BIOL 212 LAB

Introduction to Primary Scientific Literature

Required Reading: 1. Karp pp. 492-498. Overview of nucleocytoplasmic transport.
2. Gorlich et al. (1994) "Isolation of a protein that is essential for the first step of nuclear protein import." Cell 79: 767 – 778.

See also attached to this packet:
1. Helpful Hints for Reading Primary Journal Articles.
2. Glossary of Terms for Gorlich et al.
3. Model Grant Proposal.

BACKGROUND

In the first few weeks of this course we will perform some laboratory exercises that have been done many times by others. These labs will allow us to explore some new concepts and techniques, to perform some basic data analysis, and to gain experience keeping a detailed, informative lab notebook. Later in the course, however, we will put some of those skills to work while performing some investigative labs, including an original experiment that may generate novel, publishable data about how cells move RNA within themselves. At the conclusion of this experiment we will have the opportunity to write a scientific paper describing our results and the implications they may have for understanding eukaryotic cell biology.

This week's lab will have three parts, each of which is designed to introduce you to scientific writing and investigating primary scientific literature. As an added benefit, the literature we explore will help you begin thinking about the question we will be asking in the "original experiment" lab project.

The three parts to this week's lab are:
1. "Journal Club" discussion of Gorlich et al. (1994) article
2. Introduction to the original experiment and accompanying grant proposal assignment.
3. Database searching and generation of a bibliography for use in preparing the grant proposal.

EXERCISES

1. Journal Club

Although the actual performing of experiments plays an important part in learning about a new subject or unfamiliar field, an equally important use of time for any scientist is the time spent reading scientific literature. Scientific literature can take the form of textbooks or review articles, but the most up-to-date scientific knowledge is that contained in primary journal articles. Primary journal articles are peer-reviewed publications in which an author (or authors) reports the results of original experiments he/she has performed and provides his/her ideas about how these new data advance a particular field of study. Because of the importance of primary literature and because you will be reading and writing primary journal articles before the end of the semester, we will spend the first half of this week's lab period discussing a journal article that relates specifically to molecular cell biology.

Reading primary literature is not easy (and sometimes is not fun, especially when first learning), but it is a necessity for anyone wanting to understand a field well. And, as you become increasingly adept at reading these articles, you will feel a measure of pride and excitement to know that you are exploring information at the cutting edge of cellular and molecular biology. Peer-reviewed journal articles are the initial format in which all important (and unimportant) scientific discoveries disseminated. They provide the basis for knowledge within every field of scientific study.

The paper that you will be reading describes the identification of a protein that is essential for the import of proteins into the nucleus of eukaryotic cells. The paper provides an excellent example of a well-written journal article, and also serves as an introduction to the original experiments we will be performing later in the semester.

The Gorlich et al (1994) paper you will be reading has been posted on Blackboard. Read the paper carefully, using the guidelines on the accompanying handout. See the "glossary" and "helpful tips for reading scientific journal articles" at the end of this packet for assistance with reading the paper and with terms and techniques that are unfamiliar. After carefully (and critically!) reading the paper, answer the questions below as your 20 pt. lab write-up. Please hand in these questions when you arrive in lab.

Questions: (20 pts.)
1. Do permeabilized cells require Ran, importin, or both Ran and importin to import a fluorescent nuclear protein into the nucleus?
2. Where does the fluorescent nuclear protein accumulate if importin is present, but Ran is not?
3. Is importin conserved between different eukaryotic species? What data is presented that supports your answer?
4. In Figure 6 the authors test the ability of "recombinant importin" to stimulate import. Why would they perform this experiment if they've already shown that importin protein purified from eukaryotic cells can stimulate import?
5. List two questions relating to nuclear import that this paper has raised in your mind.
6. Briefly describe one experiment you would perform next to examine one of the questions you listed above.

2. Introduction to the original experiment and accompanying Grant Proposal assignment

The Gorlich et al (1994) paper and the reading assigned from Karp ("Overview of Nucleocytoplasmic Transport") will give you a basic understanding of how proteins are targeted to the nuclear pore complex for import *into* the nucleus. In our original experiment later this semester (referred to as the '**Mex67/Nup1 project'** on your course syllabus), we will seek to generate some new data about how mRNA molecules are transported *out* of the nucleus using yeast as a model system. Specifically we will be testing whether a specific nuclear pore complex protein (called a nucleoporin) physically interacts with a particular soluble protein that is known to be involved in RNA export from the yeast nucleus.

The yeast nucleoporin we will be using (Nup1p) is a large protein with domains that each seem to perform a distinct function. Previous experiments have indicated that some of the domains found in Nup1p can physically associate with yeast importin protein (the same 'importin' we read about in the Gorlich et al. paper), and are likely to assist in the movement of importin across the nuclear envelope.

In our experiment, we are going to test if any Nup1p domains can bind to another protein, called Mex67p, which functions as a kind of "exportin" for mRNA export. In order to test for a Nup1p/Mex67p interaction, we will utilize a technique termed *affinity chromatography*. The molecules we will use as substrates for affinity chromatography will be specific domains of Nup1p, which we will bind to agarose beads. Once bound to the beads, the Nup1p domains will be incubated with a mixture of proteins from yeast. Any proteins in the yeast mixture that have affinity for Nup1p should bind to the Nup1p-agarose bead combination. We will then wash away any unbound proteins, leaving only our agarose beads with Nup1p domains attached, along with any yeast proteins that stick to the Nup1p parts.

Next we will separate our Nup1p-associated proteins from each other using gel electrophoresis and will test for Mex67p among those proteins. We will use a technique called Western blotting to test for the presence of Mex67p among the Nup1-associated proteins. In Western blotting, gels are incubated with specific antibodies that bind very tightly to Mex67p. We can test for the antibody-Mex67p complexes right in the gels using a simple assay that indicates the presence of antibodies. As we progress through these various steps of the experiment, we will discuss move methodology and the rationale behind each procedure that we do.

ASSIGNMENTS ASSOCIATED WITH THE Mex67/Nup1 EXPERIMENT

The five-week sequence of labs that comprise the "original experiment" will include three written assignments. First you will have to write a **grant proposal**, just as if you were applying to a federal granting agency to obtain funding to do your experiments. Second, when the labs are complete, you will generate a draft of Results, Figures, and Materials & Methods sections of a research paper reporting your results. Finally, you will write a complete **research paper** in the format of a publishable primary journal article.

Grant Proposal. (50 points) A grant proposal is a document describing an important scientific question that you would like to answer by doing experiments. These proposals are typically written to be submitted to some funding agency (National Science Foundation, National Institutes of Health, American Cancer Society, etc.) in an effort to obtain the money necessary to carry out the experiments that you are proposing. Since funding agencies have limited funds, you need to provide a strong case for your proposal. Let them know why your project deserves funding above all other proposals they receive!

A typical grant proposal (including yours) contains the following information:

1. Information about the Principal Investigator (you!). One page. For this section, include a *curriculum vita* (c.v.) describing:
    a. Your education: where you are/have been educated, the degrees you have earned, the year you earned those degrees, and any honors you received.
    b. Any research experience or lab experience that is relevant to the project you are proposing to do. Here you can list the relevant techniques with which you have experience, including those you used earlier in the semester in Biol 212 or in other courses.
    c. Any other information you think will help convince the reviewers of your proposal that you can successfully carry out the project you describe.

If you have never before constructed a c.v., now is the time to write your first one.

2. List of suggested reviewers of your proposal. Choose three names from among the authors of papers you cited in your background section and include those names with their university addresses. Try to choose reviewers you think are influential in the field of nuclear transport. Where will their names generally be in the list of authors for each paper? In addition, select reviewers from several different labs so that you get feedback from researchers with distinct scientific backgrounds and different perspectives.

3. Proposal Proper. This is the "meat" of your proposal, and it is what the reviewers will use to decide whether the project has merit. Write it convincingly and thoroughly. This will include the following sections:

a. Project Summary. A brief (one or two paragraphs) section summarizing what you plan to do, how you plan to do it, and what its overall significance will be to our understanding of nuclear transport. Think of this as being a kind of 'abstract' for your proposal, telling the reviewers of your grant proposal what to expect.
b. Table of Contents
c. Project Description (not to exceed 8 pages double spaced). This section provides the scientific rationale for the project.
    1. Background Information. About 3 - 4 pages. Here you tell the story of the project you want to do. What do we already know about RNA export from the nucleus? What proteins are involved and how do they work? What is the role of Nup1p and Mex67p? Do we know anything about how or if they interact? Where is our knowledge lacking? This section will require you to read articles from the primary and secondary literature to get a good understanding of what we understand about mRNA export. **Remember to carefully cite all information you obtain from outside sources!**
    2. A statement of the hypothesis you intend to test. One or 2 paragraphs. What idea are you testing? Be specific! This should be a natural continuation of the background section. You want to convince the reader that your experiments are filling in a major gap in our current knowledge.
    3. Methods you intend to use to test your specific hypothesis. (About 2 pages.) You will need to rely heavily on the lab handouts for the five Mex67/Nup1 labs (posted on Blackboard) to write this part, but DO NOT COPY INFORMATION FROM THE LAB HANDOUTS. Rewrite the experiments in a much shorter way, using the passive voice. Use generalities in writing this section, and assume the reader knows quite a bit of science.
    4. Possible results and what they will mean. About 2 pages. Be specific! What outcomes might there be for this experiment? What would each of these outcomes mean? Do they all support your hypothesis?
d. Literature Cited. Use the citation format used in the Gorlich et al. (1994) paper to list all of the sources you used in preparing your proposal. Make certain to only include in your Literature Cited those sources you actually cite in the text of your proposal.

**An example of a grant proposal will be posted on Blackboard to help with your synthesis of an outstanding proposal.

Results/Figures and Materials & Methods draft (30 pts.) – When the Mex67/Nup1 experiment is complete, you will write a polished description of the methods you performed and the results you obtained. Details regarding this assignment will be distributed as the experiment progresses.

Complete Scientific Paper. (100 points) Once you have obtained feedback from your instructors about your Results, Figures, and Methods sections, you will be required to write a complete scientific paper. This manuscript will include all the parts normally found in a published report, describing the background, results and significance of your study. You will be provided with more information on how to write your paper later in the semester.

3. Database searching and generation of a bibliography for use in preparing the grant proposal.

A critically important part of investigating any new scientific question is finding out what is already known about that particular subject. Thus, you will need to use your new-found skill at investigating primary literature to identify what we know (and don't know) about Mex67 and Nup1 function. But first you will have to find appropriate articles that address Mex67 and Nup1 activities.

To help initiate your preparation for the Mex67/Nup1 project, you will use Medline as a database for finding primary and secondary journal articles that are relevant to your grant proposal and scientific paper. You will use your search results from Medline to contribute to a Biol 212 database of references that you can utilize to assist you in the preparation of your grant proposal and final paper on Nup1p/Mex67p interactions. Before leaving lab, each of you will submit at least 3 sources to the on-line class database, as described below:

1. Open the Colgate University Libraries page at http://exlibris.colgate.edu.

2. From the "Find Articles" pulldown menu, select "Databases A – Z."

3. Choose "M" and then select Medline (EBSCOhost) as the database to search. In the next window, click on 'Connect to Medline.'

4. Search within Medline using the "Find" feature and keywords that seem relevant to the topic you'll be addressing in your grant proposal. Think carefully about which keywords to use based on the literature you have read and the discussion we have had in this lab. Feel free to use the pulldown menus located to the right of each search entry to limit the breadth of your search to those terms found only in the titles or abstracts of articles in the Medline database.

5. From the list of articles you generate in your search, select three to save for inclusion in the "Nuclear Export Database." Select these simply by clicking on the "Add" folder to the immediate right of each entry you select.

6. To transfer your selected references to the Nuclear Export Database, scroll back up to the top of the page and select "View Folder." A list of the articles you have selected should appear. Click on the "Save to Disk" icon at the top of your list.

7. Now select "Bibliographic Manager" and then choose "Direct export to RefWorks" from the choices on the right side of the screen. (Also select the "remove items from folder after saving" option to empty your current folder.) Once you click "save," a new RefWorks window should open.

8. Login to RefWorks by using *XXXXXX* as your login name and *YYYYYYY* as your password.

9. From the menu at the top of the RefWorks page, select "View" and choose "All References" to open the Nuclear Export Database and to download your selected references in that database. The entries from the entire class will be retained in this database for the rest of the semester.

10. Once in RefWorks, you can use the Nuclear Export Database to find full-length articles. Click on the "Article Linker" icon to the right of the database entry to obtain information on the location of the article. Information on articles located in the Science Library is found in the exlibris link. Those papers that have an "article" link highlighted next to the entry may also be available in full text online. **Be careful not to limit yourself only to those papers you can access online. Some very important papers will be available only in the journal stacks in the Science Library!

Please refer back to this RefWorks database frequently over the next few weeks as a resource for identifying articles that will provide useful background information as you prepare your grant proposal. And don't hesitate to add additional articles to the database that will be helpful to your classmates as they prepare their proposals!

A few hints on reading journal articles:

1. Read the title and abstract of the article to obtain an overview and to familiarize yourself with the subject of the paper.

2. Next, read the introduction, which provides background information on the subject of the paper and often explains why the question the researchers are asking is important. The introduction often is written so that the first paragraph is very general, and each succeeding paragraph becomes more specific to the question addressed in the paper. Read the intro section carefully and be sure to understand every point the authors make before moving on to read the results.

3. Next, read the results section. I find that the best way to approach this section is to analyze it figure-by-figure. The figures provide the original data upon which the authors base their conclusions, and the text in the results section serves to describe the data presented in the figures. This section is usually divided into smaller sections separated by subheadings, then written such that the authors begin each subsection with a sentence explaining the question, followed by a sentence or two describing the experiment, and concluding with a sentence or two stating the results. When reading the results section, you should note each question asked, the experiment performed, and the result obtained. Each experiment will correspond to a figure, and additional information on the experiment can be found in the figure legend. The figures should be scrutinized before, during, and after you read the results so that you can analyze the data critically and decide if you agree with the author's interpretation of the data.

4. Finally, the discussion section should provide a brief overview of the results and then discuss the importance of the findings, integrating all of the data reported in the paper with information published previously in other papers. Here is where the authors interpret their data in the context of what others before them have discovered, and it is here that they often provide models indicating what their discovery means within their field of science. Examine their interpretations and models carefully and critically. Do you agree with the authors? Is their model consistent with their results? Are there any additional experiments they should perform to confirm their model?

5. You'll notice that I didn't mention the materials and methods section. This is because authors almost always briefly mention their methods in the results section when describing each experiment. The materials and methods provide much more detail than generally desired when initially reading a paper, and so do not fit well with the rest of the text. Refer to this section to clarify questions about a method used or to learn about specific techniques, but don't dwell on this section when attempting to understand the general concepts or outcomes presented in the paper.

6. When you eventually write your own papers, you will also include a Literature Cited (or References) section. When you are reading someone else's paper, this section provides important sources for additional information on the topic about which you are reading. When you write your own primary journal article later in the semester, remember to use the Literature Cited section of the papers you are reading to find additional sources of information for your paper.

Reading primary journal articles will be time-consuming at first, but will become easier by following these guidelines, remembering to note each important point the authors make as it is encountered, and mentally integrating all of the information after completing the article.

Glossary: Gorlich et al. (1994), Isolation of a protein that is essential for the first step of nuclear protein import. Cell 79: 767-778.

Bipartite Signal: An NLS having two separate parts, both of which are required for import into the nucleus.

BSA-NLS Conjugate: Bovine Serum Albumin protein with nuclear localization signals (NLSs) attached. Any protein containing a NLS will be targeted to the nucleus in a properly functioning cell. In this paper, this NLS-BSA conjugate is attached to a fluorescent molecule (fluorescein) and used in permeabilized cells to assay for conditions under which protein import into the nucleus occurs.

cDNA cloning: Isolation of a DNA sequence that is complementary to a specific mRNA. Since mRNA is translated into protein, the DNA sequence of a cDNA is basically the DNA sequence of the region of a single expressed gene that encodes a protein.

Digitonin: A detergent that selectively permeabilizes the plasma membrane, but does not disrupt intracellular membranes, including the nuclear envelope.

Energy-regenerating system: Permeabilized cells no longer produce much ATP, and nucleotides are essential for some activities related to nuclear protein import. So the nuclear import assay is performed with molecules (the "energy regenerating system") added that maintain a high concentration of nucleotides in the solution.

Karyophilic: Directed to the nucleus ("nucleus loving").

NLS: nuclear localization signal (or sequence). A region of positively charged amino acids within a polypeptide that is required for that polypeptide to be translocated into the nucleus.

Nucleoplasmin: A protein normally found in the nucleus. In this paper the authors covalently bound a fluorescent molecule (fluorescein) to nucleoplasmin, then used a microscope to determine what conditions stimulated nucleoplasmin to enter the nucleus.

Nucleoplasmin Core: A fragment of nucleoplasmin protein that does not contain an NLS.

Permeabilized Cells: Cells incubated with the mild detergent digitonin. Digitonin permeabilizes the plasma membrane of living cells, but does not alter the nuclear envelope.

Q-Sepharose, IMAC, Superose 6, Mono-Q, and Sephadex columns: Columns of specialized beads that bind to some proteins, but not others, allowing the separation of proteins from one another by mass, charge, or some other chemical characteristic.

Ran/TC4: A small GTP-binding protein that hydrolyzes GTP in the process of transporting proteins across the nuclear pore complex.

Recombinant protein: Protein expressed from a gene that has been cloned (isolated) from one organism, then artificially transferred to another organism. Recombinant proteins are often expressed in bacteria (*E. coli*), even when the gene encoding them was cloned from eukaryotic cells.

BIOL 212 LAB
EXERCISE

Mex67/Nup1 Original Research Experiment

Over the next five weeks you will use the experiments you proposed in your grant proposal to examine how proteins interact to mediate mRNA export from the nucleus. Please think carefully about the experiments you described and how they will be used to determine if specific domains of the nucleoporin Nup1 and the mRNA export factor Mex67 physically interact *in vitro* and to identify which region of Nup1 is responsible for that interaction. Also keep in mind the information you gleaned from the primary literature examining Nup function and RNA transport. How does each method used over the next five weeks allow us to ask a new question about RNA transport? This packet provides a week-by-week description of the experiments and their background. **Arrive in lab prepared to ask questions about parts of the experiment that you do not understand!**

Week 1: Yeast Fractionation

Several weeks ago we discussed the Gorlich et al. (1994) article in which the authors used cellular fractionation techniques to purify a protein from a sample of cytosol. In this lab you will follow a simpler fractionation protocol designed to separate yeast proteins and soluble molecules from cell walls, intact cells, and other insoluble material. The fractions prepared in this week's lab will be used in subsequent weeks for your original experiment examining the interactions between proteins involved in nuclear transport. Please follow the directions carefully and KEEP THE CELLS COLD to prevent proteins from denaturing and degrading.

The yeast cells that you will be using this week have been genetically engineered to allow us to more easily ask the questions we would like answered: 1) Does Nup1 physically interact with Mex67 as a step in mediating mRNA export from the nucleus? 2) Is a specific region of Nup1 responsible for associating with Mex67? These yeast cells have been altered so that the *MEX67* gene, instead of encoding a normal Mex67 protein, encodes Mex67 fused to a polypeptide called GFP (which stands for Green Fluorescent Protein). The recombinant Mex67-GFP protein can be more easily detected biochemically than can wild-type Mex67, so we will use these yeast extracts containing the recombinant Mex67-GFP protein to assay for a Nup1/Mex67 interaction.

Week 1 Protocol:

This week you will isolate soluble proteins from yeast cells, using the protocol described below. Since the entire fractionation procedure takes longer than three hours, including several long incubations to allow the cells to grow to an appropriate abundance, the first several steps of the protocol will be completed prior to your arrival. These steps simply involve growing the yeast cells in large cultures, pelleting the yeast out of the growth medium by centrifugation, washing the cells with an osmotic and pH buffering solution, and freezing the washed cells in eppendorf tubes.

You will complete the fractionation procedure by lysing the cells and separating the soluble proteins from the insoluble material using the protocol below. Remember, keep the cells as cold as possible!

1. Each pair of students should thaw one yeast cell pellet on ice. Vortex for a few seconds to loosen pellet.

2. Resuspend each pellet in 400 ul Sorbitol Lysis Buffer by pipetting gently up and down. Sorbitol lysis buffer maintains physiological pH and ion concentrations in the extracts and also contains synthetic protease inhibitors to prevent degradation of proteins in your extracts.
3. Add 300 ul glass beads (425-600um in diameter) using the measuring device provided at each bench.
4. Vortex cells 1 min., then place on ice 1 min. Repeat until your tube has been vortexed five times. During this vortexing, the collisions between the glass beads and the yeast cells lyse the cells, releasing their contents.
5. Using a p1000, transfer the liquid in the tube to a SECOND eppendorf centrifuge tube.
6. Add another 400ul Sorbitol Lysis Buffer to the FIRST tube to rinse remaining cell extract off of the beads.
7. Vortex the FIRST tube 1 time for 5 seconds to mix.
8. Again, transfer the supernatant from the FIRST tube to the SECOND tube. Discard the first tube (containing the glass beads).
9. Add 80ul 10X Lysis buffer to the solution in the SECOND eppendorf tube. The 10X lysis buffer contains two mild detergents that help to degrade the yeast membranes while still maintaining the extracted proteins in roughly their native conformation.
10. Add 8ul 10% SDS to the SECOND eppendorf tube. SDS is an additional detergent used to solubilize membranes and complete lysis of the cells. Invert tube to mix solution.
11. Incubate tube on ice 15 minutes. Invert again to mix. Keep the tube on ice until the moment you begin the centrifugation in step 12.
12. Spin tube in microcentrifuge in cold room for 10 minutes to pellet the cellular debris and any beads that may have been transferred. The supernatant contains soluble proteins (including Mex67-GFP) and other soluble cellular material.
13. Using a p1000 transfer your supernatant to a clean Eppi tube, being careful not to disrupt the pellet. Avoid pipetting any of the insoluble material found in the pellet. (It is more important to avoid transferring material from the pellet than it its to salvage all of the supernatant!)
14. Label tubes containing supernatant and place this soluble yeast protein fraction in rack in the –20°C freezer for use in the affinity chromatography experiments to be performed over the next several weeks. Discard the pellet in the waste bag on your benchtop.

Think carefully about the following two questions:
1) What is present in the supernatant fraction you have saved?
2) How will this fraction be used in subsequent weeks to test for an interaction between Mex67p and Nup1p?

Week 2: Induction, Extraction, and Affinity Purification of Recombinant Nup1 Proteins

This week we will continue our preparation of samples to be used to test for an interaction between the yeast "nucleoporin" Nup1 and the mRNA transport factor Mex67 in order to investigate whether such an interaction potentially occurs during the translocation of mRNA across the nuclear pore complex. We have already prepared yeast protein samples containing Mex67-GFP. This week we will prepare recombinant Nup1 protein samples to be used for affinity chromatography.

As we discussed briefly when introducing our original experiment, the nucleoporin we will be working with is encoded by the yeast gene *NUP1*. However, the Nup1 that we will be using in our experiments will actually be expressed in *E. coli* bacterial cells. Why express a yeast gene in bacteria in order to test for an interaction between yeast proteins? The answer is simply because recombinant proteins can be more easily expressed in and extracted from bacteria than from yeast. In order to express a eukaryotic protein in bacteria, the gene encoding that protein must be inserted into a bacterial plasmid, and that plasmid DNA must be placed into bacterial cells. The bacterial cells can then be induced to transcribe mRNA from the newly introduced plasmid DNA and translate protein from the RNA message, thus producing a eukaryotic protein in a bacterial cell!

In this lab we will be generating soluble protein fractions from bacterial cells that express a fragment of Nup1. These fractions will contain a high concentration of bacterial proteins, as well as the recombinant Nup1 we will use for our affinity chromatography experiment. We have engineered the experiment so that only a specific domain of Nup1 will be expressed in each bacterial culture because we would like to investigate if a specific domain of Nup1 associates with Mex67. In addition, large proteins are often not well tolerated by bacteria and may be quickly degraded by bacterial proteases. The expression of short regions of Nup1 makes it more likely that the bacteria will not degrade these fragments. (See PROTOCOL for a brief description of the Nup1 domains we will use.)

After separating the soluble protein (which will contain the Nup1 fragment) from the insoluble bacterial material, we will take some of the resulting sample and determine whether it actually contains the recombinant Nup1. This determination will be done by purifying Nup1 from the remainder of the bacterial protein extract, running the purified protein on a polyacrylamide gel, and staining with Coomassie stain to observe the protein.

The actual purification of Nup1 away from the other bacterial proteins will be done by affinity chromatography. The Nup1 that we are expressing in bacterial cells has been genetically engineered so that it contains not only polypeptide sequence from Nup1, but also amino acid sequence from the enzyme glutathione-S-transferase (GST). The resulting recombinant "fusion protein" has both Nup1 and GST domains, and will be referred to as GST-Nup1. The reason for expressing Nup as a fusion with GST is that GST has a very high affinity for another molecule called glutathione. As a result, a GST-Nup1 fusion protein can be easily purified using beads (made of a gel-like substance called sepharose) that are covalently bound to glutathione.

The genes encoding these GST-Nup fusion proteins were generated by taking a region of DNA encoding a particular domain of Nup1 (see Week 2 protocol for a description of these domains) and inserting it into a 'plasmid' DNA molecule (Figure 1). A plasmid is a small, circular piece of DNA that can be maintained in an organism in much the same way as a chromosome. The plasmid used for this exercise can be maintained in *E. coli* bacteria and replicated and inherited similarly to a bacterial chromosome. Importantly, this plasmid also contains the gene encoding GST. We have modified this plasmid by inserting DNA encoding specific regions of Nup1 into the plasmid DNA immediately

adjacent to the GST gene. We have designed these Nup1 DNA fragments so that when inserted into the plasmid, a single long gene that encodes a polypeptide including amino acids from both GST and Nup1 is generated. Thus, when this gene is expressed (transcribed and translated) in bacteria, a GST-Nup1 fusion protein is produced in the bacterial cells.

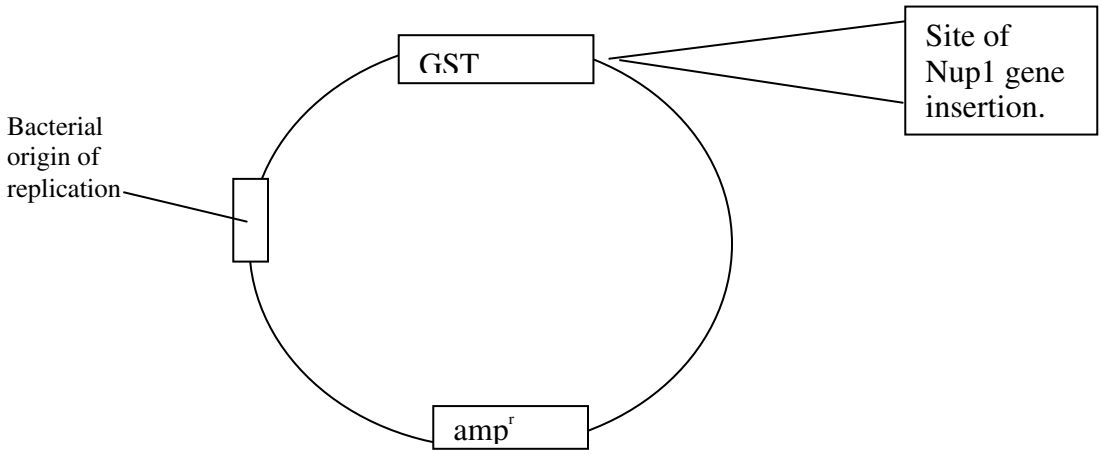

Figure 1. Map of GST-containing plasmid DNA. Depicted on the circular plasmid are the location of the ampicillin resistance gene, bacterial origin of replication, and DNA sequence encoding the GST fusion protein. DNA regions encoding the Nup1 domains used in this lab exercise were inserted into the plasmid immediately adjacent to the GST gene.

We will express in *E. coli* the GST-Nup1 fusion proteins encoded by each plasmid and will isolate "total soluble protein" fractions from the bacteria expressing these fusions. We will use glutathione-sepharose beads to affinity-purify GST-Nup from our bacterial protein fractions. The affinity-purified protein will be run on a polyacrylamide gel and stained with Coomassie stain in order to estimate the quantity and purity of nucleoporin protein that was purified from the bacterial cells.

<u>Week 2 protocol:</u>

We will be using six different fragments of Nup1 (each fused to GST) to explore the association of Nup1 with Mex67. These fragments (and their molecular weights) are:

Nup1-Rep$^{432\text{-}816}$ (80 kD) = Nup1 residues 432-816 containing most of the "FXFG-repeats" of Nup1.

Nup1-C$^{778\text{-}1076}$ (60 kD) = amino acids 778-1076 including the entire carboxyl-terminus of Nup1. Contains some FXFG and FG repeats.

Nup1-C$^{778\text{-}999}$ (51 kD) = amino acids 778 – 999, including only the FXFG repeats closest to the C-terminal end of Nup1.

Nup1-C$^{1002\text{-}1076}$ (35 kD) = amino acids 1002 – 1076, including some FG repeats and some sequence lacking FGs, but lacking any FXFG repeats.

Nup1-C$^{778\text{-}1041}$ (56 kD) = amino acids 778 – 1041, comprised entirely of FXFG and FG repeats.

Nup1-C$^{1041\text{-}1076}$ (32 kD) = amino acids 1041 – 1076 completely lacking any FXFG or FG repeats.

See figure 2 for a cartoon depicting the regions of Nup1 we will be testing for interaction with Mex67.

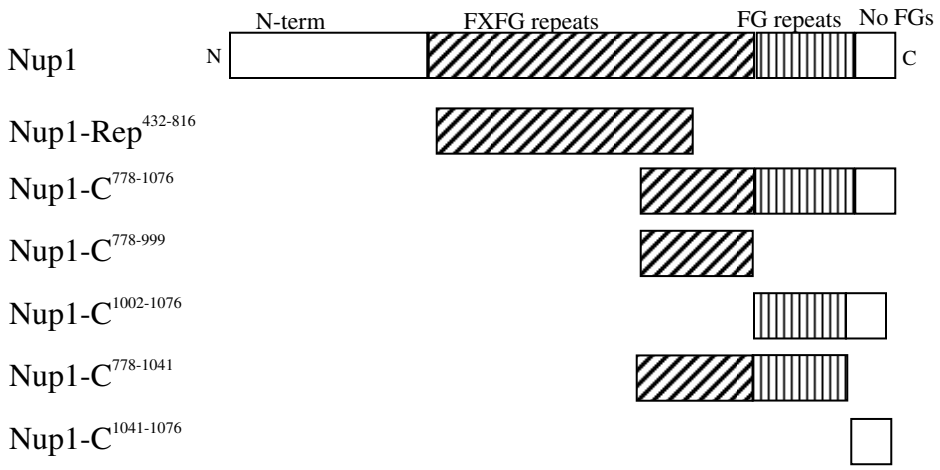

Figure 2. Representation of the regions of Nup1 present in each GST fusion protein. The top panel (Nup1) depicts the domain structure of the entire Nup1 protein. Each of the subsequent panels shows the region of the protein included in the GST fusion protein with that name.

In addition, several groups will generate a sample containing GST (26 kD) alone (not attached to a Nup1 fragment) to use as a control in the remainder of the original exercise. Why is "GST alone" an important control in this experiment?

The bacterial cells found in the ice buckets on your benchtop were prepared by being grown in a 1 L liquid culture, induced to express the GST-Nup protein by adding IPTG, incubated for 2.5 hours after induction, then pelleted using a centrifuge. The resulting samples were divided into four different tubes for purification by different lab groups. Each bench should have four samples in ice on the benchtop. Each student pair should select two samples on which to perform the fractionation.

Using the procedure below, you will purify these recombinant proteins from the pelleted bacterial cells.
**Keep cells cold (on ice) at all times to keep proteins from being degraded.

Procedure:

1. Resuspend bacterial cell pellet in 500ul "TSE + PIs" buffer by gently pipetting up and down. (TSE contains Tris-buffer that maintains neutral pH, sucrose to maintain an osmolarity similar to that inside bacterial cells, and EDTA to bind cations that acts as cofactors for some proteases. The "PI"" are small peptides that act as protease inhibitors.)
2. Transfer resuspended cells to round-bottomed centrifuge tube. Add another 2 ml of TSE + PIs to this cell suspension.
3. Add 75ul lysozyme solution. Swirl gently to mix. (The lysozyme digests the bacterial cell wall so we can lyse the bacterial cells.)
4. Incubate 5 minutes at room temperature.
5. Add: 35ul $MgCl_2$
   3.5ul $MnCl_2$
   35ul DNase I
   (DNase I digests DNA and Mn^{++} and Mg^{++} are cofactors for this enzyme.)
6. Incubate 15 minutes at 37°C.
7. Add: 425ul PBS solution

200ul 20% Triton solution
200ul 20% Tween solution
45ul 1M DTT

(PBS provides a pH buffer and optimum salt concentration to prevent proteins from denaturing. Triton and Tween are detergents that solubilize the bacterial cell membranes and DTT is a reducing agent that breaks disulfide bonds in the bacterial cell wall. They work together to complete the breaking open of the bacterial cells.)

8. Mix well by inverting tube several times. Place on ice.
9. Pellet cell debris by spinning samples 12,500 rpm for 20 minutes at 4°C. Be sure to balance your tubes by adding an appropriate amount of TSE to equalize their mass before placing them in the centrifuge rotor.
10. While the tubes are spinning, label four microfuge tubes per sample with your group name and the plasmid you are purifying. After labeling, place the tubes on ice.
11. After centrifugation, transfer 75ul of supernatant to one labeled microfuge tube and save on ice. Transfer remaining supernatant to additional microfuge tubes in 1 ml aliquots and freeze samples immediately.

The 75ul deposited in the first microfuge tube will be used to determine how much GST-Nup fusion protein has been isolated from your bacterial prep.

<u>Purification of GST-Nups using glutathione-sepharose beads:</u>

Thus far, you have isolated all soluble proteins from a sample of bacterial cells and have saved a portion of this soluble fraction on ice on your benchtop. In this part of the procedure, you will separate the GST fusion proteins from the remainder of the soluble bacterial proteins by taking advantage of the affinity of GST for glutathione. In other words, you will affinity-purify the GST-Nups using sepharose beads bound to glutathione. Finally, you will analyze the precipitated proteins (which should consist primarily of GST-Nup1) using polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie staining.

12. Add 500ul ELB buffer to one clean (new) microfuge tube per GST-Nup1 extract. (Thus, two microfuge tubes per group.)
13. Mix glutathione-sepharose beads well by inverting bottle several times, then add 30ul beads to the ELB in each eppi tube using a yellow pipetman tip with the end of the tip snipped off. Invert several times to mix.
14. Pellet beads in microcentrifuge by spinning for 5 seconds.
15. Carefully remove most of the buffer from the beads, leaving a small amount of buffer behind so that the pellet of beads remains intact. DO NOT SUCK THE BEADS INTO YOUR PIPET TIP!
16. Add 500ul cold ELB + PIs to the tube containing the beads.
17. Add 50ul GST-Nup (or GST) total extract to the eppendorf tube containing the beads + ELB. Save the remaining extract on ice.
18. Incubate the GST-Nup+beads+ELB mixture for 30 minutes in the cold room, mixing constantly on the rotator. During this incubation you will also make several “drawn pipettes” for later use in separating your GST-sepharose beads (and associated proteins) from the soluble material in your sample (see protocol step 23).
19. After the 30 minute incubation, pellet the beads in microcentrifuge by spinning for 5 seconds.
20. Carefully remove most of the buffer <u>without disturbing the beads</u>.
21. Add another 500ul cold ELB to the tube. Invert several times to wash.
22. Repeat spin in microcentrifuge and removal of buffer.

23. Add another 500ul ELB to the tubes. Spin again for 5 seconds.
24. Carefully remove most of the buffer with a pipetteman. Then remove the remainder of the buffer with a thinly drawn glass pipette. The pipette tip should be so thin that the beads cannot enter the pipette.
25. Add 25ul ELB and 25ul Laemmli sample buffer to the precipitated GST-Nup1+beads samples. Also add 25 ul Laemmli sample buffer to 25ul of your "total extract" samples saved after step 17.
26. Heat the samples to at least 70°C for 5 minutes using the heat block on your benchtop.
27. Load 5ul of Mol Wt Standard in lane 1 of your 10% polyacrylamide SDS-PAGE gel. Be certain to carefully keep track of which sample you loaded in each lane.
28. Load 10ul of each sample per lane in the remaining lanes of your gel. (After loading, clearly label the tubes containing the remaining sample with your name and tube contents. Save in -20°C freezer for use in subsequent labs.)
29. Run the samples on SDS-PAGE and stain with Coomassie stain to observe total protein.

Your gels will be fixed, stained, and destained after you leave lab. A photograph of each gel will be available for you to pick up the day following completion of the lab. Examine your photo carefully, comparing the affinity purified vs. total extract lanes. Use the Molecular Weight Standards in lane 1 to determine the size(s) of the protein(s) in your samples. Before next week's lab, think carefully about the following questions:

1. Did you get expression of your GST-Nup?
2. Was your recombinant Nup1 protein purified by incubation with the glutathione-sepharose beads? (Remember that Coomassie stain binds to <u>all</u> abundant proteins in a gel. Thus, Coomassie staining should reveal a strong band of protein at the molecular weight of the GST-Nup1 protein in the fractions you affinity purified.)
3. What molecular weights are the most abundant proteins in each lane on your SDS-PAGE gel?
4. Is this the size protein you expected in these lanes?

We will use the information from your stained gel to determine how much of your extract we should use in our experiment to test if GST-Nup1 associates with Mex67 *in vitro*.

Week 3: Affinity Chromatography to Identify Nup1/Mex67 Interactions:

This week we continue our experiment to determine if the yeast nucleoporin Nup1 can associate with the mRNA transport factor Mex67. Over the past two weeks, we prepared yeast protein fractions containing recombinant Mex67 fused to GFP and *E. coli* protein fractions containing recombinant GST-Nup1. In today's lab exercise you will incubate the Mex67-GFP-containing yeast extracts with the Nup1-containing bacterial extracts to determine if specific regions of Nup1 can associate with Mex67.

In actuality, the whole bacterial extract containing the GST-Nups will never be mixed with the yeast extract containing Mex67-GFP. Instead, the bacterial extract will first be incubated with glutathione-sepharose beads, allowing the GST found in the GST-Nup fusions to associate with the beads via glutathione. Once the GST-Nups are bound to the beads and the unbound proteins are washed away, the yeast fractions containing Mex67-GFP will be added to the beads. If the Mex67 has an affinity for Nup1, the Mex67-GFP from the yeast extract will associate with the GST-Nups already bound to the beads. With GST-Nup as an intermediary, the Mex67-GFP will also be associated with the beads. This bound Mex67-GFP will remain in the pellet formed when the beads are spun to the bottom of the tube. Using this technique, those yeast proteins associating with Nup1 (some of which we hypothesize are Mex67) will be affinity-purified from a solution containing many different proteins (such as our yeast protein extract isolated in Week 1 of this exercise).

We are actually not certain which of our recombinant GST-Nup1 proteins will precipitate Mex67 from our yeast cell extracts. In order to determine if Mex67 (fused to GFP) has been precipitated by a particular GST-Nup fusion, we will perform a "Western blot"; the precipitated proteins will be run on SDS-PAGE and transferred to nitrocellulose, then detected using anti-GFP antibodies. (See next week's lab description for background information and the Western blotting protocol.) If a band appears on the Western blot at the molecular weight of Mex67-GFP, then we know that Mex67-GFP was affinity purified from the yeast protein fraction by binding to the GST-Nup, indicating that Mex67 can physically associate with Nup1 *in vitro*!

This week we will perform the precipitation portion of the experiment. The Western blotting and analysis of your data will be performed during the last two weeks of this exercise.

Week 3 Protocol:
In the first part of this procedure, you will be incubating your GST-Nup-containing extracts with glutathione-sepharose beads as you did in the latter half of last week's experiment. Once the GST-Nups are bound to the beads, you will then add the yeast protein extracts containing Mex67-GFP (and all other soluble yeast proteins), and will allow the Mex67 (and other proteins) to associate (or not associate) with the Nup1 fusions on the beads. The beads will then be washed thoroughly to rinse away all unbound proteins, leaving only those proteins associated tightly with GST-Nup1. The proteins that remain associated with the beads will be resuspended in sample buffer and frozen to later be loaded on an SDS-PAGE gel in preparation for Western blotting.

Procedure:

1. Last week you placed several tubes containing 1 ml of GST-Nup1 extract in the freezer. As soon as you come into lab this week, take one tube of each extract and place it in an ice bucket on your benchtop. Set this sample aside until step 7.
2. Get out one clean microfuge tube for each of the GST extracts you generated last week. (Most student pairs will thus be adding ELB to two tubes.) Place 500 ul ELB in each of the clean tubes. Label your tubes to indicate which GST-Nup1 extract you will add to each.
3. Using a yellow tip with the tip snipped off, add 60ul glutathione-sepharose beads to the ELB in each of the tubes. Invert several times to mix.
4. Pellet beads in microcentrifuge by spinning for 5 seconds.
5. Carefully remove most of the buffer from the beads, leaving a small amount of buffer behind so that the pellet of beads remains intact.
6. Add 500ul of ELB + PIs to the tubes containing the beads.
7. Add 200ul GST-Nup extract to the eppendorf tubes containing the beads + ELB. Use the GST-Nup extracts (either Rep, C, a C-terminal region, or GST) you generated last week as the source of GST-Nup1.
8. Incubate the tubes on the roller in the cold room for 30 minutes. During this incubation, remove your yeast extracts (from Week 1 of this exercise) from the freezer, thaw them on ice, spin them for 1 minute in the microcentrifuge in the cold room, and then gently place them on ice until they are used in step 14.
9. At the end of the 30 min incubation, pellet the beads in a microcentrifuge by spinning for 5 seconds.
10. Carefully remove most of the buffer without disturbing the beads.
11. Add another 500ul ELB to the tubes, invert to mix well, pellet beads, and remove buffer.
12. Repeat step 10 one more time to thoroughly rinse away unbound bacterial proteins.
13. Add 500ul ELB + PIs to the beads.
14. Add 200ul yeast extract (thawed and centrifuged in Step 8) to each tube, being careful to avoid any pellet that may have formed in the bottom of the tube during the centrifugation you performed in Step 7.
15. Incubate tubes for 1 hr on the roller in the cold room. During this incubation, prepare two drawn pipettes for use in step 19.
16. After the 1 hr incubation, pellet the beads in microcentrifuge and carefully remove most of the buffer.
17. Add 500ul ELB Wash Buffer, invert to mix, pellet beads in microcentrifuge, and carefully remove buffer, making certain not to remove the pellet of beads.
18. Repeat step 16 four more times, being very careful each time not to remove the pellet of beads when removing the buffer.
19. After the fifth wash with ELB Wash Buffer, use a drawn pipette to carefully aspirate all of the buffer off of the beads.
20. Add 25ul ELB Wash Buffer and 25ul Laemmli buffer to each of your samples. Label your tubes clearly and store in the freezer until the next lab period.

Questions:

1) What do you predict is in each of the samples you have prepared? Why?
2) How will you detect the presence (or absence) of these proteins in your samples?
3) Could there be more than one protein associated with Nup1 in your sample? How?

Weeks 4 and 5: Western blotting to detect Mex67 precipitated by Nup1.

Western blotting utilizes antibodies that recognize a specific protein to determine if that particular protein is present in a sample that may contain thousands of different proteins. In this lab, we will be performing Western blots to determine if Mex67-GFP is present in the yeast protein fraction you affinity purified last week using recombinant GST-Nup1. If Mex67 bound to Nup1 during your affinity purification, probing your extracts with antibodies that bind to Mex67-GFP and that can be detected colorimetrically should result in the appearance of a distinct "band" on your Western blot. For this lab we will use two different antibodies to probe our GST-Nup1 precipitated samples. One pair at each bench will probe with an anti-GFP Ab to detect the Mex67-GFP fusion. The other pair will probe their blots with anti-Srp1 Ab as a positive control for a protein that should be precipitated by specific GST-Nup1 fusions.

Performing a Western Blot:
The principle behind Western blotting is quite simple. Basically, if you have an antibody that binds to a specific protein, you can use that antibody to see where the protein is. In the case of Western blotting, you can use that antibody to identify the location of a specific protein on a piece of filter paper that contains polypeptides separated using SDS-PAGE.

The main steps in the Western blotting procedure are:
Protein separation: Once samples containing proteins have been obtained (in our case by fractionation of yeast cells and affinity purification of the fraction using GST-Nup1), it is important to separate the proteins by size. This is particularly true if you are probing for a protein with a known molecular weight. We will separate our proteins by size using SDS-PAGE, exactly as performed on our GST-Nup fractions several weeks ago.

Transfer to nitrocellulose: After the proteins are separated by size on a polyacrylamide gel, they must be transferred to a material that can be handled easily. The material we will use is nitrocellulose filter paper. Nitrocellulose is quite durable and adheres tightly to proteins (and nucleic acids – the same paper can be used for Northern and Southern blots in which probing is done for specific RNA and DNA sequences). Because of the stability and durability of the filter and the inability of molecules to move once adhered to the filter, nitrocellulose provides an excellent matrix to which to transfer proteins during Western blotting.

The electrophoretic transfer of proteins from polyacrylamide to nitrocellulose takes advantage of the fact that the proteins in the SDS-PAGE gel are still associated with SDS, and thus still carry a negative charge. The polyacrylamide gel will be laid on top of the nitrocellulose, and a current will be applied so that the proteins migrate toward a positive pole, out of the polyacrylamide and into the nitrocellulose. Because nitrocellulose binds proteins tightly, very few proteins pass through the filter. When the transfer is done, most of the proteins have left the gel and are bound to the filter. The proteins on this filter are stable for several months at 4^{o}C.

Probing the blot with antibodies: Once the proteins have been transferred to the nitrocellulose, the filter is often called a "blot". We can now use antibodies to detect specific proteins on the blot. Since proteins associate tightly with nitrocellulose and antibodies are proteins, the blot must be "blocked" prior to probing with antibody. The blocking solution usually contains either nonfat dry milk (NFDM) or bovine serum albumin (BSA), which coats the nitrocellulose with protein so that the antibodies – which will be added next – do not bind directly to the filter.

After the blot has been blocked, it is incubated in a solution containing the primary antibody. This antibody should bind only to the protein against which it was raised. (In our case, this will be either GFP or Srp1.) That protein will most often be present in only a single band on the blot, corresponding to the distance the protein migrated on the polyacrylamide gel. After incubation in primary antibody, the blot is rinsed to wash away any unbound antibody.

Once the primary antibody is rinsed away, the blot is incubated in a secondary antibody. The secondary antibody is a protein that binds to the primary antibody with very high affinity. Thus, wherever the primary antibody is present on your blot (which should correspond to where your protein of interest is located on the blot), the secondary antibody will bind. Importantly, the secondary antibody is covalently bound to another molecule (in our case, an enzyme), which allows the detection of the location of the secondary antibody on the blot (see below).

Detecting bound antibodies: As mentioned above, the secondary antibodies we will use in our Western blotting experiment have been conjugated to enzymes - either horseradish peroxidase (HRP) or alkaline phosphatase (AP) - to facilitate their detection. These enzymes catalyze chemical reactions in certain substrates that can be detected by observing a change in the color of the substrate. Thus, wherever there is appropriate enzymatic activity on the blot (i.e. wherever 2^{o} Ab is bound) a purple/brown colored band will appear upon incubation with substrate. Using this color change as a marker, the location of bound antibodies (and thus the Mex67-GFP) on the blot should be apparent.

Week 4 Protocol:
Weeks 4 and 5 of this exercise complete your original experiment designed to test whether specific regions of the Nup1 protein physically interact with yeast Mex67 *in vitro*. You have already performed the affinity chromatography part of the experiment in which you precipitated proteins that bind to Nup1 by incubating GST-Nup1-bound beads with a yeast protein extract. You will now complete the experiment by performing a Western blot to determine if Mex67 is among those proteins that associate with Nup1.

Over the next two weeks, the samples you have generated will be used to perform two Western blots. One blot will be probed with anti-GFP in an effort to detect the Mex67-GFP fusion that is expressed in your yeast extract. The presence of a band on this blot indicates the presence of Mex67-GFP protein in that lane. The second blot will be probed with anti-Srp1 antibody to detect the presence of Srp1 protein (yeast importin-α). Srp1 has been shown previously to bind to Nup1-C regions (seemingly to a site between amino acids 1041 and 1076), so the anti-Srp1 blot will serve as a positive control for the precipitation of any proteins by the GST-Nup1 beads.

Procedure:
1. Heat the two samples you prepared in the last lab, your "purified" GST-Nup1 samples from week 2, the "Mex67 yeast" sample you generated in week 1 (mix 15ul of yeast extract with 15ul Laemmli buffer to make a sample you can load), and a "WT yeast" sample supplied on your benchtop to 95^{o}C for 3 min. Remove the tubes from the heat block carefully so that the lids do not pop open. Expansion of the air in the microfuge tube can cause the lid to snap open and some of the sample to spill out.
2. After heating, spin the sample briefly in a microfuge and place on ice.
3. Working with the other pair of students at your bench, load your samples onto two polyacrylamide gels. Load 5ul of Mol Wt Standard in lane 1 of each gel. Then load 10ul of each of your GST-Nup1

samples in the order indicated below. Finally, load 10ul of Mex67-GFP yeast extract and 10ul of non-recombinant (WT) yeast extract Kathleen has placed on your benchtop in lanes 10 and 11.

Lane 1: Molecular Wt Standard
2. Nup1-Rep$^{432\text{-}816}$ + yeast*
3. Nup1-Rep$^{432\text{-}816}$ alone#
4. Nup1-C$^{778\text{-}1076}$ + yeast
5. Nup1-C$^{778\text{-}1076}$ alone
6. Nup1-C^{truncation} + yeast
7. Nup1-C^{truncation} alone
8. GST + yeast
9. GST alone
10. Yeast extract containing Mex67-GFP
11. Yeast extract lacking GFP (WT)

*The "+yeast" indicates that this sample contains GST-Nup bound to beads and incubated with yeast protein extract (week 3).
#The "alone" indicates that this sample contains only GST-Nup bound to beads (week 2).

4. Run the gels approximately 45 minutes at 200V to separate proteins by size.

Next, transfer the proteins from your SDS-PAGE gel to nitrocellulose filter paper by carefully following the steps outlined below. Wear gloves for all subsequent steps, as the protein on your fingers may contaminate your blot and residual polyacrylamide may be present.
5. While the gels are running, cut two pieces of thick blotting paper (or six pieces of thin Whatman filter paper) and one piece of nitrocellulose to the size of your polyacrylamide gel (~6cm x 9cm).
6. Soak the blotting paper and nitrocellulose filter paper in a clean tray of "Western transfer buffer" until saturated. (Use a pencil to label your piece of nitrocellulose with your group's initials in a corner of the filter paper.)
7. Once the SDS-PAGE is completed, carefully remove the gels from between the glass plates using a spatula.
8. Place each gel in a clean tray containing "Western transfer buffer."
9. On the BioRad semi-dry trans-blotter, lay one piece of thick blotting paper (or three pieces of thin paper), the nitrocellulose filter paper, a protein gel, and one more piece of thick (or three pieces of thin) blotting paper in that order. [*Note: Reverse the order of these layers if your bench has the black Fisher transblotter.]
10. Roll a glass pipette firmly across the resulting stack to force out any bubbles that may remain between the layers.
11. Pour just enough additional transfer buffer on the stack to keep it saturated. Using a Kimwipe, wipe up any excess buffer that accumulates on the electrode.
12. Place the lid on the semi-dry blotter.
13. Run the BioRad blotter at 20V for 1h and the Fisher blotter at 160 mA for 1 h to transfer protein from the gel to the nitrocellulose.
14. After shutting off the power supply, lift the lid off the semi-dry blotter. Then carefully lift the blotting paper and gel off of the nitrocellulose filter.
15. Place the nitrocellulose into a tray containing 10 mls of Ponceau stain. Incubate 5 minutes with rocking, then rinse briefly three times with dH_2O. Use a pencil to gently mark the location of your lanes and markers on the nitrocellulose filter.

16. Between the two pairs at each bench, decide who will probe their filter with anti-Srp1 and who will probe with anti-GFP. Then use the pencil to label one filter "anti-GFP" and the other "anti-Srp1." You will probe these two identical filters with two different antibodies – one that binds to GFP and another that binds to Srp1 – when completing your Western blot next week.
17. Dump the water out of your tray and add ~20 ml TBST+NFDM blocking solution. Snap the lid on your tray and place your blot on a shelf in the cold room.

Week 5: Completion of Western blotting and data analysis.
Upon arrival in lab, Kathleen will have added appropriate primary (1^{o}) antibodies (Ab) so that one blot will have been incubating in anti-GFP antibody and the other in anti-Srp1 antibody overnight. Make certain to note the label on your blot to recall which antibody your filter has been probed with.
1. Remove your blots from the primary antibody solution. (Do not discard this solution. It is expensive and can be reused, if necessary.) Place blot in a clean tray and immediately cover with TBST solution.
2. Wash unbound 1^{o}Ab from the blots by adding enough TBST to cover them completely, and rocking for 3 min. After 3 minutes, dump the TBST into the sink. Repeat this wash step two more times.
3. Transfer each blot into goat anti-mouse 2^{o}Ab in Ab buffer. Incubate 30 minutes at room temperature with rocking on the orbital shaker.
4. After incubation, remove the blot from the secondary antibody solution and wash three times with TBST as described in step 2.
5. Rinse the blot two additional times with TBS to rinse away any detergent remaining from the TBST.
6. Pour off the TBS, shake as much buffer as possible off the blot, and cover with enzyme substrate. Add just enough enzyme substrate (<10 ml) to cover the blot completely. Incubate blot in substrate until bands reach desired intensity. This can take from 1 – 30 minutes, depending upon the amount of protein present on the blot and how well the 1^{o} Ab recognizes the protein.
7. The reaction should be terminated when the band of the desired size reaches an easily detectable level of color, but the "non-specific" background bands are still very lightly colored. Stop the reaction by pouring off the substrate solution and washing the blot 3 times for 3 minutes with ddH_2O.
8. Once you have washed your blot, wrap it in Saran wrap. The color should be quite stable even when the filter dries, although it will not be as intense as when the blot was wet. We will photograph your blots to be used as figures in your Primary Journal Article.
9. Use the molecular weight standards to estimate the molecular weight of the protein(s) you detect with your antibody.

Take a careful look at your blot and ask yourself the following questions:
Does the most prominent band on your Western Blot correlate with the expected size of the protein that your antibody recognizes?
If not, is there a less prominent band that is of the expected size?
What might cause "non-specific" bands to appear on a Western Blot?

We will use the remainder of the period to discuss your results and observations. Think carefully about what your data (and the published data from other labs) suggests about Mex67-Nup1 interactions.

Summary

You have just completed a novel, unpublished experiment examining the *in vitro* interaction between two specific proteins. The goal of this experiment was to determine if Nup1 physically interacts with Mex67. As you examine your Western blots, think carefully about what pattern of bands you would see on your blot if Mex67-GFP does bind to a specific region of Nup1. Then compare what you would expect to see if binding between Mex67 and Nup1 occurred with what you actually observe on the blot. Did you detect binding between the two proteins in your experiment? Be certain to look carefully at your control lanes to make certain that the bands you observe are due to an interaction between GST-Nup1 and Mex67-GFP.

If you do detect binding, what implications might this have for our understanding of the mechanism by which mRNA is exported from the nucleus? What experiments do you propose should be performed next?

If you do not detect binding between Mex67-GFP and GST-Nup1, what reasons can you think of for an inability to detect an interaction? Think about both procedural and cell physiological explanations for the results obtained, paying particular attention to the results observed on the Srp1 control blots. What experiment(s) do you propose should be performed next?

Writing assignments:

Please remember that you have **two writing assignments** associated with this exercise due in the upcoming weeks.

1. "Draft" of your Results, Figures, and Materials & Methods sections.
2. Final Paper, including all sections typically found in a primary journal article.

Refer to your handouts and lab notes for information and due dates for each of these writing assignments.

Glossary of solutions used in Probing Western Blots:

Western transfer buffer: Similar to SDS-PAGE buffer, but with lower conductivity to prevent proteins from migrating through the nitrocellulose.

TBS: Tris-buffered saline. This is just a neutral pH salt solution used to gently wash nitrocellulose blots.

TBST: Tris-buffered saline + Tween20. Tween20 is a mild detergent that is added to the TBS solution in order to decrease the amount of non-specific interaction between the antibodies and proteins on the blot. It coats the blot with a uniform charge that is not well recognized by the antibodies.

TBST + NFDM: TBST + Nonfat Dry Milk. The NFDM is simply a readily available, inexpensive protein that can be obtained in a very pure form. NFDM is the blocking agent that coats the surface of the nitrocellulose so that the filter will not stick to antibodies present in subsequent solutions.

TMB: Substrate for horseradish peroxidase. The color resulting from the oxidation of TMB by horseradish peroxidase is a dark blue to purplish brown.

Primary Journal Article: Results and Methods from Mex67-Nup1 Experiment
Lab Report "Draft" (30 pts.)

As you wrote your Mex67-Nup1 grant proposal you gained experience applying the principles of scientific writing to the generation of a paper in which you described the research you planned to do in the future. The final lab report you generate using your Mex67-Nup1 data will also be written in a format used for the presentation of scientific findings, but now will focus on providing a description and analysis of the data you have collected. You will present these data in the format of a scientific journal article.

As you have read primary journal articles this semester, you have probably observed that the format for such a report is not identical from journal to journal. Although the exact format is not universal, the eight sections we discussed during our Journal Club lab earlier in the semester are found in nearly all primary journal articles. These sections include:

1. Title
2. Abstract
3. Introduction
4. Results
5. Figures
6. Discussion
7. Materials and Methods
8. Literature Cited

Your next two writing assignments will be based upon the original research experiment you performed to determine if Mex67 associates with specific domains of Nup1p. The "draft" lab report described in this handout will include only the Title, Results, Figures, Materials and Methods, and Literature Cited sections of your paper. The report should contain these sections in the order indicated above.

Note: We cannot emphasize strongly enough the importance of constructing your paper using YOUR OWN WORDS AND IDEAS. Copying material from other sources is plagiarism, even if you cite the source. Any incidences of plagiarism will be dealt with through the appropriate University procedures. If you are struggling with the material or struggling with synthesizing the material to generate a cohesive paper, please come and talk to one of your instructors. We will be more than happy to help you work your way through this assignment in an effort to familiarize you with the reading and writing of scientific literature.

While your grant proposal described experiments you proposed to do, this primary journal article will describe an experiment you have already performed. Thus, the sections included in this paper have a slightly different format than those in the grant proposal. When generating your journal article, **please use the format for each section that is described below**.

Title

The Title should describe or summarize your results from the experiments you are reporting in the paper. Remember, a simple indication of the technique you used is not a suitable title for a primary journal article. Indicate your most significant results.

Example: *Nucleotide triphosphates are required for microtubule assembly.*

Figures

The figures are presented to serve as the raw data for a reader to see in order to make his/her own interpretations of your results. Make sure your figures include both your experimental data and any controls you have run. Each figure should be described by a single figure legend, placed **underneath** the figure. This legend should begin with a title sentence that summarizes the data in the figure. The remainder of the figure legend should carefully describe what is presented in the figure. Your figures should be understandable by a reader of your paper who does not refer to the text of the Results section. Thus, the figure legend can sometimes get quite long. Don't hesitate to be descriptive. In addition, the figures should be clearly labeled to indicate what is present in each lane (if the figure is a gel or blot) and/or in each panel. The labels can be as simple as numbers or can be a bit more descriptive (i.e. GST, Nup1-N, etc.), but should be clearly described in the figure legend. The legend should begin with a short title placed under (or next to) the figure. The text in the figure legend should mention the experiment performed and briefly indicate the results obtained. An interpretation or discussion of these results is not included in the figure legend.

For this paper, you should include two Figures:

Figure 1: Your Coomassie stained gel showing your GST-Nup1 purification and
Figure 2: Your two Western blots showing the presence or absence of Mex67 and Srp1 from your samples.

**Take a careful look at some figures from the primary journal articles you have read in order to get a good idea of the information included in a figure legend and to see how figures are labeled. Other authors' papers are excellent sources of ideas for how to effectively depict your data in figure form!

Results

Written in sentence form, the Results section describes the outcomes of the experiment that you performed. In the Results, simply briefly introduce what question you're asking and how you asked it. Then state what you found, without interpretation or elaboration. Remember that your Results section should be a descriptive report of your observations. Describe what you see on your gels and Western blots. Where do you see bands? In which lanes? What are the molecular weights of the proteins that have been detected? There is no need to point out every datum, but do point out trends you may want to focus on later in the Discussion portion of the paper. **Take the time to describe your results carefully.**

The first sentence or two of this section often states why the experiment was performed and what technique was used (e.g. *In order to examine the role of microtubules in chromosome movement, we treated cells with the microtubule destabilizing drug colchicine immediately prior to entry into mitosis*.). The remainder of the Results describes what was observed as the experiment was performed. The last sentence describing the Results should summarize in a single sentence what you observed. Do not include an interpretation of why you observed these results here – the interpretation is to be saved for the Discussion section.

For this paper, your Results section will first include a description of what you observed when performing SDS-PAGE and Coomassie staining on your purified GST::Nups, and then will concentrate on the Western blot in which you attempted to detect Mex67::GFP affinity purified using the GST-Nup1 fusions.

Again, please take a careful look at some of the primary articles you have already read for additional examples of the format of Results sections.

Materials and Methods

This is where you describe in detail how you went about testing your hypothesis. You must provide enough information that another investigator could duplicate your study simply by reading your description. Chances are your study will not be duplicated exactly, but may be modified by another investigator in order to answer related questions or test similar hypotheses.

The Materials and Methods section is often divided into subsections that represent different sets of experiments. "Cell Fractionation" and "Western blotting" may be two appropriate subheadings for the set of experiments you performed. Protocols described in detail in other papers and certain procedures, such as routine lab activities like mixing solutions or weighing materials, need not be described exhaustively. If you have used another source for your protocol, cite the source of the procedure and then describe the procedure briefly. In this paper, you may include a reference to each lab handout for a description of the methods used in performing the yeast fractionation, GST::Nup1 extraction, affinity chromatography, and Western blotting experiments, and then provide a brief description of the procedure. However, if you did anything differently than the way it was described in the original handout, you must describe in detail the change you incorporated.

Example: *Fractionation of yeast cells expressing GFP-tagged Mex67 was carried out by the method of Belanger et al. (2006). Briefly, cells were grown . . .*

Again, we highly recommend referring to the papers you have read for examples of published Materials and Methods sections (as well as all other sections of your paper!).

Literature Cited

Make certain to cite within the text of your paper any material you use that comes from a published source. Your citation will contain the author's last name followed by the date, all enclosed in parentheses (Jones 1998). If two authors published the paper, include both of their names (Jones and Smith 1999). If more than two authors are on the publication, use the Latin *et al* to indicate the additional authors (Jones et al. 2000).

When listing the full citations in the References section at the end of the paper, make certain to list all authors, last name first followed by first initials, then the year of publication in parentheses, followed by the article title, journal name, volume number, and page numbers. For this paper, only cite the articles that you used in this paper (i.e. that you cite in the body of this paper).

Jones PJ (1998) Microtubules mediate chromosome movement during mitosis. *J Cell Biol* 145: 123 – 135.

Jones PJ, Smith AB, and Doe JD (2000) Colchicine treatment results in chromosome loss in *Xenopus laevis* oocytes. *Science* 255: 123 – 127.

If you are citing a lab handout, just provide the authors' names, year, and title of the handout:

Belanger KD, Geier SJ, Belanger KG (2006) Mex67/Nup1 original research experiment. *Biology 212 Laboratory Manual.*

Arrange your list alphabetically by last name of the first author of the source. Do not include other sources that were not cited, even if they are relevant and you read them from beginning to end. Only include those you have cited in the text. Remember, and ideas that are not your own MUST be credited to the author.

*Remember that citing a paper does not give you authorization to copy the author's words or ideas. Use the sources you are citing to obtain information that you can integrate into your ideas regarding nucleocytoplasmic transport. DO NOT PLAGIARIZE.

Primary Journal Article: Results and Analysis of Mex67-Nup1 Experiment
Lab Report (100 pts.)
Due XXXXXX. Please also turn in your "first draft" paper when you hand in this final lab report.

The time has (finally!) come to synthesize all of information you have learned from your Mex67/Nup1 experiments into one final primary journal article. As we have discussed throughout the semester, papers in cellular and molecular biology typically contain eight "sections." These sections include (with point values for this exercise in parentheses):

1. Title (3 pts)
2. Abstract (10 pts)
3. Introduction (15 pts)
4. Results (18 pts)
5. Figures (18 pts)
6. Discussion (18 pts)
7. Materials and Methods (12 pts)
8. Literature Cited (6 pts)

This "primary journal article" lab report will include all of these sections and will be based upon your original research experiment performed to determine if Mex67 associates with specific domains of Nup1. The report should contain these sections in the order indicated above.

Note: We'll remind you again . . . We cannot emphasize strongly enough the importance of constructing your paper using YOUR OWN WORDS AND IDEAS. Copying material from other sources is plagiarism, even if you cite the source. Any incidences of plagiarism will be dealt with through the appropriate University procedures. If you are struggling with the material or struggling with synthesizing the material to generate a cohesive paper, please come and talk to one of your instructors. We will be more than happy to help you work your way through this assignment in an effort to familiarize you with the reading and writing of scientific literature.

Your "draft" paper contained the Title, Figures, Results, and Materials and Methods sections. As a reminder of our expectations for these sections, refer back to the descriptions of each in the handout describing the first draft. Also, be certain to refer to the helpful editorial notes provided on the "first draft" paper that has been returned to you.

In this final paper, you will also be writing Abstract, Introduction and Discussion sections for the first time. When generating your journal article, **please use the format for each section that is described below**. These descriptions will help you synthesize a clear, concise, coherent, and accurate Final Paper in the format of an actual primary journal article.

Abstract
The Abstract should be a brief description of the important findings being reported in your paper. Using only a single paragraph, the Abstract should provide readers with enough information that they basically understand the question you have asked, what techniques you used to address the question, the results you obtained, and the significance of these results. Because of the space limitation, most authors present their abstract by using only one or two sentences to address each of these topics. A description of the development of a typical abstract is provided below:

The first sentence is usually a general overview of the field in which the experiments were performed.

Example: *The generation of microtubule filaments involves the assembly of tubulin monomer subunits into a long, linear array of molecules*.

The second sentence can say something specific about the aspect of the field that the experiments sought to address (i.e. the question being asked).

Example: *The mechanism by which these monomers assemble is unclear.*

Each subsequent sentence should describe an experiment performed to address the issue mentioned above, and should report the result obtained from the experiment. One or two sentences are usually included for each experiment. If many experiments were performed, the description of the experiment is often removed and only the result is present. This allows for all results to be presented in a short abstract.

Example: *In order to determine if GTP is involved in monomer assembly, tubulin monomers were incubated in presence of a non-hydrolyzable GTP analog. Microtubules failed to assemble in the presence of the GTP analog.*

If your results are significant, you should conclude the abstract with a sentence indicating how this set of experiments has contributed to our understanding of cell function.

Example*: The failure of microtubule assembly to take place indicates that GTP hydrolysis is necessary for tubulin polymerization.*

Introduction

The Introduction may be the most difficult part of a research paper to write. It is often much more difficult for an author to read the work of others (often dozens of papers) and summarize the current state of knowledge in a field in a couple of paragraphs than it is to write an entire paper describing the experiments one has just performed. You are fortunate in that you have already examined most of the background information you will need to construct a good introduction, as you have written a much more extensive introduction/background information section for your grant proposal. Feel free to use material from your grant proposal in this section! Using your own words, even if you wrote them several weeks ago, is not plagiarism. Just make certain they are your own words!

The Introduction usually begins with a paragraph providing an overview of the field of cell biology the paper will address. This paragraph often cites a review article as its reference source. Subsequent paragraphs in the Introduction then describe the findings of others that relate directly to the experiments and conclusions about to be reported in the body of the paper. These paragraphs usually contain a reference or two for each sentence, indicating that these sentences are a description of the reported results of others. The final paragraph of the Introduction is often a very brief description of the findings reported in the paper. (*See the Gorlich et al. paper you read for Journal Club for an excellent example of a concise, well-written Introduction.)

For this paper, your introduction will likely contain four or five paragraphs and should make use of the material you prepared for your "Grant Proposal" background information. (Do not be afraid to consult your grant proposal heavily for this section!)

Discussion

The Discussion usually begins with another sentence describing what experiments were attempted and why they were performed (similar to the first sentence of the Results). The second sentence often describes what results were actually observed. The remainder of each paragraph expounds upon the significance of the results. What do these results mean? How do your control samples influence your interpretation of your results? Integrate your observations with the findings of others to generate a nice

"story" about Mex67/Nup interactions. (*Remember to carefully cite the papers you use in these comparisons*.) Make certain to include not only your insights into why you obtained the results that you did, but also your thoughts on how these results influence our understanding of nucleocytoplasmic transport in eukaryotes. Remember, this section reports your insights into the data you obtained. Generate some insightful analyses! Finally, conclude your discussion with a brief description of what you would do next. Be creative!

Remember not to fall into the trap of using this section only to describe what you perceive as your own procedural errors. We began this experiment without knowing what "should" happen, and you should not write this section as though you had some specific result in mind when you began the project. Write as if the data you collected are as accurate as possible, and discuss those data in the context of the primary literature.

Literature Cited

In alphabetical order, list all of the papers you cited in your text. If you did not cite a paper in your text, do not include it in the Literature Cited section. Refer to the earlier handouts for a more complete description of this section and citation formats.

*Remember that citing a paper does not give you authorization to copy the author's words or ideas. Use the sources you are citing to obtain information that you can integrate into your ideas regarding nucleocytoplasmic transport. DO NOT PLAGIARIZE.

This paper is due XXXXXX at 5:00 p.m. Please turn in your "first draft" paper when you hand in your final lab report.