• Assigned reading
  - Choose review (or reviews) on topic that interests you from list under Hanson lectures and read critically. Challenge yourself to define the next big question in that area.
Outline

• Pathways to secretion for membrane proteins, lipids, and secreted proteins
  – ER, Golgi, plasma membrane, endosomes
  – Techniques to monitor trafficking

• General question of how secretory material moves from one compartment to another?
  – Genetic analysis of pathways in yeast
  – Biochemical analysis of reconstituted transport reactions

• Molecular mechanisms are conserved

• Bidirectional trafficking in the secretory pathway
  – Return of soluble ER resident proteins to ER
  – Transport of cargo through the Golgi apparatus
Figure 14-1
Molecular Cell Biology, Sixth Edition
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Figure 14-15
Molecular Cell Biology, Sixth Edition
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- Forming secretory vesicle
- trans-Golgi network
- trans medial
  - Golgi cisternae
  - cis
  - cis-Golgi network
- ER-to-Golgi transport vesicles
- Smooth protrusion
- Transitional elements

0.5 μm
Delineation of secretory pathway:

Pulse chase autoradiography in pancreatic exocrine cells

Experiment from Jamieson and Palade, 1967
Use of GFP and live-cell imaging to follow trafficking
Use of endoglycosidases to follow trafficking
Membrane trafficking requires that transport intermediates undergo **fission** from donor compartment and **fusion** with target compartment. None of this happens spontaneously, and is instead controlled by a highly regulated series of molecular reactions.
Machinery responsible for vesicle biogenesis and consumption

Identified by:
- Genetic analyses in yeast, *Drosophila, C. elegans*
- *In vitro* reconstitution of membrane transport reactions
- Proteomics of isolated transport vesicles: synaptic vesicle, clathrin coated vesicles, others
- Study of clostridial neurotoxins, mutations in proteins associated with human disease, etc.
Genetic dissection of the secretory pathway in *Saccharomyces cerevisiae*

- Isolation of yeast mutants with temperature sensitive defects in secretion
- Mutant strains still used today to block transport at specific steps in the secretory pathway
- 2013 Nobel Prize
• **Approach:** Isolated yeast with temperature sensitive defects in secretion, eventually identified and studied molecules involved.

• **Strategy for isolating sec mutants:** At non-permissive temperature, cells with temperature sensitive mutations in the secretory pathway will stop secreting proteins. However, the cells will continue to make secretory proteins (needed among other things for cell surface growth). These proteins accumulate inside the cells, making the cells more dense than wild-type counterparts.
• Yeast sec screen:
  - Mutagenized cells and grew at permissive temperature (24 ºC).
  - Shifted cells to 37 ºC (non-permissive temperature) for several hours.
  - Separated cells by density gradient centrifugation.
  - Plated dense cells at 24 ºC.
  - Screened individual colonies from these plates for defects in secretion.
Class A

Fate of secreted proteins: Accumulation in the cytosol
Defective function: Transport into the ER

Class B

Fate of secreted proteins: Accumulation in rough ER
Defective function: Budding of vesicles from the rough ER

Figure 14-4 part 1
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Look at phenotypes of double mutants to determine sequence of events.

Class B: Accumulation in rough ER, Budding of vesicles from the rough ER

Class C: Accumulation in ER-to-Golgi transport vesicles, Fusion of transport vesicles with Golgi

Class D: Accumulation in Golgi, Transport from Golgi to secretory vesicles

Class E: Accumulation in secretory vesicles, Transport from secretory vesicles to cell surface
Assays and Analysis

- Morphology - thin section electron microscopy

Wild type morphology

Accumulation of vesicles

Late acting protein: vesicles form, then accumulate because of failed release

25 °C  sec15-1 after 2 hr @ 37 °C
Accumulation of ER membranes after 2 hr @ 37 °C.

Proteins required early in the secretory pathway (or at many stages): ER expands, no secretory vesicles accumulate.
Assays and Analysis

- Unsecreted invertase in sec mutants accumulates proximal to secretory pathway block
- Electrophoretic mobility of immunoprecipitated invertase varies depending on glycan processing, demonstrating how far it got in secretory pathway
Classification of yeast sec mutants

Figure 6. Yeast Secretory Pathway

From pathway to molecular mechanisms...

• Cloned the \textit{SEC} genes
• In some cases, function suggested by sequence homology
  – First example: \textit{SEC4} = monomeric GTPase required for fusion of vesicles with plasma membrane, founding member of the \textit{rab} family of membrane fusion regulators
• Characterization and study of gene products
  – Localization, structure, interactions with other proteins
  – Activity in reconstituted reaction systems
• \textit{SEC} genes used as starting points for additional genetic screens to isolate further components of the pathway
  – Multicopy suppressor screens
  – Synthetic screens
Example: class B SEC genes defined COPII machinery
Other coats responsible for vesicle biogenesis throughout the cell

<table>
<thead>
<tr>
<th>VESICLE TYPE</th>
<th>TRANSPORT STEP MEDIATED</th>
<th>COAT PROTEINS</th>
<th>ASSOCIATED GTPase</th>
</tr>
</thead>
<tbody>
<tr>
<td>COPII</td>
<td>ER to cis-Golgi</td>
<td>Sec32/Sec24 and Sec13/Sec31 complexes, Sec16</td>
<td>Sar1</td>
</tr>
<tr>
<td>COPI</td>
<td>cis-Golgi to ER</td>
<td>Coatomers containing seven different COP subunits</td>
<td>ARF</td>
</tr>
<tr>
<td></td>
<td>Later to earlier Golgi cisternae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clathrin and adapter proteins*</td>
<td>trans-Golgi to endosome</td>
<td>Clathrin + AP1 complexes</td>
<td>ARF</td>
</tr>
<tr>
<td></td>
<td>trans-Golgi to endosome</td>
<td>Clathrin + GGA</td>
<td>ARF</td>
</tr>
<tr>
<td></td>
<td>Plasma membrane to endosome</td>
<td>Clathrin + AP2 complexes</td>
<td>ARF</td>
</tr>
<tr>
<td></td>
<td>Golgi to lysosome, melanosome, or platelet vesicles</td>
<td>AP3 complexes</td>
<td>ARF</td>
</tr>
</tbody>
</table>

*Each type of AP complex consists of four different subunits. It is not known whether the coat of AP3 vesicles contains clathrin.

Table 14-1
*Molecular Cell Biology, Sixth Edition
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Structural and functional principles of coat assembly and function
Biochemical study of the secretory pathway

• Cell-free assays of vesicular transport
• First used to study transport between Golgi stacks
  - Focus on how this led to identification of proteins responsible for fusion, but assay also identified proteins required for vesicle formation
  - Cell free assays remain gold standard for understanding these pathways

  “Reconstitution of the transport of protein between successive compartments of the Golgi measured by the coupled incorporation of N-acetylglucosamine” Cell 39, 405-416
Proteins are modified as they travel through the Golgi apparatus

- Mannosidase II
- GlcNAc transferase I
- Galactosyltransferase
- Sialyltransferase

Cell-free assay of vesicular transport between two Golgi cisternae

Tools:

Assay:

Figure 14-5a
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“I chose cell-free reconstitution because it had been the central experimental approach of all biochemistry since the discovery of alcoholic fermentation in yeast extracts by the Buchner brothers at the end of the 19th century. Once reconstituted, cell-free transport could be used as an assay to permit the underlying enzyme proteins to be discovered and purified according to their functional requirements. At the time of our experiment, the mechanisms of ATP synthesis, DNA replication, RNA transcription, protein synthesis, and even the genetic code, were all relatively recent trophies of the reconstitution approach. However, reconstituting intracellular transport seemed especially daunting because membrane compartments are often in intimate proximity in the living cell, and it was then common wisdom that if these spatial relationships were destroyed by homogenization then transport could no longer take place. This strong prejudice—deeply rooted in cell biology from its origin as a branch of microscopic anatomy—no doubt accounted for the skepticism with which our experiment reconstituting transport was greeted for a number of years, until we began to isolate protein machinery.”
Golgi Transport Assay in Use

What is measured?

Incorporation of $[^{3}\text{H}]\text{GlcNAc}$ into VSV G protein

INSET:
Lane a = total reaction
Lane b = IP w/ anti G serum
Lane c = IP w/ preimmune serum
Requirements for *in vitro* Golgi transport:

- Golgi membranes
- Energy
- Acyl-Coenzyme A
- Cytosolic proteins

---

*Figure 7. Effect of Cytosol Concentration on the Rate and Extent of Transport*

Each incubation (50 μl) contained 2.5 μg donor membrane fraction, 5 μg of acceptor fraction, and gel-filtered CHO cytosol in the indicated amount in the standard assay cocktail. Incubations were stopped after various times at 37°C, and the \(^3\text{H}\)-GlcNAc incorporated into G protein was determined. Inset: \(^3\text{H}\)-GlcNAc incorporated into G protein at the plateau of incorporation as a function of the amount of cytosol protein added.
Approaches used to define individual proteins required for transport

- Fractionating cytosol ... too complex, so used inhibitors to reduce complexity of search
- **Inhibitors:**
  - *N*-ethyl maleimide (NEM) reacts with cysteine sulfhydryls in proteins, often inactivating the protein
    - Led to identification of fusion proteins known as SNAREs
  - GTPgS (poorly hydrolyzable GTP analogue)
    - Led to identification of coat proteins involved in budding
- Mild NEM treatment of Golgi membranes abolished transport *in vitro*.
  - 1 mM, 15 min on ice
- What protein(s) is inactivated by NEM?
NEM inhibits transport

VSV-G
Donor Mb

Incubate with cofactors

GlcNAc Transferase
Acceptor Mb

NSF = NEM sensitive factor

NEM

VSV-G* labeled with $[^3\text{H}]\text{GlcNAc}$

+NEM

- NEM

$[^3\text{H}]\text{GlcNAc}$ incorp. (cpm)

Time (min)
Can activity be restored?

- VSV-G Donor Mb
- GlcNAc Transferase Acceptor Mb
- Uninfected (no VSV-G)
- NEM
- NSF
- Cytosol

**[3H] VSV-G**

**[3H] GlcNAc incorp. (cpm)**

- No NEM
- + NSF
- + NEM

**Time (min)**

0 20 40 60 80
NSF must be released during the course of the assay to act on donor or acceptor membranes that are participating in transport.

Transport is restored by addition of uninfected donor membranes - source of NSF.

- NSF must be released during the course of the assay to act on donor or acceptor membranes that are participating in transport.
How to purify NSF?

- Homogenize cells with buffer containing ATP/salt
- NSF is found in the cytoplasmic fraction
- Fractionate cytosol and assay for restoration of VSV-G transport activity
Purification of NSF

- **PEG precipitation**
  - Mostly to concentrate sample, starting material 120 liters CHO cell cytosol

- **Use ion exchange and hydrophobic interaction columns that do not bind NSF to eliminate other proteins**
  - DE-52 anion exchange flow-through to remove proteins that bind anion exchange resin
  - S sepharose hydrophobic interaction flow-through

- **Glycerol gradients** - to separate by size/shape, enrich for large proteins (NSF fractionates at ~500 kDa)
  - Alternative technique gel filtration/size exclusion chromatography

- **Ion exchange**
  - Mono S cation exchange (which NSF binds to)
<table>
<thead>
<tr>
<th>Step</th>
<th>Activity (U)</th>
<th>Protein (mg)</th>
<th>Specific Activity (U/mg)</th>
<th>Yield of Activity (% of step 1)</th>
<th>Fold Purification (relative to Step 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Cytosol</td>
<td>200</td>
<td>600</td>
<td>0.33</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>2. PEG precipitation</td>
<td>140</td>
<td>163</td>
<td>0.86</td>
<td>70</td>
<td>2.6</td>
</tr>
<tr>
<td>3. DE-52 flowthrough</td>
<td>140</td>
<td>54</td>
<td>2.6</td>
<td>70</td>
<td>7.9</td>
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<tr>
<td>4. S Sepharose Fast Flow</td>
<td>70</td>
<td>11</td>
<td>6.4</td>
<td>35</td>
<td>19</td>
</tr>
<tr>
<td>5. Glycerol Gradient</td>
<td>56</td>
<td>1.2</td>
<td>47</td>
<td>28</td>
<td>142</td>
</tr>
<tr>
<td>6. Mono S FPLC</td>
<td>24</td>
<td>.14</td>
<td>171</td>
<td>12</td>
<td>520</td>
</tr>
</tbody>
</table>
NSF is hexameric ATPase with chaperone-like activity

1997

NSF purification for 2015 imaging
Convergence of biochemical and genetic approaches on NSF set stage for understanding membrane fusion

**In vitro** Golgi transport assay

- Purify mammalian NSF
  - cDNA cloned

**SEC Mutants**

- Homology to **SEC18**
  - Yeast Sec18p reconstitutes activity in the Golgi transport assay.
NSF provided tool to identify additional components... including the membrane fusion machinery

• SNAPs - soluble NSF attachment proteins
  - NSF and SNAPs are general factors required for vesicular transport.

• SNAREs - SNAP Receptors
  - Large family of proteins; specific SNAREs for each compartment; mediate membrane fusion
Strategy used to identify NSF’s membrane receptors (=SNAREs)

“The meaning of the seemingly futile cycle of membrane binding and ATPase-driven release of NSF was unclear at the time Söllner joined the Rothman lab as a postdoctoral fellow in 1991. At the time, we imagined that energy from hydrolysis of ATP somehow activated the membrane-anchored SNAREs to power fusion. However, the existence of the cycle had a huge impact on our strategy for identifying the SNAREs. The assembly and disassembly of 20S particles, involving binding and release of NSF from SNAP, respectively, could be exploited as sequential affinity purification steps to isolate SNAREs. Previous experiments had shown that standard chromatographic methods and a single affinity step were inadequate for isolating SNAREs; the 20S ATPase cycle would add a second level of biological specificity.”

Jim Rothman: http://www.ergito.com/index.jsp (Great Experiments)
Scheme for Affinity Purification of SNAP Receptors

- SNAP
  - ATPγS
  - NSF
  - EDTA
  - myc

- Detergent
  - SNAP
  - NSF

- Anti-myc epitope Ig, linked to beads
- Non-specific eluate
- MgATPγS
- MgATP
- P_i

- ADP
- NSF

Detergent extract of membrane fraction
Because vesicle fusion occurs at many membrane compartments, we had suspected that cells would have a large family of SNARE proteins, related in sequence and differing in location. We were therefore surprised when the SNAREs derived from whole brain yielded a remarkably simple protein pattern consisting of only four proteins, each present in the specific (MgATP) eluate and absent from the nonspecific (MgATPγS) eluate...

The SNARE complex progressed from first discovery to final publication in a dizzying sweep lasting only 5 weeks.”

Thomas Söllner (http://www.ergito.com)

Söllner et al., 1993
Localization of first SNARE proteins:
Vesicle and target membrane in nerve terminal

Syntaxin
SNAP-25
Synaptobrevin/VAMP

v-SNARE

t-SNAREs
SNARE hypothesis

“We then went on to interpret the SNARE complex from a very broad perspective instead of limiting ourselves to the special point-of-view of synaptic vesicle exocytosis. First principles require that vesicles and targets somehow be marked to indicate which vesicles will fuse where. This, in turn, indicates that vesicle and target markers must be matched pairwise. We suggested that the simplest mechanism for matching is self-assembly, in which only matching pairs of "cognate" vesicle ("v") and target ("t") markers bind each other between membranes, thereby forming a "v-t" complex prerequisite for membrane fusion.

The primary impact of our paper stemmed from its combination of an unexpected discovery—the SNARE complex—and a broad and clearly stated concept—the SNARE hypothesis—deduced from it.”

Jim Rothman commenting on Söllner et al., 1993
Clostridial neurotoxins

‘The master of a large ship crushed the index finger of his right hand with the anchor. Seven days later a somewhat foul discharge appeared; then trouble with his tongue -- he complained that he could not speak properly...his jaws became depressed together, his teeth were locked, then symptoms appeared in his neck; on the third day opisthotonus appeared with sweating. Six days after the diagnosis was made he died.’

Hippocrates
Discovery that clostridial toxins cleave SNAREs supports role in fusion

- Toxins found to be metalloproteases
- Toxins quickly inhibit synaptic vesicle exocytosis
- Therefore targets must be important for neurosecretion
- Comparison of treated vs. non-treated synaptosomes shows cleavage of VAMP/synaptobrevin
- Injecting peptides encompassing cleavage site in VAMP/synaptobrevin inhibit action of tetanus toxin
SNAREs present on all membranes
How do SNAREs promote membrane fusion?

• Original **SNARE hypothesis**: unique matches between SNAREs on each vesicle (v-SNAREs) and target (t-SNAREs) provide specificity to membrane docking and fusion

• Biochemical and structural work aimed at understanding interactions among SNAREs and partners led from there to current model of protein mediated membrane fusion
SNARE-mediated fusion reconstituted in vitro

v-SNARE liposome (VAMP) + t-SNARE liposome (syntaxin/SNAP25)

* = fluorescent lipid
Quenched at high concentration

1. Preincubate 4°C to form SNARE complexes
2. Shift to 37°C to allow fusion
Increased signal as fluorescent lipid is diluted

Weber et al. (1998), Cell 92: 759
SNAREs in opposing membranes are sufficient to drive fusion.
Structure of core SNARE complex leads to mechanism of membrane fusion

Sutton et al., 1998 Nature 395:347
**Fig. 2.** (A) The zippering model for SNARE-catalyzed membrane fusion. Three helices anchored in one membrane (the t-SNARE) assemble with the fourth helix anchored in the other membrane (v-SNARE) to form trans-SNARE complexes, or SNAREpins. Assembly proceeds progressively from the membrane-distal N termini toward the membrane-proximal C termini of the SNAREs. This generates an inward force vector \( (F) \) that pulls the bilayers together, forcing them to fuse. Complete zippering is sterically prevented until fusion occurs, so that fusion and the completion of zippering are thermodynamically coupled. (B) Therefore, when fusion has occurred, the force vanishes and the SNAREs are in the low-energy cis-SNARE complex.
Role for NSF?
Fig. 2. A history of EM studies on 20S supercomplex. Representative images or reconstructions are shown on the time axis. They are adapted from Fig. 6 of Hanson et al. [5] (a), Fig. 2 of Hohl et al. [33] (b), and Fig. 6 of Chang et al. [34] (c), respectively. In addition, the maps of Furst et al. [31] (c) and Zhao et al. [35] (EMD-6206, state I, e) are recolored using the same scheme as in Figs. 4 and 5.

from Zhao and Brunger 2016 J Mol Biol 428, 1912
Exocytosis of synaptic vesicles (SVs) during fast synaptic transmission is mediated by soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex assembly formed by the coil-coiling of three members of this protein family: vesicle SNARE protein, synaptobrevin 2 (syb2), and the presynaptic membrane SNAREs syntaxin-1A and SNAP-25. However, it is controversially debated how many SNARE complexes are minimally needed for SV priming and fusion. To quantify this effective number, we measured the fluorescence responses from single fusing vesicles expressing pHluorin (pH), a pH-sensitive variant of GFP, fused to the luminal domain of the vesicular SNARE syb2 (spH) in cultured hippocampal neurons lacking endogenous syb2. Fluorescence responses were quantal, with the unitary signals precisely corresponding to single pHluorin molecules. Using this approach we found that two copies of spH per SV fully rescued evoked fusion whereas SVs expressing only one spH were unable to rapidly fuse upon stimulation. Thus, two syb2 molecules and likely two SNARE complexes are necessary and sufficient for SV fusion during fast synaptic transmission.
Membrane trafficking at the nerve terminal: specialized for regulated exocytosis

1. DELIVERY OF SYNAPTIC VESICLE COMPONENTS TO PLASMA MEMBRANE
2. ENDOCYTOSIS OF SYNAPTIC VESICLE COMPONENTS TO FORM NEW SYNAPTIC VESICLES DIRECTLY
3. ENDOCYTOSIS OF SYNAPTIC VESICLE COMPONENTS AND DELIVERY TO ENDOSONE
4. BUDDING OF SYNAPTIC VESICLE FROM ENDOSONE
5. LOADING OF NEUROTRANSMITTER INTO SYNAPTIC VESICLE
6. SECRETION OF NEUROTRANSMITTER BY EXOCYTOSIS IN RESPONSE TO AN ACTION POTENTIAL

Figure 13–64. Molecular Biology of the Cell, 4th Edition.
Exocytosis at the synapse is regulated by Ca$^{2+}$

- Calcium influx via voltage gated channels triggers SV exocytosis within less than 1 msec
- Occurs at synapse active zone
- Steep calcium dependence, suggests 3-4 Ca$^{2+}$ ions are required
Vesicle fusion at the nerve terminal

Frog nerve terminal at rest

5 msec after stimulation

Heuser, 1975
Ca$^{2+}$ sensor: synaptotagmin
Figure 2. Membrane Buckling by C2 Domains as a Trigger for Fusion

As the vesicle approaches the membrane in this model, the vesicular SNARE component binds to its SNARE counterparts on the target membrane, resulting in the formation of a complex that pulls the two membranes into close apposition (steps A and B). The C2 domains of synaptotagmin bind to the SNARE complex, potentially helping to complete their zipperng into a continuous helix. The C2 domains also insert into the target membrane in a Ca\textsuperscript{2+}-dependent manner, resulting in membrane buckling and an unstable membrane region optimally localized for fusion (jagged membrane in step C). As the fusion pore opens, the C2 domains would still be localized to the neck, where they might promote the early stages of fusion pore opening (step D).
Directionality of transport

Anterograde:
- ER ➔ Golgi
- Golgi ➔ plasma membrane
- Golgi ➔ endosome

Retrograde:
- Golgi ➔ ER
- Golgi cisterna ➔ Golgi cisterna
- Endosome ➔ Golgi

Requires control of:
- Vesicle delivery, docking, and fusion
- Cargo selection
Transport through the Golgi: anterograde, retrograde or both?

- Two models to debated over many years
  - Stable Compartments connected by vesicle traffic: secretory cargo (large and small) moves through these by a vesicle-mediated anterograde process
  - Cisternal Maturation: VTCs fuse into an ERGIC (ER-Golgi intermediate compartment). This matures into the cis-Golgi by gain of Golgi proteins and removal of VTC proteins via COPI vesicles that move in a retrograde direction. Cis-Golgi then matures to medial Golgi by similar mechanism, etc.
Figure 1. Anterograde vesicular transport between stable compartments. Secretory cargoes travel from the ER to the intermediate compartment (IC) and cis-Golgi in dissociative carriers. Golgi compartments are stable and biochemically distinct. Secretory cargoes move across the stack by means of COP1 vesicles that bud from one compartment and fuse with the next, whereas resident Golgi proteins stay in place by being excluded from budding vesicles. This model does not provide a mechanism for transporting secretory cargoes that are too large to fit within COP1 vesicles.
Figure 2. Cisternal progression/maturation. Secretory cargoes exit the ER in dissociative carriers, which coalesce with one another and with COPI vesicles derived from the cis-Golgi to form the intermediate compartment, which coalesces in turn to form a new cis-cisterna. In subsequent rounds of COPI-mediated recycling, the new cisterna matures by receiving medial and then trans-Golgi proteins from older cisternae while exporting cis and then medial-Golgi proteins to younger cisternae. Meanwhile, the cisterna progresses through the stack, carrying forward both small and large secretory cargoes. In the final stage of maturation, the cisterna is a TGN element that breaks down into anterograde and retrograde transport carriers.