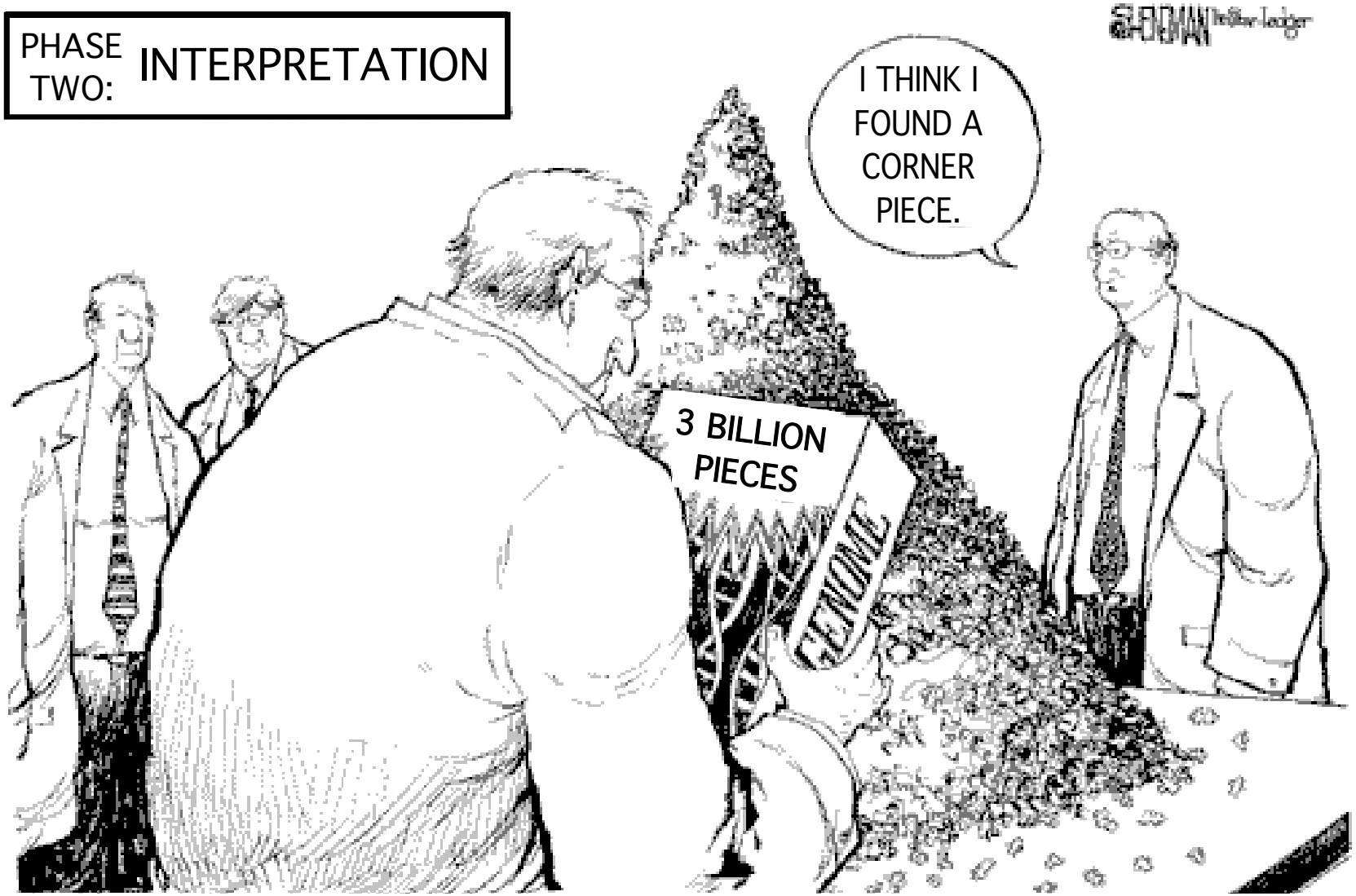
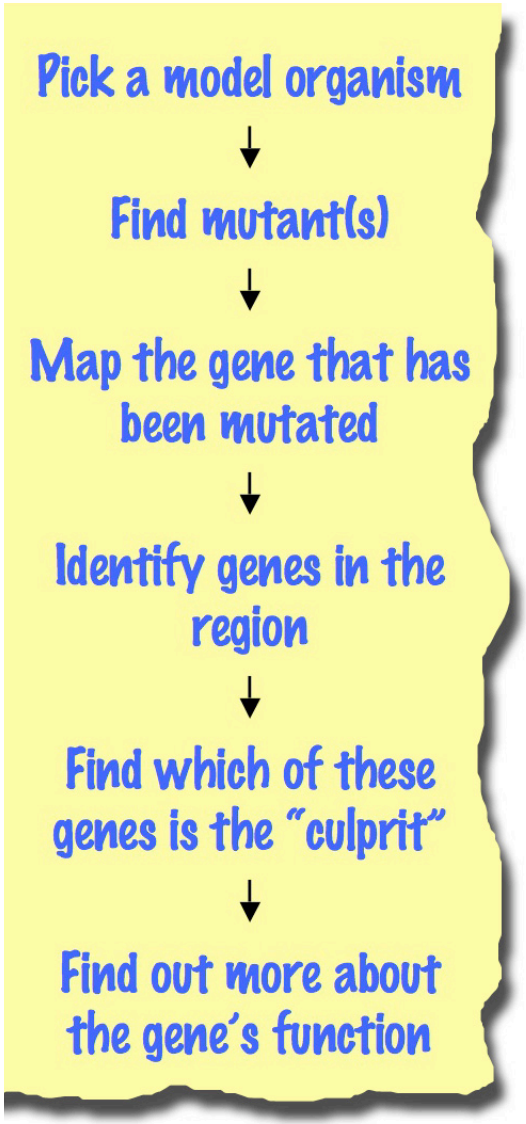
Analysis of gene function

Gene knockouts



Analysis of a disease gene



Gene knockout or "knockdown" in model systems

...does the phenotype mimic the disease?

Strategies to understand what a gene normally does…

» gene knockdown… reduce expression of the gene

» gene knockout… completely delete the gene (or a critical portion of the gene)

» "knock-in"… replace one allele (e.g., wild type) with another (e.g., a specific mutation)

What is a knockout mutation aka gene disruption

A complete loss of function allele

Usually produced by replacing a gene or portion of a gene with some kind of selectable marker

Knock-out mutations are engineered deliberately

...by modifying a gene with recombinant DNA technology, and replacing the wild type allele with the knockout ("KO") allele.

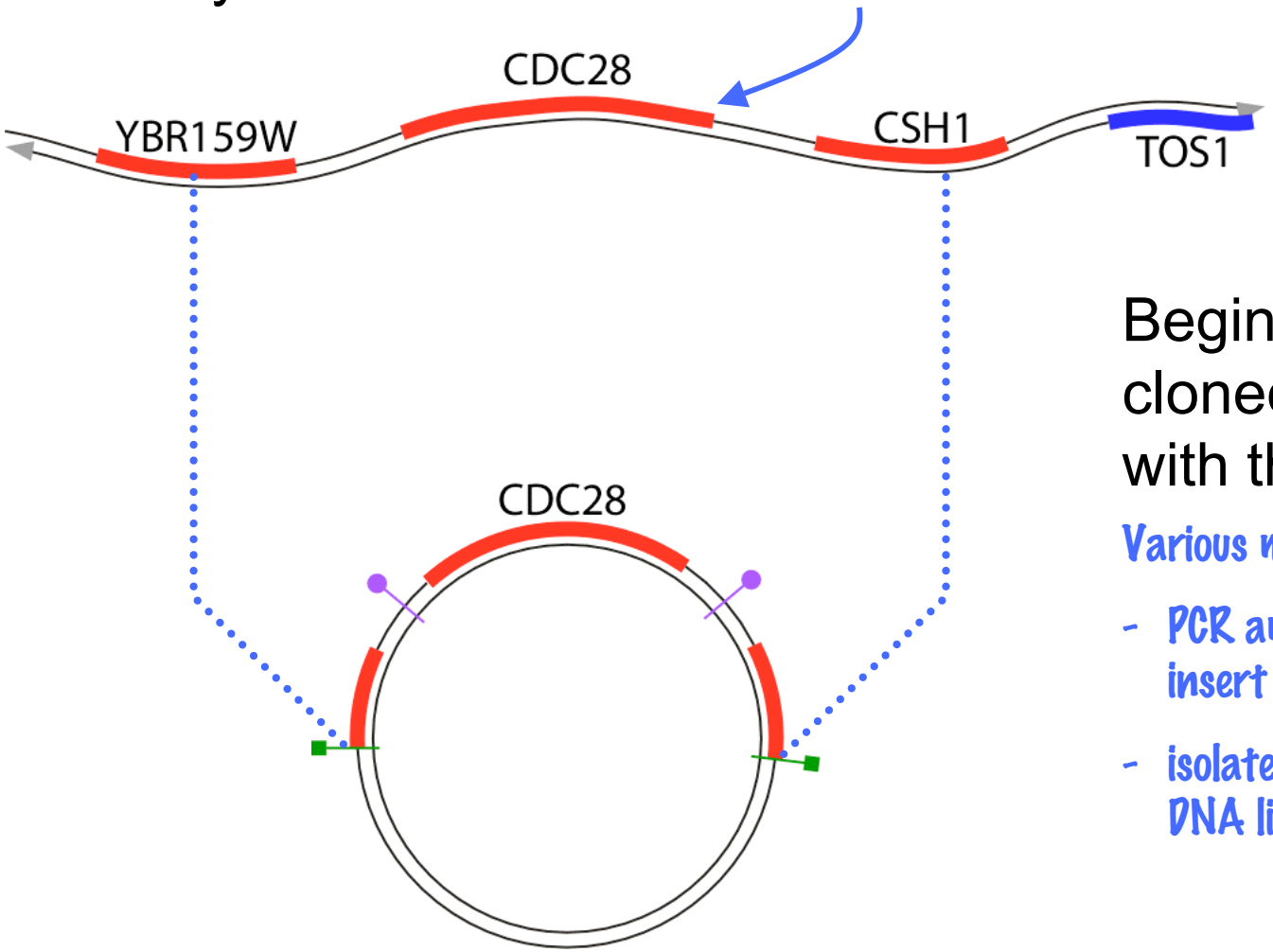
Goal?

To understand what a gene normally does, find out what happens if the gene is missing

Gene knockouts in yeast

Making a knockout ...one way to proceed:

Let's say we want to knock out this ORF



Begin with a cloned insert with this ORF

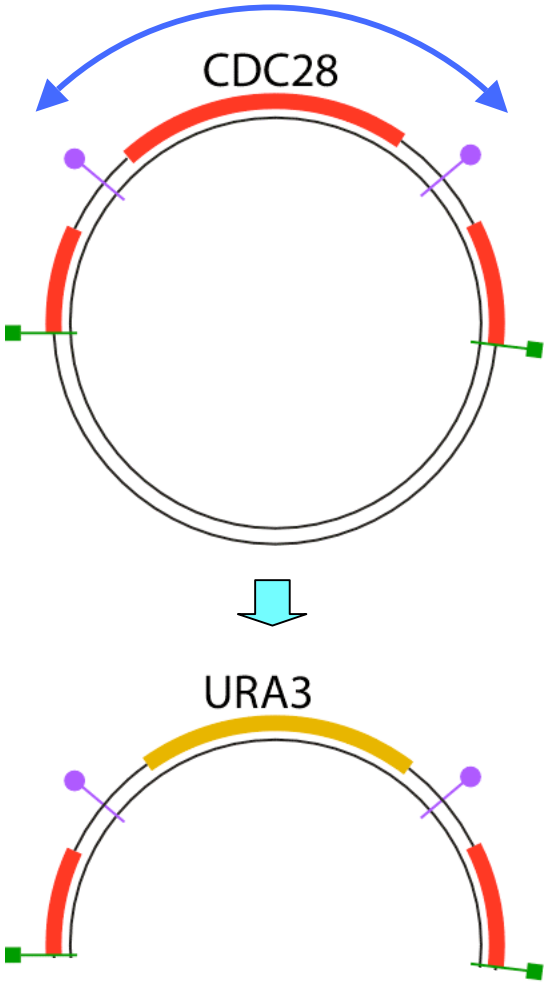
Various means...

- PCR amplify the region, insert into vector
- isolate from a genomic DNA library

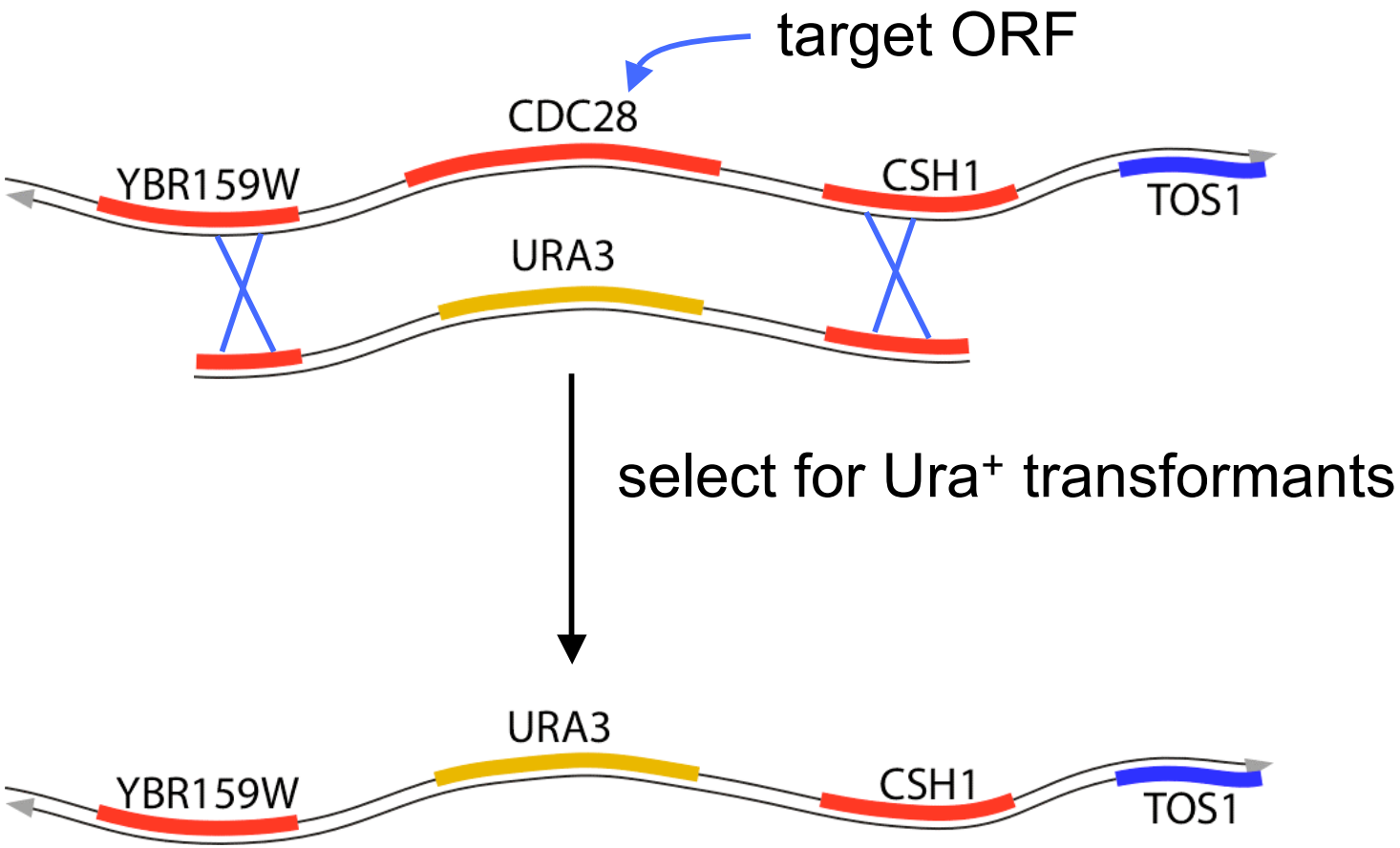
Cut out the target gene…

…and replace it with a selectable marker (e.g., URA3)

Then cut out the larger insert…



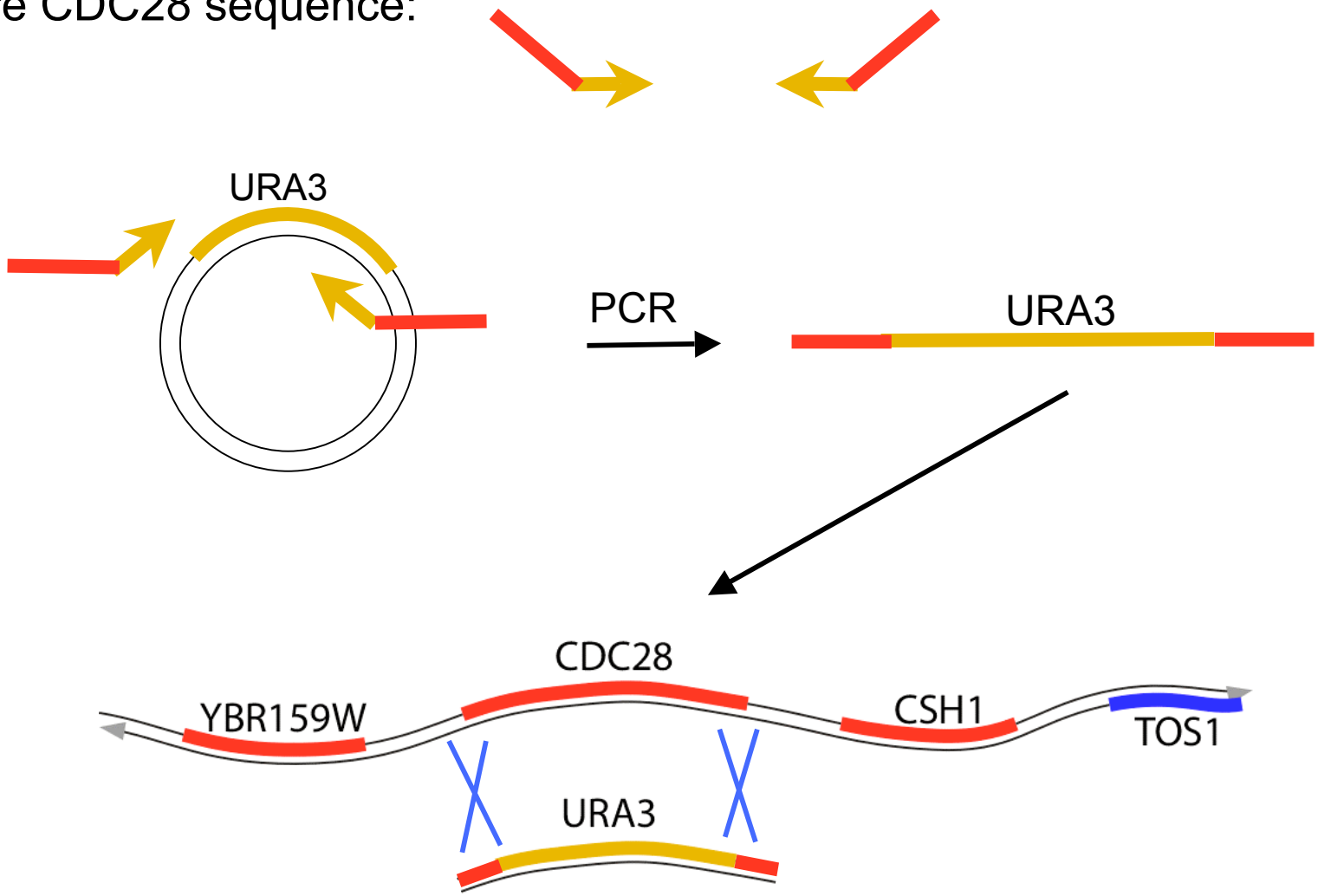
Transform **ura3** mutant yeast with this fragment



Ask… with the target gene knocked out, what phenotype do these cells have? (Besides Ura$^+$)

Another way to make the disrupted gene for a knockout

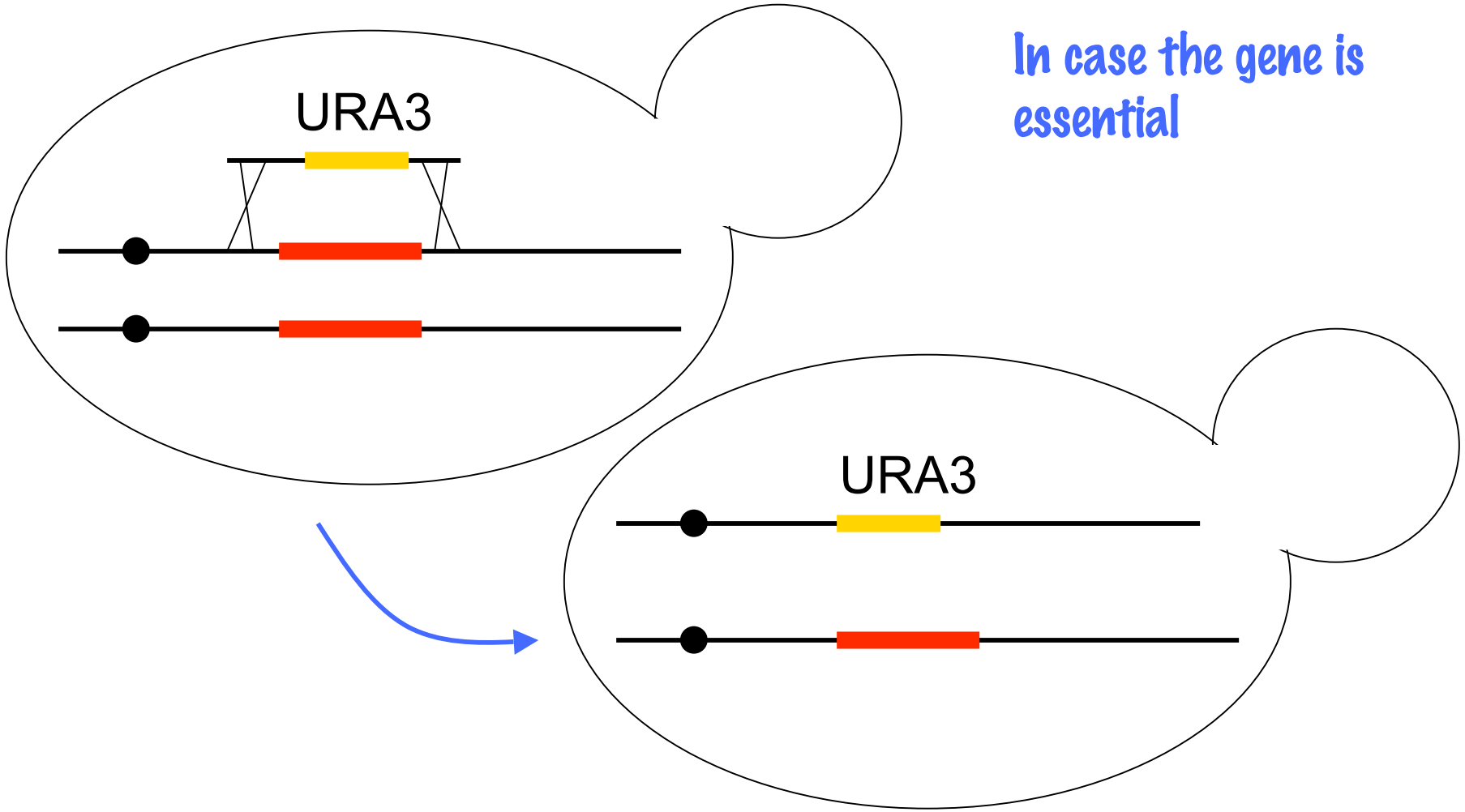
design PCR primers whose 3’ ends are URA3 sequence and whose 5’ ends are CDC28 sequence:



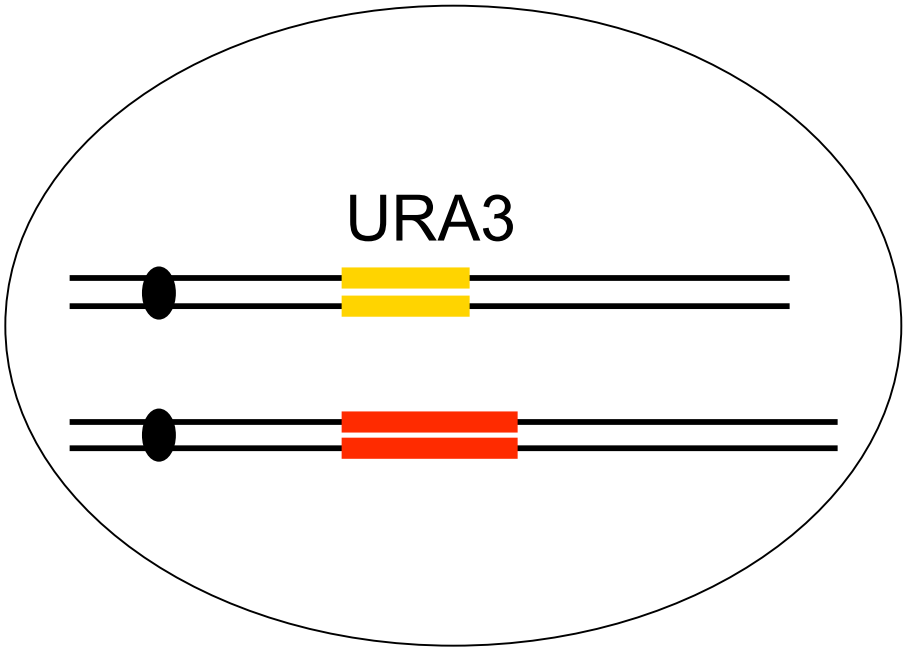
Systematic ORF deletions

The deletions must be carried out in diploids. Why?

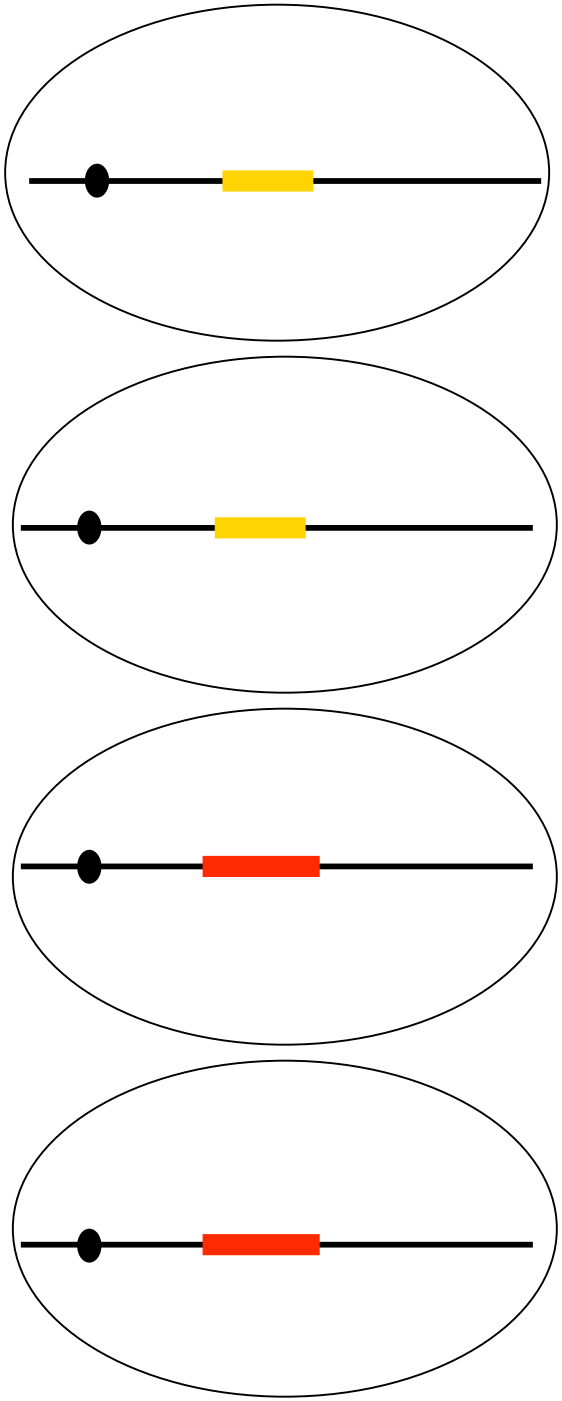
In case the gene is essential



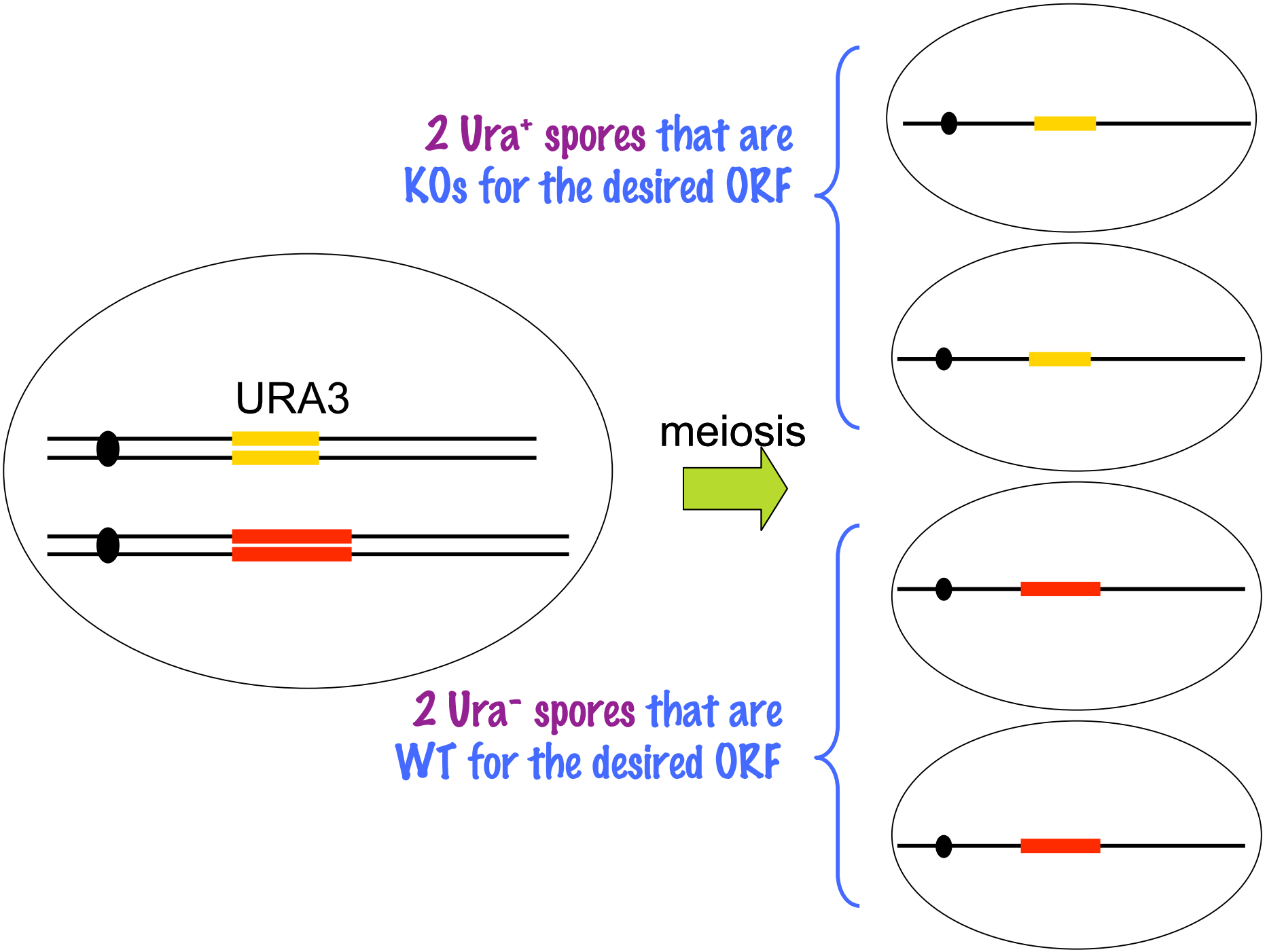
…then sporulate, look at phenotype of haploid with the knockout



meiosis



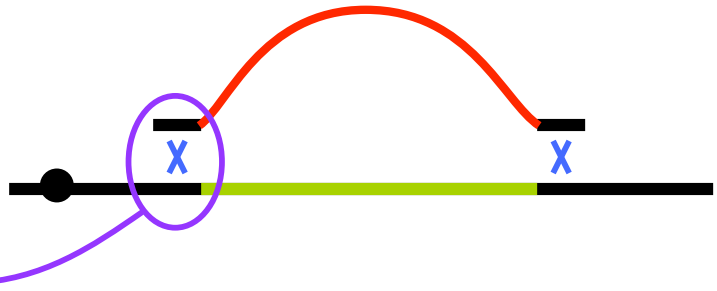
How do we know which spores have the knockout and which are wild type for the gene?


2 Ura+ spores that are
KOs for the desired ORF
URA3
meiosis
2 Ura- spores that are
WT for the desired ORF

Some notes on homologous recombination in yeast

In mitotic yeast cells . . .

1. How much homology is needed in the flanking sequences to ensure successful recombination?



~50 bp minimum

2. How perfect does the homology have to be?

Pretty close, 1 mismatch in 100 bp can reduce efficiency to 1/10 the level when no mismatches.

3. How big can the heterologous part be and still get successful recombination?

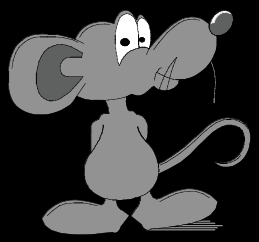
Pretty big (30 kb); but the bigger the difference the lower the frequency.

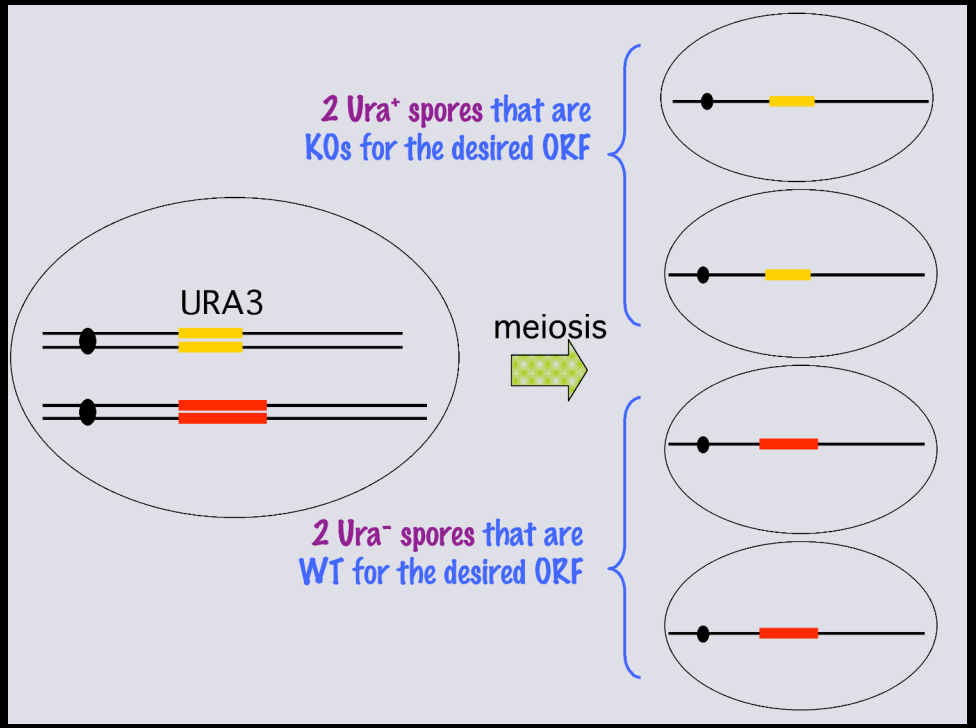
Practice question

From QS:

After analysis of many such tetrads, "good" wine production under the conditions tested was mapped to a single gene, *ASP1*, encoding an enzyme called asparaginase, which is needed for growth when the only source of nitrogen is the amino acid asparagine. The bad wine strain has a wild type allele while the good strain has a complete LOF allele of this gene. The involvement of *ASP1* in wine quality is just a hypothesis, based on a correlation between the genotype and the phenotype. Outline an experiment that you could do (perhaps using a DNA library) to test the hypothesis.

Gene knockouts in mice





Making a mouse knockout

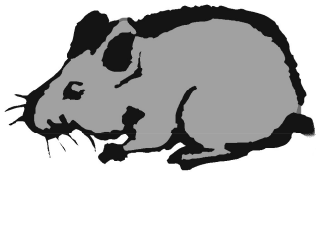
e.g., test hypothesis that gene YFG2 is needed for tail growth

(not a real gene!)

$\frac{YFG2^+}{YFG2^+}$  → $\frac{YFG2^+}{yfg2\text{-}ko}$ 

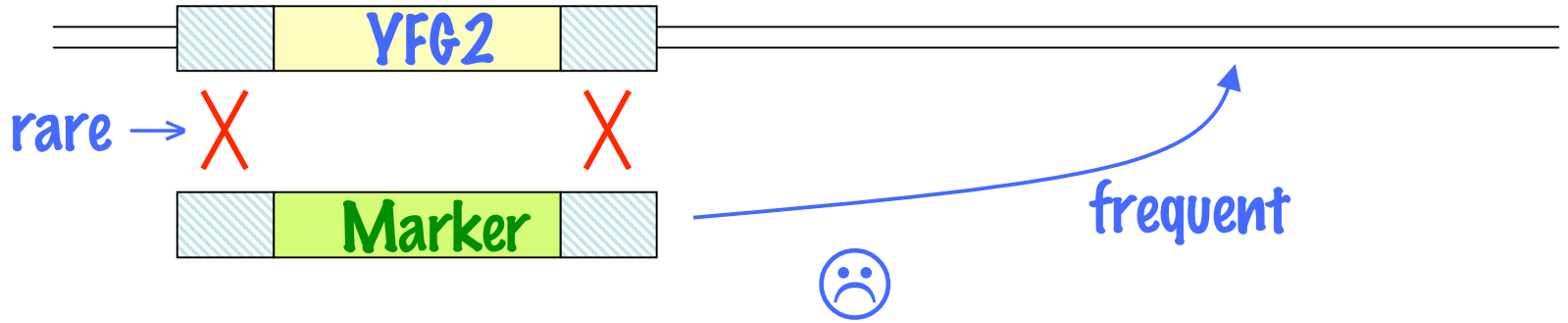
Make crosses between the heterozygotes...

identify the knockout-homozygotes, look at their phenotypes

?   ?

Problems doing a knockout in mouse

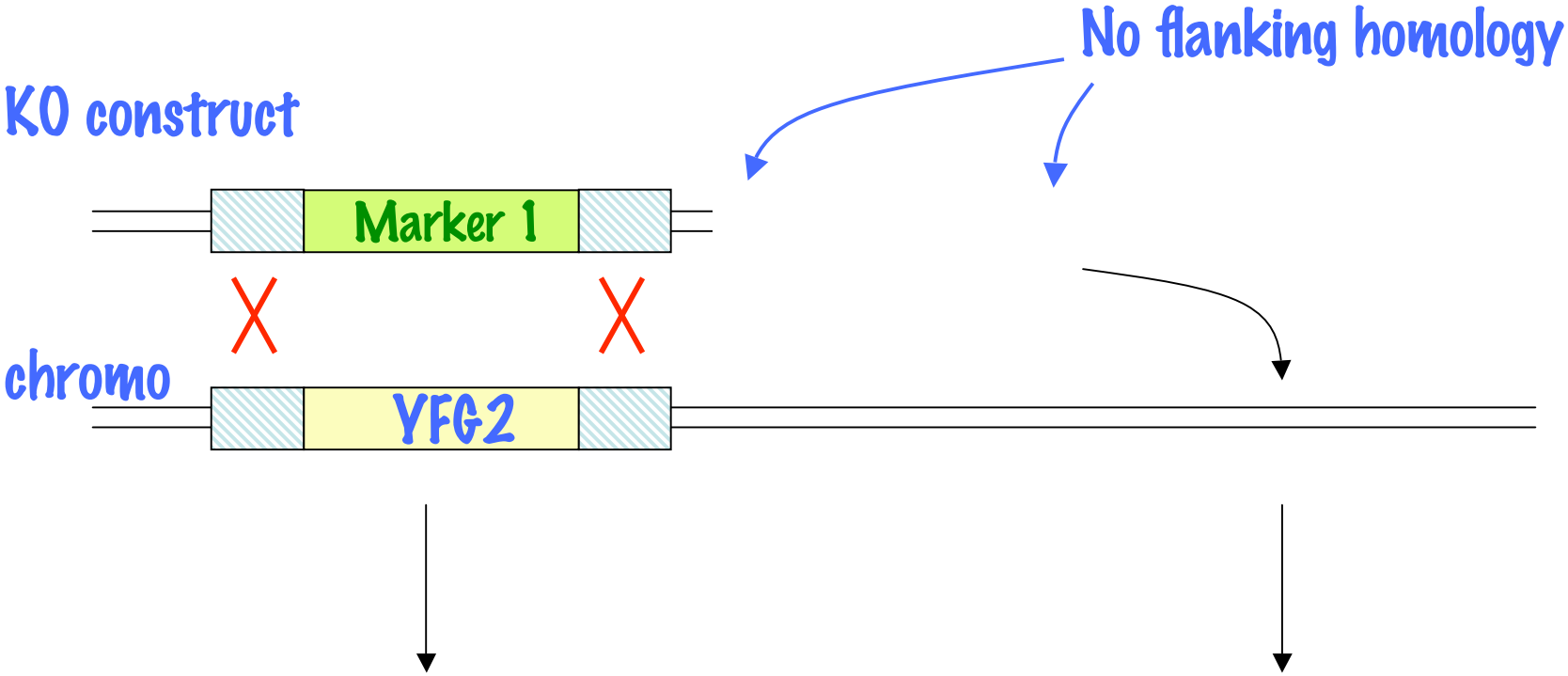
Random **insertion** of DNA much more frequent than homologous recombination



Solution?

Aside: Random insertion of the knockout allele somewhere in the genome… why is that not good enough?

How to select for homologous recombination?



outcome for homologous recombination?

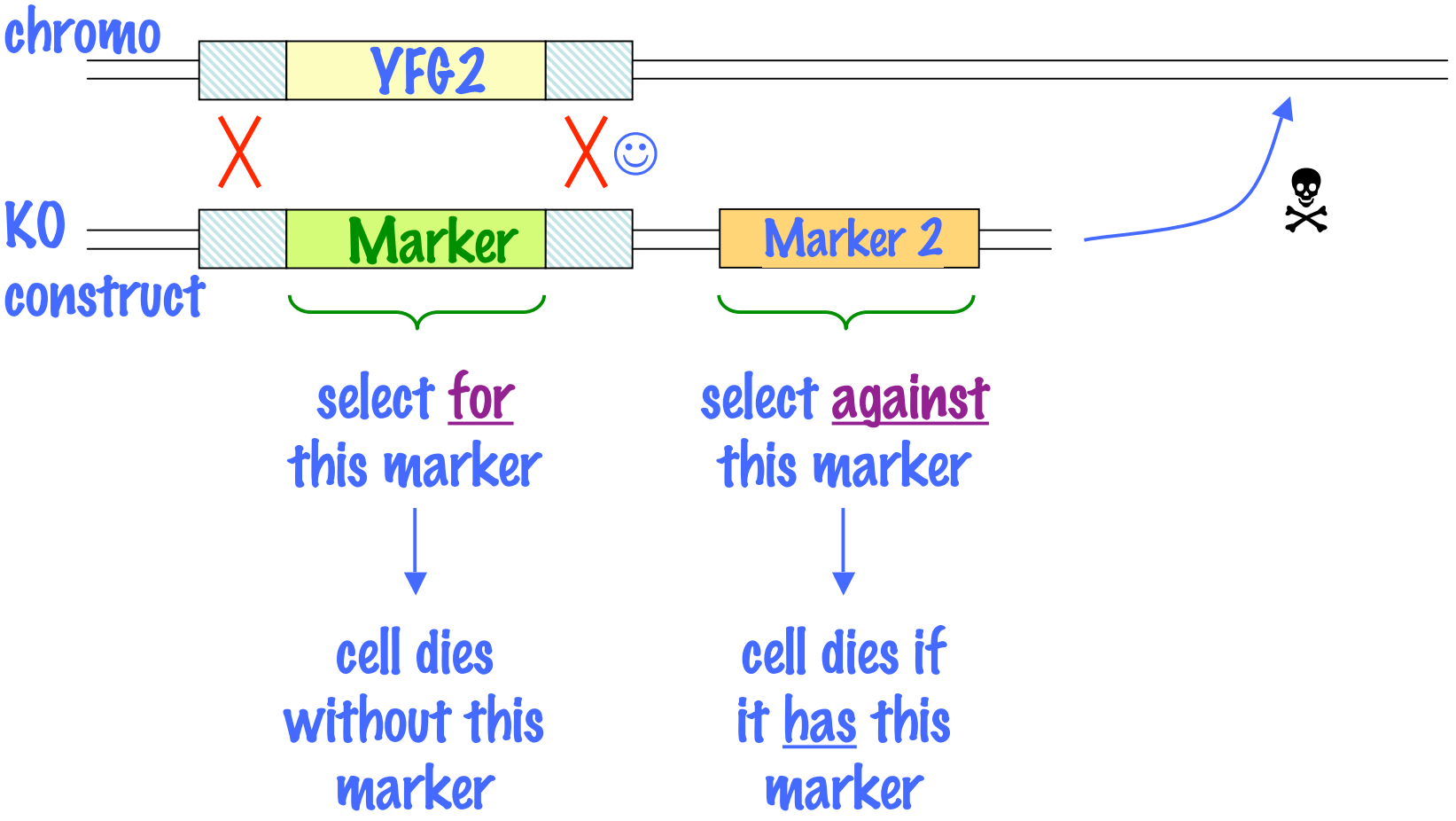
only Marker 1 is integrated

outcome for random insertion?

Both Marker 1 and Marker 2 are integrated

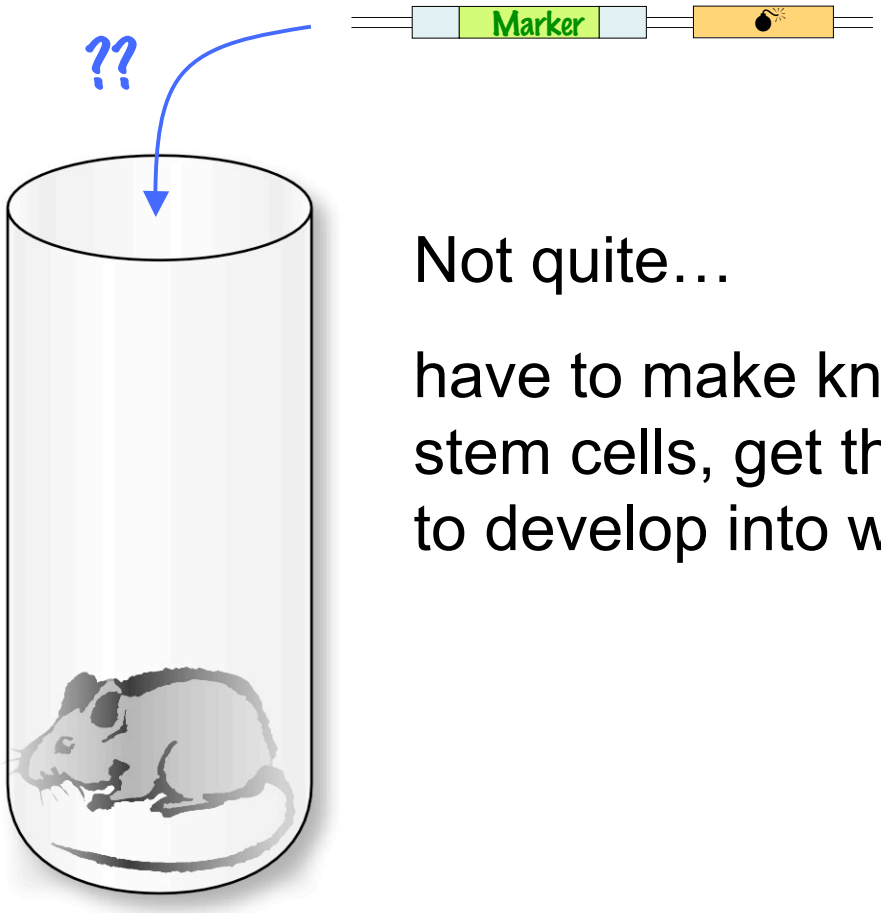
Selecting for homologous recombination

Select for homologous recombination by including **negative selection**



How to transform a whole mouse?

All cells in the mouse must have the knockout allele!

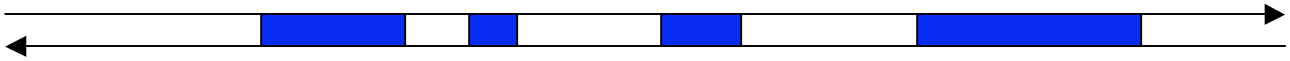


Not quite…

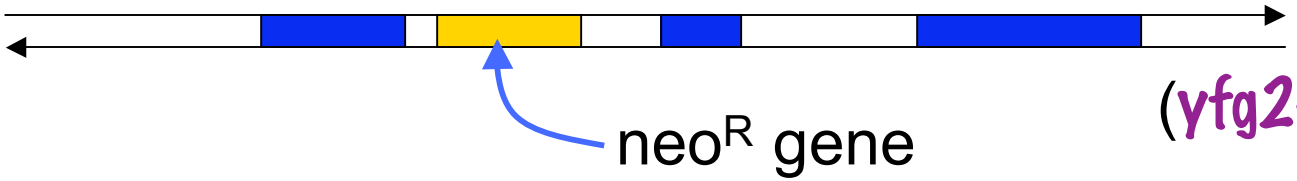
have to make knockout in stem cells, get the stem cells to develop into whole mice…

Making the mouse KO allele

Mouse gene to be knocked-out (YFG2).

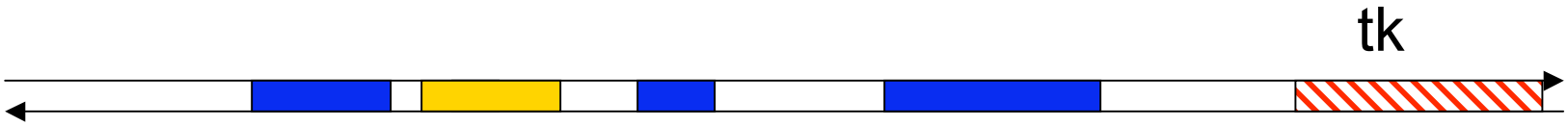


Replace part of the gene with the neo^R gene (for selection in mouse cells with the drug neomycin).



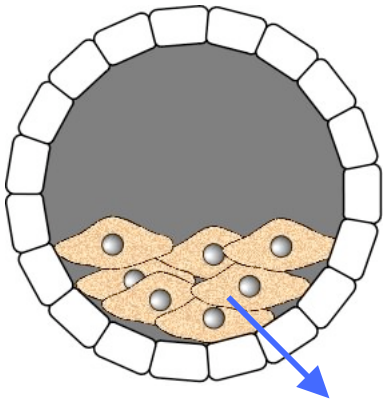
(yfg2-KO allele)

Add the thymidine kinase gene (tk) from herpes virus to the end of the construct.

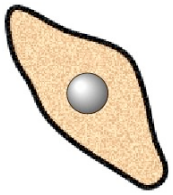


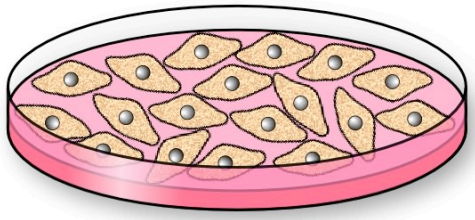
DNA is ready to go into mouse cells.

Mouse ES cells



blastocyst



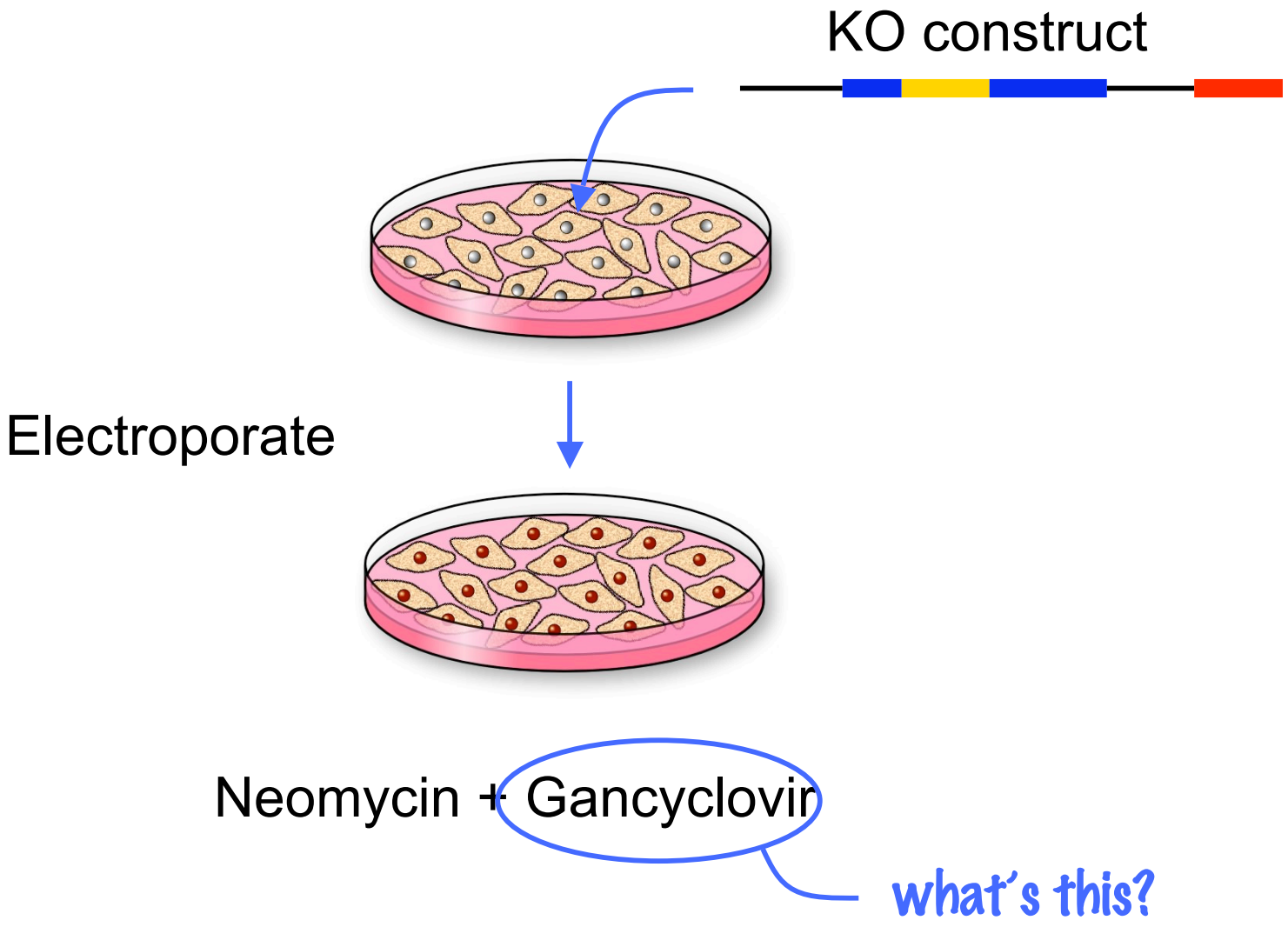


embryonic stem cells

Genotype of the ES cells is:

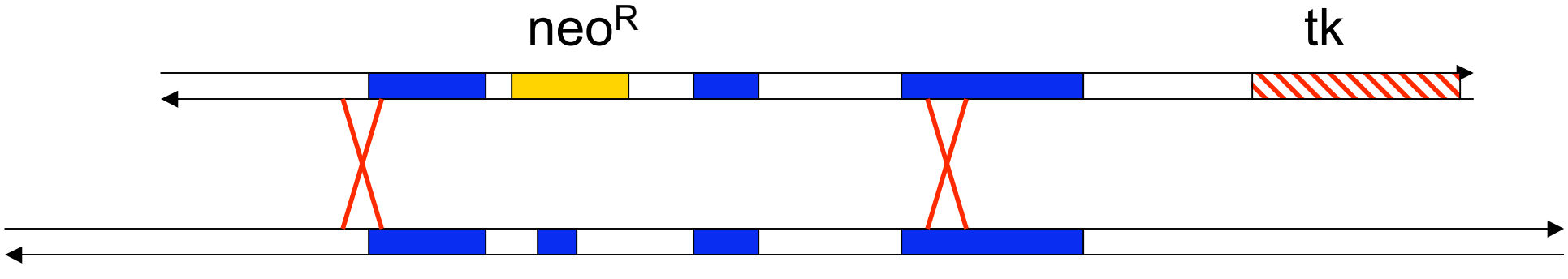
YFG2/YFG2

Transfer of DNA to ES cells



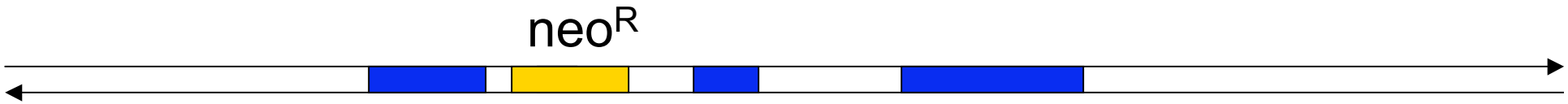
Homologous recombination in ES cells

A rare event . . .



Wild type allele in the chromosome.

Plate cells with neomycin (selection for neo^R) and with gancyclovir (selects against tk).



KO allele replaces **one** wild type allele

Genotype: YFG2/yfg2-KO

The need for positive and negative selection

Neomycin selects **for:** cells that have taken up the KO-DNA

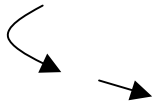
Gancyclovir selects **against:**

cells that have randomly inserted the KO-DNA

Clones of ES cells that grow in neomycin + gancyclovir are assayed by Southern blotting or PCR to see which ones have the correct gene replacement.

[How exactly would you do this test? What diagnostics would you look for?]

…and then injected into recipient blastocyst



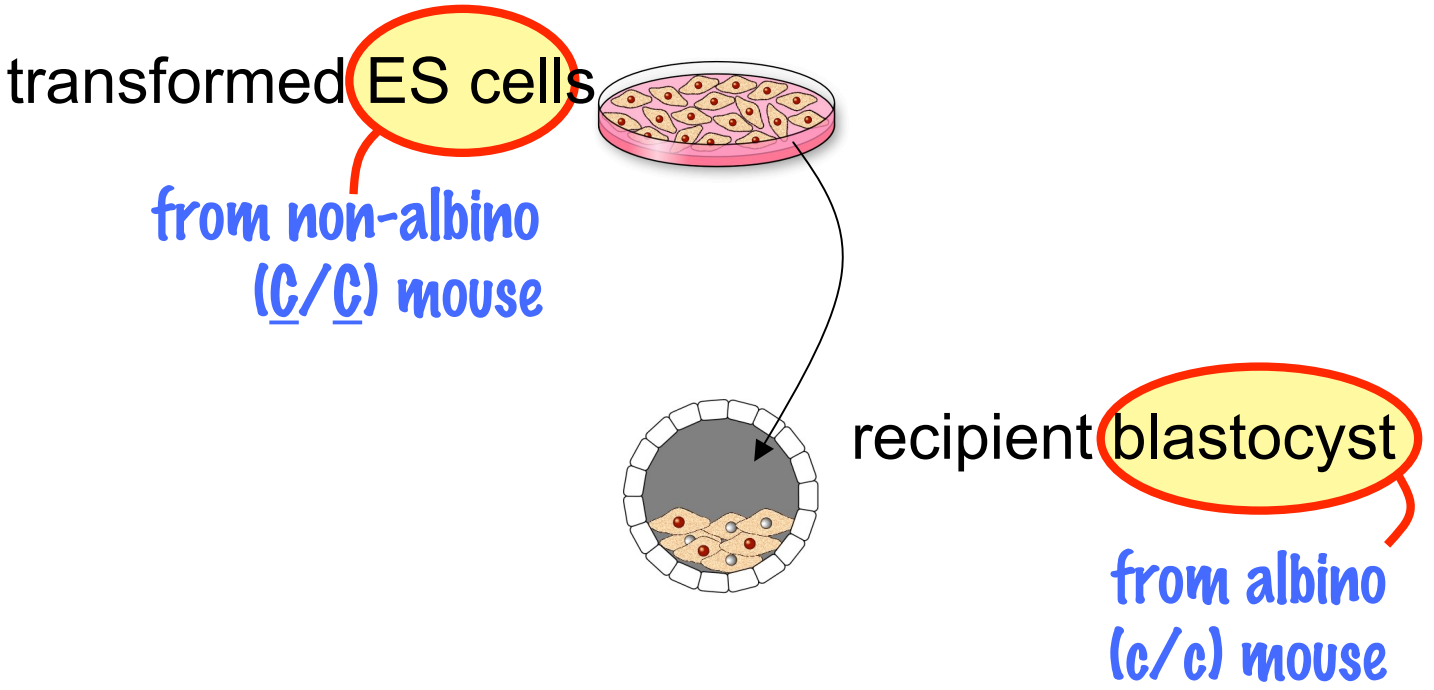
develops into mouse with yfg2-ko

Issues…

» Will all cells in the resulting mouse be transformed?

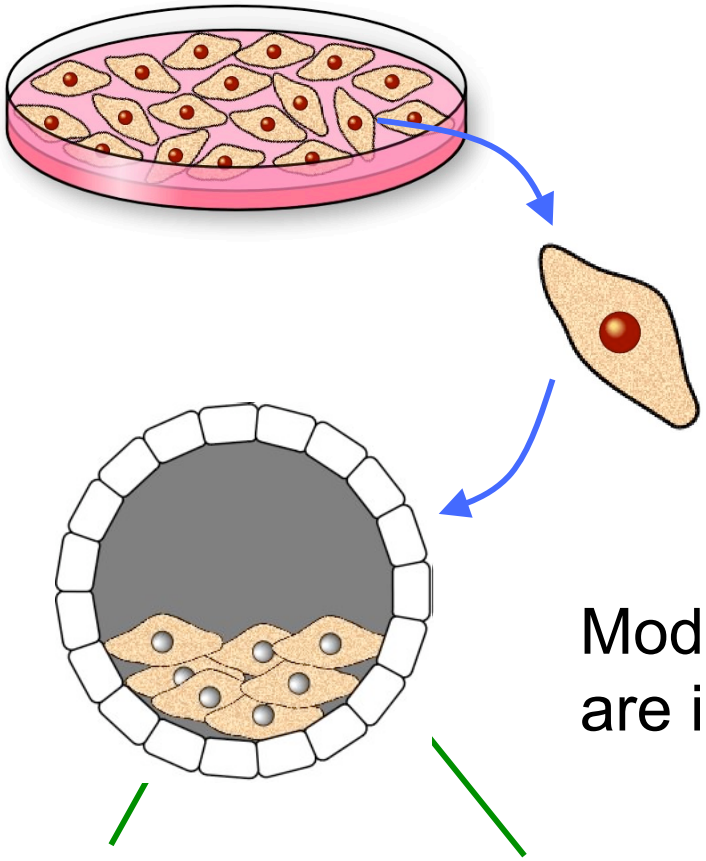
No. (The mouse will be chimeric… stay tuned.)

» How to tell which mice developed from modified blastocysts?



Returning the modified ES cells to an embryo

Two pure-breeding **albino** mice are mated and blastocysts are harvested from the pregnant female.



Modified ES cells are injected

Some cells are C/C; YFG2/yfg2-KO

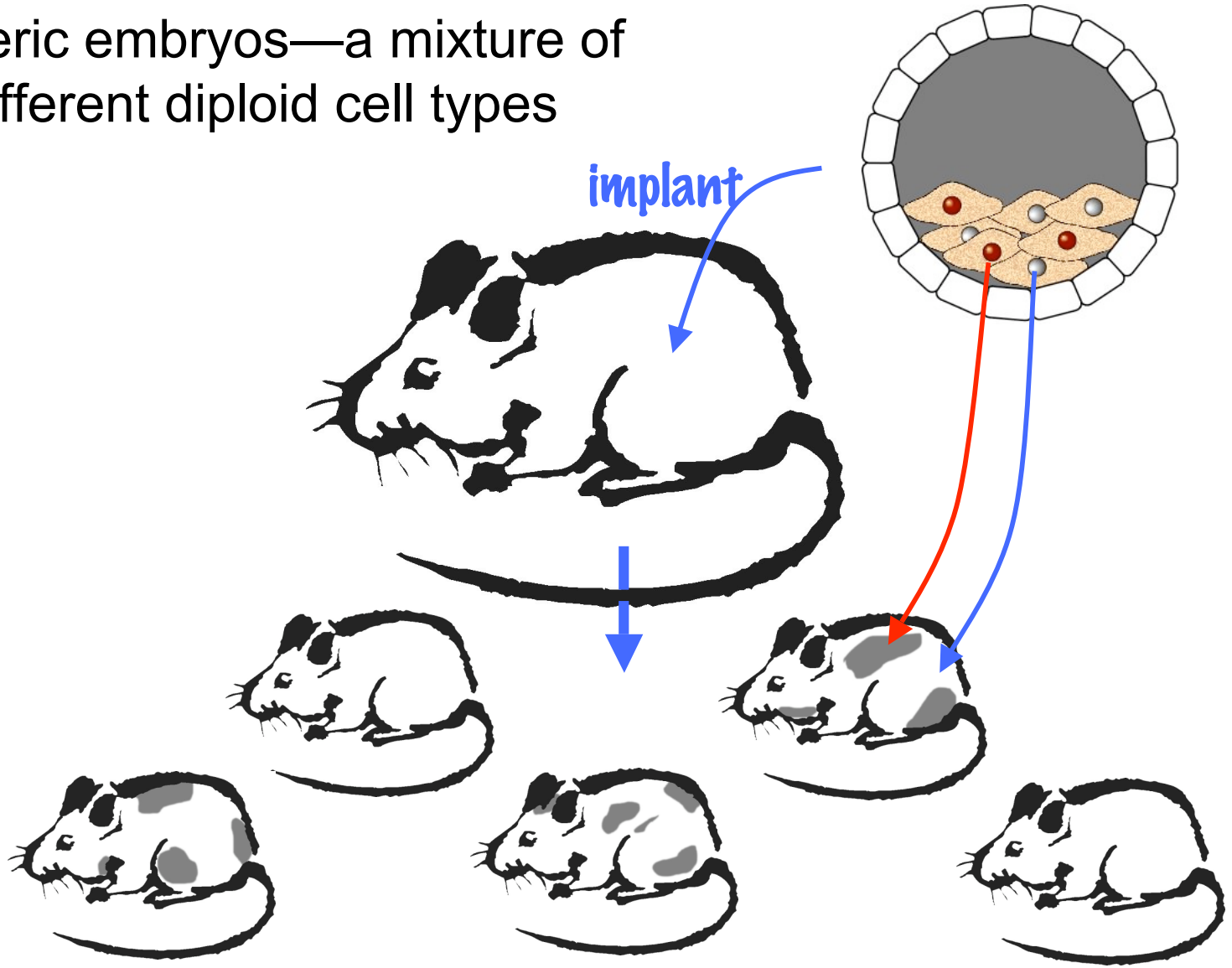
Some cells are c/c; YFG2/YFG2

Why albino?

To distinguish host from knockout products

Returning the embryos to an albino female

Chimeric embryos—a mixture of two different diploid cell types



How to get from here…

All of the tissues could be chimeric; even the germ cells!

…to here?

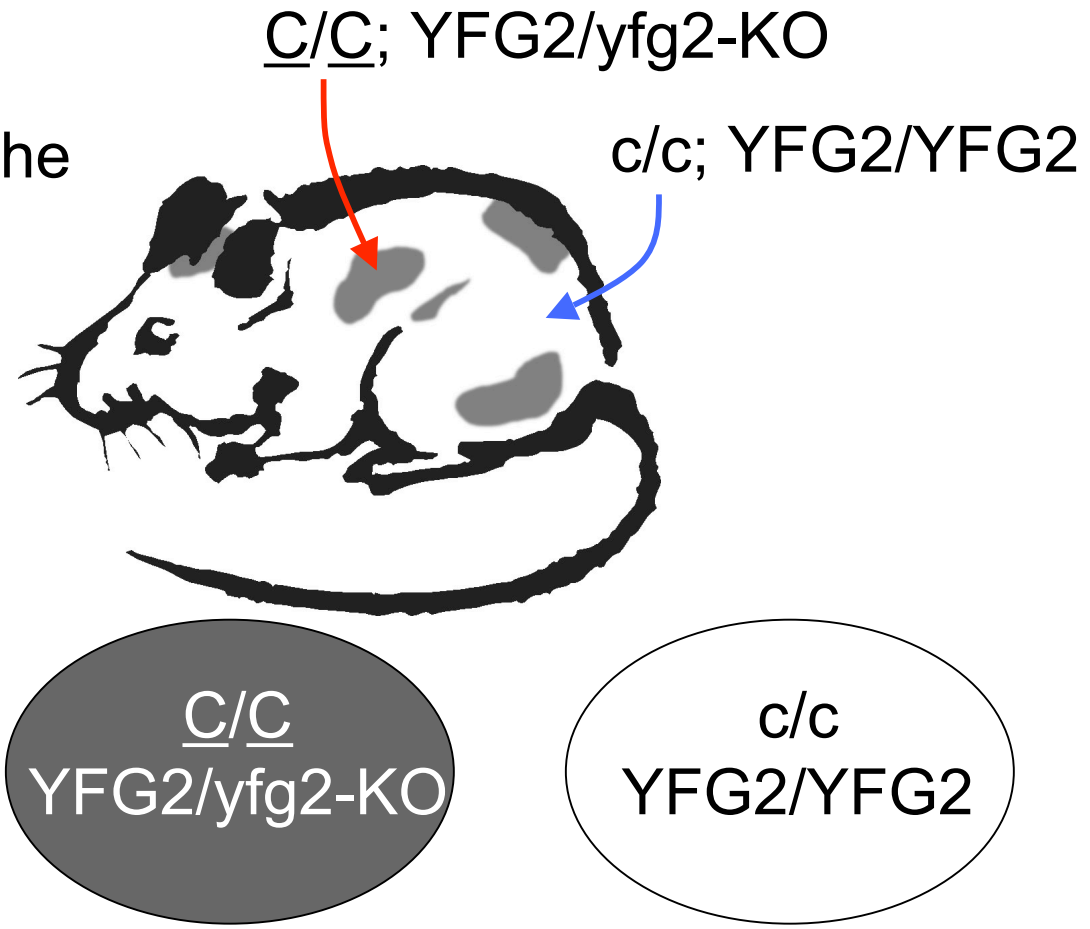
Mate the chimeric mouse to an albino →

Pick fully pigmented offspring

Looking for germ-line transmission (example)

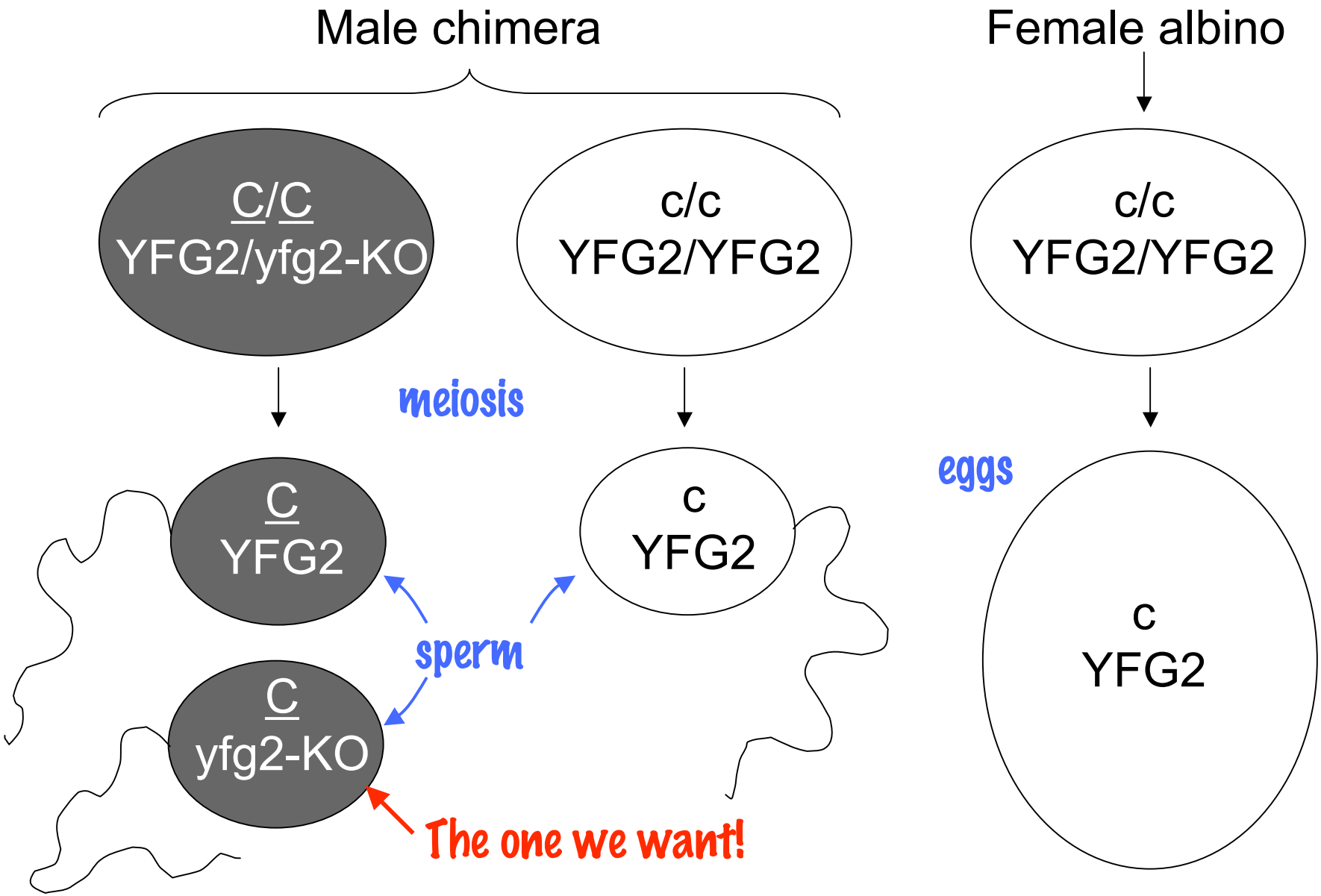
Mate this chimeric male to **albino** females and look for **non-albino** offspring.

Q: Are there KO cells in the gonads?



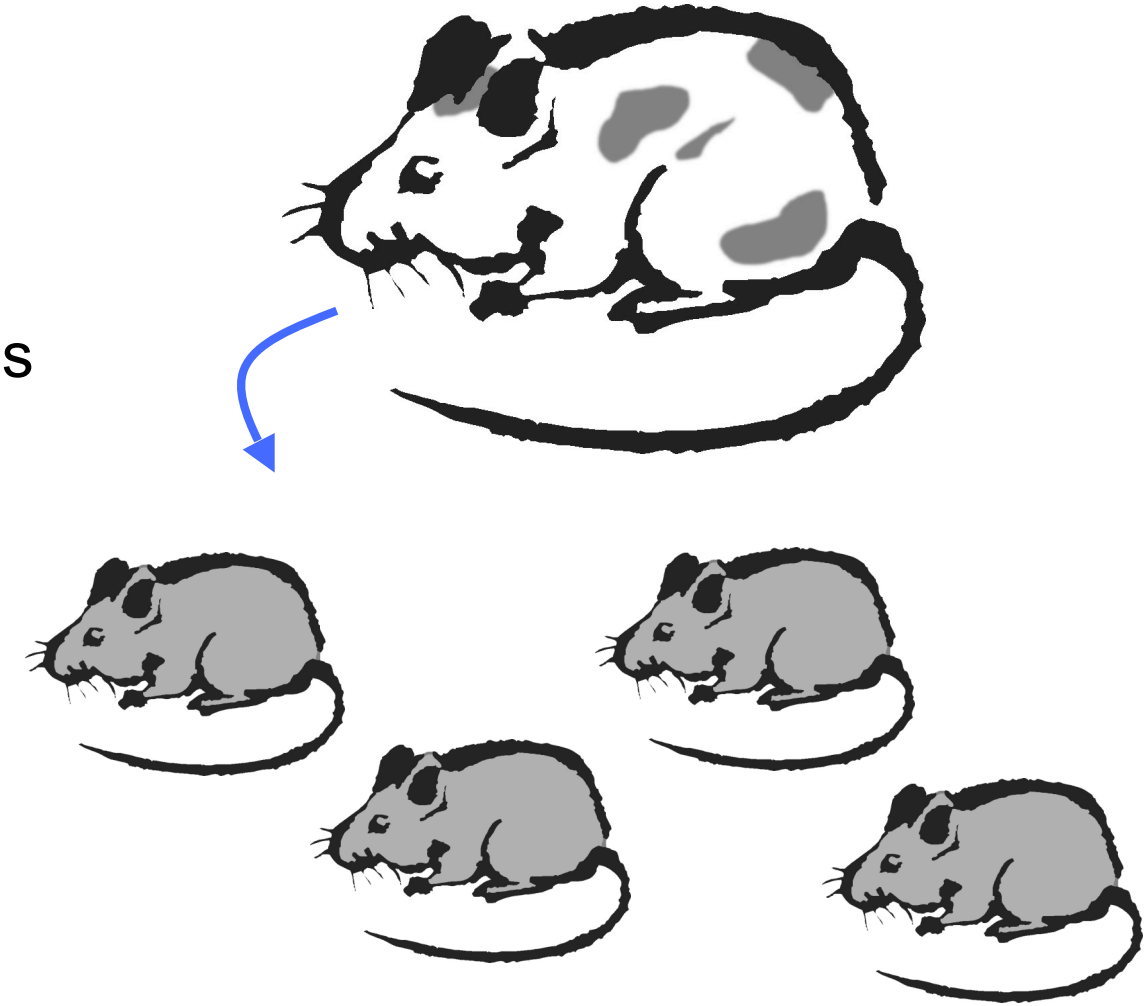
This male could have two different types of cells undergoing meiosis

Predicting the offspring from the chimera



Which of these mice have the KO allele?

Possible genotypes of the **non-albino** offspsring:



C/c; YFG/YFG

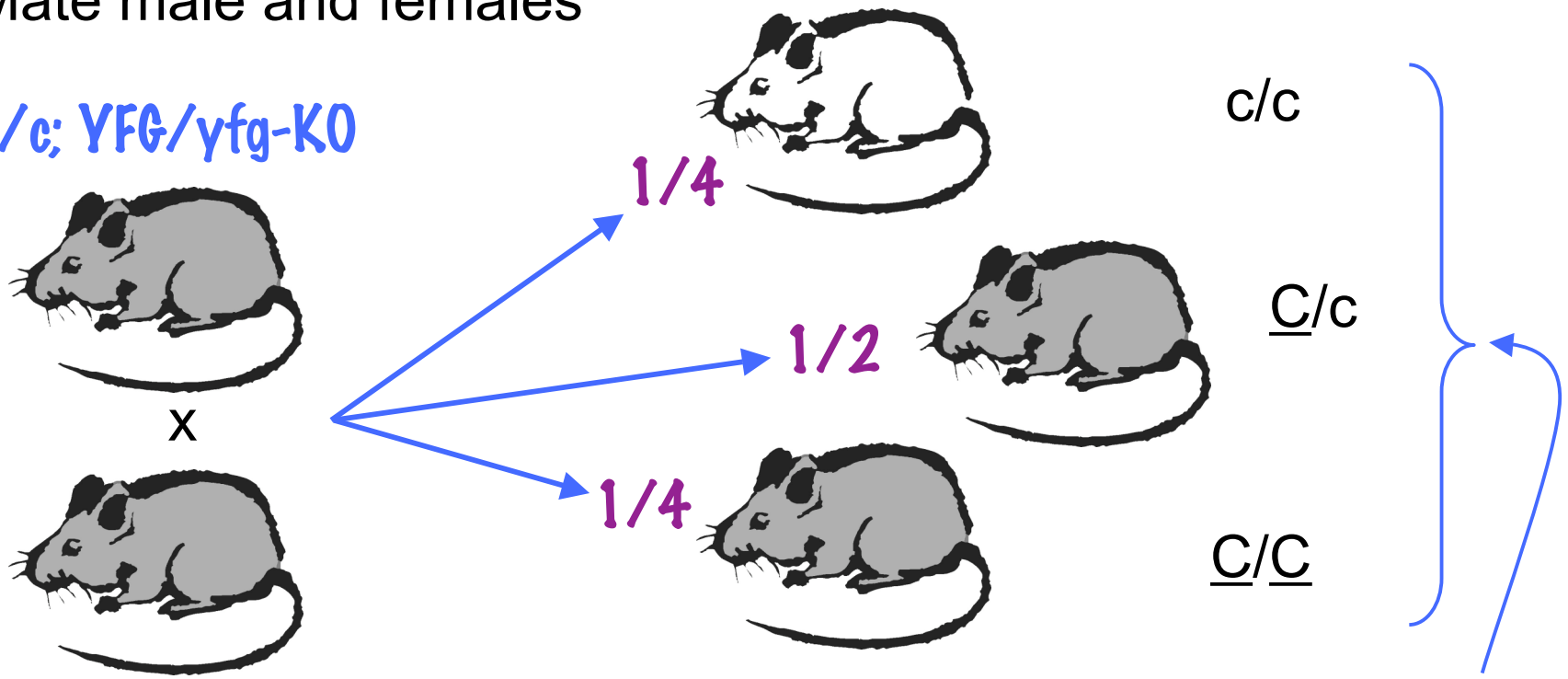
C/c; YFG/yfg-KO

Which one is which? How would you test them?

Finally . . . Homozygous knockouts?

Mate male and females

C/c: YFG/yfg-KO



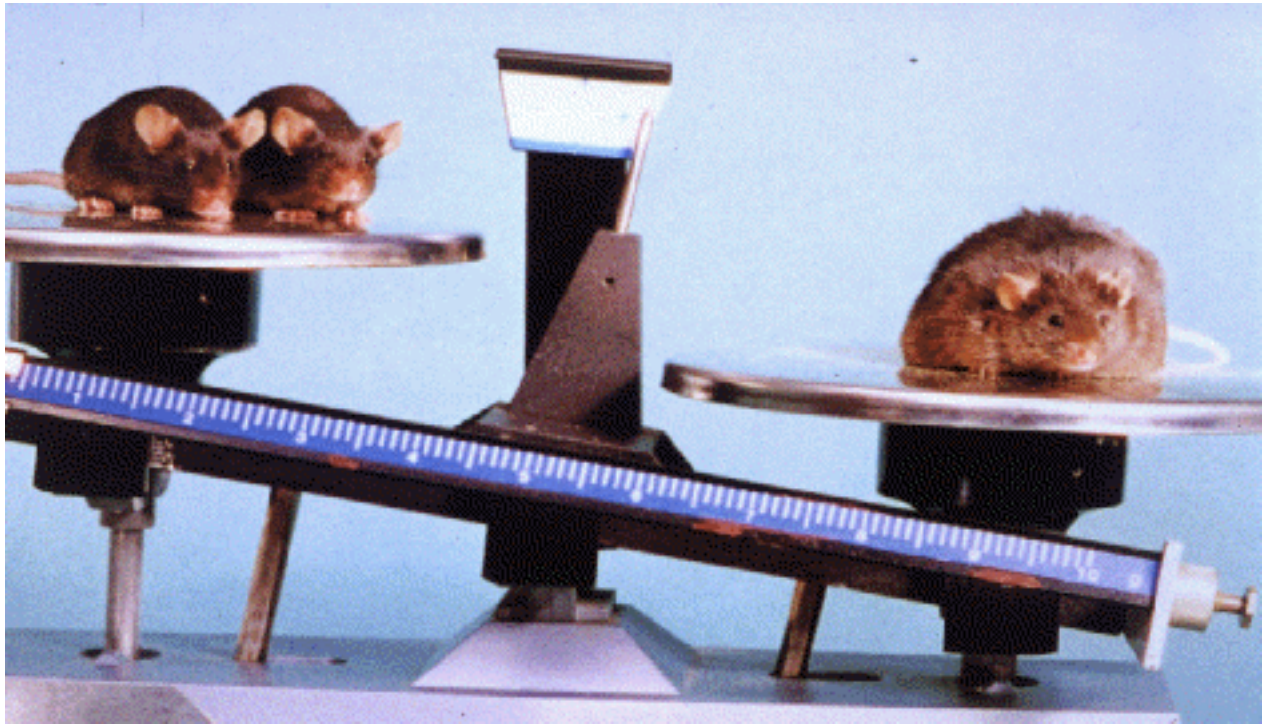
Among the offspring,
there should be 1/4 that are homozygous yfg-KO/yfg-KO

How do you find them?

Q. What phenotype results?

Some KOs have obvious phenotypes

The IL-6 KO mouse is obese.



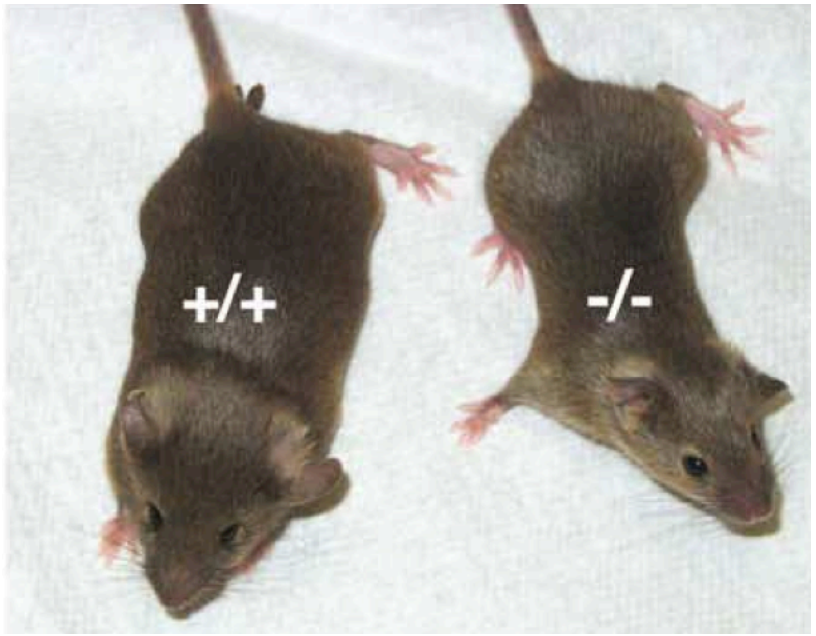
Some KOs have suprising phenotypes

Current Biology, Vol. 13, 1775–1785, October 14, 2003, ©2003 Elsevier Science Ltd

Cdk2 Knockout Mice Are Viable

cyclin gene

Cyril Berthet,[1,3] Eiman Aleem,[1,3]
Vincenzo Coppola,[2] Lino Tessarollo,[2]
and Philipp Kaldis[1,*]



...but are sterile

Working hypothesis: Cdk2 is needed primarily for meiosis, not mitosis

Some KOs have no apparent phenotypes

??

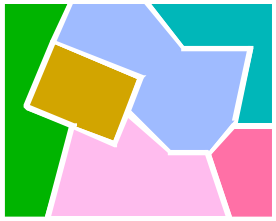
Conclusions about deletions with no phenotype?

If a knock-out has wild type phenotype, does that mean . . .

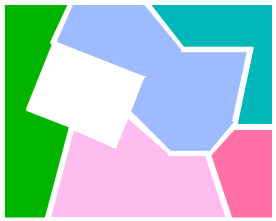
1. the gene doesn't do anything? under test conditions
2. there is a second gene carrying out the function?

3. there is a complex of proteins where any one protein is dispensable but the complex is needed?

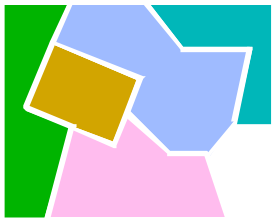
Wild type complex of 6 proteins



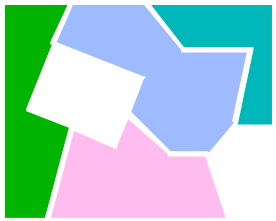
Loss of one component—complex is still functional



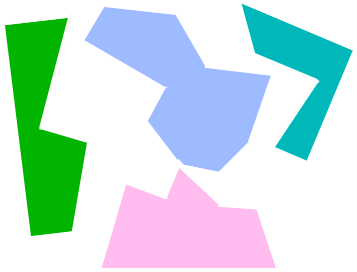
or



But loss of two components results in a nonfunctional complex



complex falls apart



Hypotheses 2 and 3 predict that certain double mutants would show a phenotype.

Testing for redundant mechanisms: "Synthetic effects"

knockout of gene 1 doesn't give phenotype

knockout of gene 2 doesn't give phenotype

knockout of both gene 1 and gene 2 results in a phenotype