Membrane Trafficking and Vesicle Fusion

Post-Palade Era Researchers Win the Nobel Prize

Riddhi Atul Jani and Subba Rao Gangi Setty

The functions of the eukaryotic cell rely on membrane- bound compartments called organelles. Each of these possesses distinct membrane composition and unique function. In the 1970's, during George Palade's time, it was unclear how these organelles communicate with each other and perform their biological functions. The elegant research work of James E Rothman, Randy W Schekman and Thomas C Südhof identified the molecular machinery required for membrane trafficking, vesicle fusion and cargo delivery. Further, they also showed the importance of these processes for biological function. Their novel findings helped to explain several biological phenomena such as insulin secretion, neuron communication and other cellular activities. In addition, their work provided clues to cures for several neurological, immunological and metabolic disorders. This research work laid the foundation to the field of molecular cell biology and these post-Palade investigators were awarded the Nobel Prize in Physiology or Medicine in 2013.

(left) Riddhi Atul Jani is a graduate student in Subba Rao's Lab at MCB, IISc. She is interested in studying the SNARE dynamics during melanosome biogenesis.

(right) Subba Rao Gangi Setty is an Assistant Professor at the MCB, IISc, Bangalore. He is interested in understanding the disease associated protein trafficking pathways in mammalian cells.

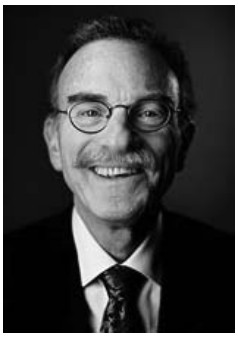
Introduction

Membrane Transport: In the eukaryotic cell, a majority of proteins are made in the cytosol. But the transmembrane and secretory proteins are synthesized in an organelle called the rough endoplasmic reticulum (ER). These proteins undergo certain chemical modifications such as glycosylation, disulfide formation, etc., either to attain their proper folding or to modify the protease cleavage sites. Further, these proteins traverse from the ER to the Golgi, and then to different cellular organelles (including the plasma membrane, PM) or are secreted out of the cell. By this process, the newly synthesized secretory proteins in the ER are transported to their

Keywords
Organelle, membrane fusion, vesicular transport, SNARE, *Sec* mutants, neurotransmission.

Nobel Laureates

James E Rothman
Yale University, USA



Randy W Schekman
University of California, Berkeley, USA

Thomas C Südhof
Stanford University, USA

respective target sites for their proper functioning. This movement of proteins is referred to as 'protein transport or trafficking'. In addition, cells also utilize the membranes or vesicles to perform this process, which is known as 'membrane trafficking' or 'vesicular transport' [1].

Vesicle Fusion and Neurotransmission: In a cell, the newly synthesized proteins transport from one place to another in the form of a transport carrier called a 'vesicle'. These vesicles are formed from the donor membrane by a process called 'vesicle budding'. They are directed towards the target membrane and undergo 'vesicle fusion' [1]. In addition, vesicle biogenesis and fusion require coat proteins and a specific fusion machinery respectively for accurate transport. This process was initially discovered in neuronal cells, where synaptic vesicles deliver their neurotransmitters through the PM in a synaptic cleft for the activation of other neurons. This process is called 'neurotransmission'[2].

The cellular components and the mechanism of regulation for the above processes which is the focus of this article were discovered in the laboratories of James E Rothman, Randy W Schekman and Thomas C Südhof.

Cell Biology in George Palade's Era

In the 1930s and 1940s, biology was more focused on the structure and function of DNA and RNA using bacteria as a model system. Very little was known about the structure of the eukaryotic cell. For the first time, in the early 1930s, Albert Claude at Rockefeller University described the ultra-structural details of the majority of the cellular organelles such as mitochondria, chloroplasts, endoplasmic reticulum, Golgi apparatus and ribosomes (except endosomes). In his discoveries, Claude used a biochemical method called 'cell fractionation[1]' developed in his laboratory on liver cell lysates, to distinguish the different components of the eukaryotic cell [3]. In addition, Claude applied electron microscopy (EM) techniques[2] to analyze the fractions separated by the above method. He revolutionized the EM

technique by treating the samples with osmium tetroxide, which preserved the cell membrane and increased the contrast of electron micrographs. Claude published the detailed structure of a cell for the first time in 1945 [4] in which he provided an insight into the structural organization of the eukaryotic cell.

During the course of time, Albert Claude's friend, Christian de Duve of Rockefeller University, described two other eukaryotic organelles. de Duve's laboratory discovered the lysosomes while analyzing the activity of enzymes distributed in different fractions obtained through rate-zonal centrifugation[3] of liver cell lysates. In addition, de Duve applied EM technique on these fractions and described the ultra-structure of the lysosome. Similarly, de Duve's laboratory identified the peroxisomes while studying the activity of an enzyme, urate oxidase [5]. Together, the studies from Claude and de Duve's laboratories illustrated the structure and organization of cellular compartments of the eukaryotic cell and these discoveries initiated the field of cell biology [6, 7]. But it was not clear how these organelles are generated and how they communicate with each other.

These questions motivated George E Palade while he was working in Claude's laboratory as a postdoc. In his own laboratory at Rockefeller University, Palade carried out integrated morphological and biochemical analysis of different organelles using pancreatic exocrine cells of the guinea pig. He also studied the structure of several pancreatic secretory proteins using EM and cell fractionation procedures. These studies illustrated the ultrastructure of the cell and, most significantly, Palade showed for the first time that the cytoplasm is packed with stacks of ER studded with ribosomes. This observation prompted Palade to investigate the role of ribosomes on the ER membrane and other subcellular organelles. Later studies from his laboratory showed that ribosomes synthesize the secretory and transmembrane proteins on the ER and translocate them into the ER lumen for further transport [8]. But, it was unknown how these proteins are secreted out of the cell.

[1] Cell fractionation is a method commonly used to separate the cell lysate into individual fractions containing different organelles or membranes. In this method, cells are lysed in a non-detergent lysis buffer which will keep the cell membranes in a native state and then separated by density gradient centrifugation (separation of molecules based on density).

[2] Electron microscopy is a technique used to reveal the ultrastructure of cells (both prokaryotic and eukaryotic), membranes, organelles, materials, etc. This method uses an electron beam as a source for illuminating the sample, which will provide an image with 200 times higher resolution than light microscopy. There are two types: transmission electron microscopy, widely used to study the structure of organelles or membranes and scanning electron microscopy, used to study surface organization.

[3] Rate-zonal centrifugation is a type of density gradient centrifugation used to separate the organelles or molecules based on size and mass instead of density. This method has the advantage that the particles with faster sedimenting rates are not contaminated with the slower sedimenting particles as would occur in the differential (density gradient) centrifugation method.

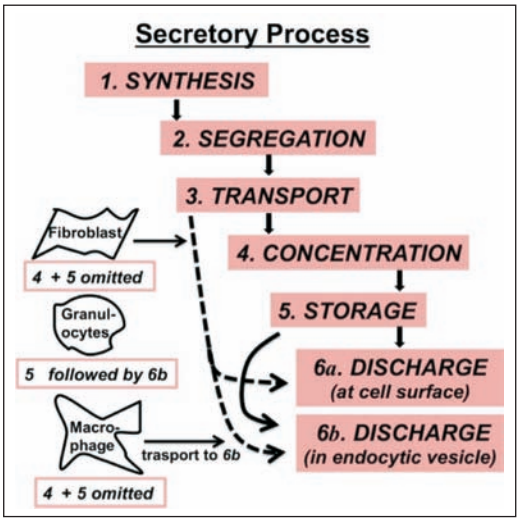


Figure 1. The key steps of the secretory process in various cell types as described by Palade.
This model was proposed by Palade in his Nobel Lecture based on the knowledge available at that time including the research findings from his own laboratory on pancreatic exocrine cells. Exceptions to this model that occur in granulocytes and macrophages are shown in the figure. He proposed that discharge in fibroblasts is likely to take place continuously, whereas it is preferentially through endocytic vacuoles in neutrophils, eosinophil granulocytes, and macrophages [8].

It was a difficult question in those days since the available techniques were not adequate for studying such processes. So Palade developed a novel method called 'pulse-chase analysis'[4] and used it to study the protein secretion process in pancreatic exocrine cells. This technique revolutionized the field and Palade was able to describe the transport of secretory proteins in pancreatic cells [8]. Based on several discoveries from his laboratory, Palade proposed a model for the cell secretory process in 1974, involving six successive steps or operations (*Figure* 1): (1) biosynthesis of new proteins; (2) segregation of proteins on the ER membrane; (3) intracellular transport between the compartments; (4) concentration of protein in a new organelle; (5) intracellular storage in organelles such as lysosomes, secretory granules and (6) discharge of contents through the PM or to endocytic vacuoles. This was the first description of the process of protein transport between cellular organelles. Palade's laboratory worked extensively on each of these six steps, and proposed hypothetical models for intracellular transport and discharge [8] which will be the focus of the remaining article.

[4] Pulse-chase analysis is a method to study the cellular processes over time. Cells are pulsed with radio-labeled amino acids, which will incorporate into the newly synthesized proteins. The lysates made from these cells are used to measure the half-life or turnover rate of any protein and the data can be correlated with cellular processes.

Intracellular Transport

By using the pulse chase method, Palade's laboratory showed that the newly synthesized glycoprotein or secretory protein in the ER

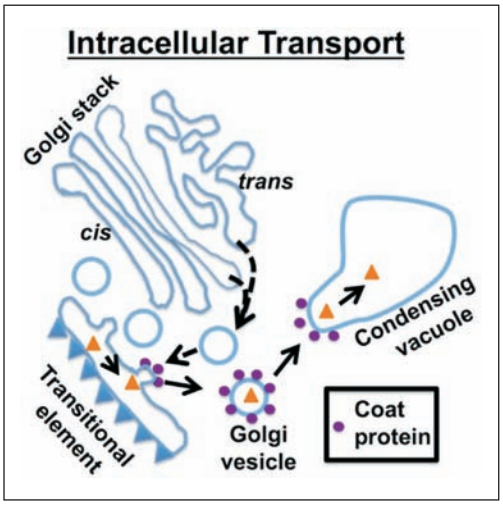


Figure 2. Model for intracellular transport proposed by Palade.
Pulse-chase experiments from Palade's laboratory showed that secretory proteins (triangles) move from the rough ER to transitional elements and to small peripheral vesicles on the *cis*- side of the Golgi complex and finally to condensing vacuoles. These transport steps require energy. Based on these studies, Palade hypothesized that the processes are controlled through a lock–gate method and are mediated through coated vesicles [8].

traverses to the condensing vacuole or the secretory compartment through the Golgi complex (*Figure* 2). Based on these studies, Palade predicted that a lock and gate machinery at the compartments would control this transport step. In addition, he also hypothesized that secretory proteins flow vectorially from one compartment to the other.

Discharge

Based on the early investigations on the secretory process, Palade also proposed that the discharge of secretory granules occurs through the PM by using a method called 'membrane fusion', later called exocytosis (*Figure* 3); this secretory discharge possesses a high degree of specificity. These experiments prompted Palade to hypothesize the existence of complementary recognition sites on each interacting membrane.

Palade's elegant studies laid the foundations for the field of cell biology. Overall, the discoveries of George Palade, Albert Claude and Christian de Duve's laboratories provided an ultra-structural view of eukaryotic cell structure and function. Their research illustrated the intracellular protein transport pathways and secretory processes of a cell. For these discoveries, they were awarded the Nobel Prize in Physiology or Medicine in 1974.

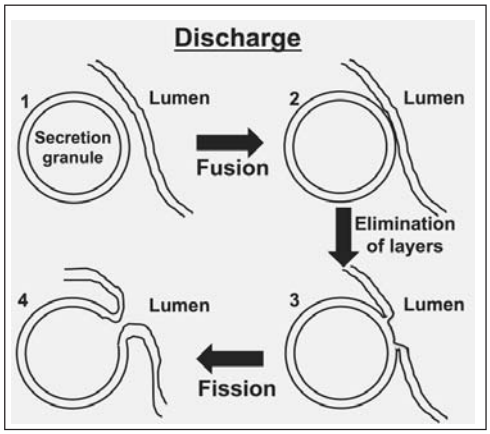


Figure 3. Model for the granule discharge or secretory vesicle fusion or exocytosis as proposed by Palade. Based on the morphological findings in their EM studies, Palade hypothesized that secretory granules discharge their contents by fusing with the PM thus establishing the continuity between the granule and extracellular medium (lumen). During this process, vesicles undergo fusion, elimination of membrane contacts and fission. Further, Palade hypothesized that fusion possesses a high degree of specificity and requires Ca^{2+} and ATP [8].

Even though Palade received the Nobel Prize, his research work was left with many questions for the other cell biologists, such as:

1. How are the polysomes (clustered ribosomes on a translating mRNA) targeted to the ER and what force drives these proteins to move out of the ER?
2. How are the connections established between ER and Golgi: Is it through continuous or intermittent tubules, or vesicles that shuttle between them? How do vesicles maintain their contents in the right concentrations?
3. How could the predicted lock–gate model regulate the vectorial transport of secretory proteins, and how is membrane fusion or discharge regulated? How does the cell redistribute the membranes after vesicle discharge?

The Post-Palade Era of Cell Biology

Despite the seminal contributions of Palade on the secretory pathway, the underlying mechanisms, especially the targeting of secretory proteins to the ER were not clear.

Blobel's Signal Hypothesis

About that time (1971), Günter Blobel at Rockefeller University (trained as a postdoc in Palade's laboratory) proposed the 'signal hypothesis' which states that the proteins secreted out of the cell contain an intrinsic signal that directs them to and across a

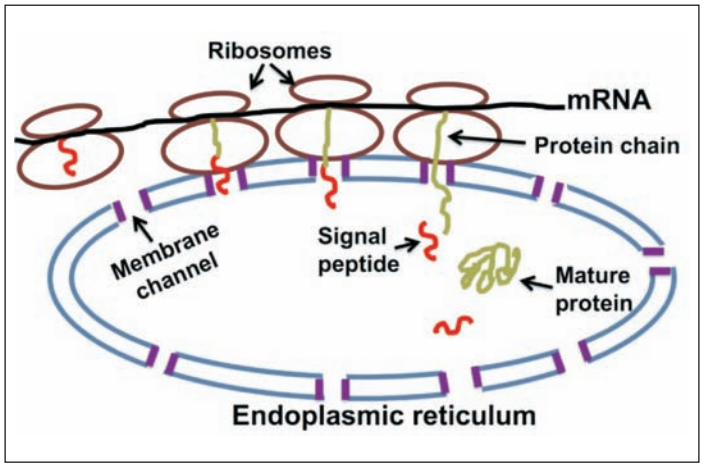


Figure 4. The model for signal hypothesis as proposed by Blobel.
Secretory proteins possess a signal peptide, which directs the translating ribosome to a channel in the ER. The growing protein penetrates the channel and is released into the ER lumen. Later, the signal peptide is cleaved and the protein is exported out of ER [9].

membrane [9]. To prove this hypothesis, they used a cell-free system in which the entire translational machinery was reconstituted with the functional ribosomes attached to the mRNA, along with initiation factors. Later studies from Blobel's laboratory showed that the intrinsic signal is a 'peptide' present in the N-terminus of each secretory protein, and this directs the translating ribosome towards the ER and inserts the protein into the ER through a channel (*Figure* 4) [9]. Eventually, research showed that the signal hypothesis is a 'universally conserved process'. Thus, Blobel's research provided an answer to Palade's first question. Further work identified another characteristic signal (located within or at the end of the protein) which acts as 'sorting signal' or 'address tag' or 'zip code' on the proteins, and directs the proteins to other intracellular organelles [9]. Overall, these discoveries demonstrated the importance of intrinsic signals for secretory proteins, showing that they govern their transport and location in the cell. For these studies, Günter Blobel was awarded the Nobel Prize in Physiology or Medicine in 1999. This was the first step in understanding protein secretion after Palade's era.

From Screening Mutants to Fusing Membrane

The answers to the remaining questions of Palade came from the research work of the cell biologists James E Rothman, Randy W Schekman and Thomas C Südhof. Interestingly, they adopted very

different approaches to address Palade's questions, such as '*in vitro* biochemistry' by Rothman, 'ingenious yeast genetic screen' by Schekman and 'curiosity to decipher the electrical activity of synapse' by Südhof. Their pioneering approaches launched a voyage of discovery for our understanding of the cell secretory transport system which revealed the basic mechanisms that govern intercellular trafficking, the regulation of membrane fusion and cell–cell communication.

Intracellular Transport

The synthesis of glycoproteins that are targeted to other intracellular organelles (e.g., lysosomes) or exported out of the cell starts at the ER. But it was not known how these proteins traverse from the site of synthesis to its target place. Previous studies from Palade's laboratory suggested that the newly synthesized proteins are transported to secretory granules or the vacuole through the Golgi (*Figure* 2). But these studies provided no insights into the nature of protein transport or its mechanism. These questions engaged James Rothman at Stanford University where he developed an *in vitro* transport assay in which he reconstituted the transport of radiolabeled secretory proteins from one set of cisternae of the Golgi to other cisternae, isolated from the mammalian cell lysates [10]. Rothman hypothesized that this transport step leads to a modification of the radiolabeled protein, occurring in the later cisternae of the Golgi. This biochemical transport assay had several advantages: experiments could be performed any number of times, it was cost effective and it could be used to identify the regulators of this reaction step. Using this elegant technique, Rothman's laboratory provided the evidence that the secretory proteins move between the compartments [11]. In addition, Rothman predicted that a new type of non-clathrin coated vesicles might mediate this transport, but the nature of those vesicles was not clear. Moreover, Rothman's laboratory identified two protein factors, NSF [NEM (N-ethylmaleimide) sensitive factor, AAA ATPase] and SNAP (soluble NSF attachment protein) that are required for the biochemical transport along with ATP [11]. This was the first time that the secretory transport step

was shown to require additional components for efficient delivery. But the precise role of these proteins in the transport was not clear at that time.

In parallel, Palade's hypotheses influenced Randy Schekman at the University of California, Berkeley and he used yeast (*Saccharomyces cerevisiae*) as a model system to study the mechanisms regulating the transport of secretory proteins to the PM. Previously, Palade had hypothesized that secretory transport between organelles could occur through either tubular connections or vesicles (*Figure* 2, Palade's second question) [8], but it was not clear how this process is mediated. In addition, yeast was not considered to be a good model to study cell biology in those days owing to its similarities to bacteria rather than to a mammalian cell, even when classified as eukaryotic. But, yeast had added advantage that cells were amenable for genetic manipulation. Further, the secretory pathways of yeast were poorly characterized. Schekman and his first graduate student, Peter Novick, focused on characterizing the vesicles observed in the tip of the developing yeast bud, as seen in their electron micrographs (*Figure* 5A) [12].These observations led Schekman to hypothesize that these vesicles might originate from the secretory pathway and he proposed that they were delivering the enzymes required for new cell wall synthesis [13]. He thought that these vesicles would accumulate in the cell if they mutated the genes of the pathways involved in their formation. In addition, he predicted that those mutations are probably lethal due to the essential nature of the pathways. At that time, Schekman used his experience as a

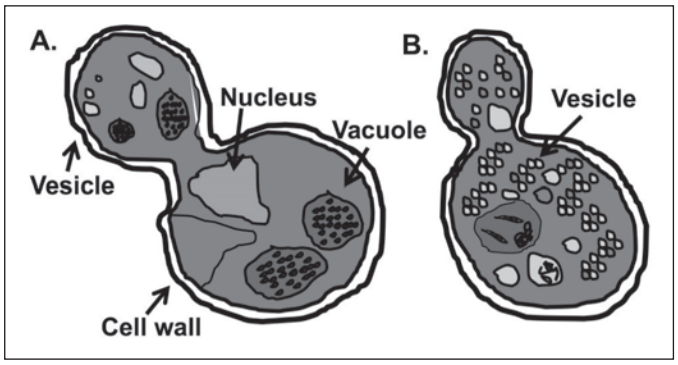


Figure 5. Schematic diagram representing the electron micrographs of *sec1* mutant yeast grown at permissive (**A**) and restrictive (**B**) temperature.

Yeast mutant cells were grown at the permissive temperature (24°C) and restrictive temperature (*ts* mutant, 37°C for 3 hr) and analyzed by EM. Note the presence of small vesicles at the tip of yeast bud neck in **A** (arrow) and accumulated vesicles throughout the cytosol in **B** (arrow).

Figures were hand drawn similar to the original electron micrographs published in [12].

graduate student in Arthur Kornberg's laboratory, where he had learnt that the function of essential genes or proteins can be studied using temperature-sensitive (*ts*) mutations[5].

Using this knowledge, Novick carried out an elegant yeast mutagenesis experiment that had two key assumptions: secretory mutants would be lethal (*ts*) and they would accumulate the secretory proteins inside the cell. The initial mutagenic screen resulted in about 87 *ts* yeast mutants, called '*sec*' mutants, among which two mutants showed defects in secretion and cell wall assembly, named as *sec1* and *sec2* [12]. Later, Novick examined these mutant cells under the electron microscope and found a large number of accumulated vesicles in the *sec1* cells that had been grown at the restrictive temperature (*Figure* 5B) [13]. Subsequently, Novick screened a total of 485 *ts* mutants based on the above criteria and also on their buoyant properties using Ludox density gradient[6] centrifugation. He predicted that secretion-defective mutants would be heavier than normal cells and should sink to the bottom of the tube. This method produced a total of 23 mutants (21 new, plus the original two) involved in the protein secretion [14]. Studies from Schekman's laboratory over several years showed that *sec* genes function in one of the three different transport routes: ER to Golgi, Golgi to PM or Golgi to vacuole (*Figure* 6) [13, 15]. This was the first time that a genetic approach had identified a large set of genes required for the intracellular transport. Further, these studies provided evidence that the essential genes regulate the secretory pathway and are mediated majorly through vesicles.

Surprisingly, two of the yeast *ts* mutants encoding *sec18* and *sec17* genes discovered by Schekman's group were homologous to the NSF and SNAP proteins, identified by Rothman's group [13, 16]. This suggested that the genes required for the secretory pathway are universally conserved from yeast to humans. Furthermore, the discovery of the analogous genes by the two different groups using two different approaches provided strong evidence that the transport mechanisms are also conserved.

[5] Temperature-sensitive mutations (*ts*): Certain mutations in a protein are temperature sensitive and these proteins retain their function at the'permissive' temperature but not at the 'restrictive' temperature. This strategy is used to study the function of essential proteins (or genes) and the absence of these proteins causes cell death. These mutations can be obtained through a conditional mutagenesis method.

[6] Ludox density gradient separation is a method in which Ludox particles form a density gradient during the centrifugation process. Ludox (HS-30 or -40) is a colloidal glass suspension that contains different sized particles and is capable of producing self-generating gradients in a centrifugal field.

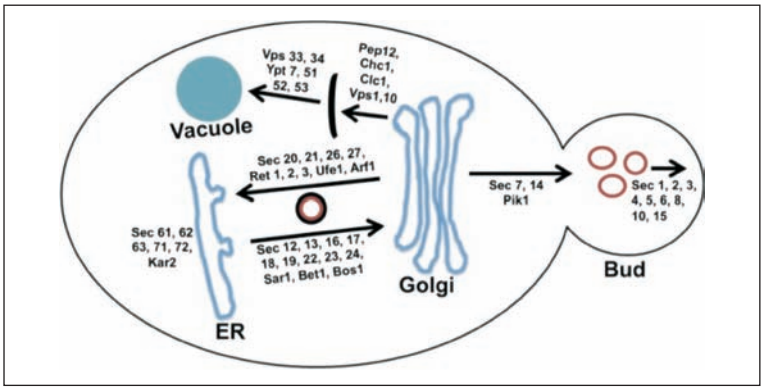


Figure 6. Overview of the *sec* genes function in different yeast secretory pathways. Novick identified these genes by using a yeast mutagenesis screen. The diagram represents the function of *sec* genes in various secretory routes: ER→Golgi; Golgi→ PM; or Golgi→vacuole [13].

Transport is Mediated Through Vesicle Carriers

Around the mid 1980s, the majority of the *sec* genes had been cloned and sequenced but the function of these genes remained unclear. While Schekman's laboratory focused on characterizing the *sec* genes, Rothman's laboratory found small coated vesicles at the tip of the Golgi cisternae in their biochemical preparations. Initially, they compared these vesicle coats with the known clathrin-coated vesicles[7], derived through endocytosis. Later, in collaboration with Lelio Orci, Rothman showed that the Golgi vesicle coats are morphologically distinct from the clathrin coats and they named them 'fuzzy coats' [17]. But, Rothman's laboratory failed to isolate these coated vesicles from their *in vitro* preparations. Meanwhile, Peter Novick at Yale University characterized the function of *sec4* gene as a Rab GTPase and showed that Sec4p function can be blocked using a non-hydrolyzable GTP analog [11]. These results prompted Rothman to hypothesize that an unknown GTPase might recruit the fuzzy coats on Golgi vesicles and if that was true, these coats could be blocked on the vesicles with the help of a non-hydrolyzable GTP analog. Rothman's laboratory treated the cells with this compound and isolated the Golgi vesicles along with their fuzzy coats.

Further studies identified the composition of the vesicle coat which contains 7 novel proteins, called COPI coats or coatomers (*Figure* 7). Later studies from the laboratory characterized a GTPase, ARF1, which belongs to the Ras superfamily[8] and is required for the assembly of these coats on the Golgi membrane. Now, it is known

[7] Clathrin-coated vesicles: Clathrin is a protein, which assembles into triskelion structures, and forms a coat around vesicles during endocytosis processes at the plasma membrane. Similar coat assemblies are also observed on the membrane of trans-Golgi network and early endosomes. Clathrin associates with other accessory proteins, which interact directly with signals on cargo proteins,and sorts them into vesicles.

[8] Ras superfamily is a protein family of small GTPases, which utilizes GTP for their activity. This family represents nine sub families: Ras, Rad, Rab, Rap, Ran, Rho, Rheb, Rit and Arf GTPases and these control a wide range of cellular features including cell morphology and proliferation, nuclear and vesicular transport, cytoskeletal dynamics.

that these coatomers help in membrane bending and vesicle formation, and also in protein sorting [11].

In parallel, Schekman's laboratory was interested in characterizing the yeast mutants in which secretion from ER to Golgi is blocked. Surprisingly, several of their *ts* mutants showed an accumulation of ER-derived vesicles with a distinct coat on them. In collaboration with Lelio Orci, Schekman's laboratory characterized these ER vesicle coats and found that these coats are different from the COPI coats and named them as COPII [18]. Later, Schekman's laboratory identified the COPII coat machinery by isolating the vesicles using an *in vitro* ER budding reaction. Interestingly, this set of coat proteins contained several of their previously described *sec* proteins [13]. Further studies identified a GTPase, Sar1p which regulates the recruitment of COPII coat onto the ER membranes [19]. These coat proteins (COP1 and COPII) disassemble before the vesicles fuse with the target membrane (*Figure* 7) [11, 19]. Thus, the coat proteins play an essential role in membrane transport. In summary, the studies from these two laboratories provided evidence that vesicles mediate the communication between organelles. The discovery of two different coat proteins by two different groups further expanded our understanding of the secretory protein transport.

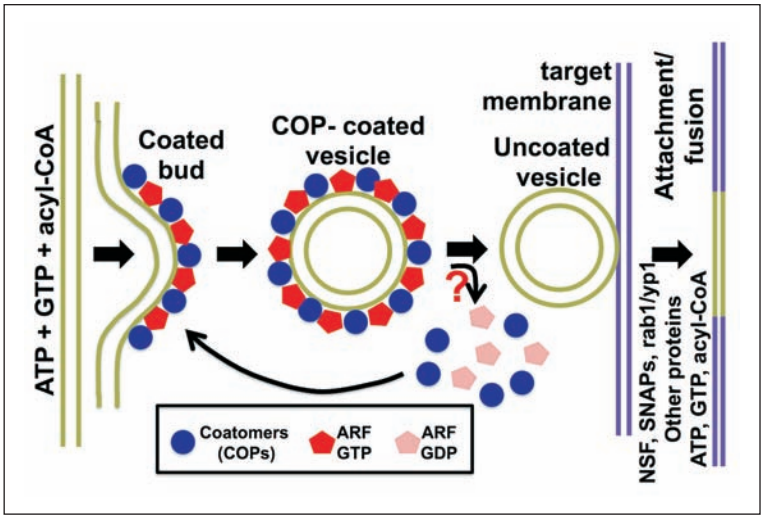


Figure 7. Vesicular transport model by James Rothman. ARF1 GTPase is proposed to recruit the COPI subunits (coatomers) onto the Golgi membranes (left) thus generating COPI-coated vesi-cles. These coats would be disassembled by an unknown mechanism (?) and utilized for the next round. Uncoated vesicles fuse with the target membrane (right) with the help of NSF and SNAP [11].

Vesicles Require SNAREs for Membrane Fusion

In the 1980s, Rothman's and Schekman's laboratories identified NSF/Sec18p and SNAP/Sec17p proteins and showed that these proteins were essential for protein transport between the ER and Golgi or for intra-Golgi transport. The addition of NSF to Rothman's biochemical assay increased the efficiency of membrane transport. But it was not clear how these proteins mechanically regulate protein transport and it was also very difficult to predict their function from the biochemical assay. Interestingly, inactivation of NSF using NEM (N-ethylmaleimide) resulted in the accumulation of uncoated vesicles and few vesicles were associated with the Golgi stacks [16]. This observation led Rothman to predict that NSF helps in the membrane fusion process. He hypothesized that accumulation of uncoated vesicles might be due to failed membrane fusion events. Later studies from Rothman's laboratory found that NSF requires another protein, SNAP for its recruitment onto the membranes [11, 16]. It was not clear how these proteins were recruited onto the membranes, but it was known that their membrane recruitment requires ATP. These observations provided a clue to the existence of SNAP receptors on the membrane (*Figure* 8) [11]. Later, these SNAP receptors were called SNAREs (soluble NSF attachment receptor proteins).

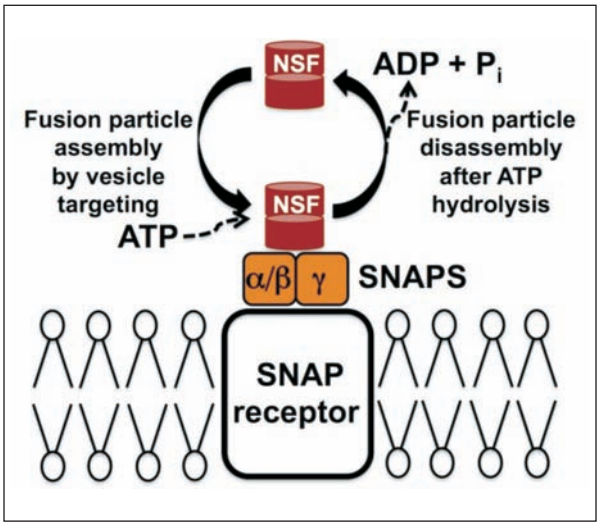


Figure 8. Model for recruitment of NSF and SNAP by Rothman.
NSF and SNAP subunits assemble and interact with the membrane localized SNAP receptor or SNARE on the vesicle which is destined for fusion. NSF and SNAP proteins will disassemble after ATP hydrolysis. Initially, Rothman hypothesized that this assembly is required for membrane fusion, but later findings showed that this process occurs post-fusion [11].

In search of the SNAP receptors, Rothman's laboratory used a large-scale brain cell lysate to study the NSF/SNAP interaction with the membranes. These studies showed that SNAP is selectively bound to syntaxin (STX), synaptobrevin (VAMP) and NSF. In addition, these interactions were sensitive to the addition of ATP, which causes these proteins to disassociate [20]. Surprisingly, these NSF interacting proteins were identical to the synaptic vesicle fusion proteins VAMP (on synaptic vesicle), STX (on PM) and SNAP-25 (on PM) in neurons discovered previously by Richard Scheller, Thomas Südhof and Michael Wilson (*Figure* 9). These research laboratories had shown that this protein machinery is required for the release of neurotransmitters from synaptic vesicles in the neuronal synapse [2, 21]. But, it was not clear how these various proteins mediate fusion of vesicles with the membrane. Later, elegant studies from Cesare Montecucco's laboratory found that two bacterial toxins, tetanus and botulinum toxin, cleave the SNAP receptors (SNAREs) on the synaptic vesicle by their protease activity and block neurotransmitter release. These studies prompted Rothman to propose the 'SNARE hypothesis', wherein a vesicle containing a specific v-SNARE forms a complex with a specific set of t-SNAREs on the opposing target membrane and this minimal unit is necessary for membrane fusion [16]. Later, Rothman's laboratory provided evidence for this hypothesis by incorporating the appropriate combination of v- and t-SNAREs on

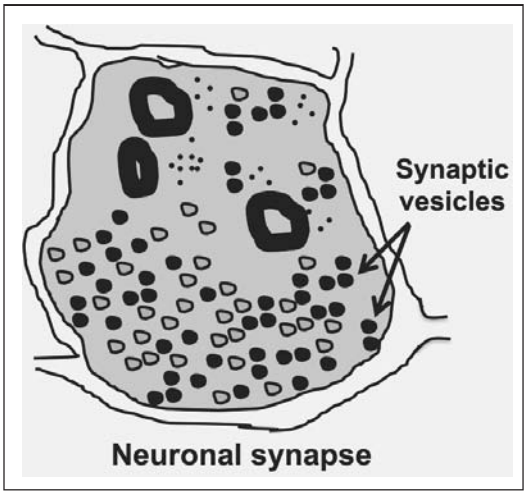


Figure 9. Schematic diagram representing the electron micrograph of a nerve ending with numerous neurotransmitters.

Neurotransmitter-filled synaptic vesicles are accumulated at the neuronal synapse. These vesicles fuse with the PM in a calcium dependent manner. Forty years later, research of Thomas Südhof and other laboratories identified the molecular mechanism of neurotransmitter release.

Figure was hand drawn similar to the original electron micrograph published in [24].

two different populations of liposomes and studied their fusion events *in vitro*. These studies showed that only 9 out of 275 combinations of SNAREs successfully lead to fusion [22]. Now we know that these protein combinations correspond to the known fusion complexes of different transport pathways in a cell. Subsequent studies identified several SNARE family members that localize to different cellular organelles in human and similar homologues in yeast. This suggests that the mechanism of SNARE fusion is universally conserved.

It was not clear how NSF regulates SNARE proteins during membrane fusion. Studies from William Wickner's laboratory now showed that both NSF and SNAP are involved in unwinding the SNAREs from the post-fusion SNARE complex (*Figure* 9) [23]. This suggests that the inactivation of NSF will block the SNAREs on the target membrane, and thus SNARE recycling and fusion is inhibited. Interestingly, these results were consistent with Rothman's studies in which NSF inactivation resulted in the accumulation of uncoated vesicles, which had failed to fuse with the target membrane. Overall, these studies clearly showed that SNAREs on vesicles mediate membrane fusion with the help of accessory proteins [2] thereby giving specificity of vesicle fusion in all membrane transport processes.

SNARE Fusion Regulates Neurotransmission

While Rothman's and Schekman's laboratories were trying to reveal the molecular machinery for membrane transport and fusion, at UT Southwestern University, Thomas Südhof (who received the Lasker Prize 2013, shared with Richard Scheller) was trying to understand neurotransmission at the presynaptic membranes of neurons. In the early 1970s, research studies had revealed the chemical structure of the synapse, which is filled with a large number of synaptic vesicles (*Figure* 12) [24]. Later, EM studies by Palade showed that secretory granules discharge the neurotransmitters through the presynaptic membrane by a membrane fusion process [8]. But it was not known how neurotransmission is regulated through synaptic fusion.

In their early studies, Südhof's laboratory cloned the first synaptic vesicle gene synaptophysin (*Syp*) from both rats and humans. They thought that this protein acts as a structural element for the neuron, being a part of its cytoskeleton. Later studies proposed that synaptophysin was an ion-channel protein or maybe a cholesterol-interacting protein. Since synaptophysin was known to localize to the synaptic vesicles, many researchers exploited this protein as a marker to trace the movement of synaptic vesicles in neurons. But the function of synaptophysin remained uncertain. Südhof and his colleague, Scheller, now discovered that synaptophysin interacts with synaptobrevin (VAMP1) which had been shown to regulate vesicle fusion [25]. In the following years, Südhof and his colleagues discovered two other proteins, syntaxin and SNAP-25 in neurons, which regulate the release of neurotransmitters [26, 27]. The role of these proteins in regulating synaptic vesicle fusion at the synapse was finally clarified in studies from Rothman's laboratory, which showed that VAMP1, syntaxin and SNAP-25 are the SNAP receptors or SNARE proteins. Thus, these independent studies from Südhof and Rothman's laboratories not only discovered the basic machinery required for synaptic vesicle fusion in neurons and vesicle fusion in all cells, in general, but they also suggested that this process is universally conserved.

In addition, Palade's research had indicated that neurotransmitter release also requires calcium and it was thought that calcium would trigger electrical activity at the synaptic cleft [8]. But it was not clear how neurotransmitter release was coupled to the electrical activity of the neuron and how calcium triggers this process at the molecular level. To answer these questions, Südhof tried to identify the molecular component, which senses the calcium during synaptic transmission. Elegant research from his laboratory found a calcium-sensing protein called synaptotagmin, which regulates synaptic vesicle fusion by associating with the SNARE proteins at the synapse [28, 29]. Later studies from his laboratory showed that synaptotagmin provides a trigger to synaptic vesicle fusion and also regulates the temporal synaptic transmission [2, 30]. Further,

Südhof and his colleagues identified another important regulator of synaptic vesicle fusion, Munc18, which associates with the SNARE fusion machinery [31, 32]. Interestingly, Scheller's laboratory found that Munc18 (also called n-Sec1) is Sec1p, a homologue in yeast identified by Schekman's laboratory in their yeast mutagenesis screen [12, 32]. In a nutshell, these insights into synaptic transmission indicated the complexity and tight regulation of vesicle fusion.

Implications of these Discoveries

These novel findings from the laboratories of Rothman, Schekman and Südhof not only answered Palade's key questions, but they also strengthened the field of 'molecular cell biology'. In particular, their studies identified the basic machinery required for vesicle biogenesis or budding, protein sorting, vesicle transport and fusion, and organelle identity. Further, these discoveries illustrated the secretory pathways or protein trafficking routes that regulate a wide range of eukaryotic cellular activities, including cell–cell signaling or communication. In addition, these mechanisms are universally conserved in yeast, mouse, humans and plants. A defect in any of these processes is deleterious to the cells and can be the basis of neurological (schizophrenia, autism), immunological (hemophagocytic syndrome) and metabolic (diabetes) disorders [2, 23, 33]. Mutations in the genes of the basic secretory protein machinery lead to a number of membrane trafficking diseases such as Charcot–Marie–Tooth disease, Cohen syndrome, Hermansky–Pudlak syndrome, and Griscelli syndrome [34, 35].

Current Status – Protein Trafficking, Membrane Fusion, SNARE Recycling and Regulation

The biosynthesis of proteins occurs both in the cytoplasm and the ER. The newly made cytosolic proteins either function in the cytosol or are targeted to organelles such as the nucleus, mitochondria, peroxisomes, ER and plastids (in plants). The laboratories of Blobel, Schekman and others showed that these proteins possess

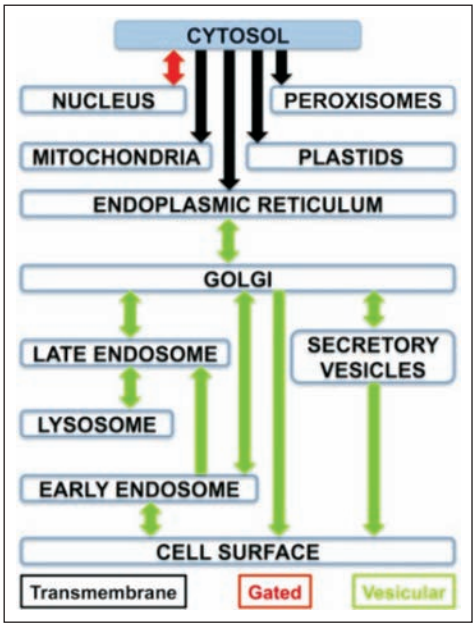


Figure 10. Overview of the secretory transport processes between organelles.
Newly synthesized proteins in the cytoplasm are transported to the target places (organelles) by three different methods. Transport of cytosolic proteins to the ER, mitochondria, peroxisomes and plastids utilizes the transmembrane transport method (black arrows); whereas transport to the nucleus uses a gated transport mechanism (red arrow). Protein transport beyond the ER, including secretion through the cell surface, degradation in the lysosome or organelle storage, uses a vesicular transport process (green arrows) [1].

a specific signal sequence, which is intrinsically integrated in the protein sequence and directs the protein towards the appropriate organelles. Now we know that the transport of cytosolic proteins to the different organelles in a cell is mediated by the following three processes (*Figure* 10):

1. Transmembrane transport: Fully made cytosolic proteins are directly transported to the organelles through the protein importers present on their membrane. Several organelles such as ER, mitochondria, peroxisomes and plastids use this method for their cytosolic protein import and it is a unidirectional process. In addition, mitochondria and plastids possess a genome which codes for certain proteins.

2. Gated transport: Cytosolic proteins enter the organelle through channels or pores present in their membrane. Protein transport to the nucleus occurs by this method and is a bidirectional process. For example, several transcription factors follow this transport route.

3. Vesicular transport: This is a common method of protein transport beyond the ER and to other organelles including the PM. The

majority of the transport steps are bi directional, except for transport from the Golgi to lysosomes or to the secretory granule.

The key machinery mediating these various transport processes is very well worked out [1]. Among the above transport processes, protein targeting to the ER is different from that to other organelles. It has been shown that biosynthesis of proteins at the ER occurs through 'co-translation protein synthesis'[9] which is a well-studied process. Newly synthesized proteins in the ER undergo post-translational modification such as glycosylation and are targeted to the Golgi. In addition, non-functional or misfolded proteins in the ER are exported and degraded by proteasome-mediated degradation. This process is known as ER-associated protein degradation (ERAD) [1].

[9] Co-translational translocation is a type of protein synthesis process in which proteins are integrated into the membrane or inserted into the lumen of an organelle while the protein is translated from mRNA. This is one of the common processes followed by all membrane proteins, and occurs on the ER membranes.

The newly synthesized proteins in the ER are transported to the Golgi for further modifications including additional glycosylation, GPI anchoring, metal loading onto metalloproteins. Protein movement between the ER and Golgi is bidirectional and it is mediated by COPII- and COPI-coated vesicles, as discovered by Rothman's and Schekman's laboratories (*Figure* 11). From Albert Claude's work, we know that the Golgi exists as multiple cisternae (*cis*-, *medial*- and *trans*- Golgi), called the Golgi complex, and it has been shown by Rothman's group that intra-Golgi transport is mediated through COPI-coated vesicles. From the Golgi, especially the trans-Golgi, proteins are targeted to multiple sites through vesicular transport (*Figure* 11). There are three major transport routes connecting the Golgi and other organelles, which include: a) transport to the cell surface through early endosomes (for example, proteins such as growth factor receptors, ABC-transporters, G-protein coupled receptors); b) transport to the lysosome through late endosomes (proteins destined for degradation); c) transport to the secretory vesicles (for example, neurotransmitters, cytokines). Research from several laboratories has shown that transport steps from the Golgi utilize different coat proteins (such as clathrin, adaptor proteins, GGA proteins) for sorting cargo selectively into these transport pathways. Protein transport beyond the Golgi is known to be multidirectional. For the purposes of

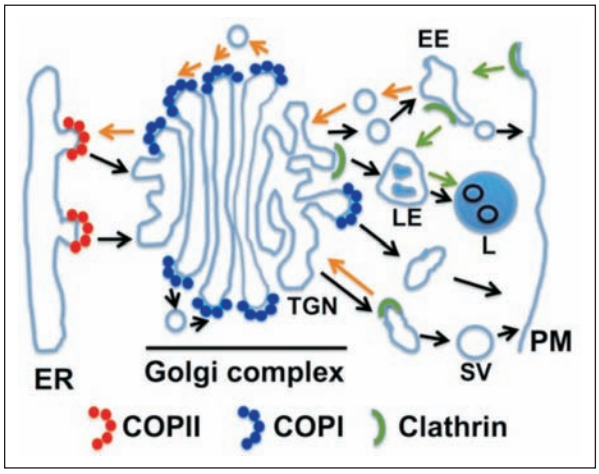


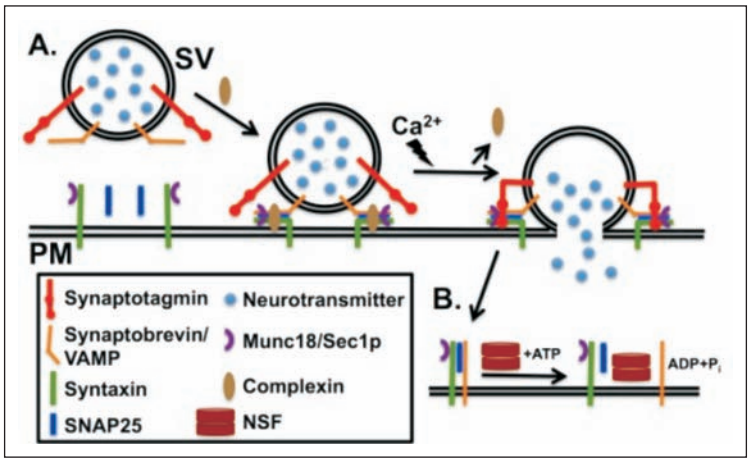
Figure 11. Overview of the secretory transport steps in the eukaryotic cell.
Newly synthesized proteins in the ER are transported to the Golgi and then to the PM or lysosome or secretory vesicles. In contrast, cell surface proteins or extracellular molecules are internalized through endocytosis and then transported either to the lysosome or to the Golgi, and sometimes a few molecules will be recycled back to the cell surface. In the majority of the transport steps, coat proteins are known to play an important role. These coat proteins are recruited from the cytosol onto the specific membrane domain of an organelle to sort the cargo into distinct vesicles for membrane transport. COPII and COPI coats function at the endoplasmic reticulum (ER) and Golgi complex respectively. Clathrin functions at multiple sites, but interacts with different adaptors. Arrows represent the flow of membrane traffic: black arrows – anterograde transport; orange arrows – retrograde transport; green arrows – transport from the PM to lysosomes (L) through early (EE) and late endosomes (LE); and transport of secretory vesicles (SV) towards PM shown separately. TGN is trans-Golgi network [1].

convention, protein trafficking from one organelle to other organelles in forward movement is called anterograde transport and the reverse process is called retrograde transport [1]. It has been shown that both pathways play important roles in maintaining organelle identity (by controlling the steady- state localization of proteins) and in the biogenesis of organelles.

In addition, cell surface localized proteins or extracellular molecules (for example, nutrients, growth factors, pathogens) are internalized from the PM by a variety of processes referred to as 'endocytosis'. This process utilizes different molecular machineries such as clathrin, caveolin, etc., for the internalization of different materials from the cell surface, including fluids [1]. Moreover, recent research suggests that special transport routes exist from the TGN or endosome to specialized organelles including the lysosome-related organelles such as melanosomes, dense granules, weibel-palade bodies in melanocytes, platelets, endothelial cells, etc., respectively [36]. Thus, the cell utilizes multiple transport routes to target proteins to the right place for their function.

It has long been a big question how these processes are controlled and tightly regulated. The research of Rothman, Schekman and Südhof clearly showed the existence of a unique fusion machinery, composed of specific SNARE proteins, which regulate vesicle fusion (*Figure* 12). It has been shown that during vesicle biogenesis a specific v-SNARE (v-vesicle) is recruited during the vesicle biogenesis from the donor membrane [37]. The SNARE of this vesicle interacts with its matching SNAREs on the target membrane (t-SNARE) and this brings the two membranes together, resulting in membrane fusion, and the delivery of the vesicle's contents to the target membrane or organelle. It has been shown that SNARE interaction or pairing is a very selective process, which brings the required specificity to the transport step. This process is known to be regulated by a metal ion, calcium, and other proteins such as SM proteins (Munc18/Sec1p) (*Figure* 12). Elegant studies from Südhof's laboratory showed that synaptotagmins sense the calcium during the vesicle fusion process, especially in neurons, which results in synaptic vesicle fusion and neurotransmitter release (*Figure* 12). Several other molecules, such as tethering factors, are known to be recruited by

Figure 12. Overview of vesicle (synaptic) fusion with the target membrane and SNARE disassembly**.** **(A)** A vesicle loaded with cargo, e.g., a neurotransmitter-filled synaptic vesicle (SV), docks nearer to the target membrane (or PM) which allows the vesicle SNARE, VAMP interaction with Munc18/Sec1p associated syntaxin and SNAP-25 complex (on PM). Complexin clamps the SNARE complex and prevents the membrane fusion. An action potential induced calcium peak triggers synaptotagmin binding to SNARE complex and displaces complexin, which will open the fusion pore. **(B)** NSF interacts with the post-fusion SNARE complex on the target membrane and dissociates them into individual SNAREs using ATP. These SNAREs are reused for another round of fusion [2].



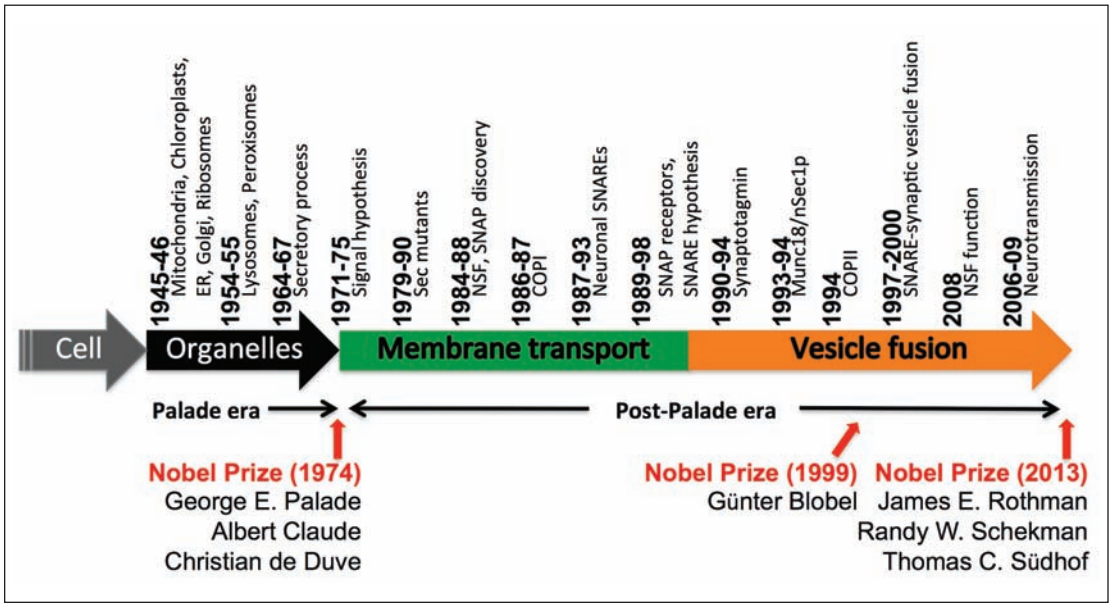


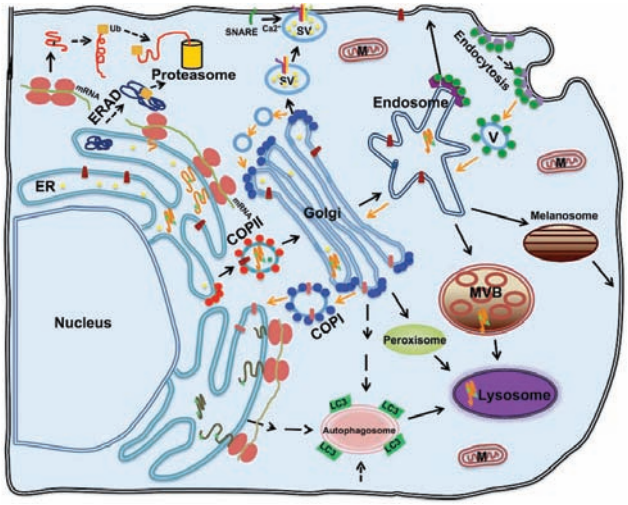
Figure 13. Timeline of the discoveries in the field of Molecular Cell Biology.

specific Rab GTPases[10] before the SNARE interactions [37]. But, the identification of additional regulatory molecules for vesicle fusion is still being pursued in many laboratories. Thus, vesicular fusion is a highly regulated and controlled process of protein delivery.

Questions for the Future

The molecular machinery required for vesicle budding, transport and fusion and their regulation are known. But it is not clear how cells maintain the appropriate concentration of protein or cargo in an organelle and how protein transport occurs against a chemical gradient. Further, how do cells maintain vesicle size and number, at steady state and after an extracellular stimulus? How do cells preserve the distinctive membrane composition of the different organelles (especially lipids)? The fusion machinery is known for a small set of transport steps but not for all the trafficking pathways. Finally, how are these fusion events linked to organelle maturation or biogenesis, especially in the case of lysosome-related organelles such as melanosomes, dense granules and other organelles? These are the challenges for the cell biologists of the post-Rothman–Schekman–Südhof era.

[10] Rab GTPases and Rab cycle: Rab family is a member of the Ras superfamily, and it contains 70 types of monomeric GTPases. Rab protein in its inactive state is bound to GDP and localizes to the cytosol. This protein is activated by another protein,GEF (guanine exchange factor) that displaces GDP with GTP and is then recruited onto the target membrane. Thus, the Rab proteins cycle between cytosol and the membrane, in the Rab GTPase cycle, recruiting downstream factors onto the membrane in each cycle. These proteins can be made constitutively active by blocking their GTP hydrolysis cycle using non-hydrolyzable GTP analogs such as GTPγs.



Front Cover Description: Intracellular protein trafficking between the organelles and their biogenesis, and degradation pathways in a eukaryotic cell.

Cellular organelles: nucleus, mitochondria (M), peroxisomes, endoplasmic reticulum (ER), Golgi, endosomes, vesicles (V), late endosomes (MVB, multi vesicular bodies), lysosomes, secretory vesicles (SV), autophagosomes and melanosomes. Cytosolic components: COPI (blue circles), COPII (red circles), clathrin (green circles) and their associated adaptor proteins (pentagon shape), proteasome, light chain 3 (LC3), ubiquitin (Ub), ribosomes and mRNA. Membrane and their associated components: SNAREs, cargo (rectangular shapes and threaded structures), neurotransmitters (yellow circles), glycosylation (green triangles). Cellular pathways: endocytosis, proteasomal, peroxisomal and lysosomal degradation, endoplasmic-associated degradation (ERAD) and autophagy. Arrows represent the flow of membrane traffic: black arrows – anterograde transport; orange arrows – retrograde transport; dotted arrows – predicted or occurs during the stress condition or clearance stage.

Acknowledgements

We thank Keerthana Ravichandran, Latha Purshotam and Geoff Hyde for critical reading of the manuscript. This work was supported by University Grants Commission (to Riddhi Atul Jani), Wellcome Trust-DBT India Alliance Senior Fellowship (to Subba Rao Gangi Setty) and Indo–French Centre for the Promotion of Advanced Research (to Subba Rao Gangi Setty and Graca Raposo).

Suggested Reading

[1] B Alberts, A Johnson, J Lewis, M Raff, K Roberts and P Walter, *Molecular Biology of the Cell*, Garland Science, Chapters 12–13, p.695–812, 2007.

[2] J H Hurst, Richard Scheller and Thomas Sudhof receive the 2013 Albert Lasker Basic Medical Research Award, *J. Clin. Invest*, Vol.123, pp.4095–4101, 2013

[3] A Claude, Fractionation of Mammalian Liver Cells by Differential Centrifugation, *J. Expt. Med*., Vol.84, pp.51–89, 1946.

[4] K R Porter, A Claude, and E F Fullam, A Study of Tissue Culture Cells by Electron Microscopy: Methods and Preliminary Observations, *J. Expt. Med*., Vol.81, pp.233–246, 1945.

[5] D D Sabatini and M Adesnik, Christian de Duve: Explorer of the cell who discovered new organelles by using a centrifuge, *PNAS USA*, Vol.110, pp.13234–13235, 2013.

[6] A Claude, The Coming Age of the Cell, *Nobel Lectures, Physiology or Medicine*, 1974.

[7] D de Duve, Exploring Cells with a Centrifuge, *Nobel Lectures, Physiology or Medicine*, 1974.

[8] G E Palade, Intracellular aspects of the process of protein secretion, *Nobel Lectures, Physiology or Medicine*, 1974.

[9] G Blobel, Protein targeting, *Nobel Lectures, Physiology or Medicine*, 1999.

[10] W E Balch, W G Dunphy, W A Braell, and J E Rothman, Reconstitution of the transport of protein between successive compartments of the Golgi measured by the coupled incorporation of N-acetylglucosamine, *Cell*., Vol.39, pp.405–416, 1984.

[11] J E Rothman and L Orci, Molecular dissection of the secretory pathway, *Nature*, Vol.355, pp.409–415, 1992.

[12] P Novick and R Schekman, Secretion and cell-surface growth are blocked in a temperature-sensitive mutant of Saccharomyces cerevisiae, *PNAS USA*, Vol.76, pp.1858–1862, 1979.

[13] R Schekman, Lasker Basic Medical Research Award. SEC mutants and the secretory apparatus, *Nat. Med*., Vol.8, pp.1055–1058, 2002.

[14] P Novick, C Field and R Schekman, Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway, *Cell*, Vol.21, pp.205–215, 1980.

[15] C A Kaiser and R Schekman, Distinct sets of SEC genes govern transport vesicle formation and fusion early in the secretory pathway, *Cell*, Vol.61, pp.723–733, 1990.

[16] V Malhotra and S D Emr, Rothman and Schekman SNAREd by Lasker for trafficking, *Cell*, Vol.111, pp.1–3, 2002.

[17] L Orci, B S Glick and J E Rothman, A new type of coated vesicular carrier that appears not to contain clathrin: its possible role in protein transport within the Golgi stack, *Cell*, Vol.46, pp.171–184, 1986.

[18] C Barlowe, L Orci, T Yeung, M Hosobuchi, S Hamamoto, N Salama, M F Rexach, M Ravazzola, M Amherdt and R Schekman, COPII: a membrane coat formed by Sec proteins that drive vesicle budding from the endoplasmic reticulum, *Cell*, Vol.77, pp.895–907, 1994.

[19] D Jensen and R Schekman, COPII-mediated vesicle formation at a glance, *J. Cell Sci.*, Vol.124, pp.1–4, 2011.

[20] T Sollner, S W Whiteheart, M Brunner, H Erdjument-Bromage, S Geromanos, P Tempst and J E Rothman, SNAP receptors implicated in vesicle targeting and fusion, *Nature*, Vol.362, pp.318–324, 1993.

[21] R C Lin and R H Scheller, Structural organization of the synaptic exocytosis core complex, *Neuron*, Vol.19, pp.1087–1094, 1997.

[22] T Weber, B V Zemelman, J A McNew, B Westermann, M Gmachl, F Parlati, T H Sollner and J E Rothman, SNAREpins: minimal machinery for membrane fusion, *Cell*, Vol.92, pp.759–772, 1998.

[23] W T Wickner, Profile of Thomas Sudhof, James Rothman, And Randy Schekman, 2013 Nobel Laureates in Physiology or Medicine, *PNAS USA*, Vol.110, pp.18349–18350, 2013.

[24] J E Heuser and T S Reese, Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction, *J. Cell Biol*, Vol.57, pp.315–344, 1973.

[25] W S Trimble, D M Cowan and R H Scheller, VAMP-1: a synaptic vesicle-associated integral membrane protein, *PNAS USA*, Vol.85, pp.4538–4542, 1988.

[26] M K Bennett, J E Garcia-Arraras, L A Elferink, K Peterson, A M Fleming, C D Hazuka and R H Scheller, The syntaxin family of vesicular transport receptors, *Cell*, Vol.74, pp.863–873, 1993.

[27] G A Oyler, G A Higgins, R A Hart, E Battenberg, M Billingsley, F E Bloom, and M C Wilson, The identification of a novel synaptosomal-associated protein, SNAP-25, differentially expressed by neuronal subpopulations, *J. Cell Biol.*, Vol.109, pp.3039–3052, 1989.

[28] M Geppert, Y Goda, R E Hammer, C Li, T W Rosahl, C F Stevens and T C Sudhof, Synaptotagmin I: a major Ca2+ sensor for transmitter release at a central synapse, *Cell*, Vol.79, pp.717–727, 1994.

[29] M S Perin, V A Fried, G A Mignery, R Jahn and T C Sudhof, Phospholipid binding by a synaptic vesicle protein homologous to the regulatory region of protein kinase C. *Nature*, Vol.345, pp.260–263, 1990.

[30] A Maximov, J Tang, X Yang, Z P Pang and T C Sudhof, Complexin controls the force transfer from SNARE complexes to membranes in fusion, *Science*, Vol.323, pp.516–521, 2009.

[31] Y Hata, C A Slaughter T C Sudhof, Synaptic vesicle fusion complex contains unc-18 homologue bound to syntaxin, *Nature*, Vol.366, pp.347–351, 1993.

[32] T C Sudhof and J E Rothman, Membrane fusion: grappling with SNARE and SM proteins, *Science*, Vol.323, pp.474–477, 2009.

[33] I Mellman and S D Emr, A Nobel Prize for membrane traffic: vesicles find their journey's end, *J. Cell Biol.*, Vol.203, pp.559–561, 2013.

[34] P Gissen and E R Maher, Cargos and genes: insights into vesicular transport from inherited human disease, *J. Med. Genet.*, Vol.44, pp.545–555, 2007.

[35] V M Olkkonen and E Ikonen, When intracellular logistics fails—genetic defects in membrane trafficking, *J. Cell Sci*, Vol.119, pp.5031–5045, 2006.

[36] M S Marks, H F Heijnen and G Raposo, Lysosome-related organelles: unusual compartments become mainstream, *Curr. Opin. in Cell Biol.*, Vol.25, pp.495–505, 2013.

[37] J S Bonifacino and B S Glick, The mechanisms of vesicle budding and fusion, *Cell*, Vol.116, pp.153–166, 2004.

Address for Correspondence
Subba Rao Gangi Setty
Department of Microbiology
and Cell Biology
Indian Institute of Science
Bangalore 560 012, India.
Email:
subba@mcbl.iisc.ernet.in