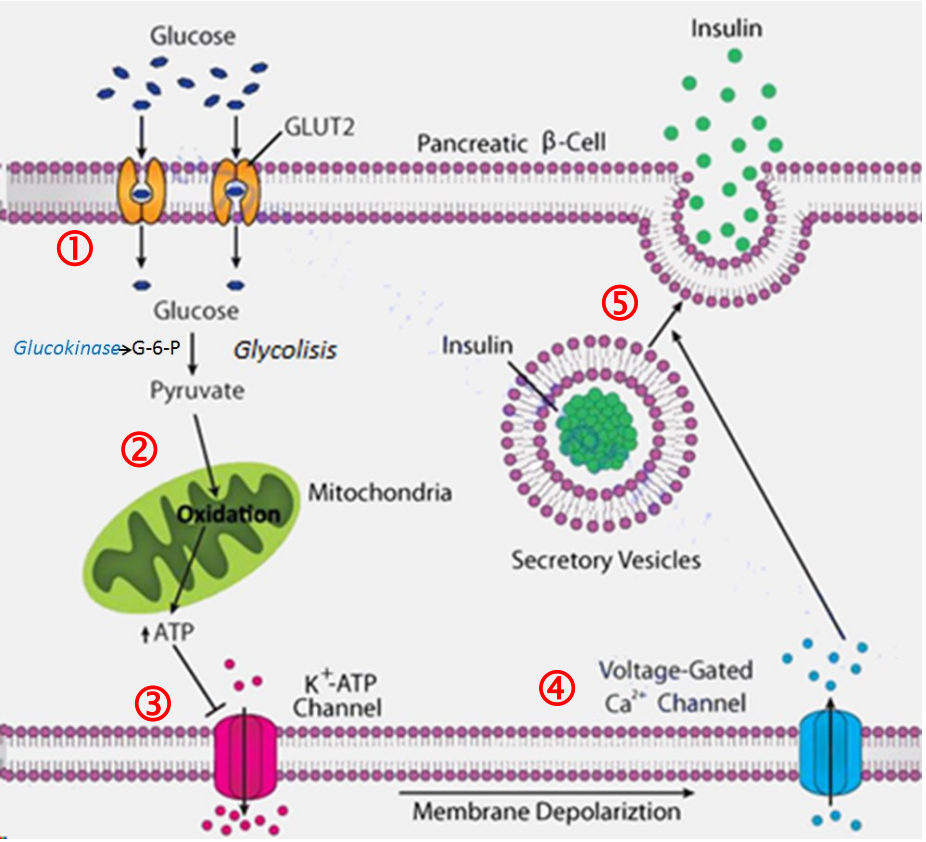
- Neuroendocrine PC12 cells have dense-core secretory granules (see Figure C – WT cells)
- Chromatogranin A (CGA) deficient PC12 cells were obtained by generating stable clones expressing antisense RNAs against CGA mRNA (see Figure A)

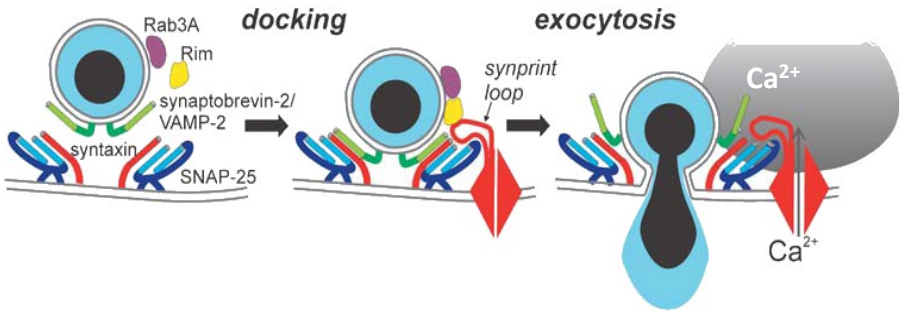


- Western blot analyses confirmed CGA expression was variably depleted in different clones compared to wild-type (WT) and vector-only control cells (WTV) (Figure B).
- Electron micrographs of these cells showed less dense-core granule formation (Figure C right), and secretory granules per unit area (μm^2) of cytoplasm correlated to CGA levels (Figure D)

PROTEIN SECRETION (cont.)

Example of regulated secretion: Mechanism of glucose-stimulated insulin secretion





- Insulin is released from RSV in pancreatic β-cells in response to elevated blood glucose.

① A *"glucosensory unit"* (GLUT2 transporter + *glucokinase*) metabolically couples glucose levels to energy production ② and membrane potential ③

Gluc ↑ ATP ↑ ATP-dependent K^+ channel ↓ membrane potential ③ ↓ voltage-gated Ca^{++} ↑ ④

- Ca^{++} influx activates vesicle fusion (SNAREs) ⑤

- There are two types of insulin secretory granules :

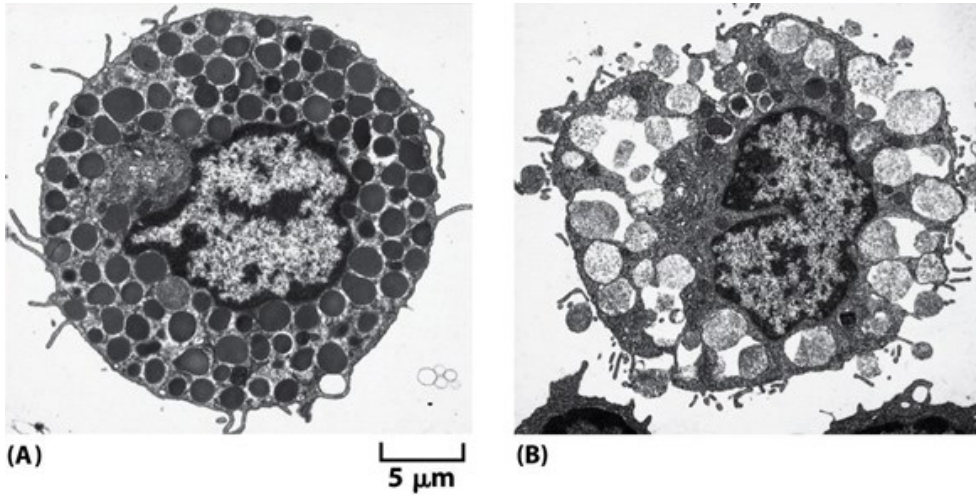
  "readily released" pool(*) (~5%) already docked at the membrane (fast, <5 min.)

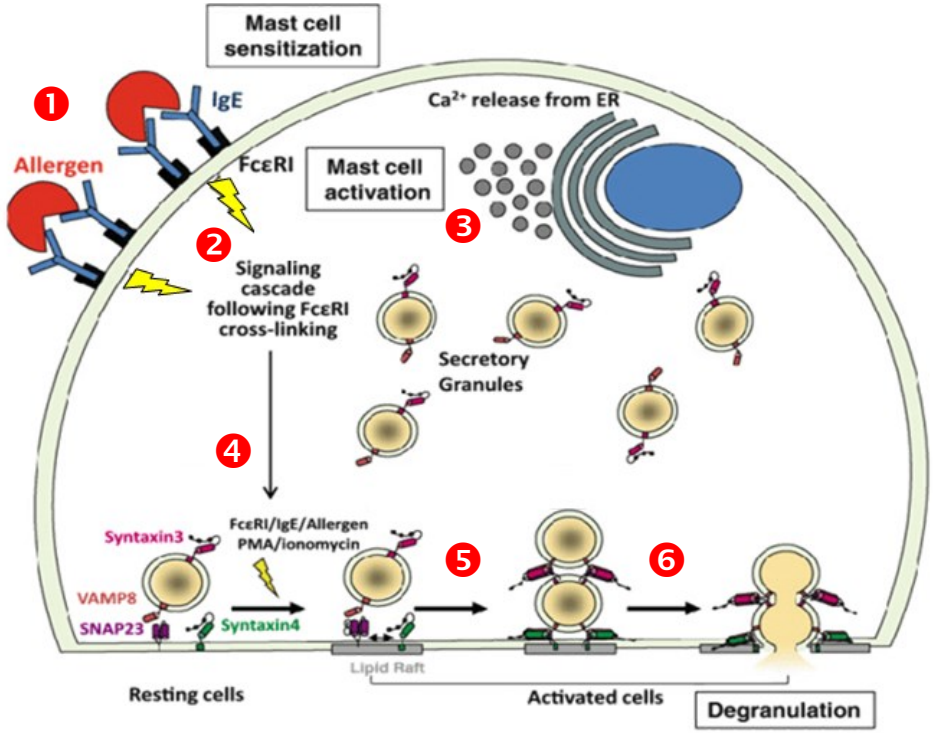
  "reserve" pool (~95%) in the cytoplasm (released after 10-60 min.)

- Ca^{2+} influx activates the vSNARE VAMP-2 (synaptobrevin) to interact with the tSNARES syntaxin and SNAP-25, allowing fusion of the insulin-loaded RSV with the plasma membrane and insulin release

(*) parco

PROTEIN SECRETION (cont.)





Histamine release from mast cells

– Electron micrographs show massive exocytosis of histamine (degranulation) from rat mast cell secretory granules.

❶ In the classical allergic response, multivalent allergens activate IgE on the surface of mast cells which cross-link FcεRI receptors.

This triggers mast cell activation and degranulation:

❷ a signalling cascade is initiated

❸ this leads to Ca^{2+} release from the ER.

❹ this in turn leads to phosphorylation of the t-SNARE SNAP23, which recruits Syntaxin 4 and binds the v-SNARE VAMP8 on RSV

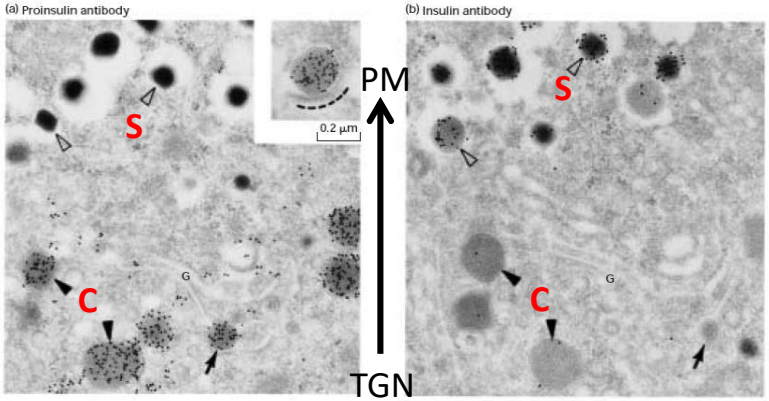
❺ The SNARE complex forms

❻ It appears that SNAP23 can relocate into the fused RSV and form a complex with the RSV-located tSNARE Syntaxin 3 to recruit another RSV via interaction with its vSNARE VAMP8, accelerating the release of histamine.

MATURATION OF REGULATED SECRETORY PROTEINS

Some secreted proteins mature inside regulated secretory vesicles (RSV)

- Many soluble secretory proteins are synthesized as longer inactive precursors (pro-proteins or pro-*enzymes*) that require proteolytic processing to generate the mature, active proteins.
- The pro-form can be relatively long-lived. Examples are insulin, glucagon and *lysosomal enzymes*, where the proteolytic processing occurs in secretory vesicles.
- Delayed activation of *lysosomal proenzymes* prevents them from digesting macromolecules in earlier compartments of the secretory pathway, before they reach the lysosome
- It has been shown also that insulin matures in secretory vesicles and not in the *T*GN (see Figure).

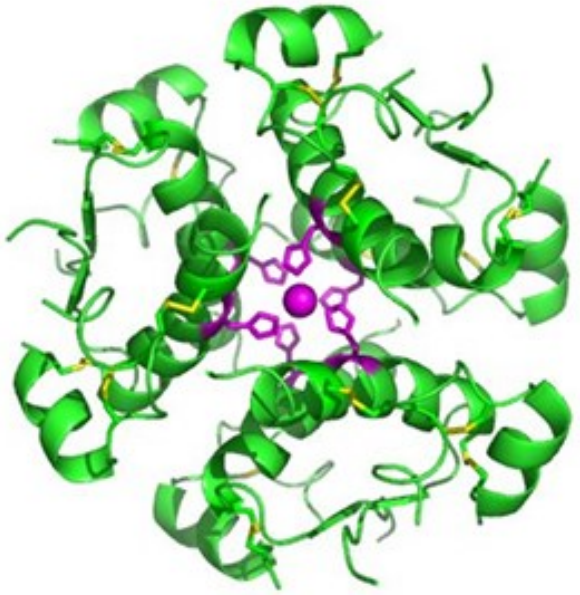


a) *Proinsulin antibody* b) *Insulin antibody*

Gold-conjugated anti-insulin antibodies appear as dark dots in electron micrographs. The presence of mature insulin (b) is visible in secretory vesicles (S) but not in *T*GN and little in condensing vesicles (C), whereas proinsulin is abundant in condensing (C) and also secretory (S) vesicles. It is still maturing

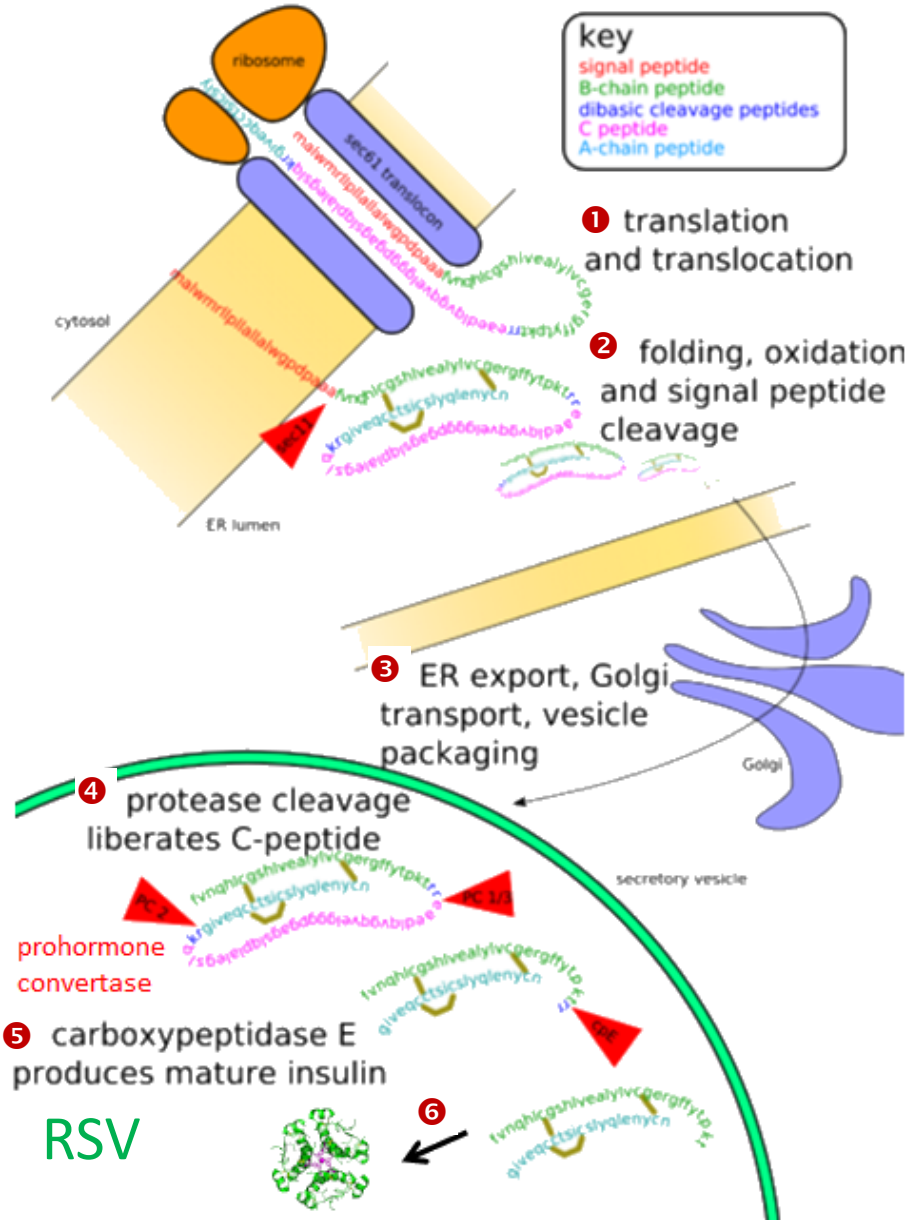
- Insulin **aggregates in RSV** via metal coordination of His residues to a Zn ion

HEXAMERIC STORAGE FORM



INSULIN MATURATION

Insulin is expressed as a pro-protein



– Proinsulin: signal peptide + A + B + C chains

❶ translation and translocation to RER

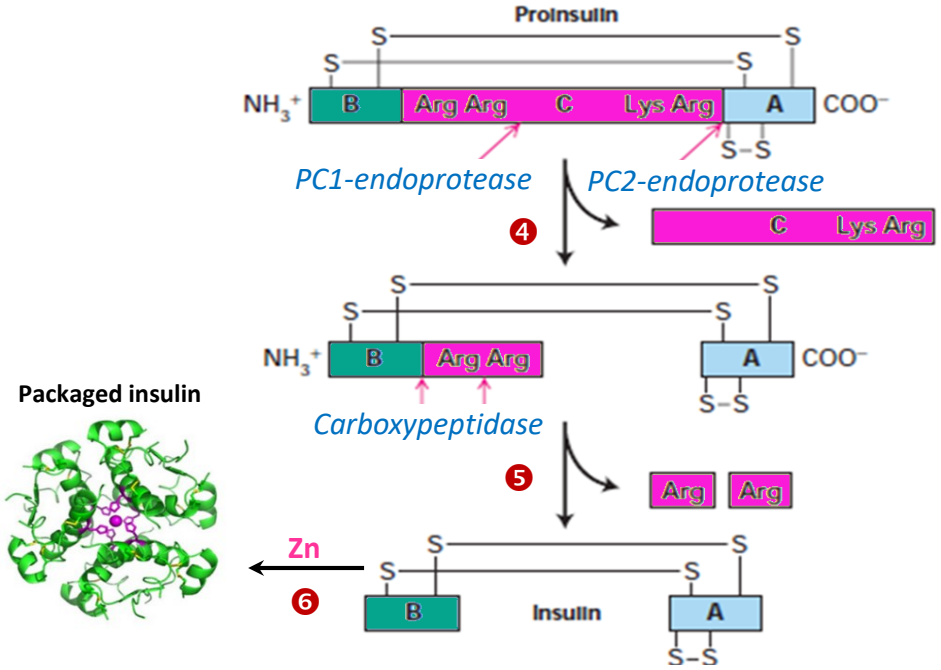
❷ the signal peptide is removed by sec11, and after folding and S-S bridge oxidation

❸ proinsulin enters the Golgi and is packaged in RSV

❹ *PC endoproteases* (*Prohormone convertases*) present only RSV process secreted proteins, cutting at dibasic sites (RR/KR), and remove peptide C from A and B.

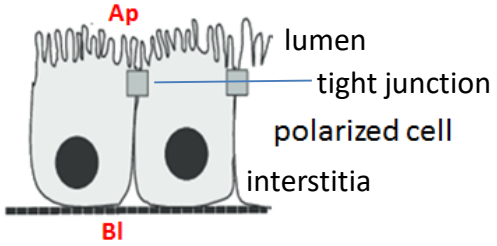
❺ *Carboxypeptidase E* then removes residual basic AA

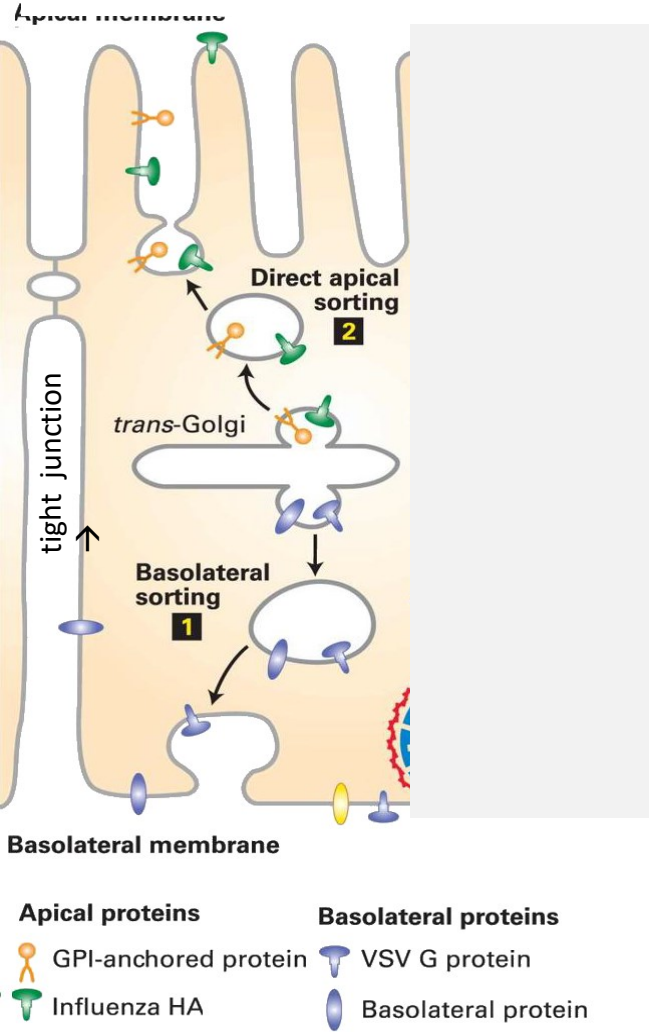
❻ Insulin is condensed by coordination to Zn



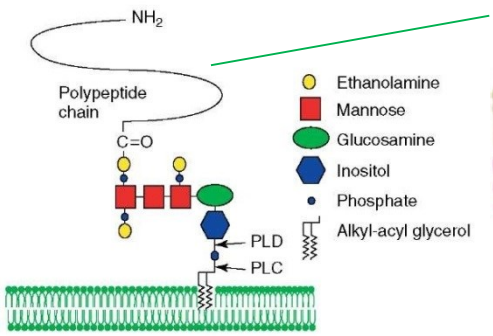
PROTEIN SORTING IN POLARIZED CELLS

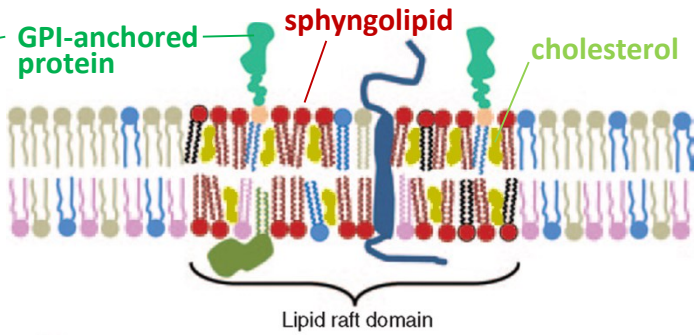
Differential sorting to basolateral (Bl) and apical (Ap) regions of polarized epithelial cells



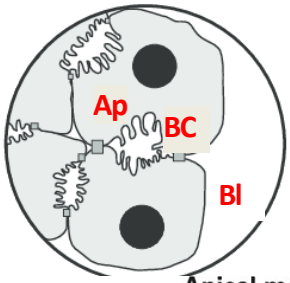


- Apical and basolateral membranes have distinct sets of MP which have to be differentially sorted.
- MPs are initially located within *t*-GN but use different transport vesicles with distinct *Rab* and v-SNARE proteins for direct basolateral ❶ or apical ❷ sorting
- Sorting systems depend on cell type and cargo protein. In virus infected epithelial cells, influenza virus HA glycoprotein buds only from the Ap membrane, while vesicular stomatitis virus G glycoprotein (VSVG) buds only from the Bl membrane.
- GPI (*glycosilinositol-P*)-anchored proteins are targeted to lipid rafts, usually in the apical membrane of epithelial cells. Cleavage by *Phospholipases* (*PLD*, *PLC*) can then release the protein from the membrane in a controlled manner.





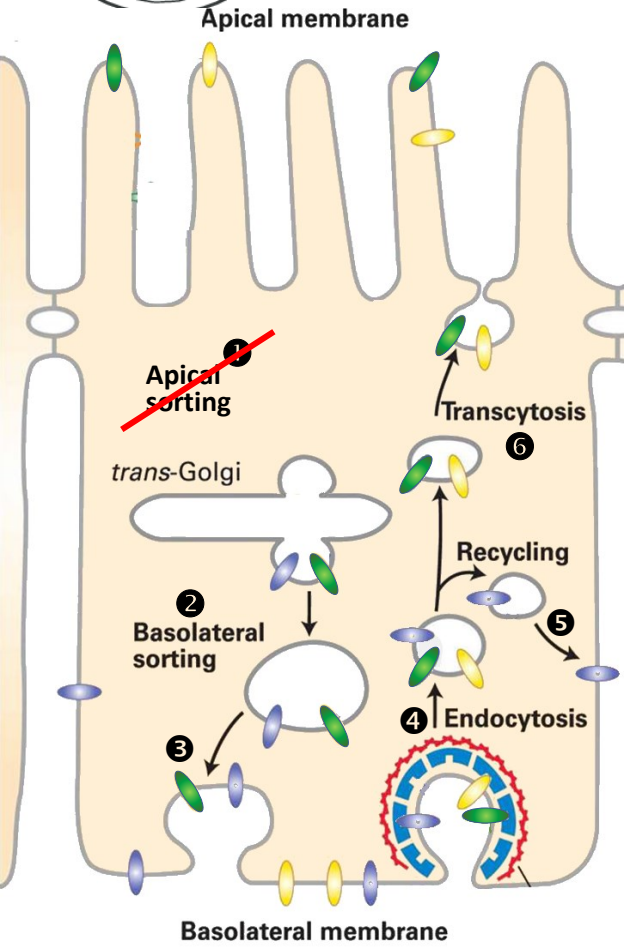
TRANSCYTOSIS



– In hepatocytes the Bl side faces blood vessels while the Ap side faces the bile channels (BC)

❶ There is no direct Ap sorting due to low activity of te required *Ras GTPase*.

❷ Both Ap and Bl proteins are transported by the same vesicle from the *t*-GN to the basolateral region. ❸ They are incorporated into the Plasma Membrane by exocytosis.



❹ Bl membrane proteins can remain in the PM or can enter the same endocytic clathrin-coated vesicles as Ap membrane proteins

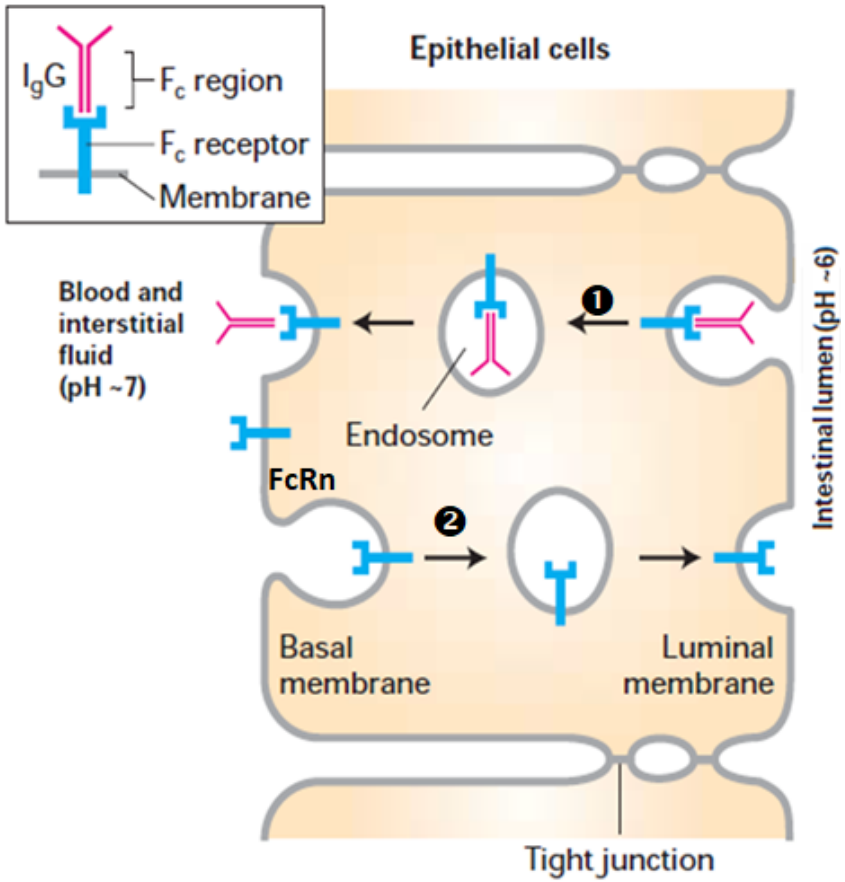
❺ Bl MP are sorted into recycling vesicles that bring them to other parts of the Bl membrane

❻ Ap MP remain in the endocytic vesicles that move across the cell and fuse with the Ap membrane, a process called <u>transcytosis</u>

- Transcytosis also <u>combines endocytosis and exocytosis</u> to move extracellular soluble proteins across an epithelial cell layer
- These are imported on one side of a cell and secreted at the opposite side.
- The process is mediated by specific receptors (Receptor Mediated Transcytosis, RMT).
- RMT is one way of crossing the blood/brain barrier

TRANSCYTOSIS (cont.)

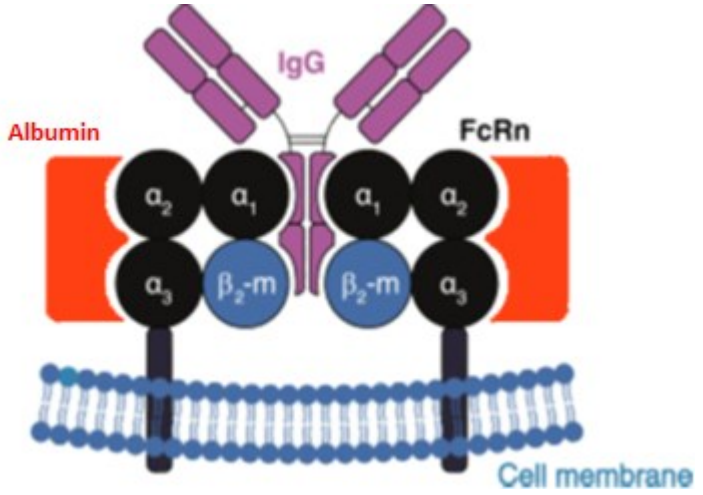
Transcytosis and transport of maternal antibodies



- Maternal IgG (antibodies) are transferred to the foetus during pregnancy and to the neonate from ingested breast milk.
- This involves transcytosis across placental or intestinal epithelia.

❶ The FcRn receptor mediates this movement by pH-dependent binding to IgG Fc.

❷It is responsible for bidirectional transport of proteins across polarized cellular barriers. It also binds albumin, effecting its transcytosis in hepatocytes.



- FcRn (Fc Receptor neonatal) an FcγR type receptor, recogniz the *crystallizable* (Fc) domain of IgG.
- It is a dimeric portien related to class I MHC.
- It binds the IgG Fc region and albumin at separate bindir sites and is active in cells throughout life.

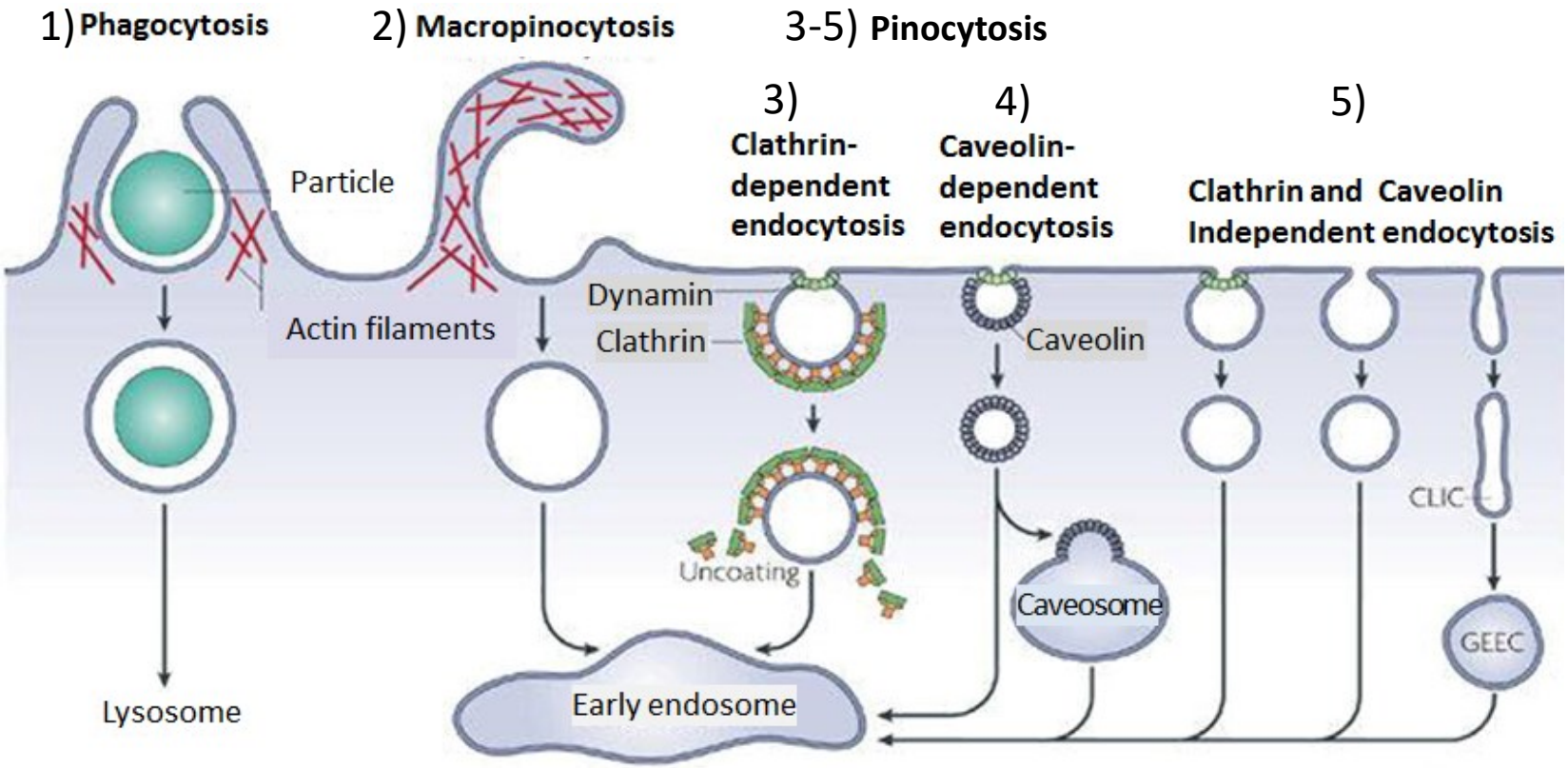
ENDOCYTOSIS

Different types of endocytosis mediate entry of material into cells

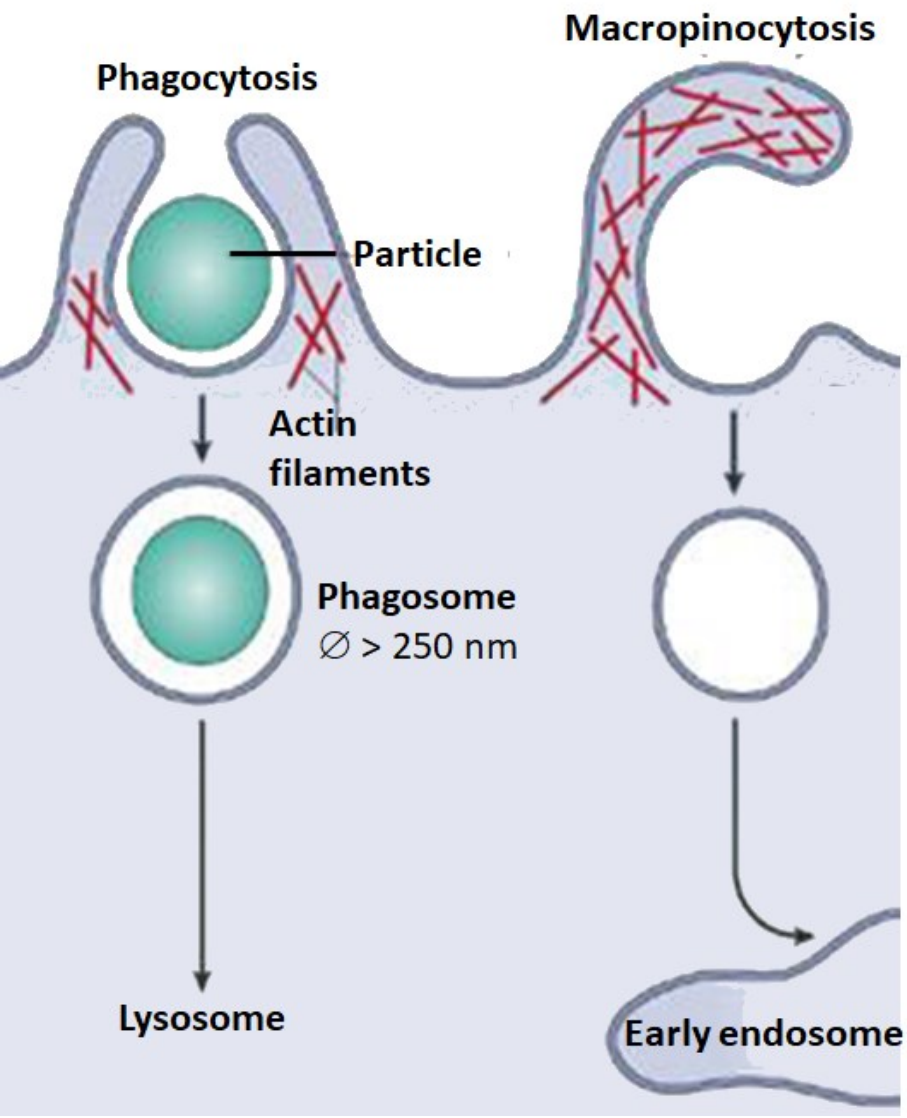
- Cells take up macromolecules, particulate substances, and in specialized cases cells or cell fragments.
- They sort the material they internalize to specific destinations.
- Several **endocytic pathways** can be employed:

  1) phagocytosis; 2) macropinocytosis; 3) clathrin-dependent endocytosis;

  4) caveolin-dependent endocytosis; 5) different types of clathrin/caveolin-independent pathways.
- Phagocytosis and clathrin/caveolin-dependent endocytosis are receptor-mediated processes



ENDOCYTOSIS (cont.)

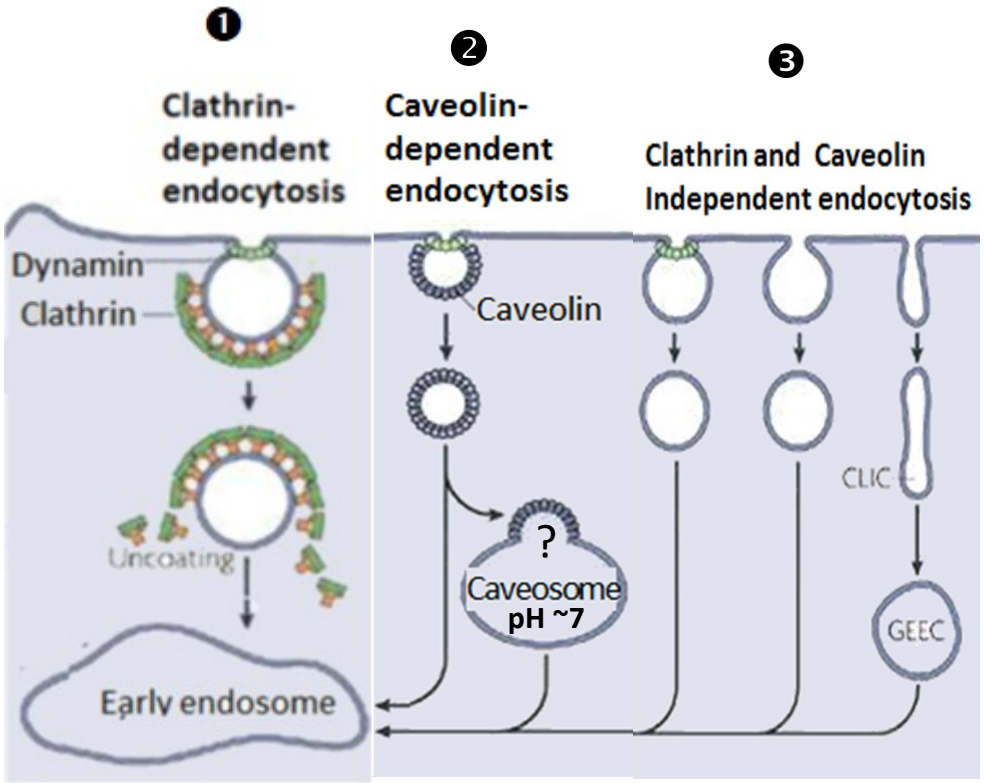


1) & 2) Actin-dependent endocytosis

- Phagocytosis: *i)* regulated *ii)* nonselective *iii)* actin-mediated process in which plasma membrane protrusions envelop and engulf a particle (forming an intracellular phagosome).
- The protrusions contain actin filaments and can curve around whole bacterial cells and large cellular fragments.
- The phagosome eventually fuses with lysosome
- Macropinocytosis: another *i)* regulated *ii)* non-selective form of endocytosis that mediates uptake of solute molecules, nutrients and antigens.
- A single membrane protrusion, filled with actin filaments, hooks around the cargo.
- It seems likely that macropinocytosis, phagocytosis and other large-scale actin-dependent uptake mechanisms are homologous processes but with different target organelles (macropinocytosis → endosome)

PINOCYTOSIS

Eukarvotic cells continually engage in a form of endocytosis known a PINOCYTOSIS.



- In this process a small region of the plasma membrane invaginates to form a vesicle ~50-100 nm in diameter.
- There are several possible pathways for pinocytosis at the cell surface:

❶ Clathrin-dependent endocytosis: a form of receptor-mediated endocytosis where extracellular macromolecular ligands bind to specific receptors on the cell surface internalization (linked to AP2) and is mediated by clathrin (*MA4-1 Slides 33-34*)

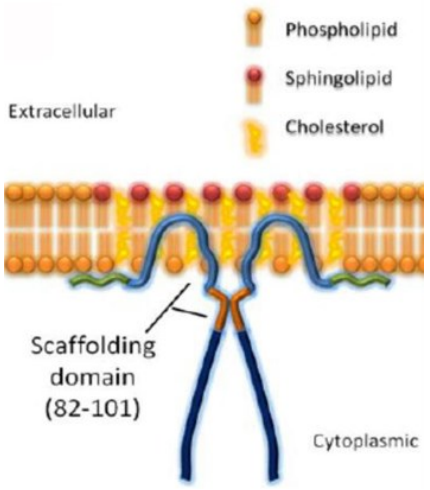
❷ Caveolin-dependent endocytosis: a slower process where small invaginations of the plasma membrane (caveolae) occur at lipid rafts in the presence of caveolin. These recruit signal receptors.

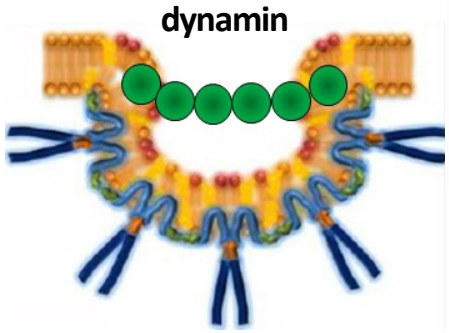
- Both clathrin- and caveolin-dependent endocytosis require *Dynamin*/GTP for vesicle budding

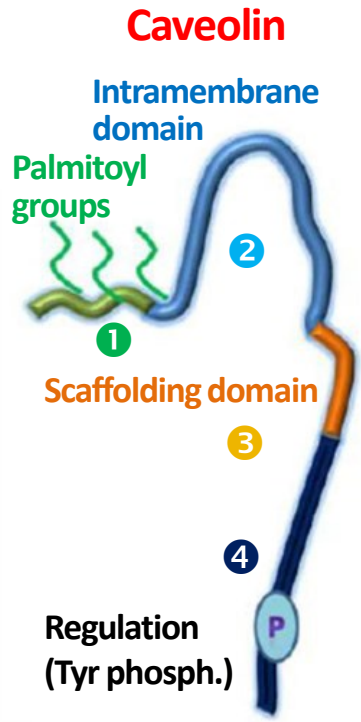
❸ Clathrin- & caveolin-independent endocytosis. Other pathways for forming vesicles that do not involve these proteins and may (or may not) involve *Dynamin* and/or Receptors.

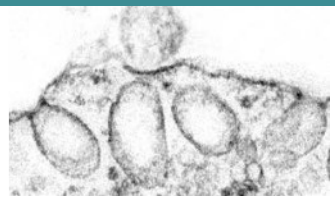
- In pinocytosis, extracellular macromolecules internalized via vesicles merge into the early endosome.

CAVEOLIN-DEPENDENT ENDOCYTOSIS







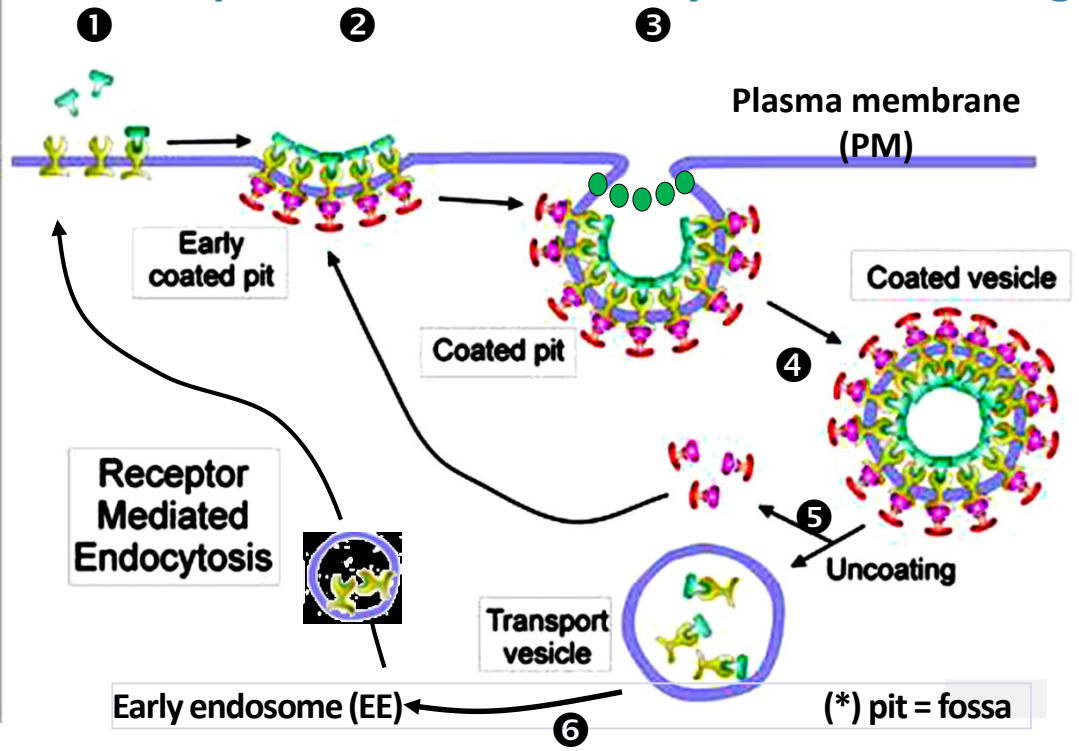


- Not as well understood as clathrin-dependent endocytosis.
- Vesicles initiate at caveolae ("*little cavities*") seen in the plasma membrane of most cell types, sometimes as deeply invaginated flasks (~50 -100 nm ∅)
- These are thought to collect cargo and invaginate by virtue of the lipid composition of the caveolar membrane.
- They occur in lipid rafts, rich in glycosphingolipids and cholesterol, where signal receptors and GPI-anchored membrane proteins preferentially locate.
- The major structural protein needed for caveolae formation is Caveolin (integral membrane protein) with:
  - ❶ membrane-anchoring domain linked to palmytoils,
  - ❷ intramembrane domain
  - ❸ scaffolding domain → oligomerization
  - ❹ cytoplasmic domain involved in regulation
- Pinching off requires the action of dynamin like in clathrin mediated endocytosis

- Possible functions:
  - signalling regulation
  - lipid homeostasis
  - regulation of PM lipid composition

RECEPTOR MEDIATED ENDOCYTOSIS

Receptor-Mediated Endocytosis and sorting of internalized proteins



- RME via Clathrin/AP2-coated pits (*) and vesicles is a process similar to trafficking of lysosomal enzymes in the *trans-Golgi network* (*see previous Module A4-1*).

- Most ligands bound to cell-surface are believed to be internalized into cells in this manner with the following steps:

❶ Binding of ligand to cognate receptor

❷ The activated receptor binds to adapter protein (AP2,) and clathrin in the pit

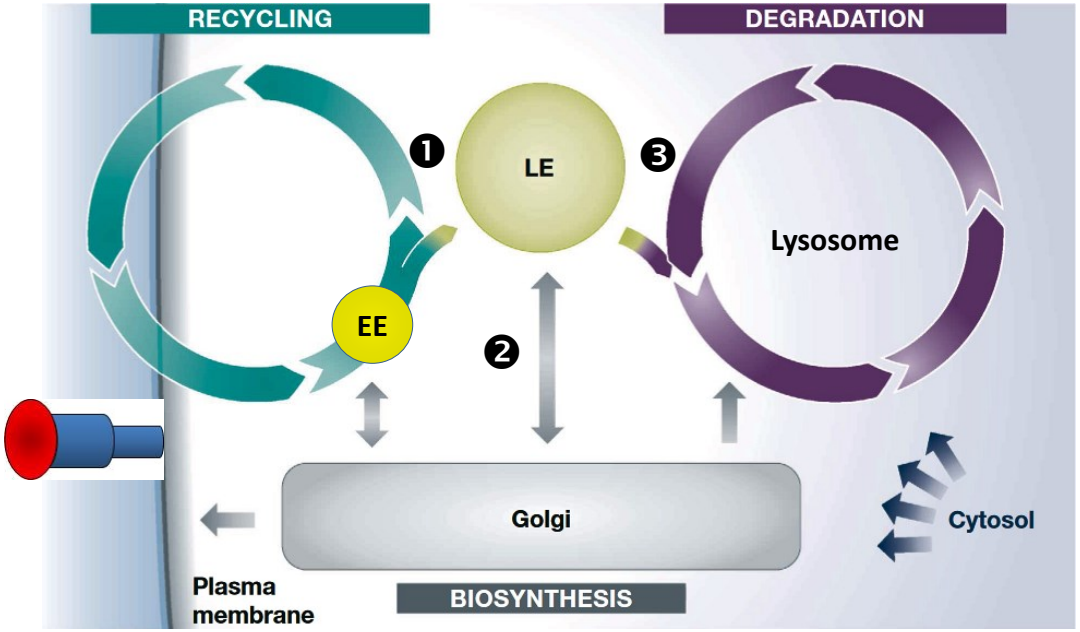
❸ Components accumulate and the pit invaginates. ❹ Dynamin concludes vesicle formation

❺ Uncoating allows Clathrin and AP-2 to return to the membrane, while the receptor complex is

❻ transported to the Early Endosome (receptor recycled to PM, ligand transported to final destination)

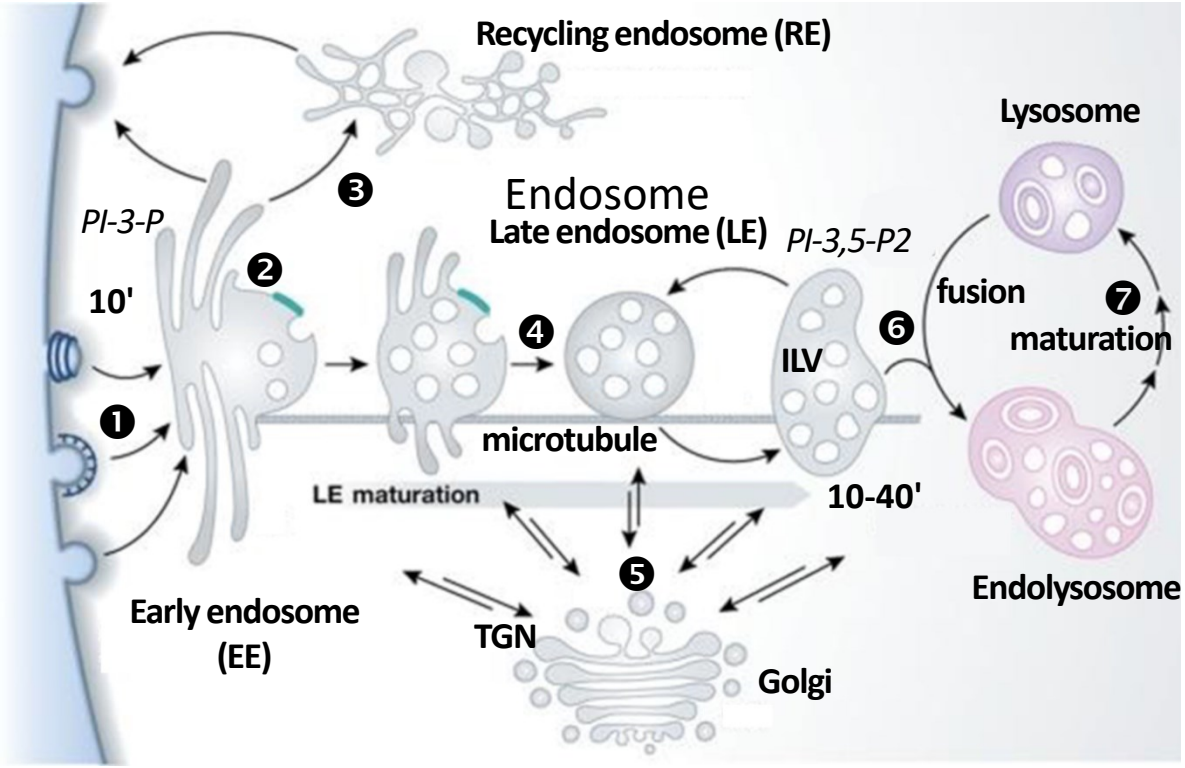
- Some receptors are already clustered in clathrin-coated pits even in the absence of ligand.
- Other receptors diffuse into pits and conformational changes in the receptor-ligand complex cause it to bind to clathrin/AP-2 and be retained there.
- TM proteins containing a Tyr-XX-Φ sorting sequence in their cytosolic domain are recruited to coated pits by the AP-2 complex (see Mod. A4-1).

The endocytic pathway carries proteins from the PM to lysosomes



- Receptors with bound ligands internalize into cells through multiple endocytic pathways
- Vesicles carrying cargoes then merge into the common endosomal network.
- Endocytic vesicles undergo multiple rounds of homotypic fusion to form early endosomes (EE).
- Internalized cargo is sorted and
- ❶ recycled back to the PM through the recycling endosome (RE) or in other ways (e.g. receptors)
- ❷ sent to the trans-Golgi network (TGN) via retrograde trafficking mechanisms
- ❸ trafficked to the lysosome through the late endosome/multivesicular body (MVB) for degradation.
- Most of the PM components (receptor proteins and lipids) that are endocytosed are continually returned to the cell surface by exocytosis (up to 50% / hr in some estimates).

Role of the endosome in vesicle trafficking



- The endosome is central to vesicular trafficking from the PM.
- Endosomes mature as they progress.
- The regulation of membrane traffic and cargo sorting is achieved through a tight spatial and temporal control of endosomal identity.
- This is defined by specific phosphatidyl inositol phosphates (PIPs) & *Rab GTPases*.

❶ Endocytic vesicles from PM → EE (~10min)

❷ Recruits *Rab5* & *PI(3)kinase*. PI-3-P forms in EE

❸ Vesicles from EE tubular membrane → RE (recycling of receptors → PM);

❹ EE → LE which travels along microtubules (MT) in the perinuclear space. pH ↓ (*v-type H^+ ATPase*);

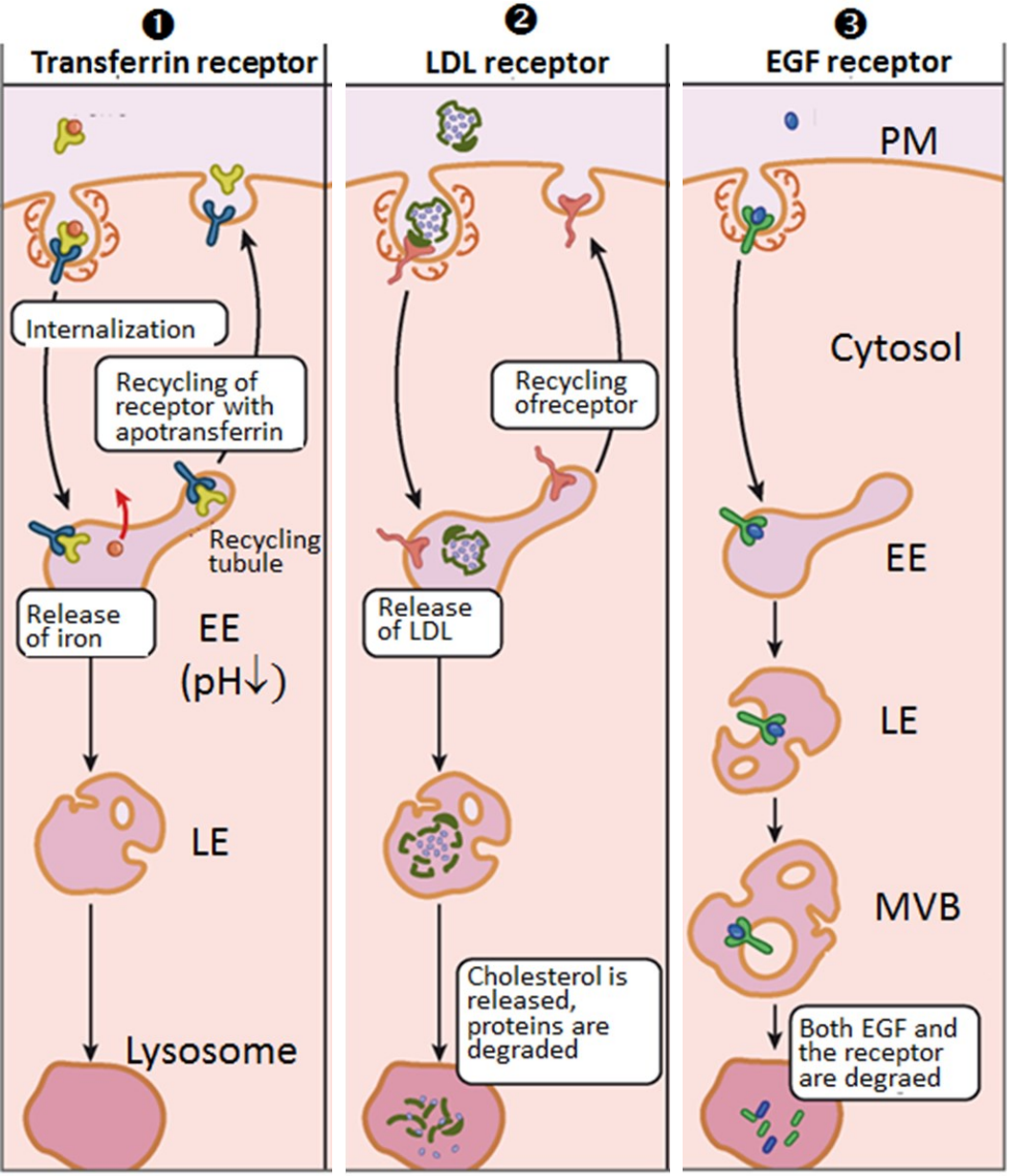
❺ ILV (intralumenal vesicles) form. Vesicles ←→ TGN; (retrograde transport via *Arf*/COPI vesicles), anterograde transport via Clathrin/AP1 vs). *Rab5* switches with *Rab7*; PI-3,5-P_2 forms

❻ LE fuses with lysosome (delivers endocytic & secretory cargo) forming endolysosome (transient hybrid organelle). Cargo degradation begins (after 10-40 min.)

❼ Maturation to lysosome (denser)

ENDOCYTIC PATHWAY (cont.)

Internalized receptors may be recycled or degraded – 3 cases



- EE sorting depends initially on endosomal acidification leading to ligand dissociation.
- Internalized ligand/receptor complexes have different pH sensitivities for dissociation.
- This allows for the differential recycling of cell surface receptors.

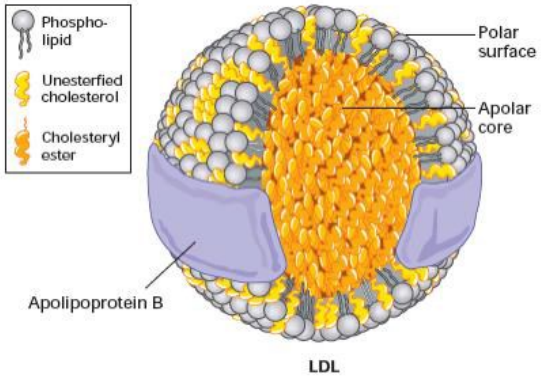
❶ Iron-binding transferrin binds extra_ cellularly to its receptor. At the lower endosomal pH Fe is released, but apotransferrin remains attached. Both receptor and ligand are recycled to PM

❷ LDL receptor releases LDL in EE, which is sent to the lysosome for degradation. Only the receptor is recycled to PM

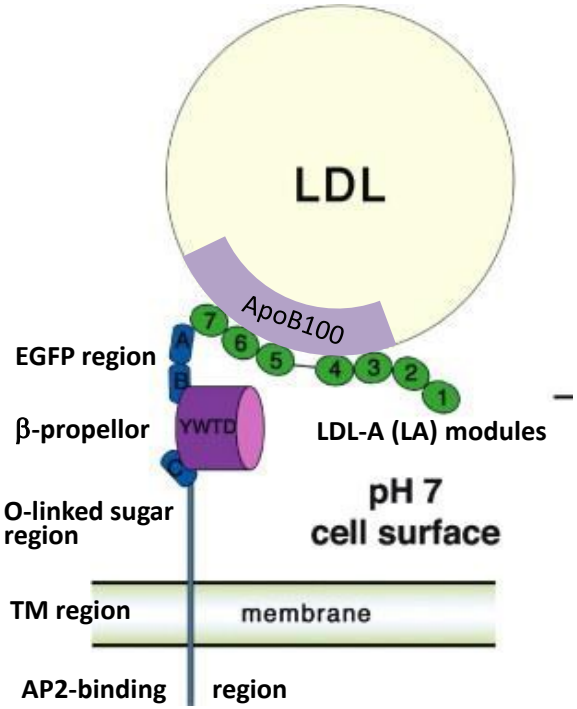
❸ EGF receptor does not released EGF in the EE. Both receptor and ligand are degraded

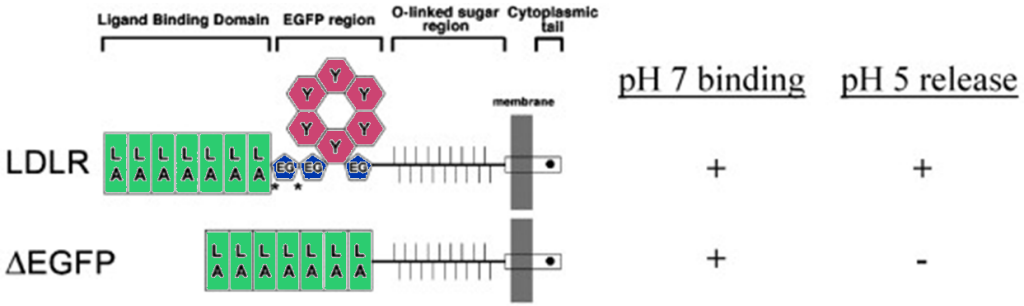
NB. Two or more receptors of different type can be carried in the same vesicle

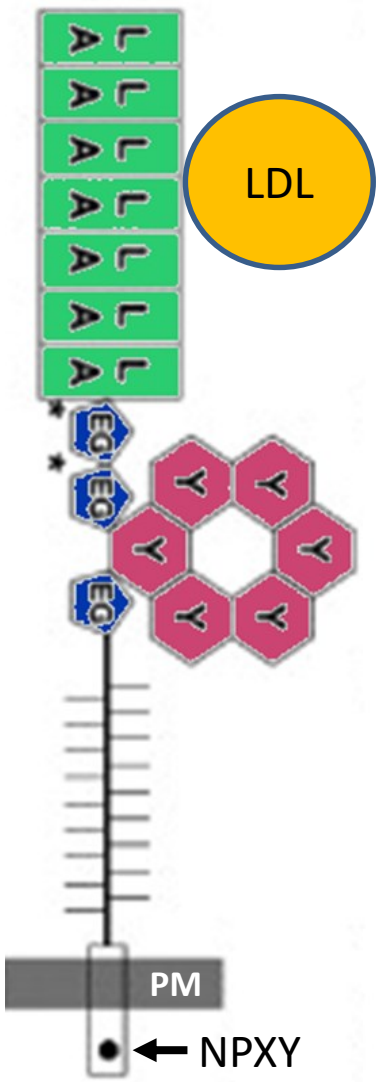
CASE 1: LDL Receptors



- The LDL particle is a sphere of ~20–25 nm ∅ (see *Mod. 2_3*)
- The outer shell is composed of phospholipids, the apoB-100 protein and cholesterol, the core of cholesteryl esters.
- Most cells have cell-surface receptors that specifically bind to apoB-100 and internalize LDL particles by receptor-mediated endocytosis.
- ApoB-100 receptor: an 839-residue glycoprotein with:
  - a single transmembrane segment
  - a short C-terminal cytosolic segment
  - a long N terminal exoplasmic segment consisting of:
    - ~ a ligand-binding domain (7 Cys-rich imperfect repeats several of which bind to Apo B-100)(*see Mod. 2_3*)
    - ~ a β–propeller domain (with YWTD motifs)
      → confers pH sensitivity of ApoB100 binding





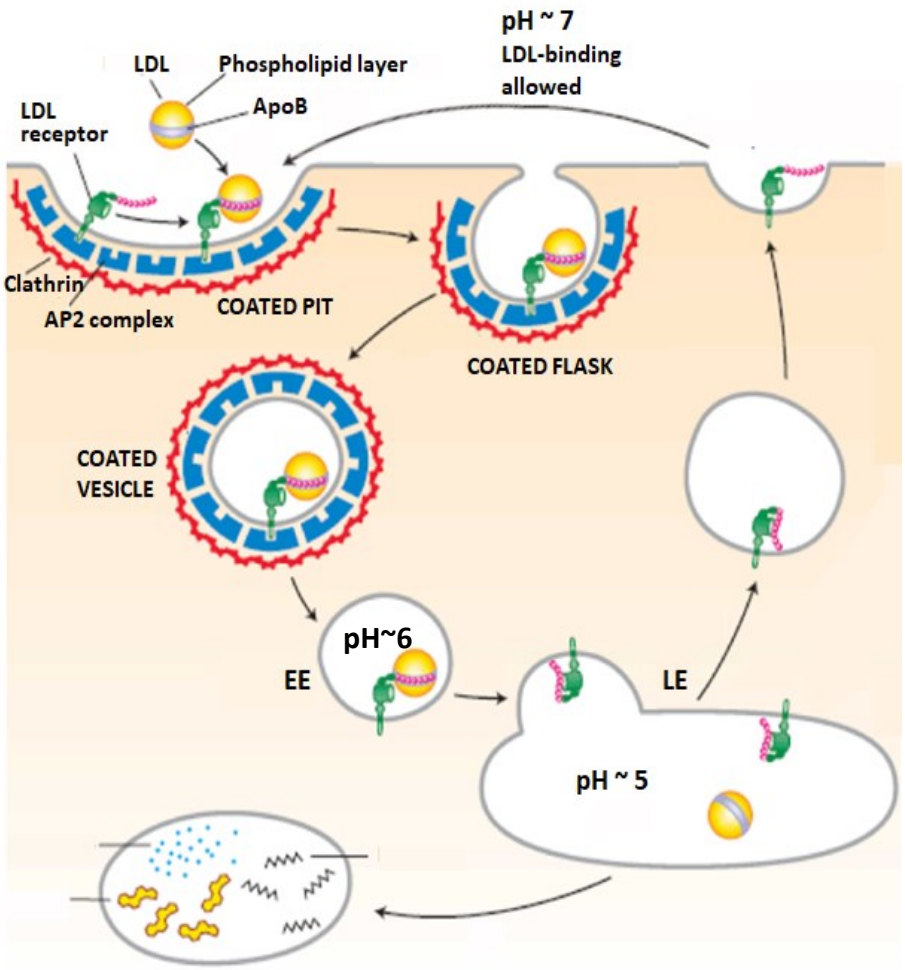


The LDL receptor has a sorting signal for endocytosis

- An initial understanding of the endocytic pathway was gained from studies of the inherited disorder *familial hypercholesterolemia* (high cholesterol serum levels). It led to the discovery of the LDL receptor
- Individual with this disorder have one of several mutant forms of the LDL receptor that causes impaired endocytosis:
- Mutant Apo-B100 receptors bind to LDL particles normally, but are not internalized and remain distributed over the cell surface rather than being confined to clathrin/AP2-coated pits
- This led to the identification of a motif in the cytosolic segment of the receptor that is crucial for internalization: [FY]X**NPX[Y**F] (in particular the residues **NPXY),** recognized by Adapter Protein 2 (AP2).
- A mutation in any of the conserved residues of the NPXY sorting signal abolishes the ability of the LDL receptor to be incorporated into coated pits.
- The gene encoding for the AP-2 adapter protein (adaptin), that binds the NPXY sorting signal, may also be defective giving a similar phenotype.

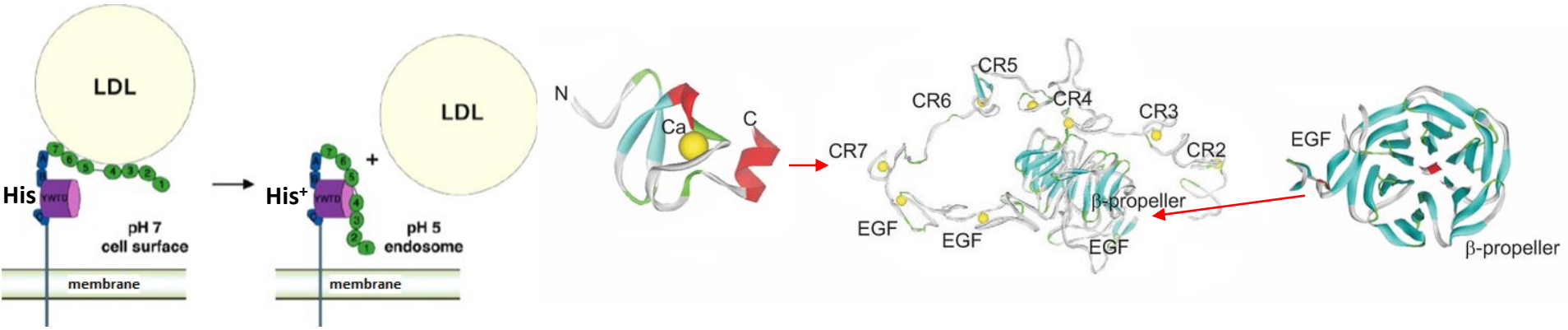
LDL/Receptor dissociation in the Endosome

- The acidic pH of Endosomes causes most receptor/ligand complexes to dissociate
- Surface receptors that bind ligands and undergo endocytosis do so repeatedly, recycling to the membrane in 10-20 min.



- The LDL receptors starts dissociating from LDL in the Early endosomes (begin to acidify)
- The process continues in the Late endosome which is even more acidic (*v-type H^+ ATPase*).
- The receptor recycles back to the PM. The Late endosome fuses with a Lysosome
- Proteins and lipids of the free LDL particle are broken down into their constituent parts by enzymes in the lysosome (AA, FA, Chol)
- On returning to the cell surface, the receptor migrates to the coated pits and the neutral pH allows binding to LDL and another cycle of LDL uptake.

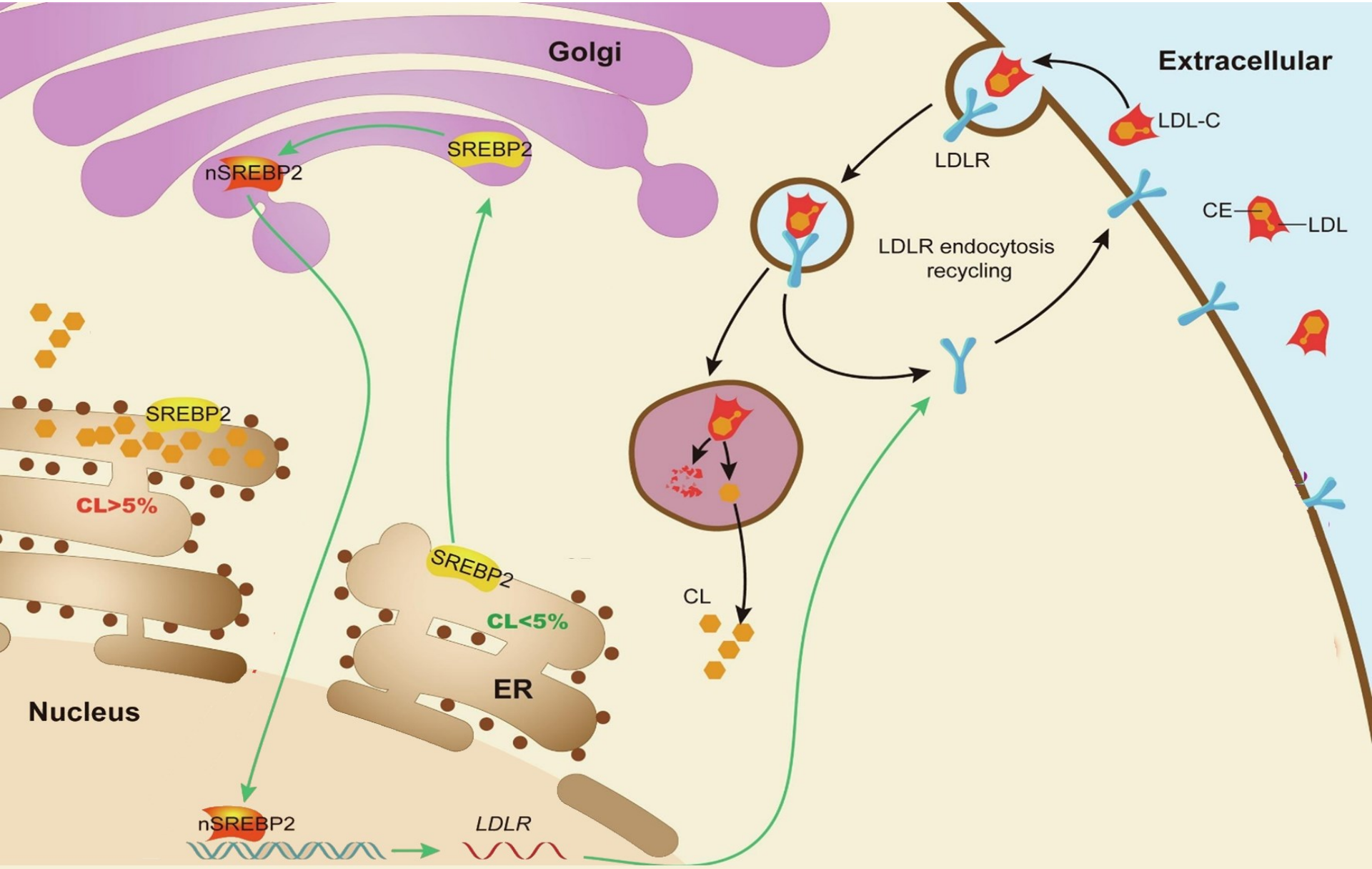
ENDOCYTIC PATHWAY (cont.)



LDL release mechanism

- The Cysteine rich CR modules in the LA domain of the LDL receptor bind strongly to LDL at neutral pH but release it at acidic pH
- At the Endosomal pH of 5.5–6.0, histidine residues in the β-propeller domain of the receptor become protonated. They form a positively charged site that can bind with high affinity to **negatively charged repeats** in the ligand-binding domain (CR-LA domain).
- The CR-LA repeats have a rigid structure due to structural Ca^{++} ions that coordinate with Asp and Glu residues (see CR7 in figure). When a positively charged docking site forms in the β-propeller domain, at the reduced pH, this binds the negatively charged aspartate and glutamate residues rigidly constrained in the CR4 and CR5 (LA4, LA5) 'calcium cages' (low PKa)
- This intramolecular interaction sequesters the LA/CR repeats in a conformation that cannot simultaneously bind to apoB-100, causing release of the bound LDL particle.

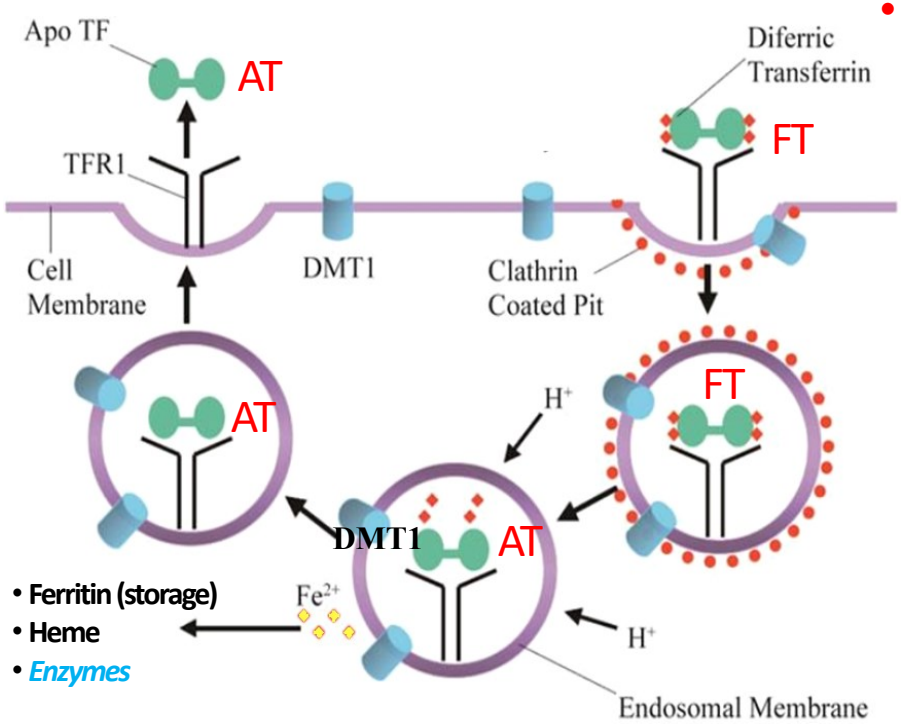
ENDOCYTIC PATHWAY (cont.)



LDL uptake is regulated by cholesterol levels

CASE 2: Endocytic delivery of iron to cells

Iron is delivered to cells **without dissociation** of the receptor-transferrin complex



- **Transferrin** (blood glycoprotein) transports iron to all tissue cells from the liver and intestine (required for iron homeostasis)

Ferrotransferrin (FT) binds $2Fe^{3+}$ ions very tightly

Cell-surface receptors (TFR1) bind FT at neutral pH

TFR1/FT complex → endocytosis

In the LE, at pH < 6.0:

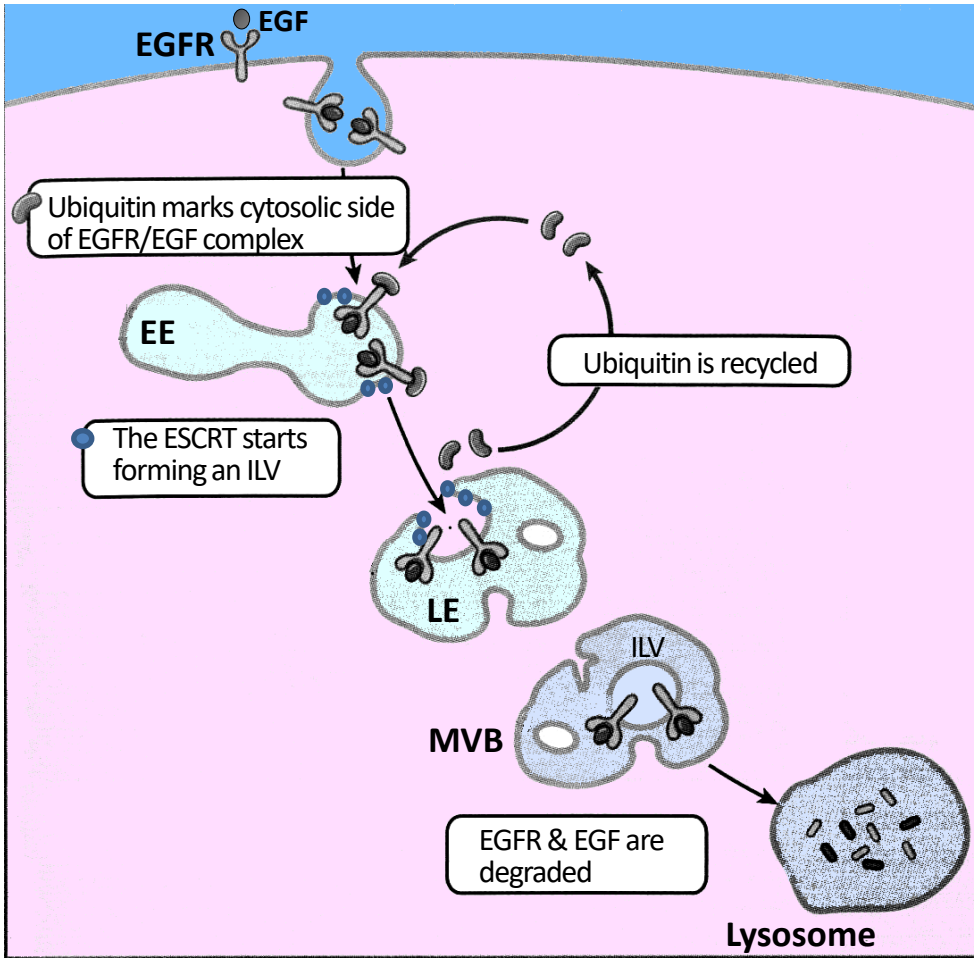
- Fe^{3+} dissociates from FT → **Apotransferrin** (AT)
- Fe^{3+} is reduced to Fe^{2+} by a *ferrireductase*
- Fe^{2+} → cytoplasm (DMT1, *divalent metal transporter*)

- Apotransferrin **does not dissociate** from TFR1

- The TFR1/AT complex is recycled to the PM within a few minutes and exposed to the EC medium
- AT binds to TFR1 at pH = 5.0 - 6.0 but dissociates at pH = 7 (on exocytosis).
- Affinity of the receptor for **apo**transferrin at pH = 7 is lower than that for **ferro**stransferrin so that the two exchange, apotransferrin returns to the blood stream and ferrotransferrin is bound

CASE 3: EGFR long term signalling attenuation by degradation

- Endocytosis decreases the number of receptors on the cell surface (a form of desensitization)
- It down-regulates the sensitivity of a cell to a particular signalling molecule

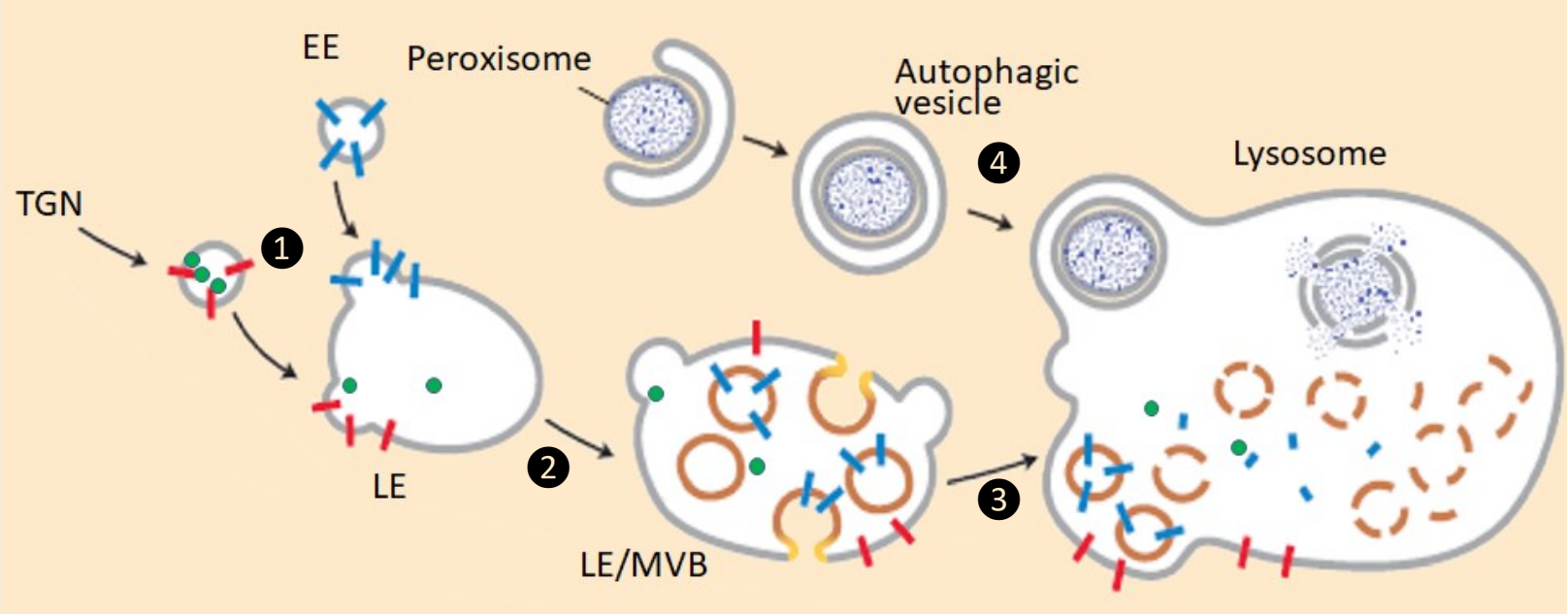


- Clathrin-mediated endocytosis is the major pathway for **epidermal growth factor receptor** (**EGFR**) internalization.
- Other tyrosine kinase receptors (e.g. insulin receptor) are also internalized in this manner
- **Recycling** results in **short-term attenuation** (INSR) while **degradation** results in **long-term attenuation** (EGFR) of signalling
- The EGFR/EGF complex is internalized and sent to the early endosome. Here, **ubiquitin** (that regulates protein degradation) marks the receptor on the cytosolic domain.
- Ubiquitin recruits the *Endosomal Sorting Complex Required for Transport* (ESCRT), a cytosolic protein machinery that induces inward budding. Ubiquitin is removed and recycled.

- This sorts EGFR/EGF complex into **ILV**s (**intra luminal vesicles**) in the late endosomes and the multivesicular body (**MVB**) forms.
- ILVs enter the lysosome where complete degradation of the EGFR/EGF complex occurs.

ENDOCYTIC PATHWAY – INTRALUMINAL VESICLES (ILV)

- ILV are specialized vesicles in the late endosome (LE) with which endocytosed proteins are associated for delivery to the lysosome. Their composition is different to that of the surface membrane.
- Similar in size and appearance to cytoplasmic transport vesicles (TV), they are topologically different. TV *bud outward* from the surface of a donor organelle into the cytosol, ILV *bud inward* (luminal)



❶ Proteins are endocytosed and delivered to EE, which fuses with TVs from *T*-GN that carry cargo proteins destined for the lysosome.

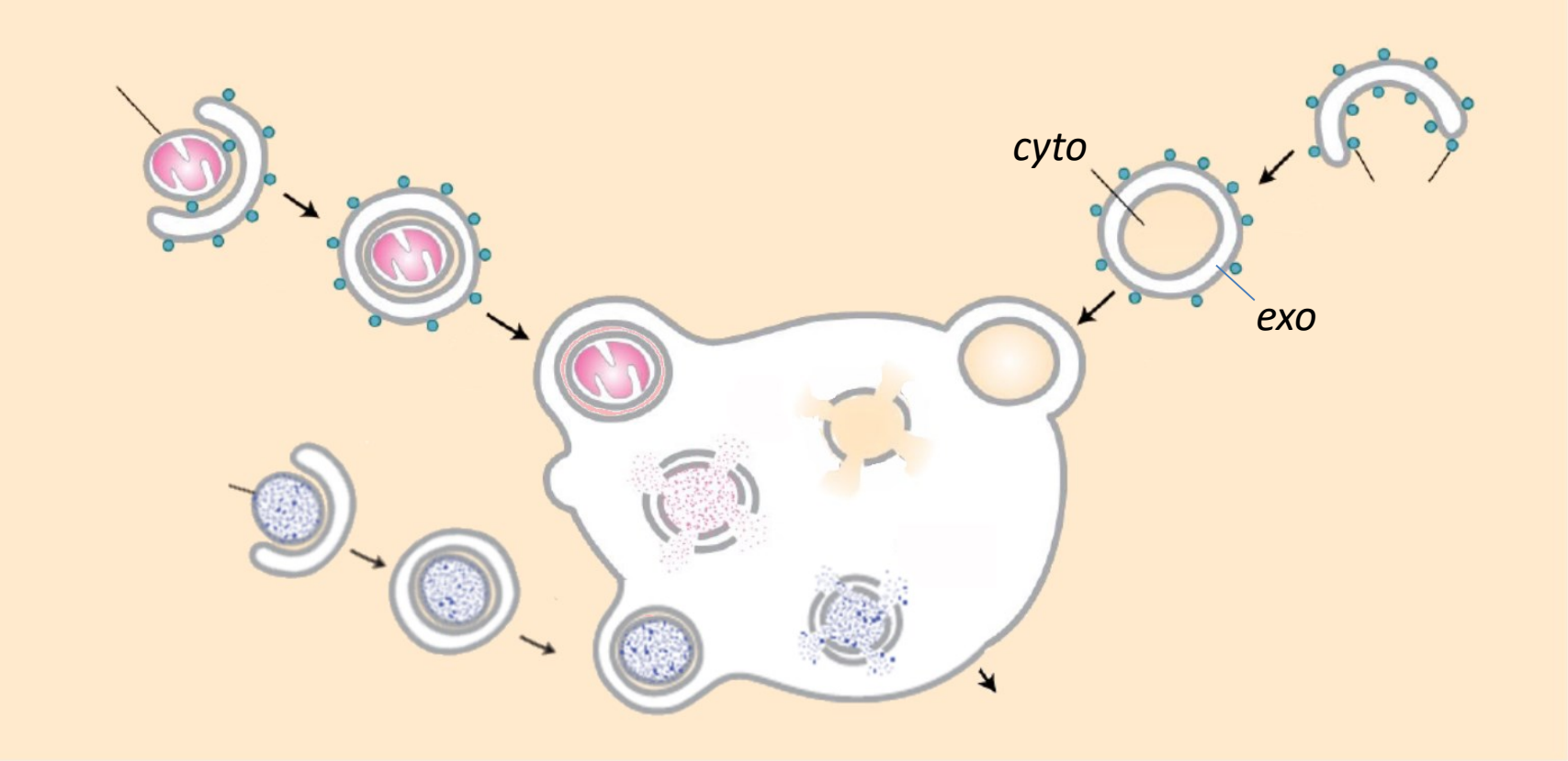
❷ Some endocytosed proteins are inserted into ILV and form the MultiVesicular Body (MVB); Lysosomal protein cargo remain in the LE/MVB membrane or its lumen.

❸ Only proteins incorporated in ILV are degraded.

❹ Autophagic vesicles follow a similar path.

AUTOPHAGIC VESICLES

Autophagic Vesicles deliver cytosolic content and organelles to the lysosome



- The delivery of bulk amounts of cytosol, or entire organelles, to lysosomes for degradation is known as autophagy ("eating oneself").
- This occurs in cells deprived of nutrients or subject to other types of stress (e.g. defective organelles).
- An autophagic vesicle (AV) envelops a region of the cytosol or an entire organelle
- The origin of the initially cup-shaped section of membrane that results in an AV is as yet unknown, likely the ER. They form vescicles with *a double membrane*, with cytosol in their lumen.
- The outer membrane of the AV fuses with the lysosome, delivering the large inner vesicle.