The Cell Cycle, Part I

I. Introduction

Yeast Cyclin
The eukaryotic cell cycle is divided into four phases, G1, S-phase, G2, and M-phase. Cdk complexes, composed of a regulatory cyclin subunit and a catalytic cyclin dependent kinase subunit, regulate progression of a cell through the cell cycle. Mitotic cyclin-cdks regulate the events associated with M-phase such as chromosome condensation, nuclear envelope breakdown, and spindle assembly. S-phase cyclin-cdks regulate the process of DNA synthesis. G1-cdk complexes regulate the passage from M-phase to S-phase by inactivating the anaphase promoting complex, activating transcription of S-phase cyclin-cdks, and inactivating the S-phase cyclin-cdk inhibitors.

Much of what we know about regulation of the cell cycle comes from studies using yeast as a model organism; these studies include both the fission yeast S. pombe as well as the budding yeast, S. cerevisiae. For our experiment today we will be using S. cerevisiae. S. cereivisiae expresses a single cyclin-dependent protein kinase, cdc28, which interacts with several different cyclins during different phases of the cell cycle. Three cyclins are active in G1, Cln1, Cln2, and Cln3. Clb5 and Clb6 are S-phase cyclins and Clb1 and Clb2 are M-phase cyclins. Clb3 and Clb4 are considered to be M-phase cyclins but are expressed in late S-phase.

Growing Yeast in the Lab
Yeast is an excellent model system to study a variety of cellular processes in addition to the cell cycle. The cells are inexpensive and easy to grow in the laboratory, and can be maintained as either haploid or diploid strains. At 30°C they have a doubling or cell cycle time of 90-140 minutes. Haploid cells exhibit either of two mating phenotypes, mating type a or $\text{a}^\text{\textdagger}$ and can reproduce vegetatively by a mitotic cell cycle. However, when cells of the opposite mating type
are mixed, they mate to produce diploid cells. The diploid cells can reproduce vegetatively by mitosis, or they can undergo meiosis to produce four haploid cells.

The mating process requires that a and a cells communicate with each other and interact physically, so that nuclear fusion can take place. Communication is achieved via the cell-type specific production of secreted mating factors and receptors for those factors. Only a cells secrete a factor and only a cells have receptors for a factor. Likewise, only a cells secrete a factor and only a cells have receptors for a factor. Binding of the appropriate mating factor to either strain results in the activation of a signal transduction pathway that elicits physiological changes that enable the responding cell to mate efficiently. These changes include cell cycle arrest in G1 and elongation of the cell towards its partner. As a result, the cell cycles of the mating pair are synchronized and the proteins that act during fusion are activated. When cells are exposed to mating factor in the absence of a mating partner, the cells arrest in G1 and the cellular elongation becomes exaggerated leading to pear shaped cells that are termed schmoos. Exposing a homogeneous population of cells to the appropriate mating factor is a technique commonly used to synchronize cultures of actively dividing cells. Once the cells are placed in fresh media with no mating factor present, the cell cycle resumes.

In this laboratory exercise we will cell cycle arrest (in G1) two different strains, both mating type a, by exposing them to a factor. The synchronized cultures will be used to examine the expression of two different cyclins acting during the yeast cell cycle, Clb5 and Clb2.

II. Objective

In this laboratory exercise you will examine the expression of two different cyclins that regulate passage through the cell cycle in the budding yeast S. cerevisiae. Yeast strains expressing a specific epitope-tagged cyclin are synchronized by a factor arrest in G1. After release from G1 arrest, you will collect a sample of cells every 20 minutes for 140 minutes. A small aliquot of the cells will be viewed using the light microscope, and the remaining cells will be pelleted in a microfuge and stored on ice until all samples are collected. After all time points are met, the frozen cells are lysed and prepared for SDS-PAGE. In Lab 7 you will perform the final detection steps of a western blotting procedure to identify cyclin.

The two strains you will have available are SBY454 and PY147. SBY454 contains a Clb5 construct tagged with Myc. Myc is a protein that stimulates entry into the cell cycle. In this construct, Myc does not affect the activity of Clb5 but only serves as a molecular tag to identify Clb5 by western blotting using an antibody that recognizes the Myc tag. Similarly, the PY147 strain contains a Clb2 construct tagged with the hemagglutinin protein (HA) from influenza virus. Like the Myc tag, HA does not affect the function of Clb2 but only serves as a tag to identify Clb2 by western blotting using antibodies that recognize HA. In this exercise, you will collect your samples and perform SDS-PAGE. The proteins will be transferred for you onto a nylon membrane. You will complete the steps for detecting the tagged Clb proteins in Lab 7.

In addition to these two strains we also have strain SBY379. This strain contains a construct in which one of the proteins comprising the spindle pole body is tagged with GFP (green fluorescent protein). This strain can be viewed at any time during the course of the lab using the
demonstration fluorescent microscope. This cell culture is not synchronized, allowing you to see the spindle pole bodies at various stages of the cell cycle.
(Note: The three yeast strains used for this laboratory were generously provided by Steven I. Reed, Ph.D., Department of Molecular Biology, MB-7. The Scripps Research Institute La Jolla, CA 92037)

III. Procedures

The following steps have been done before lab:

The night before lab, yeast cultures were set up. 50 mls of YPD media was inoculated with 40µl of an overnight yeast culture. Cultures were incubated overnight at 22°C. The morning of lab, the yeast cells were pelleted and resuspended in 20 mls of YPD media to give the desired optical density or O.D. (cells should be in log phase).

40µl of alpha factor was then added to the 20 ml yeast culture to arrest the cells. Cultures were incubated at 22°C on a shaking platform for 3-3.5 hours.

You will begin this exercise with a yeast culture in which cell cycle arrest has been induced. The first step involves washing away the alpha factor by pelleting the cells in the centrifuge. You will do two washes with YPD media.

Before you start this protocol, there are a number of things for you to label.

- Label 8, 2 ml microfuge tubes with 0, 20, 40, 60, 80, 100, 120 and 140. These are the tubes you will use to pellet and then freeze your cells at the various time points.

- Label 8 microscope slides with 0, 20, 40, 60, 80, 100, 120 and 140. You will use these to make wet mounts at each time point and then view under the microscope.

Releaseing the cells from α factor arrest.

1. Before beginning this exercise make a note as to which yeast strain you are given. Transfer the yeast culture from the flask into a labeled 50 ml conical tube.

2. To pellet the cells, spin for 3 minutes at 4000 rpm in the centrifuge. Your TA will instruct you how to operate the centrifuge.

3. After the spin, you will see the cell pellet at the bottom of the tube. Carefully pour the supernatant into the "Waste" beaker.

4. Resuspend the pellet in 20 mls YPD media. Vortex the tube to resuspend the pellet.

5. Spin the suspended cells again for 3 minutes at 4000 rpm.
6. Carefully pour the supernatant into the "Waste" beaker.

7. Resuspend the pellet in 20 mls YPD media. Vortex the tube to resuspend the pellet.

8. After the pellet has been resuspended, transfer the 20 mls of culture into a sterile culture flask. Be sure to label the flask with your name.

**Time point collection.**

9. Take a 1.6 ml aliquot of this culture and transfer into a 2 ml microfuge tube labeled time "0". This will be your "0 time point" sample.

10. Also, take a 20µl aliquot from your culture to make a wet mount. This will be your "0 time point" to examine the cells under the microscope.

11. Shake your culture in the 30°C incubator and set your timer to count "up". You will take samples every 20 minutes.

12. During the 20 minute incubation period, take your 1.6 ml "O time point" sample and pellet the cells by spinning this sample for 30 seconds at top speed in the microfuge. Be sure to include a "balance" tube in the rotor of the microfuge. The balance tube should be placed in a slot directly across from your sample.

13. Carefully pour the supernatant into the "Waste" beaker. Small amounts of supernatant remaining in the tube may be removed using a pipettor.

14. Freeze the pellet in the ice bucket containing dry ice. WARNING: Dry ice can cause severe burns. Avoid skin contact with the dry ice.

15. You should also have time to look at your "O time point" sample under the microscope using first the 10x and then the 40x objective. Record your observation as a description or drawing that you can refer to throughout the lab. Obtain a sample of the non-arrested asynchronous culture of cells from the teaching assistant. Compare the appearance of your factor arrested sample with that of the asynchronously growing cells.

16. At the 20 minute time point, remove your flask from the shaker and transfer 1.6 ml sample from your culture to a 2 ml microfuge tube labeled time "20". This will be your "20 minute time point" sample.

17. Remove a 20µl aliquot to make a wet mount.

18. Return your flask to the shaker in the 30°C incubator.

19. Take your 1.6 ml "20 minute time point" sample and pellet the cells by spinning the sample for 30 seconds at top speed in the microfuge.
20. Carefully pour the supernatant into the "Waste" beaker.

21. Freeze the pellet in the ice bucket containing dry ice. Small amounts of supernatant remaining in the tube may be removed using a pipettman.

22. Observe your "20 minute time point" sample under the microscope. Record you observation as a description or drawing.

23. Repeat steps 14 - 22, every 20 minutes up to 140 minutes.

24. **At this point you should begin wearing gloves.** After you have collected all of your samples and viewed them all under the microscope, add 150 µl of YPER to each of your frozen samples. YPER will lyse the frozen cells.

25. Vortex each sample one at a time after adding YPER.

26. Incubate the samples in YPER for 20 minutes at room temperature. During this incubation, label the piece of PVDF transfer membrane with your names and the yeast strain you were given. Write on the PVDF using a pencil. Only write on one edge of the membrane. The label should be small so as not to interfere with the western blotting procedure. **IMPORTANT:** When handling the transfer membrane, always wear gloves and use forceps. The western blotting procedure is extremely sensitive and will detect fingerprints.

27. Prepare your samples for SDS PAGE by adding 37.5µl of 4X sample buffer. While one lab partner adds sample buffer to the samples, one lab partner should prepare the acrylamide gel for loading, including writing your names on the glass plates.

28. Perform SDS PAGE as instructed in Lab 3. (If you reach the end of the laboratory period before loading your samples, laboratory assistants will perform SDS-PAGE for you.)

29. Follow the detailed steps on how to boil your samples, as outlined in Lab 3.

30. In this Lab, you will be using 10 well gels. Load 20µl of prestained standard in lane 1.

31. Load 40µl of your time-course samples in lanes 2-9 in order of increasing time.

32. When the electrophoresis is complete, and the proteins have been separated by SDS-PAGE, the laboratory assistants will electrophoretically transfer the proteins from the gel onto a nylon membrane. This technique will be discussed in more detail in Lab 7.
The Cell Cycle, Part II

I. Introduction

Western blotting (or Immunoblotting) is a powerful technique for detecting a particular protein of interest. It is widely used in determining the presence, quantity, and molecular weight of a desired protein.

There are a number of steps involved in western blotting:

1. A mixture of proteins is first separated by size using gel electrophoresis (SDS-PAGE).
2. The separated proteins are then transferred from the gel onto a membrane, thus making a replica of the separated proteins. The transfer of the proteins from the gel to the membrane occurs by placing them in direct contact and placing this gel/membrane sandwich in an electric field. This drives the proteins from the gel to the membrane. All the proteins are bound non-specifically to the membrane.
3. The membrane is then subjected to a series of treatments to detect the protein of interest.
4. First, it is important to "block" all areas of the membrane which do not contain the blotted proteins from the gel. Just as proteins transferred from the gel can non-specifically bind to the membrane, so can the antibodies used for protein detection. Blocking the membrane prevents the antibodies from non-specifically sticking to the membrane, causing a false positive result or high background during subsequent detection steps. Blocking solutions typically contain proteins which do not react with the antibodies you're using. Two common examples of blocking agents are non-fat dried milk and bovine serum albumin.
5. The membrane is next incubated with a solution containing the primary antibody which specifically recognizes the protein of interest. After incubation with the primary antibody, the membrane is washed to remove any unbound antibody. This wash usually involves changes of a buffer containing a low concentration of detergent.
6. The washed membrane is then incubated with a secondary antibody. This antibody recognizes the primary antibody and is conjugated to an enzyme. For example, our primary antibodies which recognize either Myc or HA were generated in a mouse. The secondary antibody we will use recognizes mouse antibodies, and are conjugated to the enzyme alkaline phosphatase. After this incubation, the membrane is washed as before to remove any excess secondary antibody.
7. The last step is detection. In this experiment we are using a secondary antibody
conjugated to alkaline phosphatase. We can detect the antibody conjugated to alkaline phosphatase by supplying the substrate bromochloroindolyl phosphate/nitro blue tetrazolium (BCIP/NBT). When the membrane is incubated with this chromogenic substrate, a deep purple precipitate forms wherever the secondary antibodies have bound. This method is termed colorimetric detection since a colored reaction product is formed.

II. Objective

In last week's lab, we synchronized two yeast strains, SBY454 and PY147 (each of which express a specific epitope-tagged cyclin) and collected cell samples after release from G1 arrest. Samples were collected over a 140 minute time period, then run on an SDS-PAGE gel and transferred onto a membrane for western blotting.

In today's lab, you will proceed with developing the western blots in order to observe the expression of the two tagged cyclin proteins, Clb5 and Clb2, in your time-course samples from Lab 6.

As a reminder, SBY454 contains a Clb5 construct tagged with Myc, which enables Clb5 to be identified by western blotting using an antibody to the Myc tag. PY147 contains a Clb2 construct tagged with the hemagglutinin protein (HA) from influenza virus. Similarly, HA serves as a tag to identify Clb2 by western blotting using antibodies to HA. We will divide the lab section so that some groups will perform colorimetric detection and some groups will perform chemiluminescent detection.

III. Procedures

The following steps will be performed for you:

33. Block the nitrocellulose membrane for 2 hours in 5% milk in TBS-Tween (0.1% Tween).  
34. Wash membrane in TBS-Tween for 2 minutes on a shaking platform. Repeat once.  
35. Incubate the membrane in primary antibody at room temperature for 2 hours.  
   The primary antibodies are:  
   - Anti-HA at 0.2µg/ml in 5% milk in TBS-Tween (0.1% Tween) - to be used for blots containing samples from strain PY 147.  
   - Anti-myc at 1:5000 dilution in 5% milk in TBS-Tween (0.1% Tween) - to be used for blots containing samples from strain SBY 454.

During lab, continue with the following steps:

All of these steps should be performed wearing gloves. We are not protecting your skin. Rather, we are protecting the blot from your fingerprints. Even while wearing gloves, always handle the blot by the edges with forceps.

1. Wash membrane in TBS-Tween for 15 minutes on a shaking platform.  
2. Wash membrane in TBS-Tween for 5 minutes on a shaking platform. Repeat once.
3. Incubate with the secondary antibody for 45 minutes on a shaking platform. The secondary antibody is a 1:10,000 dilution of an anti mouse-AP in 3% milk, TBS-Tween (0.1% Tween).
4. Wash membrane again in TBS-Tween for 15 minutes on a shaking platform.
5. Repeat 3 washes in TBS-Tween for 5 minutes each.

**Colorimetric Detection**
6. Wash twice in AP buffer for 2 minutes each.
7. Prepare the colorimetric substrate:
   - Add 44µl of NBT and 33µl of BCIP to 10ml of AP buffer.
8. Add this solution to your blot and incubate for up to 30 minutes or until the color develops.
9. Stop the reaction by pouring out the substrate and adding dH₂O for 10 minutes.
10. Place membrane between filter paper to dry. Label filter paper clearly with both students’ names and yeast strain.