Cholesterol Homeostasis and Sensing

In this recitation, we will discuss how cholesterol levels are sensed in the ER. Low cholesterol levels trigger transport of SREBP to the Golgi, where it is cleaved to release a transcription factor domain. It then activates expression of the cholesterol biosynthetic and uptake genes. The mechanism of cholesterol sensing is complex and depends on cooperative interactions between several proteins and cholesterol.

Reading: "Switch-like Control of SREBP-2 Transport Triggered by Small Changes in ER Cholesterol: A Delicate Balance" Radhakrishnan, A.; Goldstein, J. L.; McDonald, J. G.; Brown, M. S. *Cell Metabolism* **2008**, *8*, 512

Background

Cholesterol is an essential component of mammalian cell membranes and also the precursor to steroid hormones and bile acids. Cholesterol is obtained both from biosynthesis and from the diet. Elevated cholesterol levels, however, lead to atherosclerotic plaques and cardiovascular disease. Elaborate systems are responsible for sensing and regulating cellular cholesterol levels.

Cholesterol levels are sensed in the endoplasmic reticulum (ER). Low cholesterol levels trigger transport of the ER-localized membrane-bound sterol regulatory element-binding proteins (SREBPs) from the ER to the Golgi, where they are proteolytically cleaved to release a N-terminal basic helix-loop-helix (bHLH) transcription factor domain. The bHLH domain enters the nucleus and activates expression of the cholesterol biosynthetic and uptake genes. Thus, cholesterol homeostasis is maintained by increasing cholesterol synthesis and uptake when membrane cholesterol levels fall, and by decreasing synthesis and uptake when cholesterol accumulates in the membrane. At least two proteins, Scap and Insig, are involved in sensing cholesterol levels in the ER membrane (Figure 1A, 1B). Scap is a transmembrane protein that triggers transport of SREBPs to the Golgi by targeting SREBPs to CopII coated vesicles. Cholesterol promotes binding of Insig to Scap, which prevents the transport.

Notably, most of the cholesterol is located in the plasma membrane, but the sensing occurs in the ER membrane. Likely, sensing cholesterol in the ER serves to increase the sensitivity to changing cholesterol levels. This mechanism requires cholesterol transport between the plasma membrane and the ER.

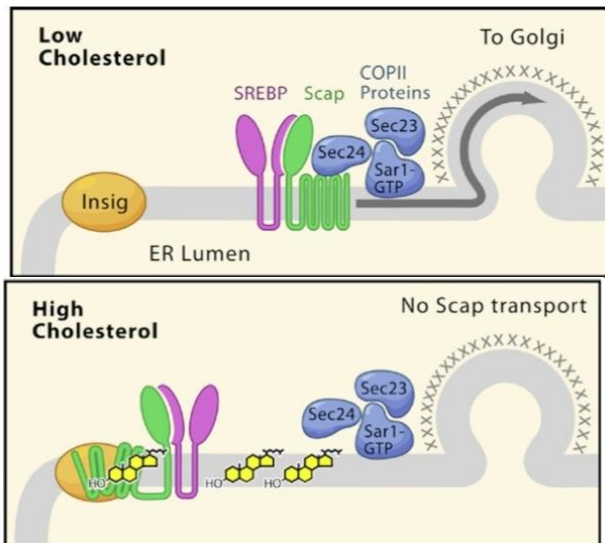

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Figure 1: Regulation of SREBP transport by Scap and Insig. (**A**) At low cholesterol levels, Scap binds to SREBP and to CopII proteins, which targets the SREBP/Scap complex to CopII coated vesciles for transport to the Golgi for subsequent proteolytic activation. (**B**) Cholesterol mediates binding of Insig to Scap, which prevents binding to CopII proteins. As a consequence, SREBP remains in the ER.

In this study, Goldstein and Brown sought to examine what **changes in ER cholesterol levels** need to occur to regulate SREBP transport. To do these experiments, they first needed to develop a **method to purify ER membranes** by subcellular fractionation to accurately determine cholesterol levels in the ER membranes. They then demonstrated that suppression of SREBP processing by cholesterol exhibits a **sharp transition indicative of cooperativity**.

Subcellular Fractionation to isolate ER membranes

The researchers used a three-step fractionation scheme (Figure 2, left panel) to isolate ER membranes from mammalian cells (chinese hamster ovary cells, CHO). This fractionation scheme relies on density gradient centrifugation, which separates organelles and membranes based on size, density, and/or mass, depending on the type of gradient used. A sample is layered on top of a density gradient, for example a sucrose gradient, where solutions of different sucrose concentrations (here 45, 30, 15, and 7.5 % (w/v) sucrose) are layered on top of each other. The higher the sucrose concentration, the denser the solution. As centrifugal force is applied, the particles start to sediment, according to their size (in general, larger and denser particles pellet faster and at lower centrifugal force). Particles of the same size will migrate through the gradient in distinct bands until they sediment at the bottom of the tube. If the centrifugation is performed for an appropriate amount of time, different particles will have migrated to different positions in the gradient and can be isolated by removing the corresponding band from the tube.

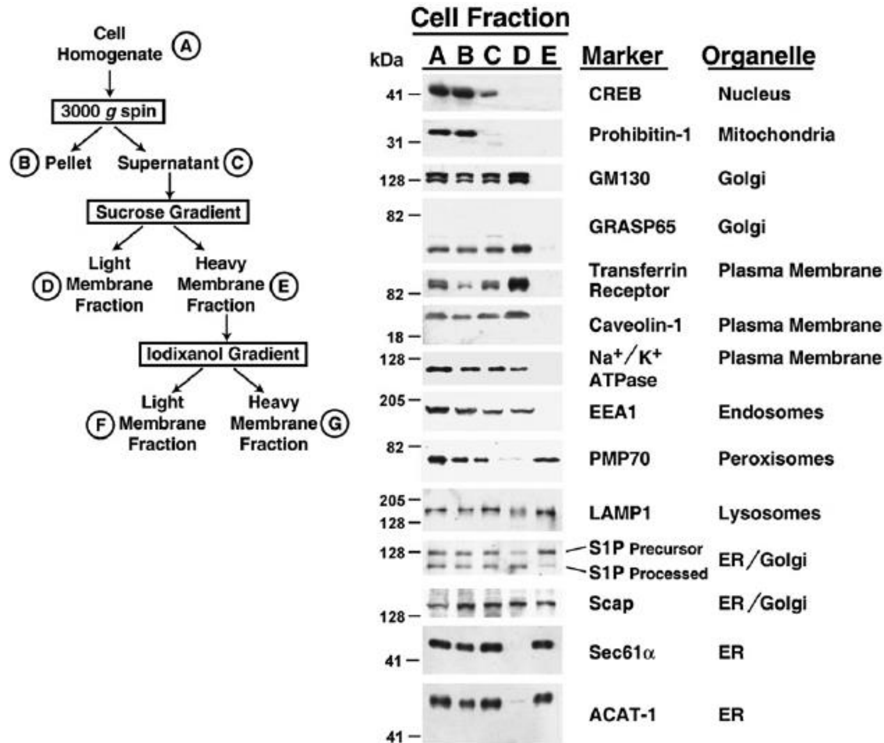

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Figure 2: Subcellular fractionation for isolation of ER membranes. **Left**: Three-step fractionation scheme employed in this study. ER membranes accumulate in fraction G. **Right**: Western Blot detection of organelle- or membrane-specific proteins in fractions A-E of the fractionation protocol. ER markers accumulate in fraction E, along with peroxisomal and lysosomal markers. All other markes accumulate in fractions B or D. Note that there are also significant amounts of ER markers in fraction B.

In this paper, the cells were lysed using a ball-bearing homogenizer. Three centrifugation steps were then used to isolate ER membranes:

1. A 3000 × *g* centrifugation step to remove unbroken cells, nuclei, mitochondria, and other cellular debris.
2. Sucrose gradient centrifugation with a discontinuous sucrose gradient (100,000 × *g* for 1 hour) to separate light membranes (Golgi and endosomal membranes) and heavy membranes (peroxisomal, lysosomal, and ER membranes).
3. Iodixanol gradient centrifugation with a continuous iodixanol gradient (110,000 × *g* for 2 hours) to separate ER membranes (heavy) from all other remaining membranes (light).

A potential issue is that membranes are not static, but dynamic. Membranes can fuse or their composition can change depending on solution conditions, which can affect the outcome of the subsequent experiments depending on the kinetics of these processes.

Western Blots (immunoblots) to follow membrane fractionation

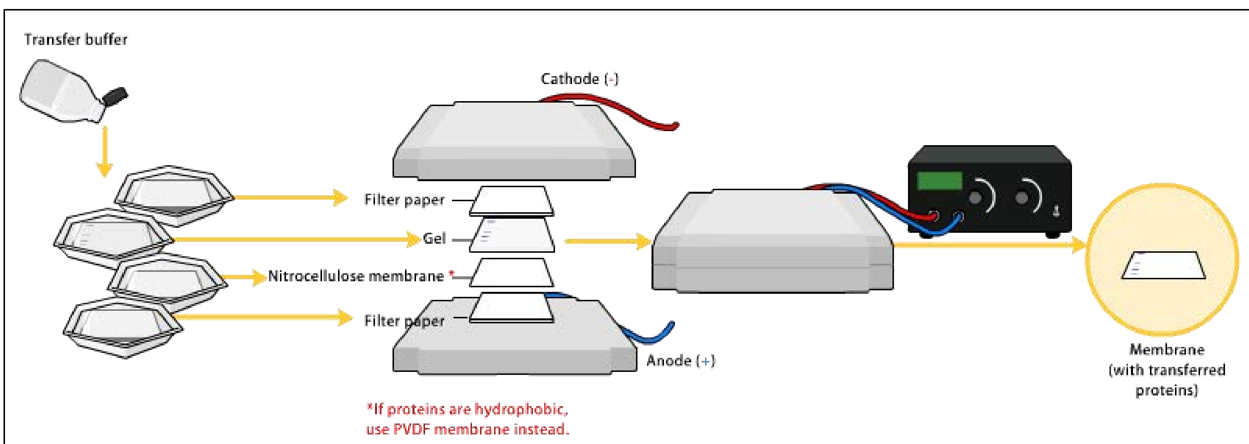
The researchers followed the fractionation using Western Blots, detecting proteins specific to individual organelles and markers. A Western Blot is an analytical technique to detect specific proteins using antibodies. Antibodies are produced by animal immune systems to remove foreign substances (antigens). In particular, antibodies bind to a

specific three-dimensional structure, often a region of a protein, known as the epitope. Nowadays, antibodies can be raised against almost any protein of interest by immunizing rabbits or other mammals.

The specific binding of an antibody is used to detect a protein of interest among a mixture of proteins after gel electrophoresis. Recall that one can stain a gel using a non-specific stain such as Coomassie to visualize all proteins. Although this non-specific detection is very useful, it can be hard to visualize and quantify a given protein of interest. The antibodies used in a Western Blot provide specificity in detection.

The general procedure for a Western Blot is as follows:

1. Subject a mixture of proteins, for example from tissue homogenate, to **gel electrophoresis** such as SDS-PAGE to separate the proteins.
2. **Transfer** proteins from the gel **to a membrane** (nitrocellulose, polyvinylidene difluoride (PVDF)) so that they are accessible to antibodies (Figure 3). Transfer is often done using electric current ("electroblotting").
3. **Block** non-specific antibody binding sites on the membrane using bovine serum albumin (BSA) or dry milk. (the membranes are designed to bind proteins non-specifically, but non-specific antibody binding would give lots of noise.)
4. **Detect** the protein of interest by adding an antibody specific to it (the so-called **primary antibody**). This binding provides no direct readout. To visualize the protein, a **secondary antibody** is added that binds to the primary antibody. The secondary antibody is coupled to a molecule that allows for quantification, often by colorimetric readout using enzymes such as horseradish peroxidase (HRP) or alkaline phosphatase (AP). Both enzymes can convert uncolored substrates into colored products. One can also use other methods such as radioactivity or fluorescence for quantification.



Western blot transfer, by Bensaccount (talk), Wikimedia Commons. License CC-BY.

Figure 3: Schematic depiction of the transfer process, where proteins are moved from the gel to the membrane by an electric current.

Potential Issues:

1. Different proteins transfer with different efficiency, so that comparing levels of different proteins by Western Blots is difficult (and even comparing levels of the same protein in different experiments can be difficult).
2. The primary antibody needs to be very specific for the protein of interest. Most primary antibodies will have some amount of cross-reactivity that manifests itself in the form of additional bands (that you never see in papers because they are cut out of the figure). But you need to be sure that you are actually looking at the right band in the Western Blot.

In the right panel of Figure 2, results from Western Blots at different stages of the fractionation process are shown. The researchers picked a number of "marker proteins" for the various organelles that they used to determine which organelles were in which fractions.

Analyzing this Western Blot closely is critical to assess the efficiency of their fractionation process. Note, for example, that fraction B contains a significant amount of the ER markers (*What could this observation indicate?*). Based on the Western Blots shown, the researchers determined that the ER membranes accumulated in fraction E after the sucrose gradient centrifugation, but that there was significant contamination by lysosomal and peroxisomal membranes. Therefore, they performed the iodixanol gradient, which afforded pure ER membranes in fraction G (Figure 4A). Note again that fraction F also contains ER proteins in addition to lysosomal and peroxisomal proteins.

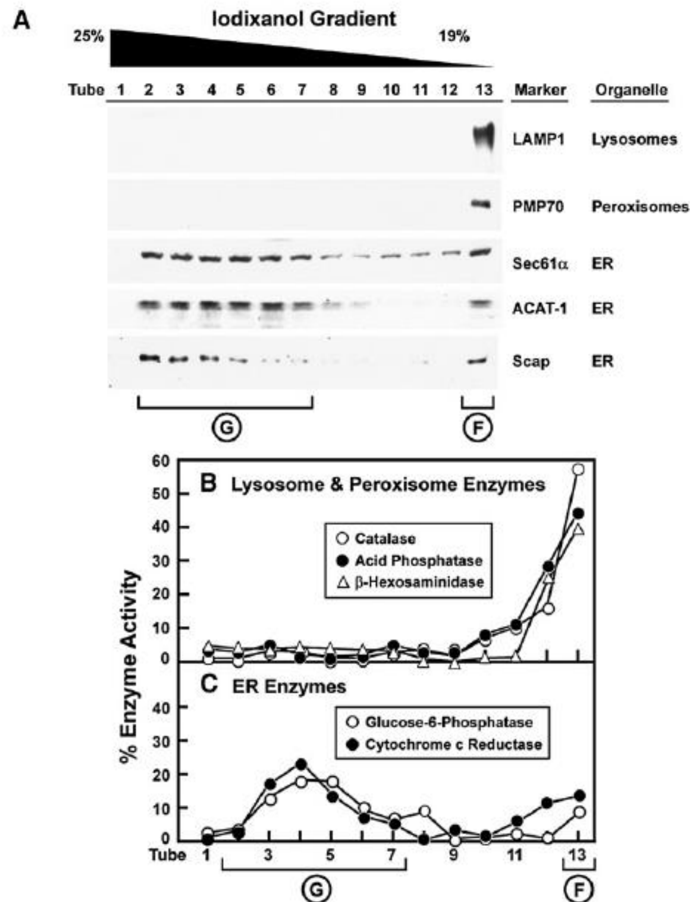


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Figure 4: Iodixanol Gradient centrifugation separates ER membranes (fraction G, tubes 2-7) from lysosomal and peroxisomal membranes (fraction F, tube 13). (**A**) Western Blot analysis of the iodixanol gradient fractions. (**B,C**) Enzyme assays for (**B**) lysosomal and peroxisomal enzymes as well as (**C**) ER enzymes in the different iodixanol gradient fractions.

To validate these results, the authors also performed enzyme assays for enzymes specific to the different membranes (Figure 4B, 4C). Activity of lysosomal and peroxisomal enzymes was localized exclusively to tubes 10-13, while activity of ER enzymes was maximal in tubes 2-7 (fraction G). Thus, the authors concluded that they had obtained ER membranes with <10% other membranes as impurities.

Cholesterol Sensing

Having purified ER membranes allowed the researchers to quantify the cholesterol levels in the ER membranes and to relate the cholesterol levels to SREBP transport and activation. Membrane cholesterol levels were quantified by mass spectrometry (MS) techniques, which we will discuss in a later recitation. (Note that mass spectrometry usually does not allow for absolute quantification, so standards need to be added at defined concentrations.) SREBP cleavage was followed by Western Blots and the amounts quantified by densitometry (Figure 5A-C).

First, cells were depleted of cholesterol by adding hydroxypropyl-β-cyclodextrin, which sequesters cholesterol. As shown in Figure 5A, reduced levels of cholesterol are correlated with accumulation of the cleaved transcription factor domain of SREBP.

Addition of cholesterol as a methyl-cyclodextrin complex to cholesterol-depleted cells causes an increase in cholesterol concentrations and a concomitant decrease in the processed form of SREBP (Figure 5B). Note that addition of 5-hydroxycholesterol at low levels has the same effect on SREBP, but does not lead to an increase in cholesterol (Figure 5C). Thus, 5-hydroxycholesterol can block SREBP processing even at low cellular cholesterol levels.

How accurate do you think the cholesterol quantification is?
What part of SREBP does the Western Blot antibody need to bind for these experiments?
Why not add free cholesterol instead of the methyl-cyclodextrin complex?

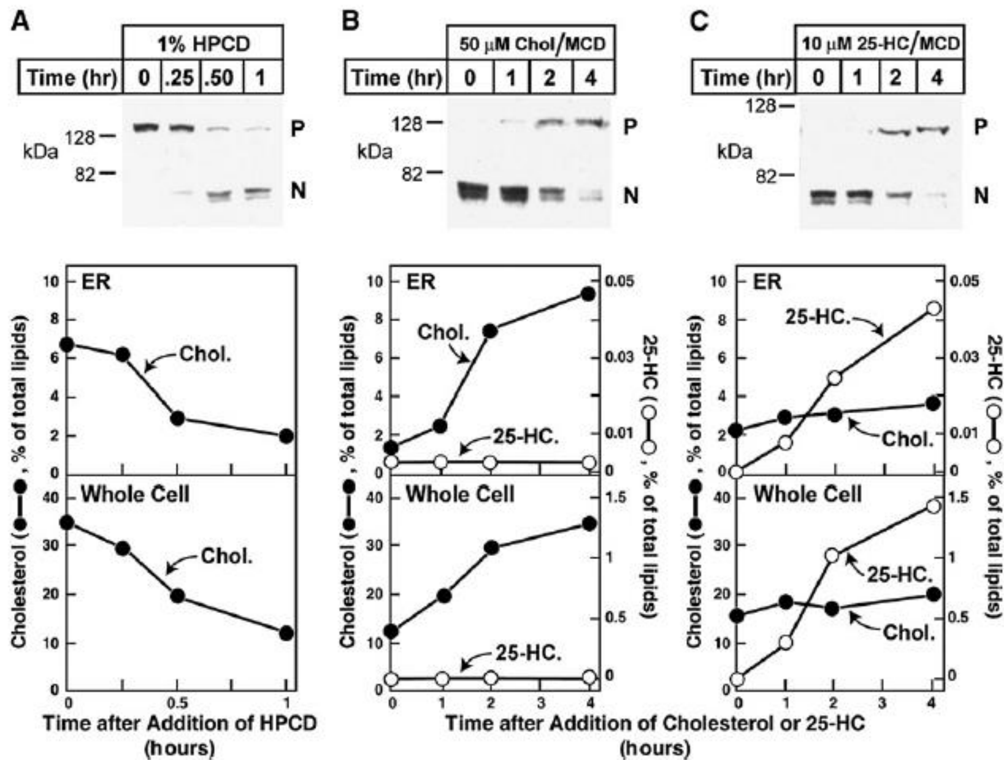

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Figure 5: SREBP processing (top panels) and cholesterol levels in ER membranes (middle panels) and whole cells (bottom panel) as a consequence of (**A**) cholesterol depletion, (**B**) cholesterol addition to cholesterol-depleted cells, or (**C**) 25-hydroxycholesterol addition to cholesterol-depleted cells.

Next, the researchers sought to quantify the extent of SREBP activation at different cholesterol concentrations. They supplemented cholesterol at defined concentrations to cholesterol-depleted cells via two different methods, either via the cholesterol•methyl-cyclodextrin complex described above or via lipoproteins, and again determined the ER cholesterol concentration and the fraction of activated SREBP. For both delivery methods, they observed a sigmoidal relationship between the fraction of active SREBP and the ER cholesterol concentration, indicative of a cooperative transition with a threshold concentration below which activation occurs (Figure 6, left panel). The threshold was about 5% cholesterol in the ER membranes, with slight variation depending on the delivery method.

What factors could be responsible for the cooperativity?

To test the proposed model of cholesterol sensing by Scap and Insig, the authors performed an additional experiment in cells that overexpress Insig. As expected, increased Insig lowered the threshold concentration below which activation occurs (Figure 6, right panel).

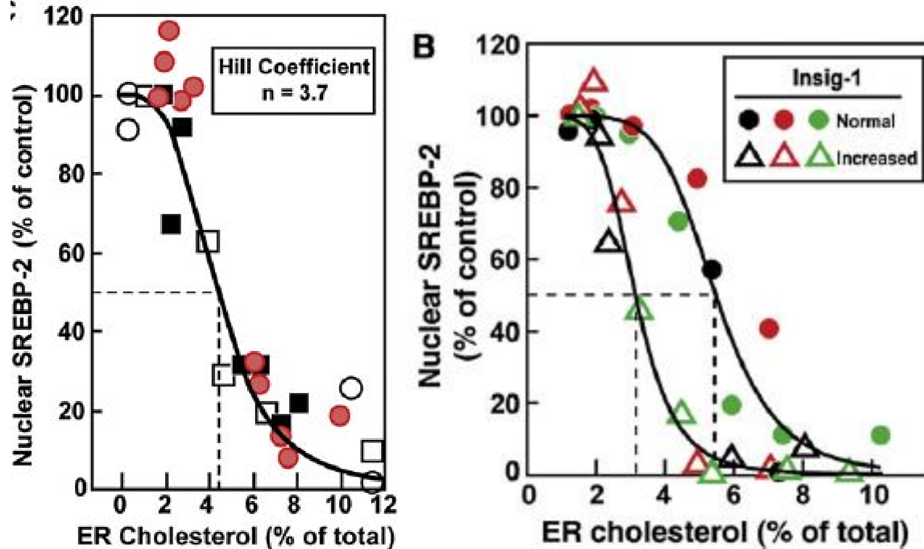

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Figure 6: Relation of SREBP processing to cholesterol content. **Left**: Fraction of activated (nuclear) SREBP at varying cholesterol concentrations, delivered in a complex with methyl-cyclodextrin. There is a sharp transition centered around 4.5% cholesterol. **Right**: Fraction of activated (nuclear) SREBP at varying ER cholesterol concentrations in cells with normal (closed circles) or elevated (open triangles) Insig concentrations. The transition shifts from 5.5% cholesterol at normal Insig levels to 3.1% cholesterol at elevated Insig levels.

Conclusion

Taken together, the authors established a method to isolate ER membranes from mammalian cells. Obtaining purified membranes allowed them to determine ER membrane cholesterol levels and to correlate these levels to SREBP activation. The results presented are indicative of a cooperative transition from SREBP activation at low cholesterol concentrations to block of activation at high cholesterol concentrations, with a threshold ER cholesterol concentration of approximately 5%. Overexpression of Insig lowered the threshold concentration because Insig is a repressor of the activation. The observed cooperativity affords great precision in responding to changes in cholesterol levels.

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