APPENDIX A
KEY for yeast secretory pathway spotting experiment predictions

Please use the following key to enter your predictions for the experiment you are setting up today into the chart below:
AG (abundant growth), G (some growth) or NG (no growth)
Please also include all appropriate symbols to explain your growth choice:
PT = permissive temperature; **NPT**=non-permissive temperature; **BPT**= borderline permissive temperature
-S= lacks an enzyme required to synthesize essential compound(s) missing from media—name which one(s): U, H, UH
+S= able to synthesize all essential compounds missing from media—name which are missing: U, H or UH
-SR= unable to synthesize missing compound(s) because the required enzyme(s) is(are) removed from the proper cellular compartment—include the symbol for the missing compound(s): U, H or UH

	SD-U, 25°C	**SD-U, 30°C**	**SD-U, 37°C**	**SD-U-H, 25°C**	**SD-U-H, 30°C**	**SD-U-H, 37°C**
WT + YEp24	AG PT +S (U)	AG PT +S (U)	AG PT +S (U)	NG PT -S(H)	NG PT -S(H)	NG PT -S(H)
WT + pRSB203	AG PT +S (U)	AG PT +S (U)	AG PT +S (U)	AG PT +S(UH)	AG PT +S(UH)	AG PT +S(UH)
WT + pRSB204	AG PT +S (U)	AG PT +S (U)	AG PT +S (U)	NG PT -SR (H)	NG PT -SR (H)	NG PT -SR (H)
***sec61* + YEp24**	AG PT +S (U)	G BPT +S (U)	NG NPT	NG PT -S (H)	NG BPT -S (H)	NG NPT -S (H)
***sec61* + pRSB203**	AG PT +S (U)	G BPT +S (U)	NG NPT	AG PT +S (UH)	G BPT +S (UH)	NG NPT +S(UH)
***sec61* + pRSB204**	AG PT +S (U)	G BPT +S (U)	NG NPT	NG PT -SR (H)	G BPT +S (UH)	NG NPT +S (UH)
***sec18* + YEp24**	AG PT +S (U)	G BPT +S (U)	NG NPT	NG PT -S(H)	NG BPT -S(H)	NG NPT -S(H)
***sec18* + pRSB203**	AG PT +S (U)	G BPT +S (U)	NG NPT	AG PT +S (UH)	G BPT +S (UH)	NG NPT +S (UH)
***sec18* + pRSB204**	AG PT +S (U)	G BPT +S (U)	NG NPT	NG PT -SR(H)	NG BPT -SR(H)	NG NPT -SR(H)

Key to Symbols: **WT** = Yeast strain LY527 *MATα SEC+ ura3 his4*; ***sec61*** = yeast strain LY651 *MATα sec61-1 ura3 his4*; ***sec18*** = yeast strain LY689 *MATα sec18-1 ura3 his4*; **YEp24** = empty vector with *URA3* but no *HIS4*; **pRSB203** = YEp24 vector plus WT *HIS4* (not fused to an ER signal sequence); **pRSB204** = YEp24 vector plus *HIS4* fused to an ER signal sequence (*ssHIS4*).

APPENDIX B1

YEAST SECRETORY PATHWAY LABORATORY II: ANALYZING SECRETION DEFECTS BY WESTERN BLOTTING

(Pre-pro-α-factor experiment, Week 2)

Your Tasks Today

1. Scoring the results of the *ss-HIS4* reporter gene assay
2. Performing the initial steps of a Western blot, using a different reporter, to determine which forms of a normally secreted protein accumulate in the two *sec* mutant strains

BACKGROUND INFORMATION

Protein Maturation in the Secretory Pathway

Proteins that travel through the secretory pathway usually undergo several types of modifications before they reach their mature forms (Karp, 2005). Usually, the signal sequence that targets secreted proteins for co-translational insertion into the ER is removed by a signal peptidase even before the entire protein has been synthesized by the ribosome. Second, most secreted and plasma membrane proteins are modified by the addition of carbohydrates in a process called *glycosylation*. Branched chains of sugar molecules (oligosaccharides) are initially added to proteins in the ER in a process called glycosylation. Some trimming of these chains occurs before proteins leave the ER and proceed to the Golgi. In the Golgi, additional carbohydrate modifications often occur. As a result, multiple, differently glycosylated forms of a particular secreted or cell surface protein are present in a cell at any one time as different molecules of the protein travel through the secretory pathway. Since they have different molecular weights, these various forms of a protein can be separated by SDS-PAGE and, if an antibody is available, can be detected by Western blotting.

Pre-pro-α-factor

In this experiment you will perform a Western blot to detect the various forms of a normally secreted protein, α-factor, that are present in the wildtype and *sec* mutant yeast cells. Alpha-factor is a yeast mating pheromone. Yeast can exist as both haploid and diploid cells. Haploid yeast come in two mating types (*MAT*): **a** and **α**. *MATa* yeast secrete a pheromone called a-factor, while *MATα* cells secrete α-factor. These pheromones allow yeast cells to sense cells of the opposite mating type that are in close proximity and to initiate events that prepare them to mate (i.e. to fuse into a diploid cell). We will talk more about yeast mating factors in the cell signaling portion of the course, but for now, the important thing is that they are secreted proteins that must travel through the ER, the Golgi, and subsequent compartments before being released at the cell surface by exocytosis.

The mature, secreted form of α-factor is a polypeptide of only 13 amino acids, but it is synthesized as a larger precursor protein that is processed to its mature form in several glycosylation and proteolysis steps. Using the nomenclature that is generally applied to secreted polypeptides derived from larger precursors (e.g. many hormones), the form of α-factor that is translated by the ribosome is referred to as "pre-pro-α-factor" (pp-α-F). This form includes the ER signal sequence and other "spacer" sequences that are eventually removed to generate the mature α-factor polypeptide. The term "pro-α-factor" (minus the "pre-") refers to an

intermediate form of the final product, after cleavage of the signal sequence (which occurs after insertion into the ER) but prior to removal of spacer sequences. In the case of α-factor, the signal sequence was originally thought not to be removed in the ER, but during a later proteolytic processing step further along the secretory pathway (Julius, 1984); a later paper proved that this conclusion was erroneous, however, and that the pp-α-F signal sequence is indeed cleaved by the ER signal peptidase, as occurs for most proteins inserted into the ER (Waters, 1988).

A polyclonal antibody raised against pp-α-F would be expected to recognize all forms of the protein as it progresses through the various glycosylation and proteolysis steps in its processing sequence. The molecular weight of the unglycosylated, translated form (including the signal sequence) is approximately 18.6 kD. In the ER, N-linked core oligosaccharides are added to three sites in the protein, causing its molecular weight to increase significantly; the fully modified ER form of pp-α-F is 26 kD (Julius, 1984). Many secreted proteins undergo additional carbohydrate modifications in the Golgi, but glycosylation of pp-α-F is completed by the time it leaves the ER. As pp-α-F moves through the Golgi and into the secretory vesicles that bud from the Golgi, endo- and exo-proteolytic steps eventually produce the mature, secreted form of α-factor, reducing it to 3.4 kD (see Fig. 7 from Julius, 1984).

Using pp-α-F to investigate the steps blocked in *sec18-1* and *sec61-1* strains

For the Western blot analysis you will probe for precursor forms of α-factor in WT, *sec18-1*, and *sec61-1* yeast cells that were either grown continuously at room temperature (RT, 25°C) or grown at RT then shifted to 37°C for one hour. Note that all of these strains are haploids of mating type α (*MATα*): if they were *MATa* haploids or diploids, they would not produce α-factor! Recall that the *sec18-1* and *sec61-1* strain each have a temperature sensitive defect in a different step in the secretory pathway. The Western blot will allow you to determine the molecular weight of the form of α-factor that accumulates in each of these strains due to the block in secretion. The WT strain, as well as a WT *MATa* strain that does not express any α-factor will serve as controls. *What should happen to α-factor in a cell with a normal secretory system?* (Note that the mature form of α-factor will be too small to see on our gels since it will run off the bottom.)

REFERENCES

Caplan, S., Green, R., Rocco, J., and Kurjan, J. (1991). Glycosylation and Structure of the Yeast *MFα1* α-Factor Precursor Is Important for Efficient Transport through the Secretory Pathway. *J. Bacteriol.* **173:**627-635.

Julius, D., Schekman, P., and Thorner, J. (1984). Glycosylation and Processing of Prepro-α-Factor through the Yeast Secretory Pathway. *Cell* **36:**309-318.

Karp, G. (2005). Cytoplasmic Membrane Systems: Structure, Function, and Membrane Trafficking. In: Cell and Molecular Biology: Concepts and Experiments, 4th ed. John Wiley & Sons, Inc., Hoboken, NJ. p. 279-333.

Waters, M.G., Evans, E.A., and Blobel, G. (1988). Prepro-α-factor Has a Cleavable Signal Sequence. *J. Biol. Chem.* **263:**6209-6214.

PROTOCOLS

1. SDS-PAGE

1. Label your six tubes of yeast cells with your initials and make sure the cells are thawed. To each tube of cells, add 200 µl of 1X SDS sample buffer lysis buffer and approximately 200 mg of glass beads. You will be given six microfuge tubes, each containing enough glass beads for one sample, so you can just pour the beads from these tubes into the screw-capped tubes that contain your yeast cells.
2. Make sure the caps are tightly closed. Process the samples at room temperature in the FastPrep bead beater for 20 seconds (speed = 6.0). Your instructor will assist you in loading and unloading the samples from the machine. Yeast cells have a tough cell wall, so the glass beads are necessary to lyse the cells. The samples will be very foamy at this point, but the foam should dissipate with heating.
3. Boil the samples for 3 minutes at 100°C in a heating block. Let the tubes cool briefly on your bench. Before loading the samples onto the gel, spin the tubes for about 30 sec at max speed in a microcentrifuge to dissipate any remaining foam and collect all the beads at the bottoms of the tubes.
4. The gels you will be using are 12.5% Tris-HCl polyacrylamide gels. Each group will run one gel, but two groups can use a single electrophoresis apparatus. When you pipet your samples to load on the gel, draw from the top of the liquid, trying to avoid sucking up the glass beads. Load your samples as shown below:
    - Lane 1 — 10 µl of pre-stained MW standards (these do not need to be boiled)
    - Lane 2 — 10 µl of WT, *MATa* lysate (already prepared for you)
    - Lane 3 — 10 µl of WT, 25°C lysate
    - Lane 4 — 10 µl of WT, 37°C lysate
    - Lane 5 — 10 µl of *sec18-1*, 25°C lysate
    - Lane 6 — 10 µl of *sec18-1*, 37°C lysate
    - Lane 7 — 10 µl of *sec61-1*, 25°C lysate
    - Lane 8 — 10 µl of *sec61-1*, 37°C lysate
5. Run your gel at 200 volts until the dye front reaches the bottom of the gel. Be sure to stop the gel before the 15 kD marker runs off the bottom.

2. Western blot transfer

Turn the power source controlling your gel electrophoresis to OFF. Remove your gel from the apparatus and separate the plates. Carefully cut off the wells at the top of the gel and just the thicker part at the very bottom of the gel with a razor blade. Be careful not to tear the gel. Take it over to the blotting apparatus.

The holder for the blotting apparatus is color coded so the black side should end up facing the cathode (black electrode) and the clear side facing the anode (red). The proper Western blot "sandwich" will be made in the following manner, keeping all parts wet in the blotting buffer in the plastic container provided at your bench:

1. Wear gloves when handling the nitrocellulose membranes. The membrane is white, and will be given to you sandwiched between two pieces of blue protective paper. Remove the top piece of blue paper. In pencil, label the top left corner of your piece of nitrocellulose (NC) with your initials. Be careful not to tear the membrane with the pencil tip. Immerse the NC in the blotting buffer so that it is wet evenly.

2. Place the holder in the plastic container after filling the container half full with blotting buffer.
3. Wet one of the sponges with blotting buffer and place it on the **black side** of the holder.
4. Wet 2 pieces of 3 mm paper and place them on top of the sponge.
5. Wet your gel in blotting buffer and place it on top of the 3 mm paper. To make the left-most lane (Lane 1) of the gel come out on the *left* side of the blot, orient your gel so that Lane 1 (containing the stained MW ladder) is on the *right* side of the blot sandwich.
6. Place your wet NC on top of the gel after removing the bottom blue protective paper.
7. Add 2 more pieces of moistened 3 mm paper on top of the NC. Use a broken plastic pipet like a rolling pin to gently "roll" out any air bubbles out from between the gel and the NC.
8. Place a second moistened sponge on top of the 3 mm paper.
9. Close the clear side of the holder, pushing the clasp down and along the top of the holder.
10. **Place the holder into the blotting tank so that the clear side faces the red pole and the black side faces the black pole.**
11. Two blots can be run in each tank. Place the frozen ice compartment into the tank. Fill the tanks so the buffer is up to the top of the gel. Connect the top of the tank tightly. Connect the power supply and run the blots at 100 volts for one hour. Be sure the stir bar is free to stir to keep the buffer cold. Watch the current. It should read 0.15-0.20 amps during the run.
12. After an hour, disconnect the power and remove the holders. Remove the NC, rinse it with distilled water, allow it to dry, then wrap it in plastic. Store the NC in the refrigerator until the next lab.

APPENDIX B2

YEAST SECRETORY PATHWAY LABORATORY II: ANALYZING SECRETION DEFECTS BY WESTERN BLOTTING

(Myc-tagged invertase experiment, Week 2)

Your Tasks Today

1. Scoring the results of the ss-HIS4 reporter gene assay
2. Doing the first part of a Western blotting procedure to determine what forms of a protein that is normally found on the cell surface accumulate in the two *sec* mutant strains

BACKGROUND INFORMATION

Protein Maturation in the Secretory Pathway

[See Appendix A1.]

Invertase

We will be performing our Western blots using an antibody that recognizes an epitope-tagged form of the protein invertase. Invertase is an enzyme encoded by the yeast *SUC2* gene that is required for yeast to be able to derive energy from fermentation of sucrose. Sucrose is a disaccharide composed of glucose and fructose joined by a glycosidic bond, and invertase is the enzyme that hydrolyzes this bond during the process of sucrose catabolism. Yeast express two types of invertase: one that is cytoplasmic and one that travels through the secretory pathway to reach the cell surface (Dodyk, 1964; Esmon, 1981). *(Both forms of the protein are encoded by the same SUC2 gene—what might be the basis for their different localizations within the cell?)* For your Western blotting experiment, you will be using WT, *sec18-1*, and *sec61-1* strains in which the normal *SUC2* gene has been replaced with a copy of the gene that has been modified with a sequence that encodes 13 copies of the myc epitope tag appended to the C-terminus of the normal Suc2 protein (*SUC2-13myc*). C-Myc is a mammalian oncoprotein; the epitope tag is just a portion of the c-Myc protein that happens to be particularly antigenic, so other proteins that are tagged with the Myc epitope can easily be detected using an anti-Myc monoclonal antibody. Because yeast "prefer" to use glucose as an energy source when it is available, the cell surface form of invertase is not normally expressed when cells are growing in glucose-containing medium. In contrast, the cytoplasmic form of invertase is expressed constitutively. This regulatory feature of yeast metabolism is called *glucose repression.* For our purposes, what it means is that we must grow our yeast strains in reduced glucose medium (0.1% glucose, rather than the normal 2%) to induce expression of the secreted form of invertase.

Using invertase to investigate the steps blocked in *sec18-1* and *sec61-1* strains

Most proteins that are targeted to the cell surface (including the cell-surface form of invertase) are modified by the addition of carbohydrates (glycoslyation). Secreted and cell surface proteins are glycoslyated in a stepwise process that begins in the ER and is completed in the Golgi complex (see Karp, 2005, p. 295-297 and p. 301-302). Glycoslyation of a protein obviously increases its molecular weight and will therefore alter its mobility in an SDS-polyacrylamide gel. Consequently, we will be able to "follow" the maturation of the invertase protein as it moves through the secretory pathway based on the different molecular weight forms of the protein that are visualized in our Western blots. The molecular weight of the protein

encoded by the entire *SUC2* open reading frame is 61 kD, while the most extensively glycosylated forms of secreted invertase have molecular weights of 100-140 kD. Several of the papers listed in the References at the end of this section provide examples of the migration patterns of the forms of invertase that we may expect to see on our developed blots (Esmon, 1981; Novick, 1981; Deshaies, 1987). Note that both the cytoplasmic and secreted forms of invertase will be tagged with the Myc epitope and will therefore be detected on our Western blots. *How will the molecular weight of cytoplasmic invertase compare to that of the various forms of secreted invertase?* Also note that the 13myc tag will add 20-25 kD to the molecular weight of the Suc2 protein.

At the beginning of today's lab, you will be given pellets of WT, *sec18-1*, and *sec61-1 SUC2-13myc* yeast cells that have been treated as follows: first grown at room temperature (RT, 25°C) in high-glucose YPD ("rich") medium containing 2% glucose, then pelleted and re-suspended in YPD containing 0.1% glucose and placed either at RT or at 37°C for three hours (six cell pellets in total). You should recall that the *sec18-1* and *sec61-1* strains have temperature sensitive defects in two different steps in the secretory pathway and that those particular steps will be compromised when the cells are shifted to 37°C (and possibly also at lower temperatures to a lesser degree). The Western blot will allow you to determine the molecular weights of the forms of invertase that accumulate in these cells due to the blocks in secretion. The WT strain will serve as a control. You will also include a sample of WT cells that do not express any myc tag (prepared by your instructor) as a negative control for the antibody. Today you will prepare whole cell lysates of the yeast cells, run those lysates on a 7.5% SDS-polyacrylamide gel, and transfer the proteins from the gel to a nitrocellulose membrane (the first step in Western blotting). After Spring Break, you will probe the blots with an anti-myc primary antibody and an enzyme-linked secondary antibody to visualize the various forms of invertase that are present in the various samples.

REFERENCES

Carlson, M. and Botstein, D. (1982). Two differentially regulated mRNAs with different 5' ends encode secreted with intracellular forms of yeast invertase. *Cell* **28:**145-154.

Deshaies, R.J. and Schekman, R. (1987). A yeast mutant defective at an early stage in import of secretory protein precursors into the endoplasmic reticulum. *J. Cell Biol.* **105:**633-645.

Dodyk, F. and Rothstein, A. (1964). Factors Influencing the Appearance of Invertase in *Saccharomyces cerevisiae*. *Arch. Biochem. Biophys.* **104:**478-486.

Esmon, B., Novick, P. and Schekman, R. (1981). Compartmentalized assembly of oligosaccharides on exported glycoproteins in yeast. *Cell* **25:**451-460.

Karp, G. (2005). Cytoplasmic Membrane Systems: Structure, Function, and Membrane Trafficking. In: Cell and Molecular Biology: Concepts and Experiments, 4th ed. John Wiley & Sons, Inc., Hoboken, NJ. p. 279-333.

Novick, P., Ferrro, S. and Schekman, R. (1981). Order of events in the yeast secretory pathway. *Cell* **25:**461-469.

PROTOCOLS

1. SDS-PAGE

Steps 1-3: Steps 1-3 of the SDS-PAGE instructions for the invertase version of the experiment are identical to those described in Appendix A1 for the pre-pro-alpha-factor version of the experiment.

4. The gels you will be using are 7.5% Tris-HCl polyacrylamide gels. Each group will run one gel, but two groups can use a single electrophoresis apparatus. When you pipet your samples to load on the gel, draw from the top of the liquid, trying to avoid sucking up the glass beads. Load your samples as shown below:

Lane 1 — 10 µl of pre-stained MW standards (these do not need to be boiled)
Lane 2 — 5 µl of WT, no-myc lysate (already prepared for you)
Lane 3 — 5 µl of WT *SUC2-13myc*, 25°C lysate
Lane 4 — 5 µl of WT *SUC2-13myc*, 37°C lysate
Lane 5 — 5 µl of *sec18-1 SUC2-13myc*, 25°C lysate
Lane 6 — 5 µl of *sec18-1 SUC2-13myc*, 37°C lysate
Lane 7 — 5 µl of *sec61-1 SUC2-13myc*, 25°C lysate
Lane 8 — 5 µl of *sec61-1 SUC2-13myc*, 37°C lysate

5. Run your gel for at 200 volts until the 50 kD marker runs off the bottom and the 75 kD marker is about three-quarters of the way down the gel (approximately 45 min).

2. Finishing the SDS-PAGE & Starting the Western blot

The Western transfer for the invertase version of the experiment is performed exactly as described in Appendix A1.

APPENDIX C

YEAST SECRETORY PATHWAY LABORATORY III: PROBING AND DEVELOPING THE WESTERN BLOT

(This is the lab manual section used for the pre-pro- α-factor version of the experiment; footnotes at the end of the Protocol section indicate changes for the invertase version of the experiment.)

BACKGROUND INFORMATION

Primary and Secondary Antibodies

A variety of procedures have been developed to visualize proteins on Western blots. Most often, an antibody made against the protein of interest (*primary antibody*) is used to recognize the protein. A *secondary antibody* that binds to the primary antibody is then used to visualize the spots on the nitrocellulose where the primary antibody has bound. The secondary antibody is conjugated to an enzyme that produces a visible product. As discussed in lecture, the two-antibody scheme has two main advantages over using a single antibody: 1) the signal is amplified because more than one secondary antibody molecule can bind to each primary antibody; and 2) there is no need to generate an enzyme-conjugated version of each primary antibody. For our Western blot, we will use a primary antibody that recognizes pre-pro-α-factor (pp-α-F) to identify the various forms of α-factor that accumulated in the *sec18-1*, and *sec61-1* yeast strains grown at the non-permissive temperature (37°C)[1]. Remember that pp-α-F is the entire polypeptide that is synthesized by the ribosome, including the ER signal sequence and other sequences that are removed by proteolysis to generate the final, secreted form of α-factor. The anti-pp-α-F antibody was generated in a rabbit. We will therefore use a goat anti-rabbit antibody as our secondary antibody. This antibody is linked to the enzyme horseradish peroxidase (HRP), which will be used to visualize the immunoreactive proteins by a method called *enhanced chemiluminescence*.

Enhanced Chemiluminescence (ECL)

Luminescence refers to energy that is dissipated in the form of light when some molecules transition from an excited state to their ground state. When the excitation of the molecule is caused by a chemical reaction, it is referred to as chemiluminescence. Examples of chemiluminescence are the light produced by fireflies and the glow of Halloween light sticks. In the chemical reaction that will be used to detect the HRP-conjugated goat-anti-rabbit antibodies on our Western blots, the light releasing molecule is a compound called luminol. Luminol is commonly used in crime scene investigations to visualize traces of blood. Luminol is mixed with hydrogen peroxide (H_2O_2) and is applied to the surface that is being investigated. If traces of blood are present, the iron in the blood acts as a catalyst in the oxidation of luminol, which is accompanied by blue-green luminescence. In our case, the HRP linked to the secondary antibody will catalyze the same reaction, and we will detect the light produced by exposing our blots to blue light-sensitive film. *Enhanced* chemiluminescence refers to the fact that, in addition to the H_2O_2 and the luminol, the developing solutions we will use also contain some additional chemicals (enhancers) that increase the intensity of the light produced in the reaction.

PROTOCOL

1. Incubate your blot in 10 ml of blocking buffer at room temperature for at least 15 min. on an orbital shaker. *(The blocking buffer contains 5% milk in phosphate buffered saline with 0.25% Tween, a detergent. The proteins in the milk bind non-specifically to the nitrocellulose, "blocking" sites where the primary antibodies might bind non-specifically.)*
2. Pour off the blocking buffer into the sink (try to leave as little volume as possible so the primary antibody will not be further diluted). Add the primary antibody (rabbit anti-pre-pro-α-factor), which has already been diluted 1:2000 in 10 ml of blocking buffer[2]. Incubate on the rocker for 1 hour, then discard the primary antibody solution into the sink.
3. Pour PBST wash buffer onto the nitrocellulose so that it is submerged by approximately 0.5 cm of liquid. Incubate 10 min. on the shaker.
4. Pour off the wash buffer into the sink. Add fresh wash buffer and incubate another 10 min. on the shaker.
5. Repeat Step 4 once more (a total of 3 10-min. washes).
6. Add the HRP-conjugated goat anti-rabbit secondary antibody, which has already been diluted 1:2500 in 10 ml blocking buffer[3]. Incubate 30 min. on the shaker.
7. Pour off the secondary antibody solution into the sink.
8. Wash <u>three times</u> with PBST (10 min. each wash) as you did after the primary antibody incubation. After the last wash, remove all residual wash buffer by tilting the blot container onto a paper towel.

***Do not proceed with the following steps until your instructor is available to help you with the film exposure and developing. Leave your blot in the final batch of wash buffer until that time.**

9. Pipet 2 ml of each ECL detection reagent (1 & 2) into a 15-ml conical tube and invert to mix. *Be sure to use a clean pipet for each detection reagent—it is important for the two solutions not to mix until just before you use them!*
10. Pour the mixed ECL developing solution onto your blot. Rock the container back and forth by hand for 1 min. Discard the ECL reagents in the sink.
11. Use forceps to place your blot onto a piece of filter paper. Using another piece of filter paper, gently blot the nitrocellulose to remove excess liquid.
12. Use forceps to place the blot—protein side up—between the two plastic sheets of a page protector that has been taped into an autoradiography cassette.
13. With your instructor, take the cassette into the darkroom. Hold the open cassette up to the light for about 20 sec. to activate the glow-in-the-dark Stratagene™ logo marker. (This will help in aligning your developed film with the MW markers on the blot.)
14. Flip the switch to turn off the normal light and turn on the safe light. Also make sure the computer monitor is turned off. Open the film container and remove one piece of film. Place the film onto the blot and close the cover of the cassette (be careful not to move the film once it has been placed on the blot). Wait 1 min., then open the cassette and remove the film.
15. Feed the film into the developer and wait for it to emerge. The developing process will take 4 min. If you need to, repeat with a shorter or longer exposure time. <u>If you turn on the regular light to look at your developed blot, make sure the undeveloped film is back in its box!</u>

16. Take your developed film and the autoradiography cassette back to the lab. Align the Stratagene™ logos to properly position the film on top of the blot. Use a Sharpie marker to mark the position of the colored MW bands on the film. If you have difficulty determining the position of the gel lanes, use one of the plastic combs saved from the electrophoresis to help you.

Changes for invertase experiment:

[1] For our Western blots, we will use a monoclonal primary antibody made in mouse that recognizes the Myc epitope to identify the various forms of Myc-tagged invertase that accumulated in the *sec18-1* and *sec61-1* yeast strains when these strains were shifted to the non-permissive temperature (37°C). We will use a goat anti-mouse antibody as our secondary antibody.

[2] Add the primary antibody (mouse anti-myc), which has already been diluted 1:7500 in 10 ml of blocking buffer.

[3] Add the HRP-conjugated goat anti-mouse secondary antibody, which has already been diluted 1:2500 in 10 ml blocking buffer.

DATA INTERPRETATION

1. Estimate the molecular weights of all the bands on your developed film. Just approximate based on the MW markers—you do not need to construct a standard curve as you did for the Coomassie-stained gel in the β-gal lab series.

2. What are the dark bands that are present in each sample and what do they represent? What are the likely reasons for the differences in their sizes? Use your knowledge of the specific defects of the *sec18-1* and *sec61-1* strains to help you interpret your data. Also consider what it means if no pp-α-F signal is present for some of the samples.

THE YEAST SECRETORY PATHWAY LAB REPORT

Your paper for this module will be due on your lab day the week of April 9th. Your report should follow the same basic format as the β-galactosidase paper, and include the following sections: Title, List of Authors, Abstract, Introduction, Materials and Methods, Results (text AND figures and/or tables), Discussion, and References. You may not need an Appendix, but you should have a table of strain information at the beginning of the Materials and Methods section. You should write the report with the following experimental question in mind: What is the role of the Sec18 and Sec61 proteins in the yeast secretory pathway? This means that you should use some of the findings from reference studies to support or refute the conclusions from your data. You should also clearly explain how the two reporter systems are used in the mutants to investigate defects in the secretory pathway. Some of this information may be appropriate for the Introduction and some for the Results or Discussion section. Think carefully about where a reader most needs detailed information to avoid repetition. In this report there are no "required" tables and figures. You should think carefully about how to display your data so that the most important findings are made clear to a reader who is unfamiliar with these proteins, with the yeast secretory pathway, and with your experimental design. Part of the Discussion should explore the significance of these two proteins in a larger context. Your BLAST analysis should provide sufficient information to address the overall importance of these proteins in other organisms.

APPENDIX D

Directions for using the SGD and NCBI Databases:
(Used as part of the Week 1 lab activities)

I. Finding basic information about protein function in SGD

Go to the *Saccharomyces* Genome Database (SGD) website, http://www.yeastgenome.org/. This database is a portal through which you can access all sorts of information about yeast genes and proteins. Use the "Quick Search" feature to look up *SEC18* and *SEC61* (the search is case insensitive). You will probably be able to identify the basic functions of the two gene products relatively quickly, but take some time to explore the various links to familiarize yourself with the various types of information that can be obtained from SGD. Once you have found the function of the two yeast proteins, start on your yeast growth predictions before beginning the search described below for homologs of the gene products.

II. Finding information about potential homologs through a BLAST search

To search for proteins in other species that have sequence similarity to Sec18 and Sec61, you will perform a BLAST search. Click on VIEW next to "Protein Info" in the drop down menu called "Protein Info & Structure" on the right side of the SGD main page for the gene of interest. This link opens a homepage for the protein product of the gene that contains a lot of useful information, such as the molecular weight, isoelectric point, predicted sequence features, and full amino acid sequence. Take a look at this information before proceeding. Then, scroll down on the Protein Info page to where it says "EXTERNAL LINKS;" click on the "BLASTP (NCBI)" link under the "Homologs" sub-heading. This will open the BLAST page of the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov) in another browser window and will automatically paste the NCBI protein sequence identifier (in the format "NP_XXXXXX.X), where the Xs are numbers) into the search window. You have probably noticed that SGD has its own BLAST feature (one of the options at the top of the page), but that one only compares an input sequence to the sequences of other yeast proteins. Because we are interested in potential homologs in other organisms, you will perform your BLAST search using the more comprehensive NCBI database, which contains protein sequences from a vast range of organisms. Use the default database "nr" and the other default commands. Click "BLAST!" and follow the directions for viewing the results. You may have to wait several minutes for your search to finish.

To organize the results, click the TAXONOMY REPORTS link (two-thirds of the way down the page), then try out the different ways you can display the data. Look carefully at the human homologs by clicking on the link called "hits" next to Homo sapiens in the LINEAGE REPORT. Click on the LOCUS number (the locus number or accession number usually starts with 3 uppercase letters) of the top human "hit". Find out how many amino acids are in this protein, then close the locus window.

To determine which human protein is most similar to your yeast protein over the whole protein sequence, return to the BLAST results page by minimizing the taxonomy reports window. Scroll down to find the most promising human protein (top hit). NOTE: If you have previously clicked on its locus number, that protein should appear in a different color than the

others, which should help you find it more quickly. The homologs are arranged in order of sequence similarity, so the human ones are unlikely to be near the top.

Once you have found the human protein that exhibits the greatest sequence similarity with your yeast protein, click on number to the right of the locus and protein name in the column called SCORE BITS (DO NOT click again on the locus number). This link should take you to the section in the BLAST results containing the alignment of the yeast protein (QUERY) with the hit, the human protein or SUBJECT. LENGTH refers to the number of amino acids in the protein. Look for consensus over the whole protein. Since you know the total number of amino acids in the protein, you can look at the end of the alignment to see how far significant consensus extends. Record the name of the closest human homologs for yeast Sec18 and to Sec61. At the top of the alignment section you can find and record the % sequence identity (IDENTITY) and the % sequence similarity (POSITIVES).

Use the taxonomy report to find out the range of organisms that have similarity to the yeast proteins. Try to find the function of the human homologs using other references. Briefly summarize this information for this homework assignment; you may also find the information to be useful when you write the lab report at the end of this series.

APPENDIX E: KNOWLEDGE & UNDERSTANDING SURVEY

Instructions to students:
Please circle what you think are the correct answers to the questions below. Since this survey is designed to assess your actual knowledge, *please do not guess randomly*; if you have no idea which answer is correct, circle the choice that states "I have no idea."

(Asterisks indicate correct answers.)

1. Which of the following would definitely be a true statement about a protein that contains a signal sequence?

a) It is a transmembrane protein.
b) It will be found in the endoplasmic reticulum at some point in its lifetime.*
c) It will be secreted from the cell.
d) I have no idea.

2. How would you expect glycosylation of a protein to affect the molecular weight of that protein?

a) The molecular weight would increase.*
b) The molecular weight would decrease.
c) There would be no change in molecular weight.
d) I have no idea.

3. Which of the following would allow you to study what happens to an organism or cell when an essential gene is not functioning?

a) A knockout mutation
b) A silent mutation
c) A conditional mutation*
d) I have no idea.

4. Which of the following statements about plasmids is NOT true?

a) Plasmids are circular pieces of DNA that can be engineered to contain genes of interest.
b) Plasmids can only be used to express genes in bacteria.*
c) The retention of a plasmid within a cell requires some sort of genetic selection.
d) I have no idea.

5. In a Western blotting experiment, what is NOT a function of the secondary antibody?

a) Recognizing the protein of interest.*
b) Enabling visualization of the primary antibody.
c) Amplifying the signal from the primary antibody.
d) I have no idea.

6. Is the following statement True or False? "A polyclonal antibody used in a Western blotting experiment will only recognize covalently modified forms of the protein if those forms of the protein were present in the antigen that was used to generate the antibody."

True False* I have no idea.

7. Is the following statement True or False? "In cells that have a defect in trafficking between the ER and Golgi, some proteins may still acquire the same carbohydrate modifications that occur in normal cells."

True* False I have no idea.

8. Is the following statement True or False? "Differently glycosylated forms of a protein will appear as a single band on a Western blot because of the denaturing conditions used in the gel electrophoresis step of the procedure."

True False* I have no idea.

APPENDIX F: PERCEIVED EFFICACY SURVEY

Instructions to students:
Please respond to the statements below using the following scale: 5 = Strongly Agree; 4 = Agree; 3 = Neither Agree Nor Disagree; 2 = Disagree; 1 = Strongly Disagree.

1. Overall, this lab improved my understanding of the cellular events that make up the secretory pathway.	5	4	3	2	1
2. Actually seeing the yeast growth results helped me better understand the ss-His4 reporter assay.	5	4	3	2	1
3. If I had just listened to lectures about the two assays we used in this lab series, I would not have understood them as well as I do now.	5	4	3	2	1
4. This lab series improved my understanding of what is meant by a “genetic screen.”	5	4	3	2	1
5. Doing a Western blot myself has made me more comfortable interpreting blot figures in papers.	5	4	3	2	1
6. I now have a better understanding of the types of questions that can be answered by doing a Western blot.	5	4	3	2	1
7. Making detailed predictions about the outcomes of the two experiments before seeing the results played a significant role in my learning process.	5	4	3	2	1
8. Synthesizing the background information and experimental results in the lab report for this series was an important part of my learning process.	5	4	3	2	1