Bi 421 Advanced Molecular Genetics Laboratory Winter 2017

Instructor: Dr. Eric Selker [355D Streisinger; phone: 541-346-5193; email: selker@uoregon.edu];
Grad. TA: Erik Toraason [245 Streisinger; lab phone: 541-346-7198; cell: 262-297-2560 (for emergencies) email: etoraaso@uoregon.edu]
Undergrad. TA: Carolyn Brewster [cell: 432-254-9200 (for emergencies); email: cbrewste@uoregon.edu]
Laboratory: Room 33, Klamath Hall

Class meetings: one hour lecture/discussion and 8+ hours lab/week during class hours (M, W, F 3-6 PM); ~3 hours/week additional time outside of class; we hope to allow students 24/7 card-access to the room but for significant non-class time in lab, please email GTF to notify us of the activity/timing.

General structure of course
-students will work mostly independently but for convenience can "collaborate" with a partner for some procedures
-students will keep individual lab notebooks, which will be examined periodically (~weekly); carbon copy notebooks should be purchased before first meeting (Duckstore).
Grading: lab work/notebook (30%), quizzes (20%), oral presentations/problem sets (15%), final report (25%), effort/other (10%).

OVERALL PLAN OF QUARTER: Isolation and characterization of mutants defective in silencing genes normally repressed by H3K27 methylation. We will select for mutants that de-repress antibiotic genes *hph* and *nat-1*, which encode resistance to hygromycin ("hyg") and nourseothricin ("nor"), respectively. In the starting strain these genes are repressed by histone H3K27 methylation. We will first select for hyg-resistance and then test for nor-resistance (by "spot tests"). We will then test if the mutations segregate in sexual crosses and use progeny from crosses to perform complementation tests to see if mutations are in different genes and are in known genes (encoding PRC2 components). We will also determine the approximate chromosomal position of mutation by RFLP mapping by bulked segregant analysis by PCR using primers for different chromosome arms. Finally, if time permits, we will do whole genome sequencing to map and identify mutations.

We will take advantage of natural gaps in the lab work (e.g. while we are waiting for the crosses) to explore scientific background. In part this will involve reading and discussing primary publications. I anticipate assigning one paper to each of three groups (of 3-4 students), who will then: 1. Help formulate a few questions (for a short problem set) on the paper for the whole class to consider and 2. Lead a discussion on the paper. This will be discussed at the beginning of the course.

Brief tentative schedule:

Week 1: Mutagenesis
Week 2: Confirm mutants with spot tests, set up crosses
Week 3-4: Isolation of DNA from control strains, quantitation and testing by restriction analysis/gel electrophoresis; paper presentation.
Week 5-7: Isolation and characterization of progeny from crosses (spot tests for markers); DNA preps (Mauriceville cross); complementation tests; PCR pooled progeny DNA (Mauriceville cross)/
Week 8: RFLP mapping; prepare for whole genome sequencing.
Week 9-10: Catch up, follow up, continue complementation tests and mapping as necessary.
Week 10: Catch up, follow up, organize strains and notebooks

More detailed plan: (Please also see endnotes for additional information that will be important for the work; other information/protocols will be provided; schedule is necessarily approximate.)

Week 1

Discussion: Scientific background; laboratory orientation; use of lab notebooks; sterile technique; making solutions and media; use of microscope for determining cell density; planning; resources.
Session 1: Make media[i] for mutagenesis[ii] and filter-beaker to harvest conidia[iii]; plan mutagenesis and platings to generate mutants (these plates will later get antibiotic(s) to select mutants) and to determine survival (these will be non-selective so we'll want to plate many fewer spores; why?).

Session 2: Harvest and count conidia[iv] of strain N6279 (*his-3; mating type a*), which has antibiotic markers in H3K27m regions and requires supplementation with histidine, using a hemocytometer[1]; UV-mutagenesis (plates to select mutants and others to calculate survivorship); plan & prepare.
Session 3[v]: Add selective top-agar 18 hours after plating (you will need to come into the lab an unscheduled time to do this, but it won't take long); plan & prepare materials (e.g. make cross plates, FGS his, FGS, his, nor and FGS, his, hyg plates for spot tests). Note: there are two different approaches you/we can take to select mutants: 1. Select initially for just one antibiotic (i.e. hygromycin resistance) and then test putative mutants for resistance to the second marker (*nat-1*, conferring resistance to nourseothricin, abbreviated "nor") by "spot-testing" or 2. Select initially for resistance to both antibiotics (hyg and nor) and then confirm by spot-testing. What do you consider the pluses and minuses of these two approaches? You might want to try both methods yourself or in collaboration with your neighbor.

Week 2

Discussion: Sexual crosses; complementation testing; meiotic silencing.
Session 1: Score plates and "pick" (transfer) ~20 potential mutants to slants, grow 3-5 days at 32C (u. Consider what control strains you'll want for upcoming spot tests and make sure you have them.
Session 2: If slants of potential mutants have grown enough, suspend in water and spot test on several types of plates: 1. FGS (0.5 mg/ml fructose, 0.5 mg/ml glucose, 20 mg/ml sorbose; causes colonial growth), his; 2. FGS his, nor; 3. FGS his, hyg (include positive and negative controls; save conidial suspensions at 4C). Inoculate 8 mating plates with Mauriceville *mat A* strain (N51) for mapping and 8 mating plates with N3756 (*Sad-1*, *his-3*, *mat A*) for complementation tests. We may also cross putative mutants to a third, *trp*-requiring, strain for complementation testing, as we will discuss so make extra crossing plates. Note: for crosses we have to limit nitrogen so we use special low N Vogel's labeled SC (for synthetic cross), instead of regular Vogel's salts.
Session 3: Check spot tests and determine which strains might be "real" mutants. Pick 8 of these mutants to move forward - use these to fertilize your cross plates from last time. Also grow new slant of each of these strains for storage/future use (label carefully). Plan for next week – prepare any plates, media, solutions you may need.

Week 3

Discussion: Heterokaryon incompatibility; RFLP mapping; DNA isolation
session 1: Inoculate 5ml liquid cultures of control strains: Mauriceville (N51) and mutagenesis strain (N6279). 1st student journal presentation/discussion.
session 2: Harvest tissue and start to isolate DNA
session 3: Finish DNA isolation; quantify DNA with Qubit, run on gel to assess quality

Week 4
Discussion: Gene silencing mechanisms; Illumina sequencing. .
1. PCRs for RFLP mapping (testing with control DNAs)
2. DNA electrophoresis on PCR DNA; 2nd student journal presentation/discussion.
3. check crosses; plan for next week

Week 5
1. Make plates to germinate spores from crosses (FGS his, nor and FGS his, hyg), make 'fluffy' plates for determining mating type
2. Select at least one putative mutant for RFLP mapping (using progeny of cross with Mauriceville) germinate spores and plate onto selective media (half on FGS his, nor and half on FGS his, hyg) and germinate spores (his, nor, hyg) for all crosses with *Sad-1, his-3* strain to use for determining mating type and complementation testing
3. Pick 40 progeny of the Mauriceville cross and 10 progeny for each *Sad-1* cross to check mating type
Materials: Plates, media, fluffy strains, slants; Pre-lab prep – 200 min slants

Week 6
Discussion: Bulked Segregant Analysis; Mating Type tests
1. Prepare conidial suspensions (*Sad-1* progeny) and perform mating type spot tests and inoculate cultures for DNA preps (Mauriceville progeny).
2. Examine mating type test plates. Harvest culture for DNA and lyophilize tissue. Make buffers needed for DNA preps. 3rd student journal presentation/discussion.
3. Examine mating type test plates. Perform DNA isolation protocol through step 9 (TCA-ethanol precipitation). Set up forced heterokaryons with known PRC2 mutant strains in *trp-2* background.
Materials: Vogels sucrose + his, Salt detergent, TCA-Et-OH, NH_4OAc/RNaseA, 8:1 Isoproanol: NH_4OAc, Et-OH

Week 7
Finish DNA isolations; examine 5ul aliquot on a gel, make plates for spot tests
Spot tests forced heterokaryons and controls on min, hyg and nor
Set up PCR on pooled progeny of Mauriceville cross and controls, take pictures of spot tests of forced heterokaryons
Materials: Plates, media, polymerase, buffers, dNTPs, primers

Week 8:
Start restriction digests and gel electrophoresis to score linkage
Continue RFLP mapping
Use Nextera kit to prepare for Illumina sequencing of your pooled progeny
Materials: restriction enzymes, agarose gel and running buffer/equipment; Nextera kit

Weeks 9-10
Submit DNA for Illumina sequencing (details discussed in class); catch up, follow-up, continue mapping, organize strains into PRC2 complementation groups or potentially novel mutants; analyze data; discussion.

ENDNOTES:

[i] To grow Neurospora, you just need to supplement a diluted "salts" solution (e.g. regular "Vogel's" or modified versions for crossing) and add sugar. Regarding the sugar, typically 1.5% sucrose is used if you don't need colonies but if you need cells to grow colonially (e.g. for counting viable cells or for looking for mutants),

instead you use a combination of fructose (0.05%), glucose (0.05%) and sorbose (2%). We make a 10X concentration stock "10X") of these and add this after autoclaving since the sugars can caramelize if heated with the salts. To make 100 mls of "10X FGS" dissolve 0.5 g fructose, 0.5 g glucose and 10 g sorbose in water and adjust to 100 ml. (Note that sorbose is very expensive so just take what you need.) Always check what extra supplements strains require, e.g. histidine or tryptophan, both of which are stored as "50X" stocks. We normally use supplements at "1X". For histidine and tryptophan, 1X is defined as 1.0 mg/ml and 0.2 mg/ml, respectively. For solid medium (plates, slants, and "conidia flasks"), we include 1.5% agar (not agarose!).

[ii] Consider that for each "treatment" (untreated and three UV treatments, e.g. 0, 3, 6, 9 seconds) you should plate about 10,000 conidia/plate on four plates for selection of mutants. But you don't include the selective agent (hygromycin) right away because this would probably kill nearly all the cells, including your mutants (why?). So, instead, 18 hours after the treatments, 10 ml of hygromycin-containing agar medium (use 3X hygromycin because it will be diluted by the bottom agar, which would be ~20 ml; note hygromycin is toxic to humans and is very expensive; be careful!). To calculate total viability, you will want to put a smaller number of conidia on plates that won't receive hygromycin. A 1/50 dilution of what you put on the other selection plates (i.e. 400 rather than 10,000 cells) might be good. Thus you will need at least 20 plates, which you should be able to make from 0.5 liters of medium (use 20 ml/plate). Be sure to wrap plates in foil immediately after UV treatment to avoid photo-activated repair; the red safe light in the dark room is OK; it will not support photoreactivation. Thus, for your UV-mutagenesis, in preparation for the hygromycin selection, plate on non-selective medium containing Vogel's, 1X FGS (0.5 mg/ml fructose, 0.5 mg/ml glucose, 20 mg/ml sorbose); 1X histidine (0.5 mg/ml) solidified with 1.5% agar. This is the "BOTTOM AGAR". Use 20 ml per plate. 18 hours later, you should add 10 ml of "TOP AGAR" which has the same composition as the bottom agar except that it will contain "3X" hygromcyin when you need to select for mutants and will only have 1.0% agar. When you just want to know how many viable cells you have you don't want to select for hygromcyin and therefore will not include this drug in the top agar.

[iii] Use rubber band or tape to attach double layer of cheesecloth to top of small (~150 ml) beaker making a funnel shape by pushing cheesecloth in middle; cover with foil and autoclave.

[iv] Examine the hemocytometer under the compound microscope (use total magnification of 100X or less) and note that in the middle there are a set of 25 squares (5x5), each divided into 16 smaller squares. If you were to count all the spores in the 25 squares, this would correspond to 10^{-4} ml. But you don't really need to count all the area. We usually count the 4 corners and the middle square and then multiply by 5 x 10^4 to get the concentration of spores in the suspension. Note that you will need to dilute your stock so that you get a reasonable number of spores to count. It's sometimes necessary to do serial dilutions. Typically, dense conidial suspensions contain 10^8-10^9/ml. You might want to first try counting conidia in a 100 X dilution (dilute 10 μl of your conidial suspension into 1 ml) but it there are too many conidia to easily count, you might then want to do another 10 X dilution (e.g. mix 100 μl of the diluted conidia with 900 μl water). If you were to count conidia in this second dilution and found a total of 40 conidia in 5 of the 25 big squares, you'd calculate the concentration in your original stock to be: 40 x 5 x 10^4 x 10^2 (first dilution) x 10^1 (2^{nd} dilution) = $2x10^9$ conidia/ml.

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[v] Note that the need to add the antibitotic top-agar 18 hours after the original plating will require an extra (but short) visit to the lab on Thursday.