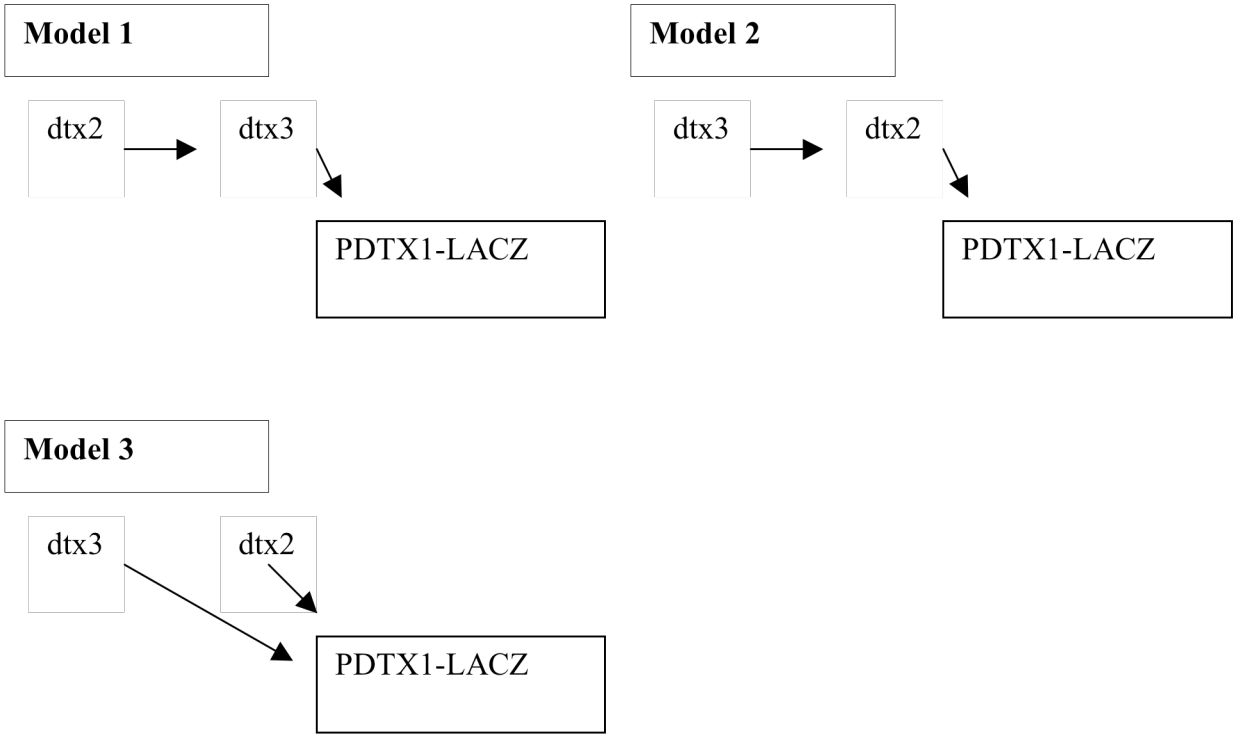
7.03 PROBLEM SET 5 KEY

BASED ON LECTURES 20-25
DUE BEFORE 5PM ON WEDNESDAY, NOVEMBER 15
SUBMIT ANSWERS DURING RECITATION OR PLACE IN BOX OUTSIDE OF THE BIOLOGY EDUCATION OFFICE

1) Genetic pathways in eukaryotes often are investigated using gene fusions. This approach could be used in yeast to study regulation of a detoxification gene, DTX1. This gene encodes an enzyme that neutralizes benzene, which is a known carcinogen. To investigate DTX1 regulation, you made a gene fusion (P_{DTX1} - LacZ) that consists of the cis regulatory region of DTX1 and the coding region of LacZ. When integrated into the yeast genome, this gene fusion shows wild-type expression. In addition, you isolated two recessive loss-of-function mutations, dtx2- and dtx3-, which show un-inducible gene fusion expression. Your analysis shows that the dtx2- and dtx3- mutations reside in different genes, are not linked to each other, and are not linked to the gene fusion.

a) Diagram three possible models that illustrate the wild-type regulatory relationships among DTX2 and DTX3 and P_{DTX1} - LacZ.



These are the three simplest models.

b) You have access to a dominant allele of the DTX2 gene, dtx2-^{D}, which causes constitutive expression of P_{DTX1} - LacZ. Describe the experiment you would perform to distinguish between two of the three models diagrammed in **part (a)**. Show all crosses, the resulting tetrads, and how each result should be interpreted.

Cross dtx2^{D} to dtx3-. The resulting diploid will be heterozygous for both dtx2-D and dtx3-. Sporulate this diploid to get the following tetrads listed below.

	Genotypes
PD	**2 uninducible, 2 constitutive**
NPD	**2 wild-type, 2 double mutant (either constitutive or uninducible)**
TT	**1 uninducible, 1 constitutive, 1 wild-type, 1 double mutant (either constitutive or uninducible)**

Take the double mutant spore from the NPD tetrad.

- **If PDTX1-LacZ is constitutively expressed in the double mutant, then discard model 1.**
- **If PDTX2-LacZ is uninducible, then discard model 2.**
- **This technique does not address parallel pathway models.**

c) Now you want to investigate the mechanism by which DTX2 and DTX3 act in the pathway. To determine if DTX2 and DTX3 can bind to each other, you decide to perform a yeast-two-hybrid assay. Which cis-regulatory regions would you put upstream of LacZ?

You would place the promoter from the Gal1 sequence in front of LacZ. The UAS sequence is essential as it is the sequence in the Gal1 promoter to which the DNA binding domain of Gal4 binds.

Suppose you engineer the protein fusion genes DTX2 : AD and DTX3 : DB on a selectable plasmid. What results from the yeast-two-hybrid assay would show that DTX2 and DTX3 interact/bind directly to each other? Complete the chart below and include the necessary controls.

Assay	Yeast strain	Reporter gene expression (expected)
Control 1	**Pgal1::LacZ**	None
Control 2	DTX2 : AD / **Pgal1::LacZ**	**None**
Control 3	DTX3 : DB / **Pgal1::LacZ**	**None**
Experiment 1 (assume no direct interaction)	**Pgal1::LacZ + DTX2:AD + DTX3:DB**	**None**
Experiment 2 (assume direct interaction)	**Pgal1::LacZ + DTX2:AD + DTX3:DB**	**++++**

The controls outlined in the table above are essential to do this experiment. There are other possible controls. For example, switch AD and DB so that DTX2 and DTX3 are attached to different domains and use this to confirm the interaction again.

d) What if one of the controls listed above shows reporter gene expression? For each control, suggest a possibility as to what is occurring in the cell if reporter gene expression is observed.

There are several possible answers for "part d". The purpose of the question was to get you to think about how the assay works and why controls are essential.

Control 1 ⟶

There is something activating expression in a strain with only the fusion gene. This strain will not be useful; it is necessary to integrate the reporter gene somewhere else, or use a different plasmid.

Control 2 ⟶

DTX2:AD is activating reporter gene expression without the DNA binding domain of Gal4. DTX2 may bind directly to DNA close enough to the reporter fusion gene to activate its expression.

Control 3 ⟶

DTX3:BD is recruiting RNA Polymerase or is recruiting other factors that promote LacZ expression without the activation domain.

e) Based on the results from the yeast-two-hybrid assay, what can be concluded about your models in **part a**?

If the proteins do interact with each other in the yeast two hybrid assay, it suggests that the proteins may interact with each other when they promote DTX1 expression. If the proteins interact, it is strong evidence that they may act in a pathway in series instead of in parallel.

If you find they do not interact, new information will be necessary.

2) Genes in the body that suppress the development of cancer are known as tumor suppressor genes. Due to its role in the repair of double-strand breaks in DNA, REP1 is regarded as a tumor suppressor gene. Individuals with loss-of-function mutations in both REP1 alleles show a much greater risk for developing some cancers. After brainstorming with colleagues, it is decided that the role of REP1 in cancer development could be investigated effectively using genetically modified mice.

a) In order to obtain an effective mouse model to study the role of REP1 in cancer development, what genotype would you generate? Explain.

REP1-/-

To model this loss-of-function mutation, we must generate a mouse that lacks two functional copies of REP1. Based on REP1's known role as a tumor suppressor, we would expect REP1-/- mice to show a greater risk of developing certain cancers.

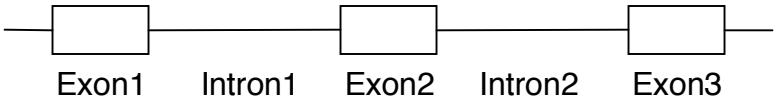
(NOTE: In certain cases, generating a homozygous mutant as we've done here is not possible. If a gene is serving an essential role, then losing the function of both of its alleles will produce unviable offspring. Here, since we are told that REP1-/- individuals are at a greater risk of developing cancers, we know that homozygous REP1 mutants are viable.)

b) Would pronuclear injection or gene targeting techniques be required to construct the desired mouse model? Explain.

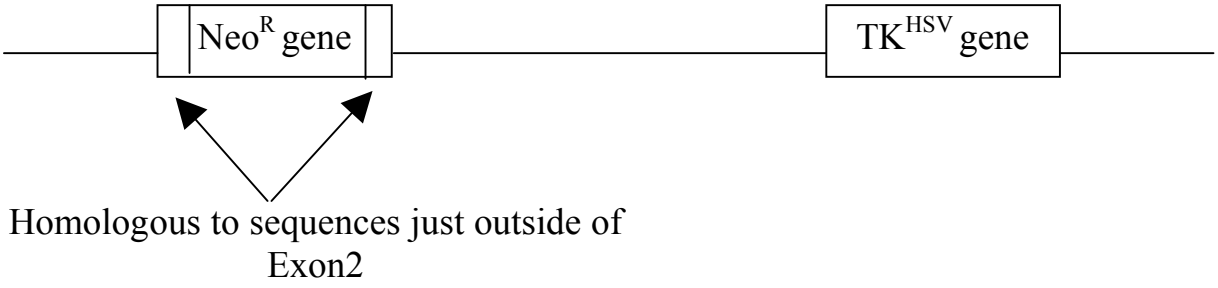
Gene targeting.

You hypothesize that the increased risk of cancer is due to two inactive versions of REP1. To model this situation in a mouse, you need to knock-out both functional copies of the REP1 gene. Gene targeting is the only method to do this.

c) Exon 2 is essential for the tumor-suppressor activity of the REP1 gene (illustrated below). Draw the DNA construct you would use to modify the mouse genome. How would it integrate into the genome? How would you detect its integration?



DNA construct:

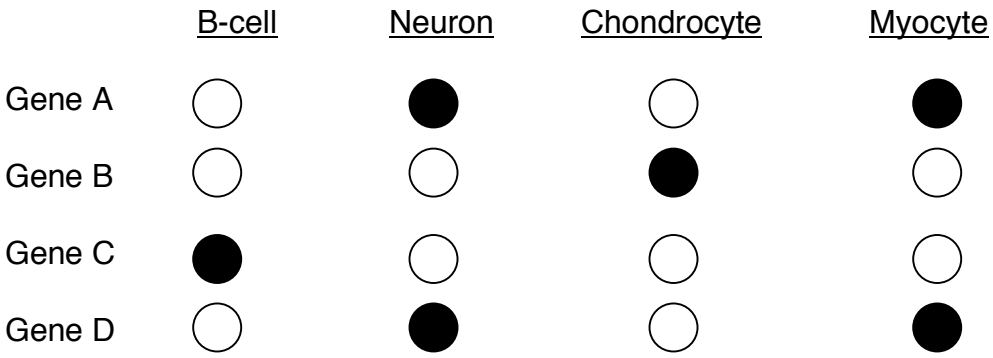


The DNA construct contains two regions that are homologous to the mouse DNA surrounding Exon 2, separated by a gene that confers antibiotic resistance (e.g., Neomycin). The construct will homologously recombine with the mouse genomic DNA at the homologous sequences and replace Exon 2 with the Neo^R gene. We can use this newly acquired drug resistance gene to positively select for ES cells that have undergone homologous recombination.

We additionally need to include a TK gene outside of the homologous sequences. This is used as a negative selection marker against integration events that occurred via *non*-homologous recombination. If the DNA construct integrates randomly using non-homologous end-joining, then the TK gene also will be integrated and the cells will be selected against with a drug (review lecture notes).

A number of genes, such as gene **X**, are involved in the normal regulation of cell growth. Oncogenes are mutated versions (alleles) of these genes that promote unregulated cell growth and lead to cancer. In one type of cancer, B-cell lymphoma, gene **X** is highly expressed. You hypothesize that the over-expression of gene **X** is the causative mutation of B-cell lymphoma.

The expression data illustrated below were generated using microarray analysis (dark circles signify unusually high expression and clear circles signify no expression).



d) Given these results, propose a strategy to generate a mouse model to investigate the role of gene **X** in B-cell lymphoma. Be sure to include the genotype you would create, whether pronuclear injection or gene targeting methods would be used, and how gene **X** would be expressed in B-cells.

We need to create a translational fusion protein consisting of the promoter region of Gene C and the coding region of Gene X (P_{GeneC}- GeneX). Gene C was chosen as it shows high levels of expression in B-cells. To add this DNA construct into the mice genome, the simplest method is pronuclear injection. After injecting into the male pronucleus of a fertilized egg, the construct will insert randomly into the mouse genome.

Every cell in the developed mouse will now possess this fusion gene, but Gene X will be expressed in a cell-type dependent manner because of its upstream promoter from Gene C. Namely, X will be highly expressed in B-cells, but not expressed in neurons, chondrocytes, and myocytes (See 3rd row of microarray analysis above).

We have created a transgenic mouse that over-expresses Gene X selectively in B cells. If over-expression of Gene X is a causative mutation of B-cell lymphoma as we have hypothesized, we would expect these transgenic mice to develop B-cell lymphoma.

3) PhiP and IQ are heterocyclic amines that are mammary gland carcinogens in mice. Both of these chemicals are present in certain food products such as cooked meats. To better understand the biology behind the carcinogenic properties of PhiP and IQ, we would like to identify genes that protect cells from their toxicity.

Wildtype yeast grow at a reduced rate in the presence of 50 mM PhiP but arrest completely in the presence of 100 mM PhiP.

a) Using the yeast *Saccharomyces cerevisiae*, design a screen to isolate mutants that are hypersensitive to PhiP. Be as specific as possible.

Mutate *S. cerevisiae* using a mutagen (example: EMS or UV light) and culture them on plates containing 0 mM PhiP. Replica plate these colonies onto plates with 50mM PhiP. Look for colonies that grow on 0, but not on 50mM PhiP. These colonies are hypersensitive to PhiP (Wild-type yeast still grow at 50mM, but not at 100mM).

From this screen you identify two mutants that are hypersensitive to PhiP. You name these mutants Mut1 and Mut2. You find that Mut1 and Mut2 both confer a recessive mutant phenotype.

b) Design an experiment to determine if Mut1 and Mut2 are alleles of the same gene. Be as specific as possible.

Cross the Mut1 and Mut2 haploid strains to produce a diploid. Plate the diploid on 50mM PhiP. If it does not grow on 50mM PhiP, then Mut1 and Mut2 are alleles of the same gene. If it does grow, then it has the wild-type phenotype, complementation occurred, and the mutations reside in different genes.

You determine that Mut1 and Mut2 define two different genes. Furthermore, you have mapped and cloned Mut1. Now that you know the sequence of the MUT1 gene you decide to make a gene fusion consisting of the cis regulatory region of MUT1 ligated to the LacZ coding sequence. A single copy of this gene fusion (P_{MUT1}- LacZ) then is incorporated into Chromosome III of the yeast genome. You perform the following experiments and test for ß-galactosidase activity:

Genotype	no PhiP	25 mM PhiP
P_{MUT1}- LacZ	-	+
Mut1; P_{MUT1}- LacZ	-	+
Mut2; P_{MUT1}- LacZ	-	-

c) What do these results tell you about Mut1 and Mut2? Why is this experiment performed with 25mM PhiP and not 50 mM PhiP?

Mut2 is a positive regulator of Mut1 expression. The fusion gene is used to monitor Mut1 expression, which seems to be induced by PhiP. These results show that Mut2 causes uninducible expression of the fusion gene, it acts in trans (it's in a different gene), and it is recessive.

25 mM of PhiP is required because at 50 mM PhiP the colonies from Mut1 and Mut2 strains would not grow.

You want to identify a mutant that constitutively expresses P_{MUT1}- LacZ. You mutagenize your P_{MUT1}- LacZ haploid strain and look for ß-galactosidase activity in the absence of PhiP. You identify a mutant that you call Mut3.

Genotype	no PhiP	50 mM PhiP
P_{MUT1}- LacZ	-	+
Mut3; P_{MUT1}- LacZ	+	+

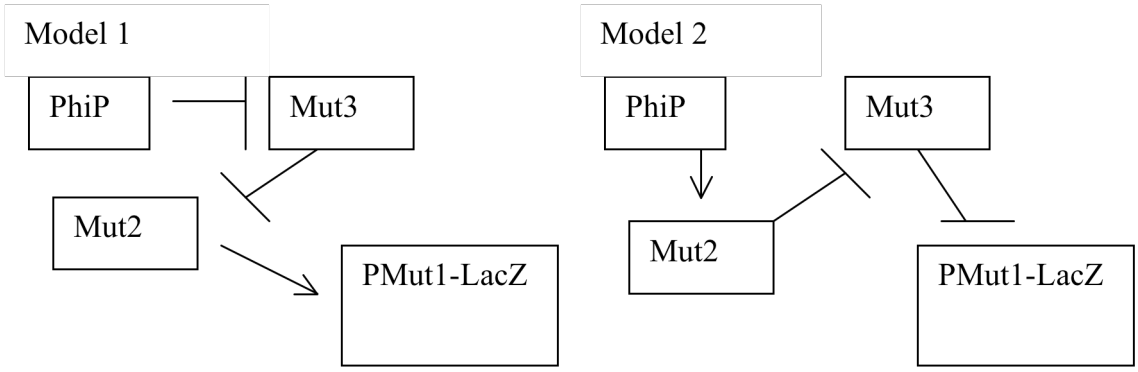
You cross this mutant to a wildtype strain (no P_{MUT1}- LacZ) and do the following experiments:

Genotype	no PhiP	50 mM PhiP
P_{MUT1}- LacZ/ +	-	+
Mut3/+; P_{MUT1}- LacZ/+	-	+

d) What conclusions can you draw from the results of the above experiments? Be as specific as possible.

You know that Mut3 has constitutive expression of the fusion gene based on the results in the 1st table. In the second cross, you find that the Mut3 phenotype is recessive. Therefore, it is a negative regulator of Mut1 expression.

e) Given the available data, draw the two most likely pathways that illustrate the involvement of PhiP, Mut2, and Mut3 in the regulation of MUT1. Assume the regulatory genes operate in series.



You cross the Mut2 strain to the Mut3 strain (both containing the P_{MUT1}- LacZ reporter), sporulate the diploid, and then measure the ß-galactosidase activity of the resulting spores grown on three different types of media. You examine 50 tetrads and observe the following:

# of tetrads		no PhiP	25 mM PhiP	50 mM PhiP
49	**3-**	+	+	+
	3-	+	+	+
	2-	-	-	Arrest
	2-	-	-	Arrest
1	**double mutant**	+	+	+
	3-	+	+	+
	2-	-	-	Arrest
	wt	-	+	+

f) Based on these data, what can you conclude about the relative positions of MUT2 and MUT3 in the pathway? Be as specific as possible.

Because two 2 types (PD and TT) of tetrads are produced, MUT2 and MUT3 must be closely linked. The 49 PD tetrads show two spores that are constitutive at all amounts of PhiP and two spores that are uninducible. In the TT tetrad, one spore must be 2-, one must be 3-, one must be wild-type, and one must be the double mutant. You find that two of the spores constitutive. Therefore, the double mutant is constitutive and MUT3 acts further downstream in the pathway then MUT2.

g) Which of the two models drawn in **part e** must be correct?

Model 2 must be correct because MUT3 is downstream of MUT2 in model 2.