Gene Inactivation

Michael Snyder

April 1, 2009

Advantage

- Arguably the best way to learn about gene function

Problems

- No phenotype
- Effects might be indirect

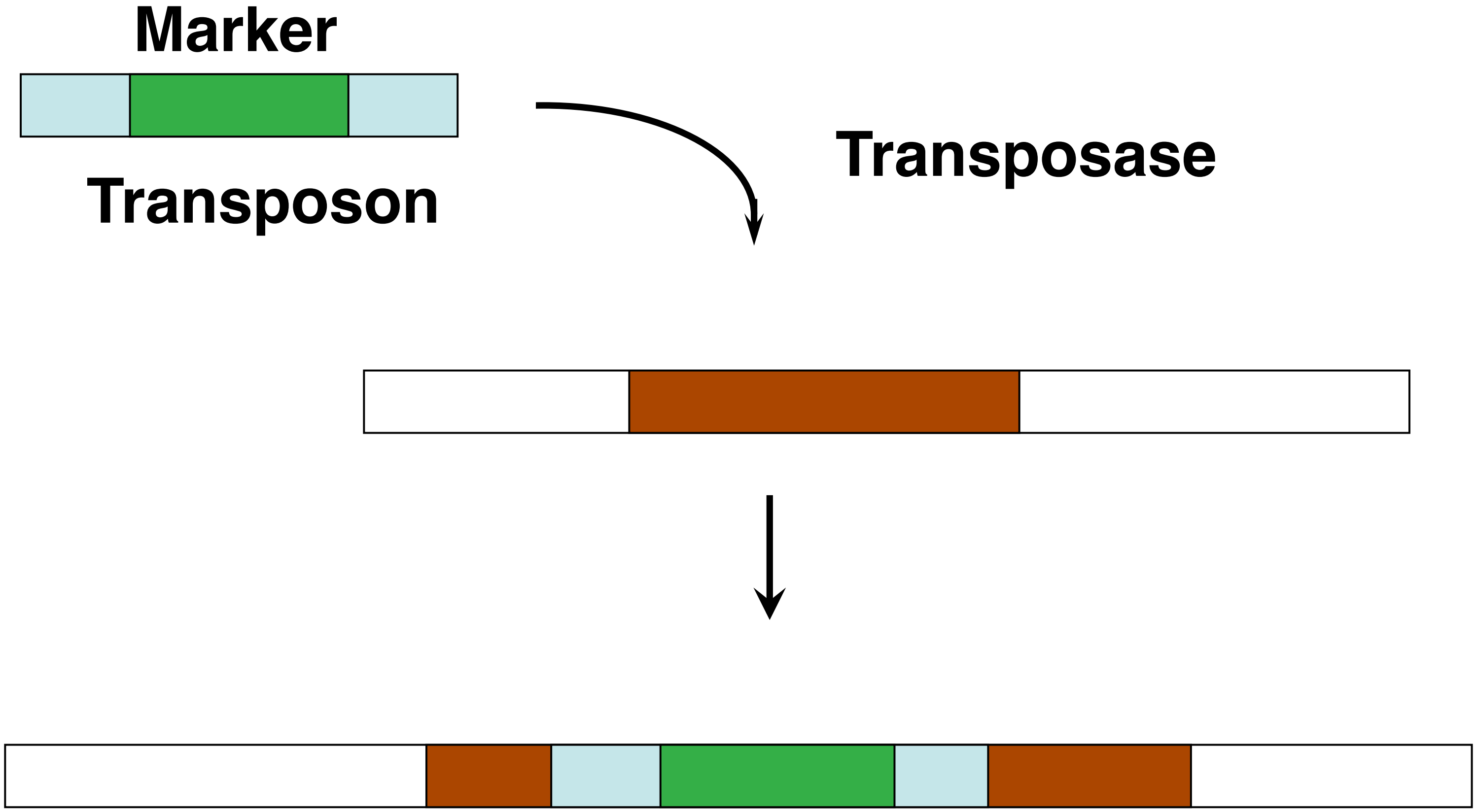
Three General Methods

1) Insertional Mutagenesis
    - Transposon Strategies
    - Insertional Mutations

2) Systematic Knockouts
    - Selectable Marker Replacement

3) RNAi

Transposon Knockouts



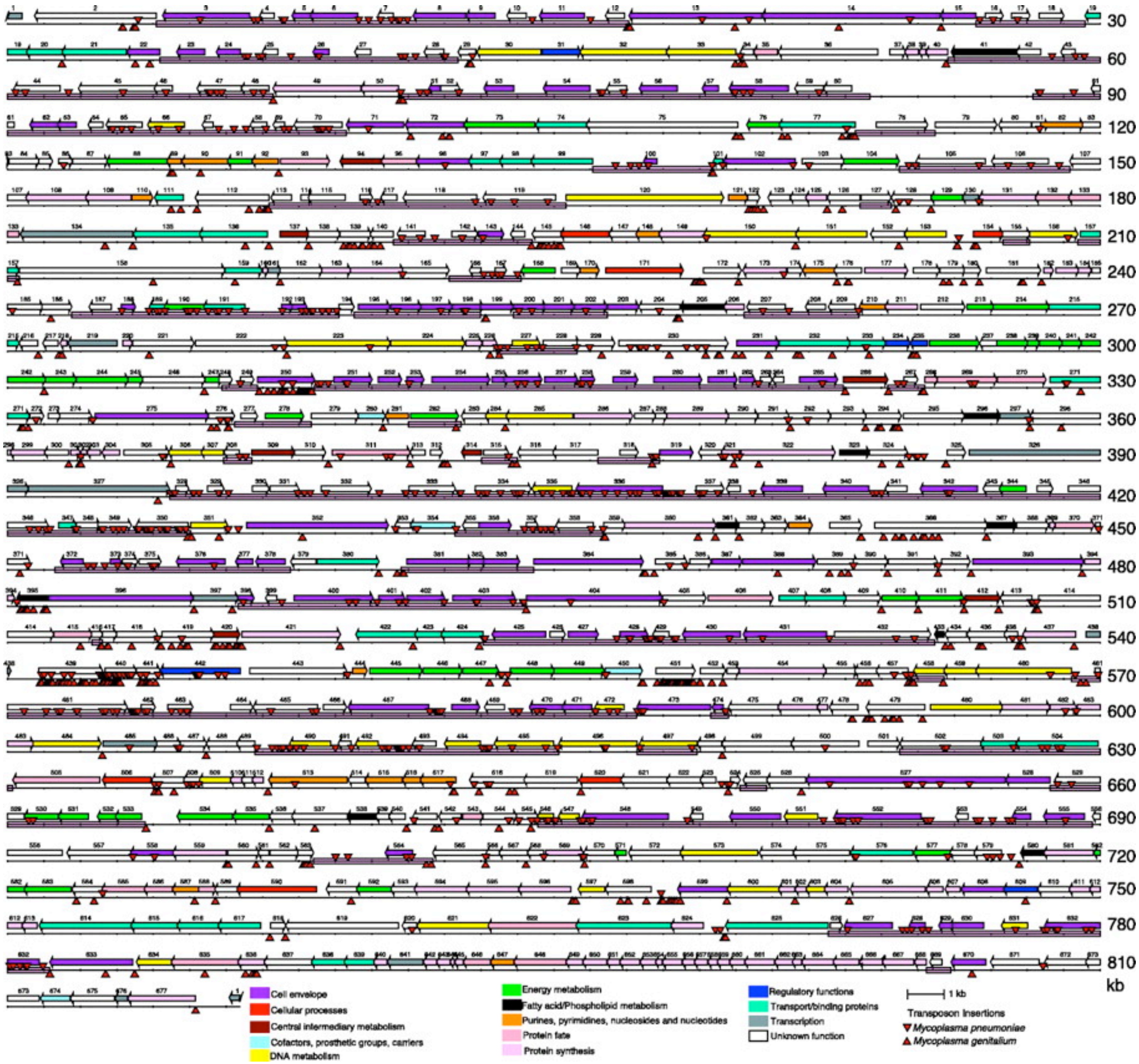
Marker

Transposon

Transposase

Tn Mutagenesis of Mycoplasma Genitalium

(480 Genes)



Method:
Mutagenize Genome
With Library of
Mutations

↓

See What Genes
Obtain Mutations

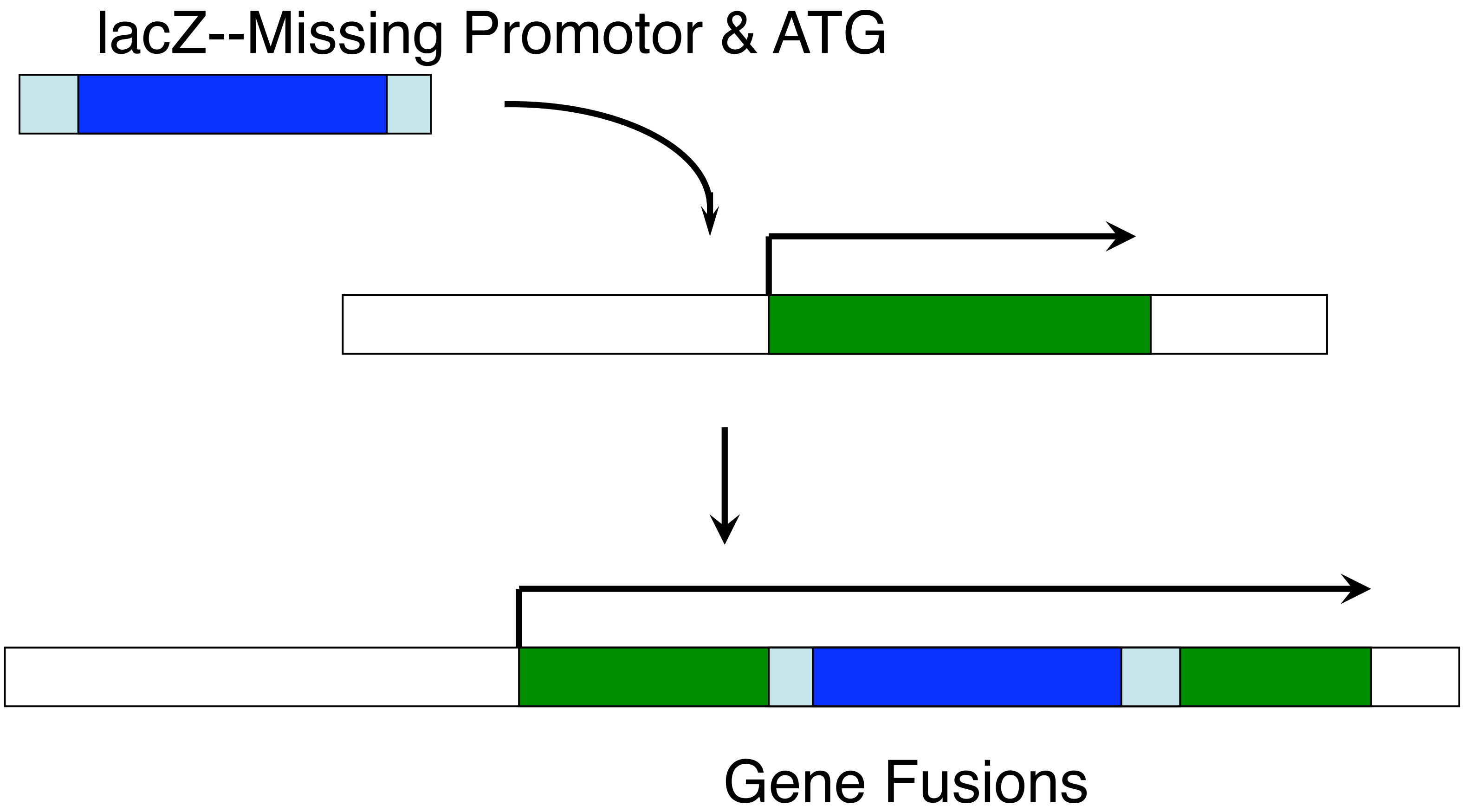
↓

Deduce the Rest
(265-350) Are
Esssential for
Viability

▲ = Insertion Allele

Hutchison et al., Science 286, 2089 (1999)

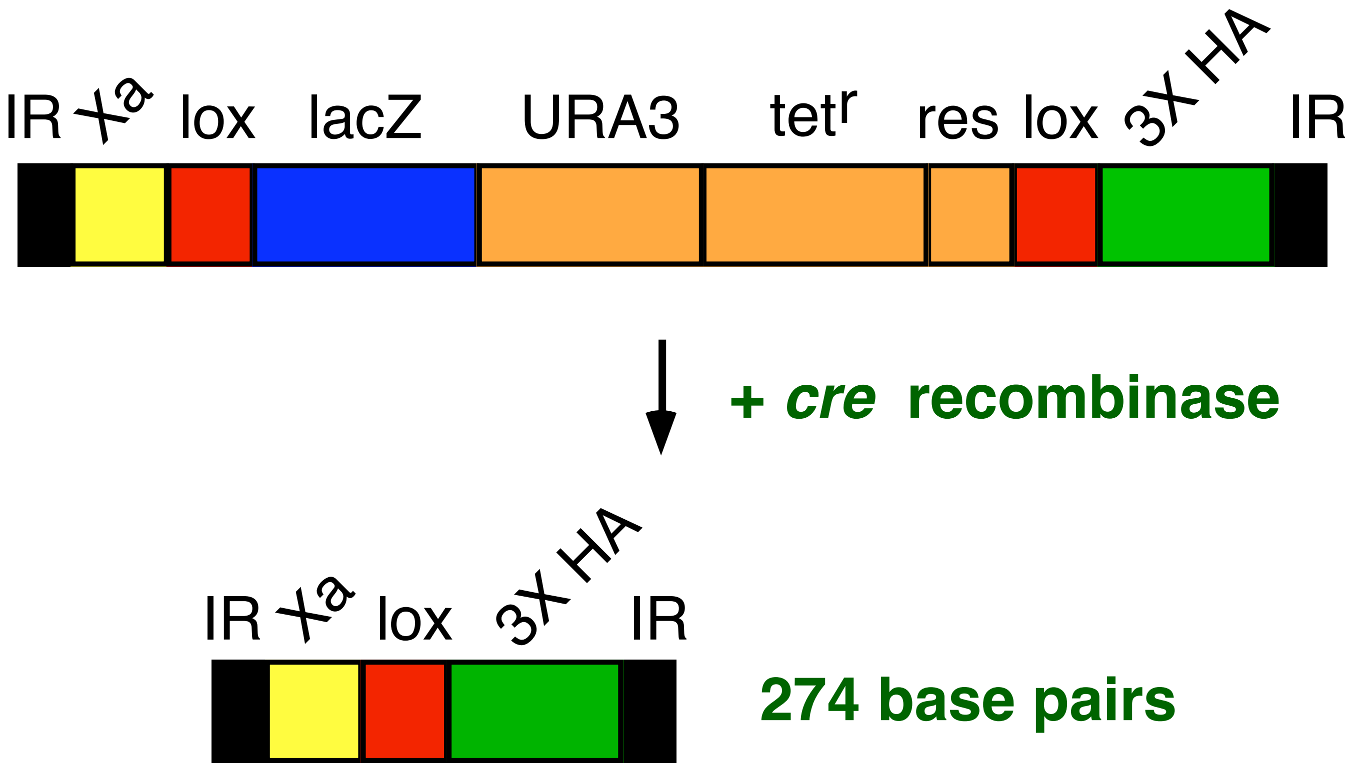
Gene Traps: Gene Fusion



lacZ--Missing Promotor & ATG

Gene Fusions

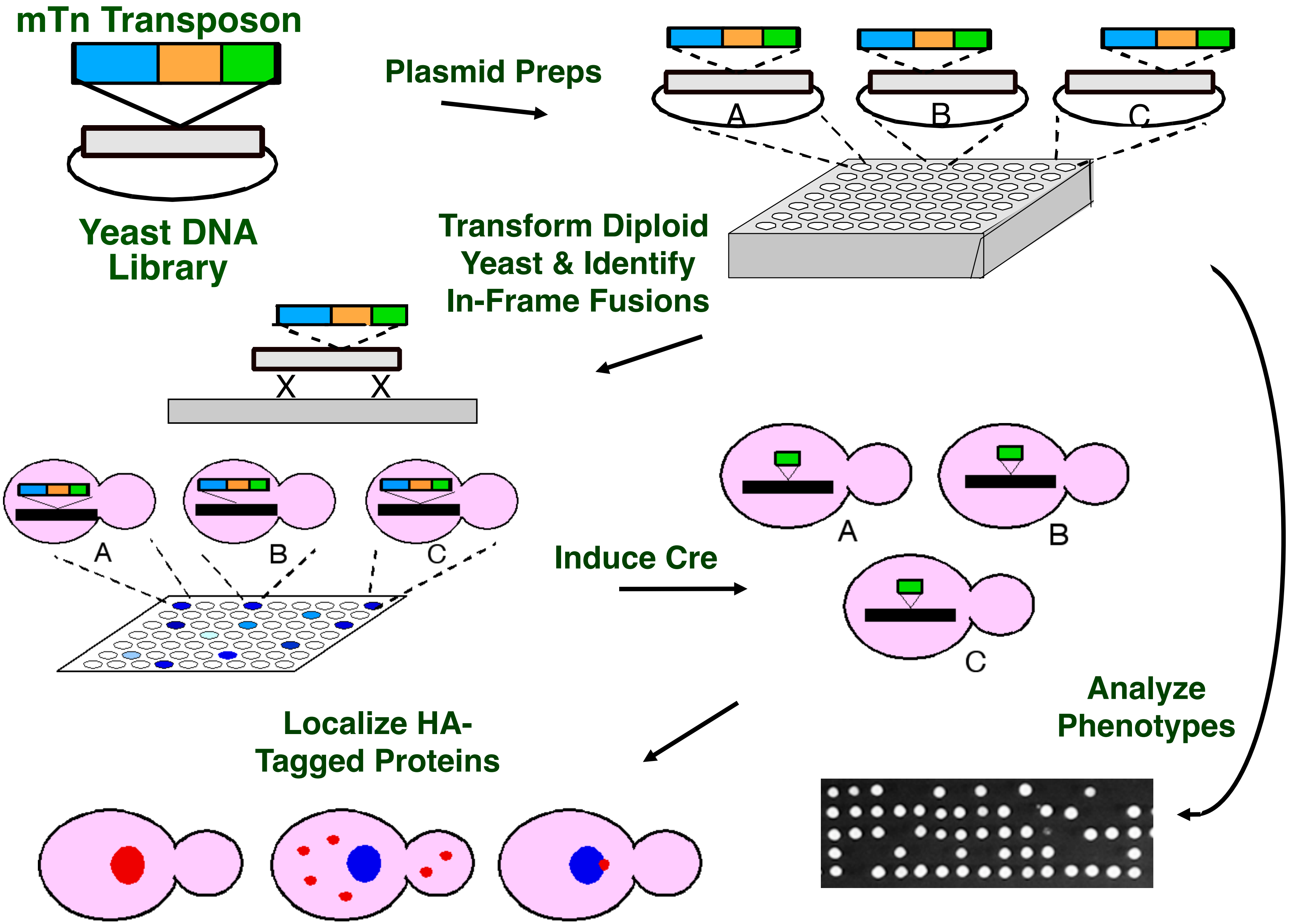
A Multipurpose Transposon



+ *cre* recombinase

274 base pairs

Screening Approach



mTn Transposon

Yeast DNA Library

Plasmid Preps

Transform Diploid Yeast & Identify In-Frame Fusions

Induce Cre

Analyze Phenotypes

Localize HA-Tagged Proteins

Screening Summary

- 25,440 Vegetatively Expressed Fusions
- 277 Sporulation Induced
- ~ 4,000 Genes Tagged

Genes Tagged

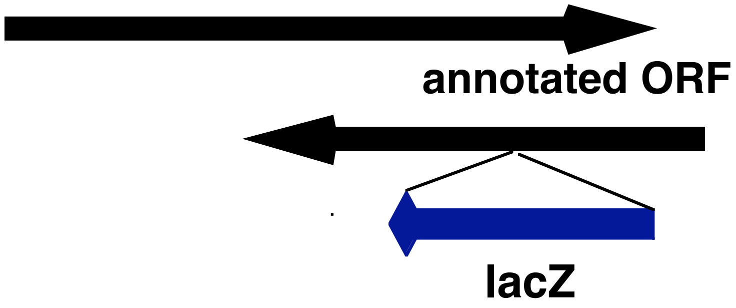
Non-annotated ORFs ("tORFs")
(>25 codons; avg. length 63 codons)

Intergenic: 72 tORFs

annotated ORF

annotated ORF

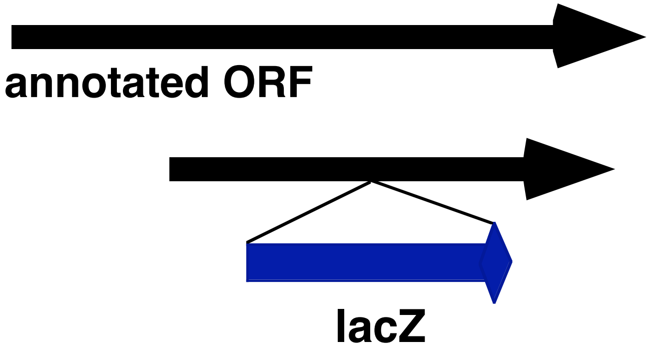
Antisense: 85 tORFs



annotated ORF

lacZ

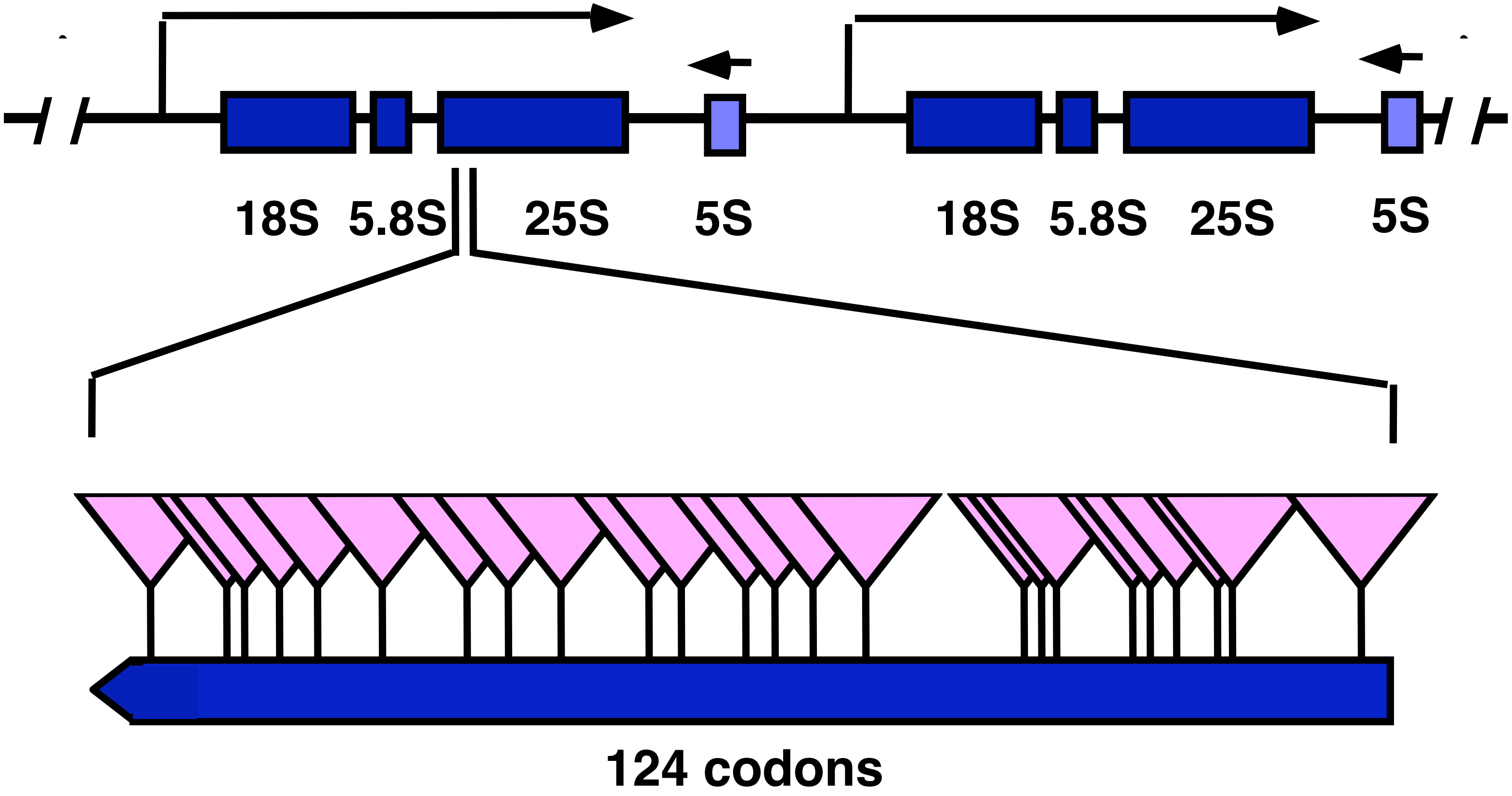
Out-of-frame: 82 tORFs



annotated ORF

lacZ

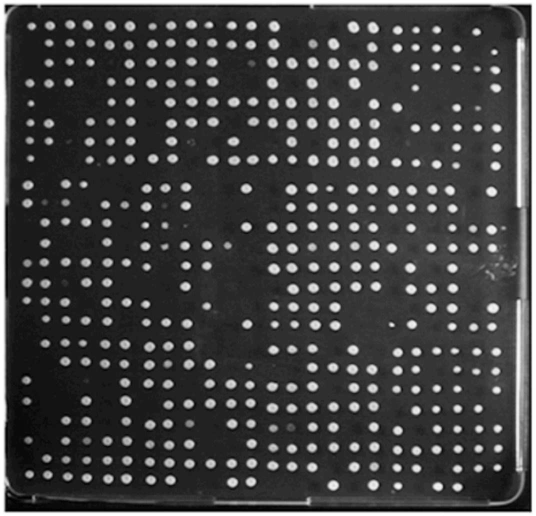
A Novel ORF Antisense to 25S rDNA



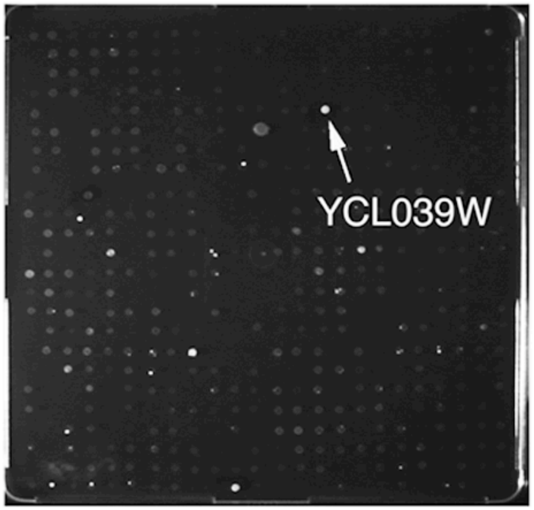
Gene Discovery

157 Additional Highly Expressed ORFs by Transposon Tagging

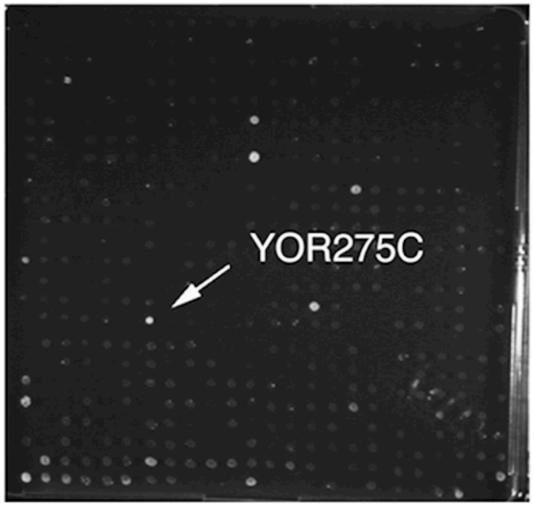
Phenotype Macroarrays



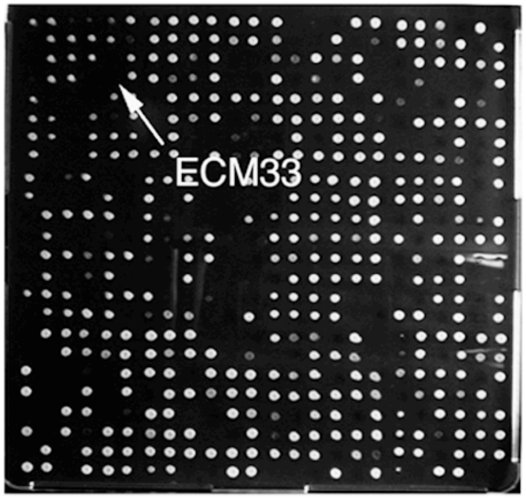
YPD



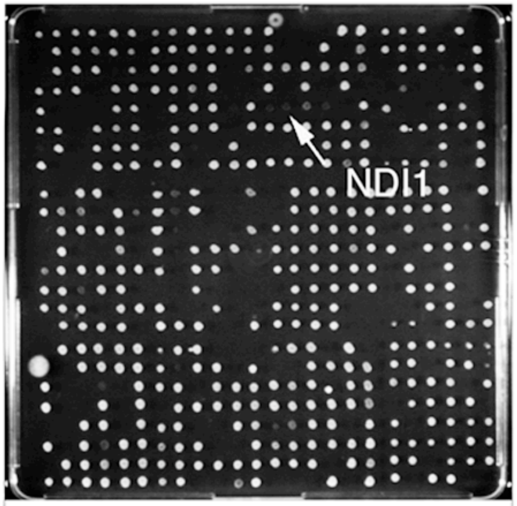
Benomyl^R



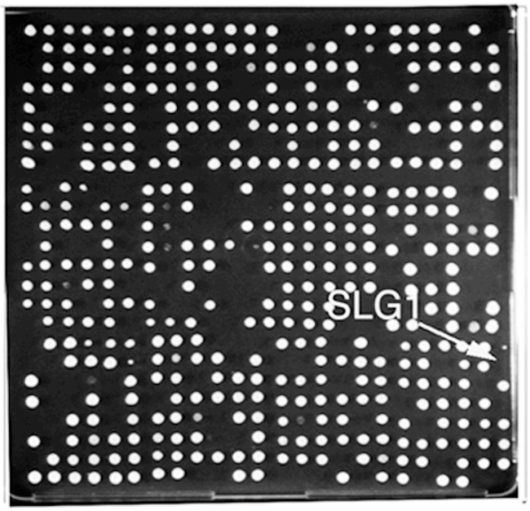
Calc^R



Hygro



Glycerol



Calc^S

Screening the Collection

Benomyl Sensitivity (2208 insertions)

Microtubule: TUB3 PAC2 DIS3 CIK1 MYO3 CIN1

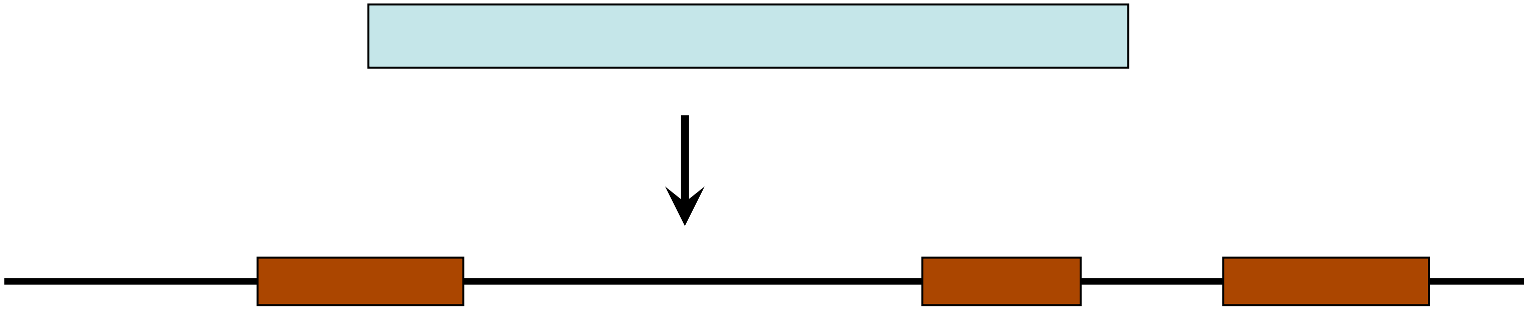
Nuclear: HTZ1 STH1 SMC3 SAE2

Known: PAN3 ALD3 SGV1 SIT4 PFK2 CDC2
RAP1 NUP116 UME6 RTS1 YPT2 BEE1
KGD2 SSH1 TFP1 FAS1 SUN1 NUP170

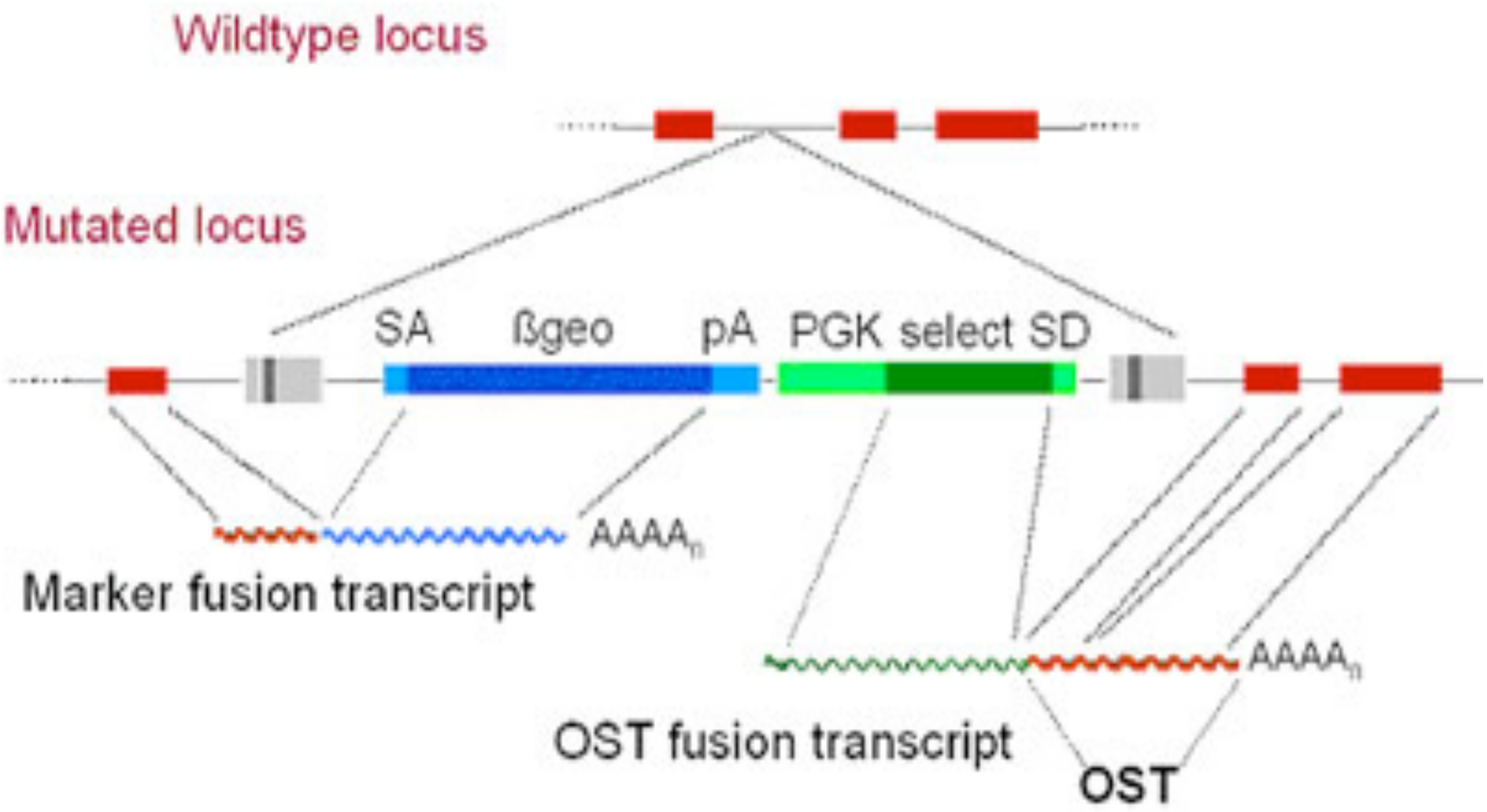
Novel: YCL060C YJL029C YKL059C YPL020
YGR068C YHR196W YGR013W
YLR162W YJR053W YBL051C

Mice

- Transposon (PiggyBac)
- Retrovirus
  - Efficient Transfer
- Gene Trapping



Lexicon Gene Trapping



SA = Splicing acceptor

Lexicon Features

- 270,000 lines affecting >20,000 transcribed regions (50% of total genes?)
- Mutagenesis is carried out in ES cells- thus can generate mutant mice

Transposon/Insertional Mutagenesis Approach

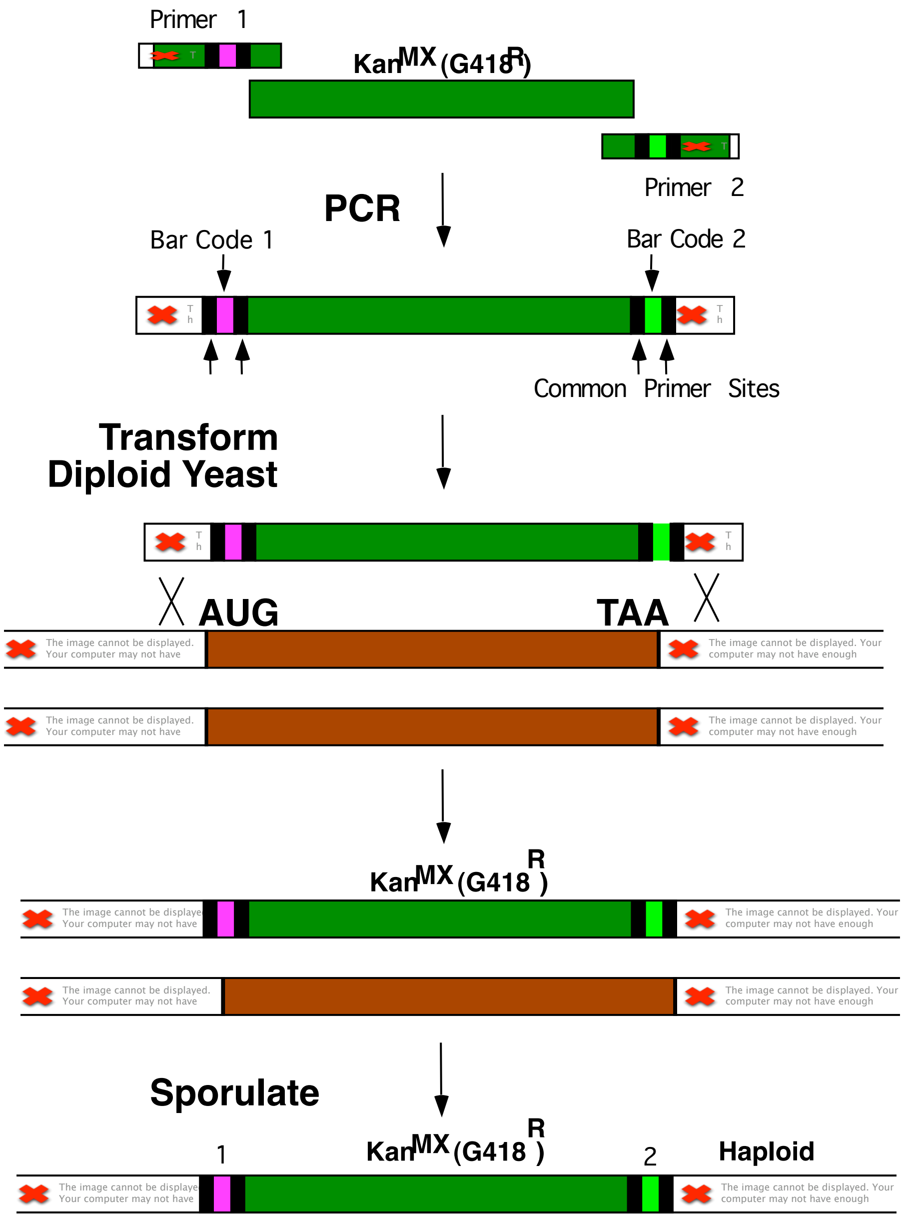
Advantages

- Simple-can generate large numbers of insertions
- Relatively inexpensive
- Can be used to find genes
- Get many alleles
- Can follow expression and tag proteins

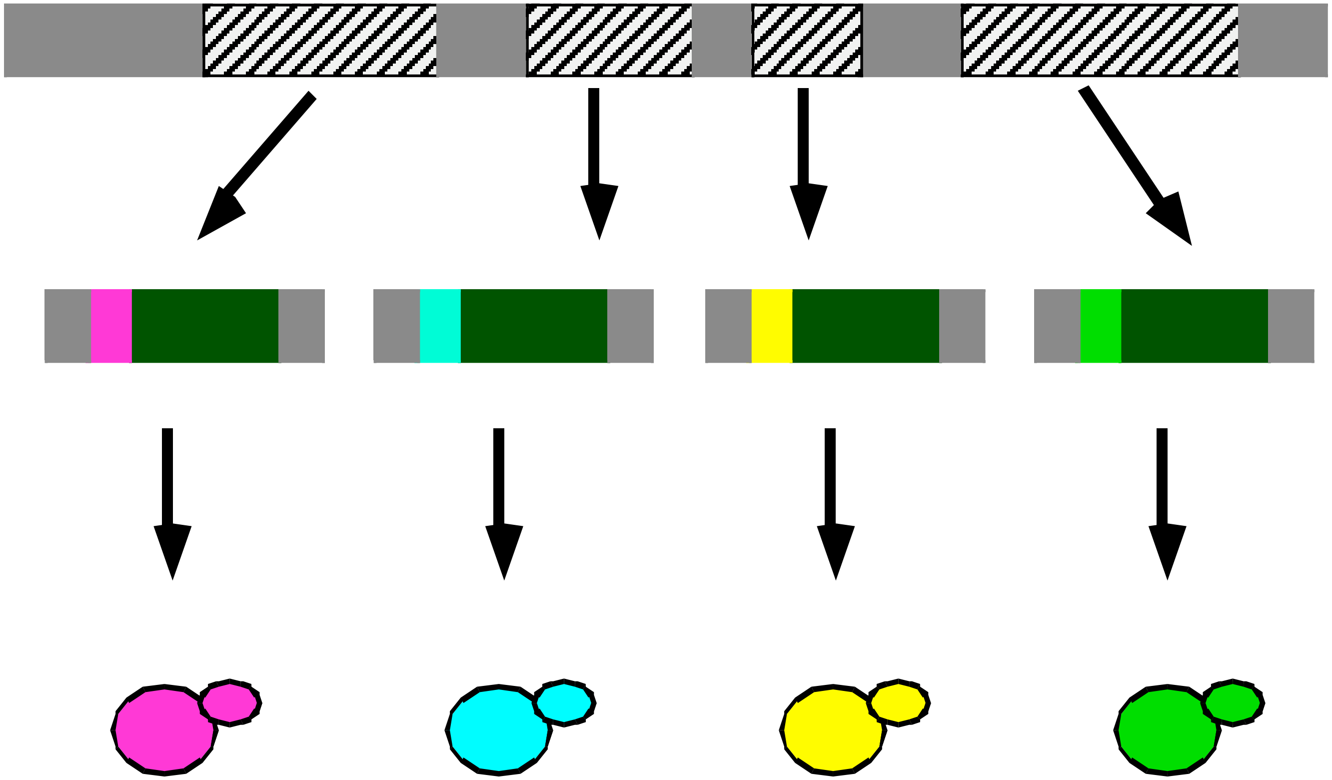
Disadvantages

- Biased-Hard to hit all Genes
- May not generate null alleles

Bar Code PCR Disruptions



Yeast Strains With Tagged Knockouts

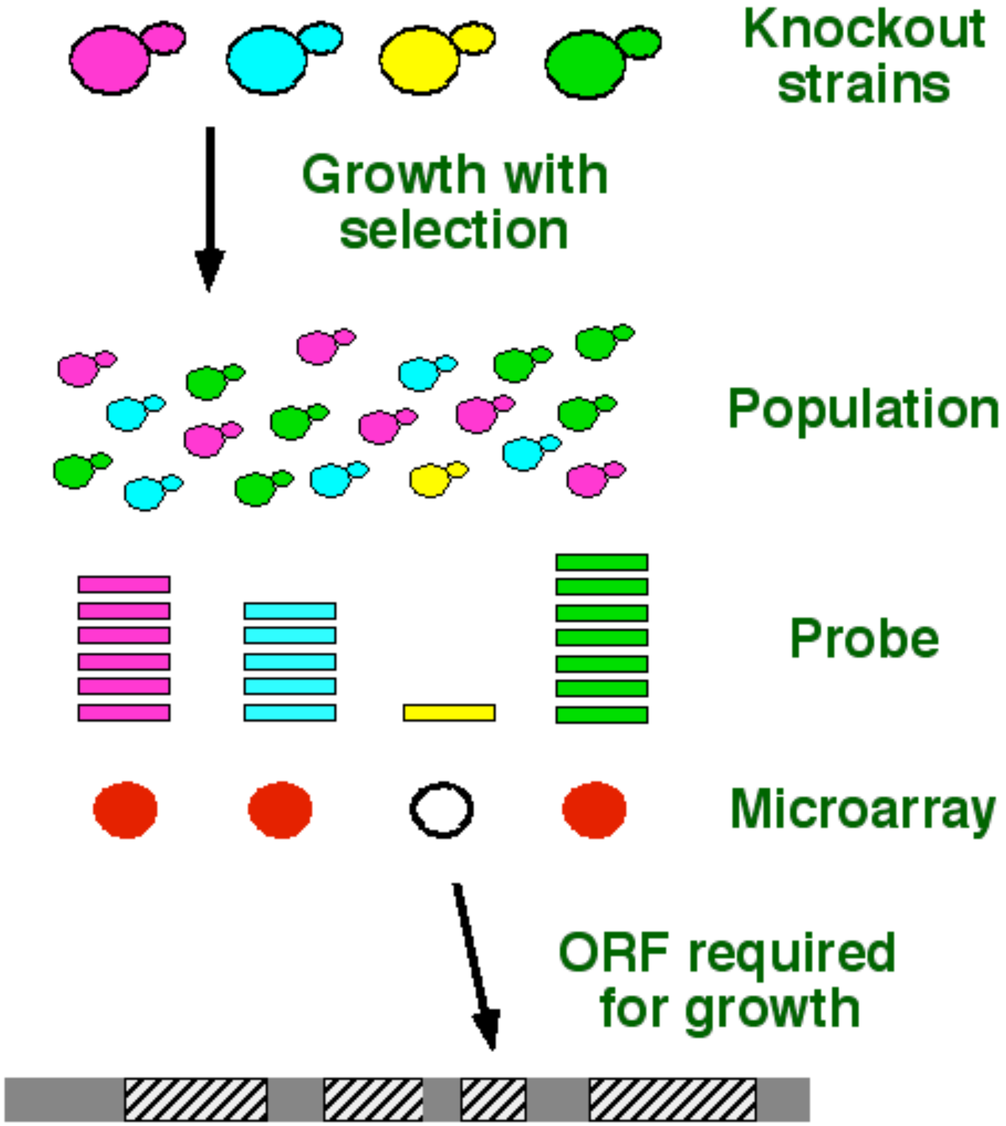


ORFs on chromosome

Tagged PCR product

Tagged yeast strains

Functional Analysis of Knockout Strains



Knockout strains

Growth with selection

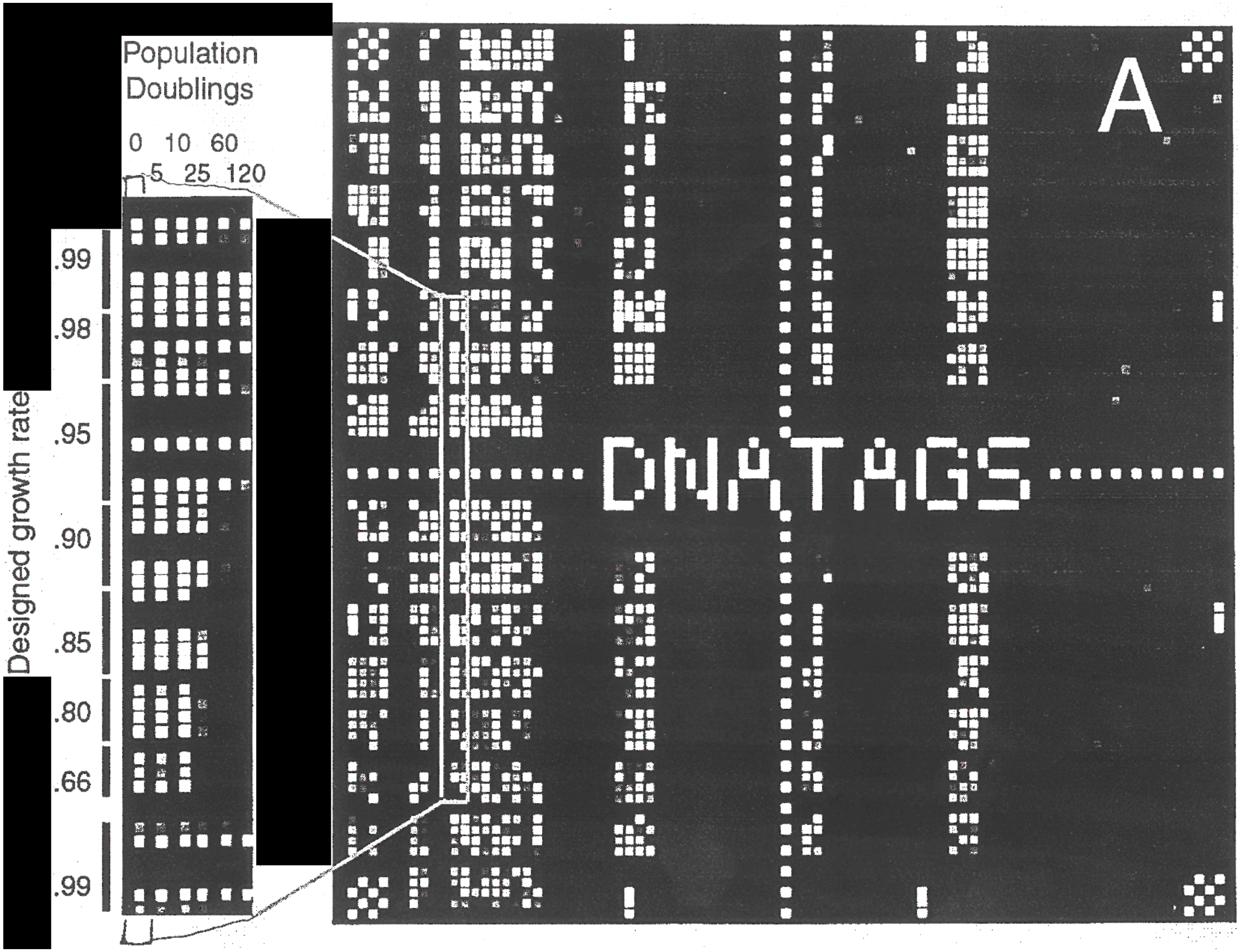
Population

Probe

Microarray

ORF required for growth

Microarray Results


[redacted]
[redacted]
[redacted]

Systematic Deletions

>95 % of Yeast Genes Disrupted

~1000 Essential Genes

~5,000 Nonessential Genes

Systematic Knockouts

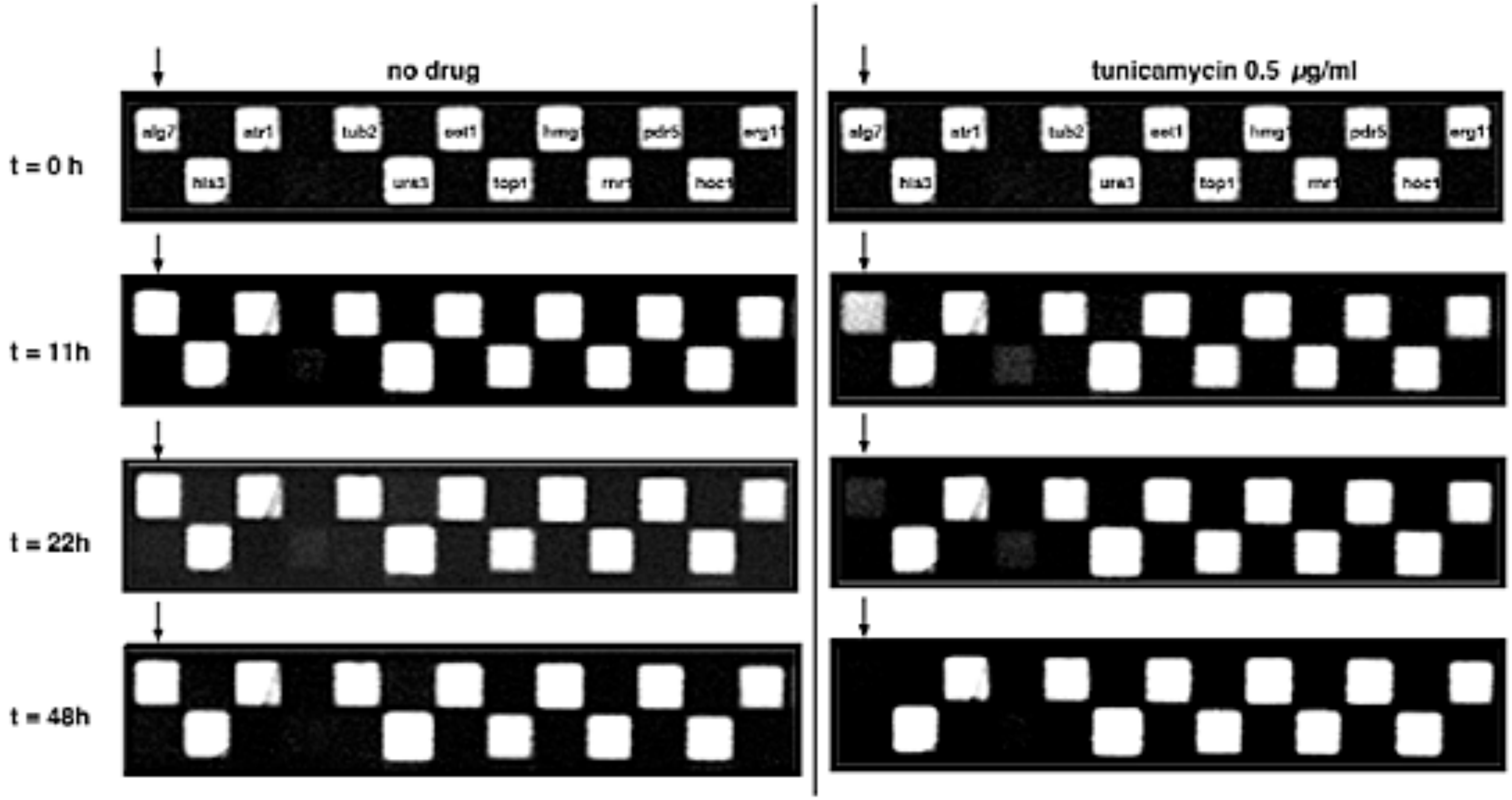
Advantages

- Gives null phenotype
- Comprehensive
- Bar Coding

Disadvantages

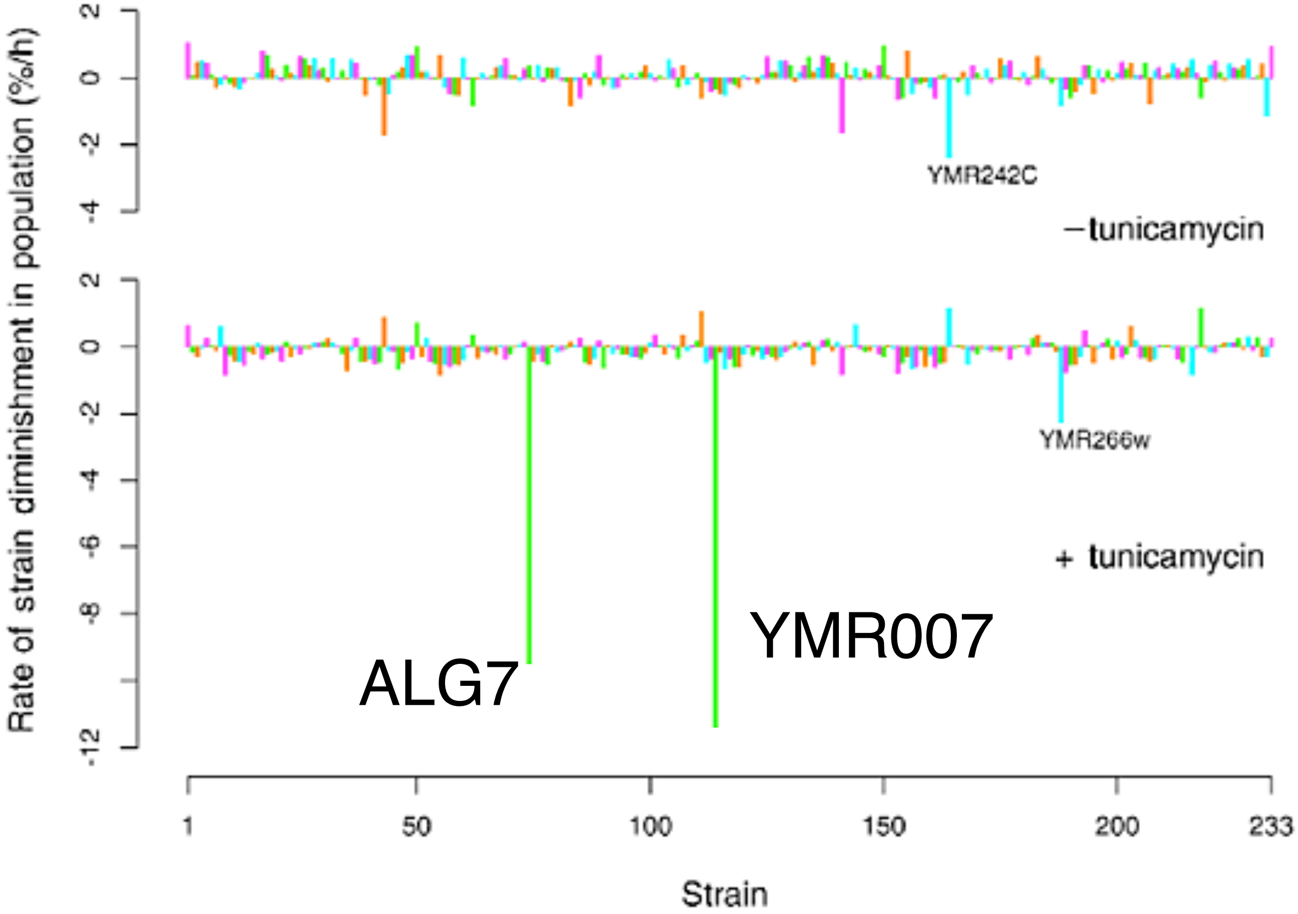
- Expensive
- Limited alleles (No reporter constructs)
- Relies on Annotated Sequence

Using Deletions to Profile Drug Sensitivity



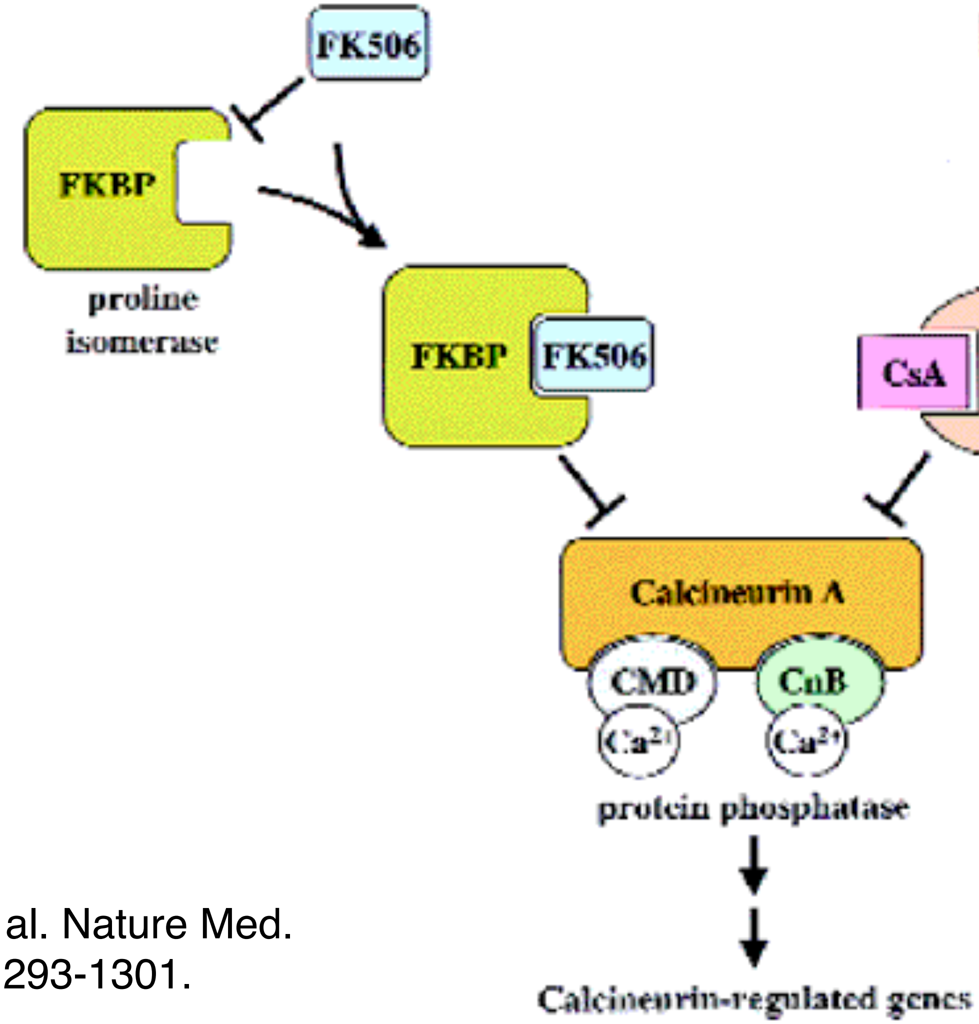
Giaever et al. Nature Genetics 1999 vol 21, 278-283

Using Deletions to Profile Drug Sensitivity



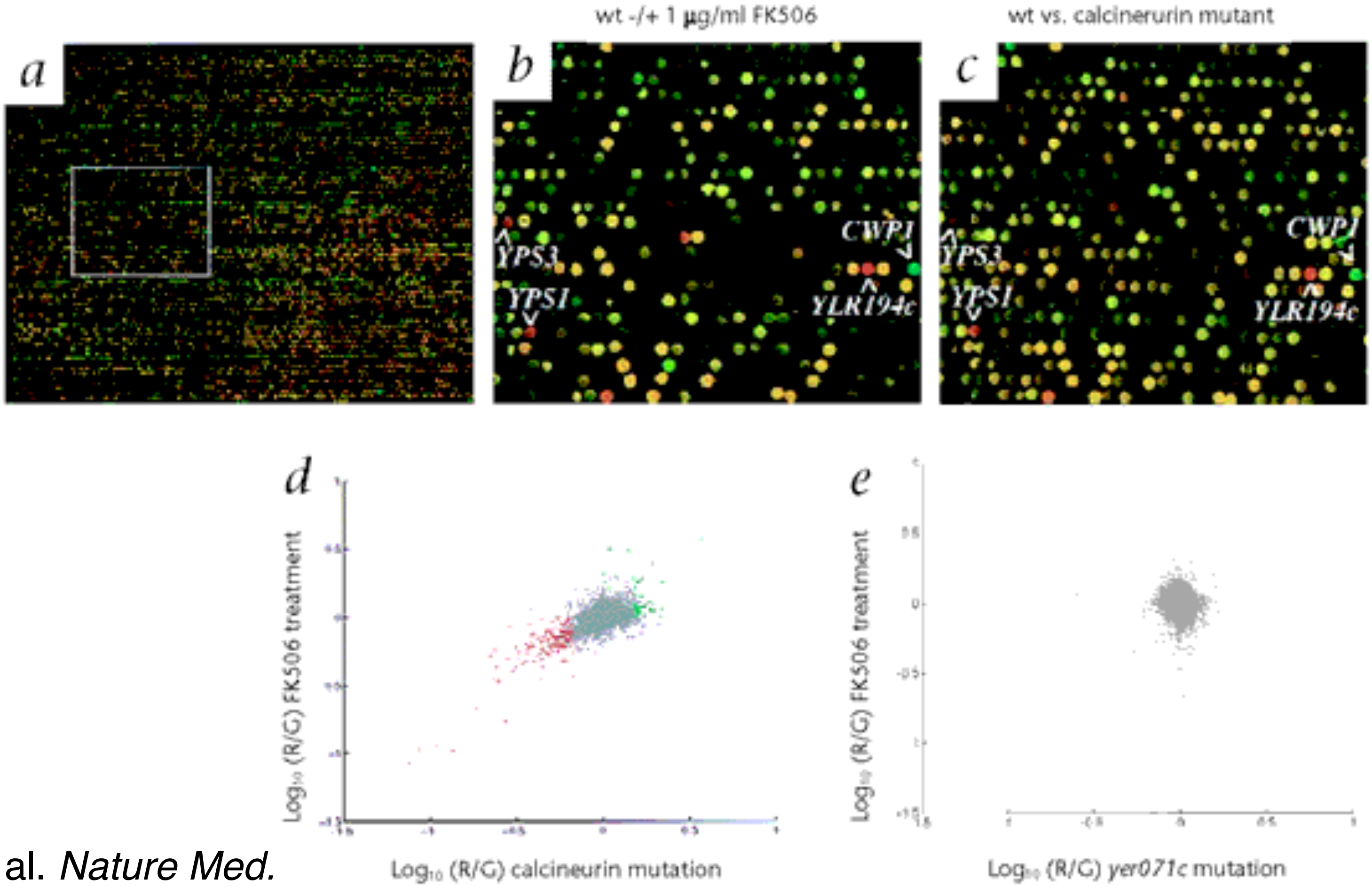
Giaever et al. Nature Genetics 1999 vol 21, 278-283

Calcineurin Signalling Pathway



Marton et al. Nature Med.
Vol 4, 1293-1301.

Drug Gives Similar Expression Profile to K/O: FK506 Calcineurin



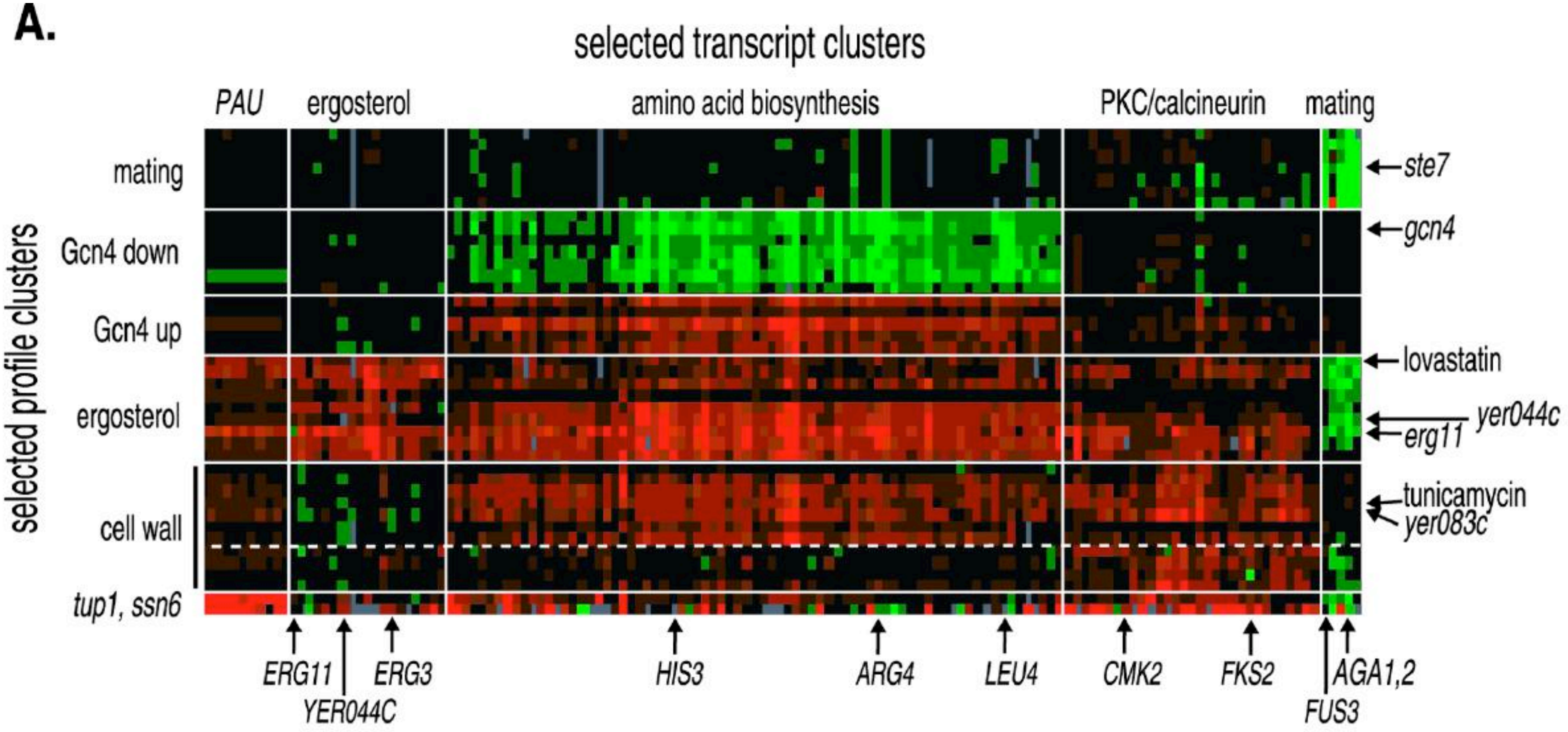
Marton et al. *Nature Med.*
Vol 4, 1293-1301.

Identification of the Dyclonine Target

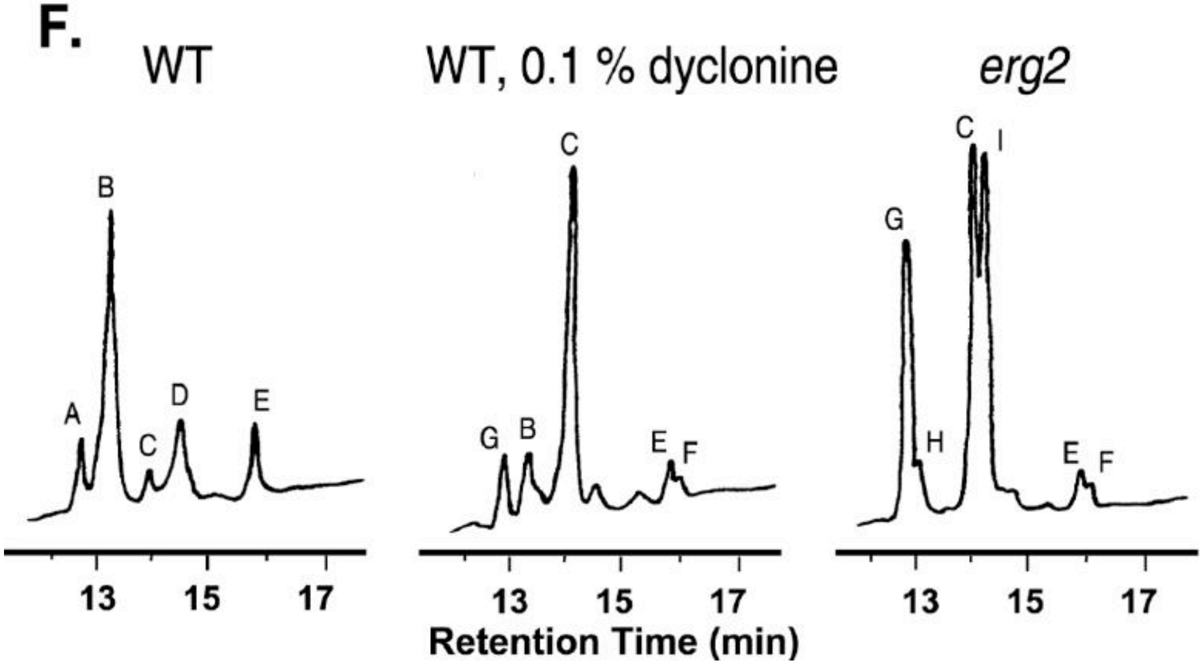
-Dyclonine: active ingredients of Sucrets
-Give a profile like Ergosterol mutant
    Phenotype similar to Erg2 (sterol isomerase)
-Human Sigma receptor is closest to Erg2
-Sigma receptor regulate K+ conductance

Model: Dyclonine reduce K+ current &
    inhibits nerve conductance

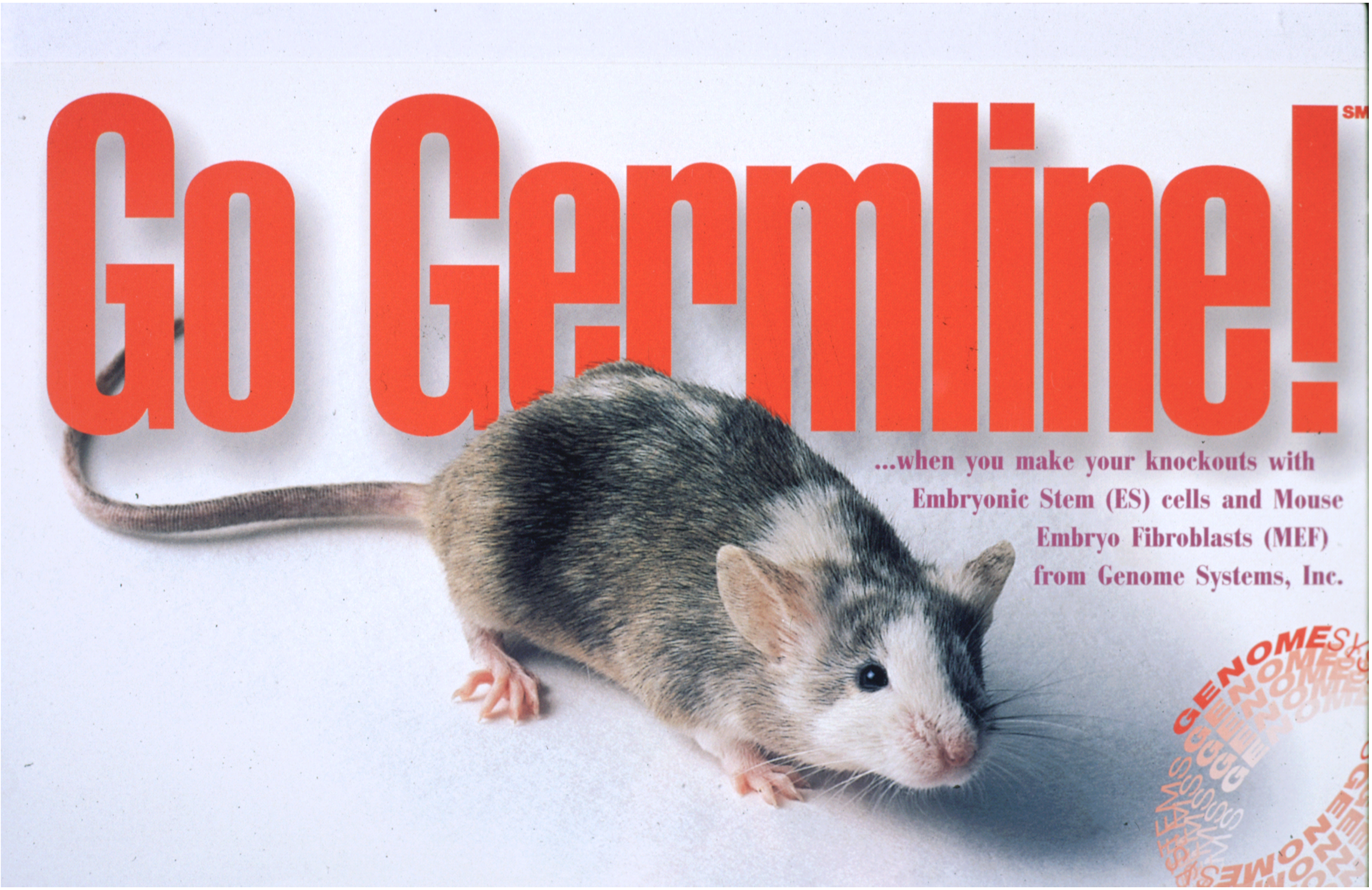
Clustering Genes



The Same Intermediate Accumulates in Dyclonine and Erg2 Mutant Cells

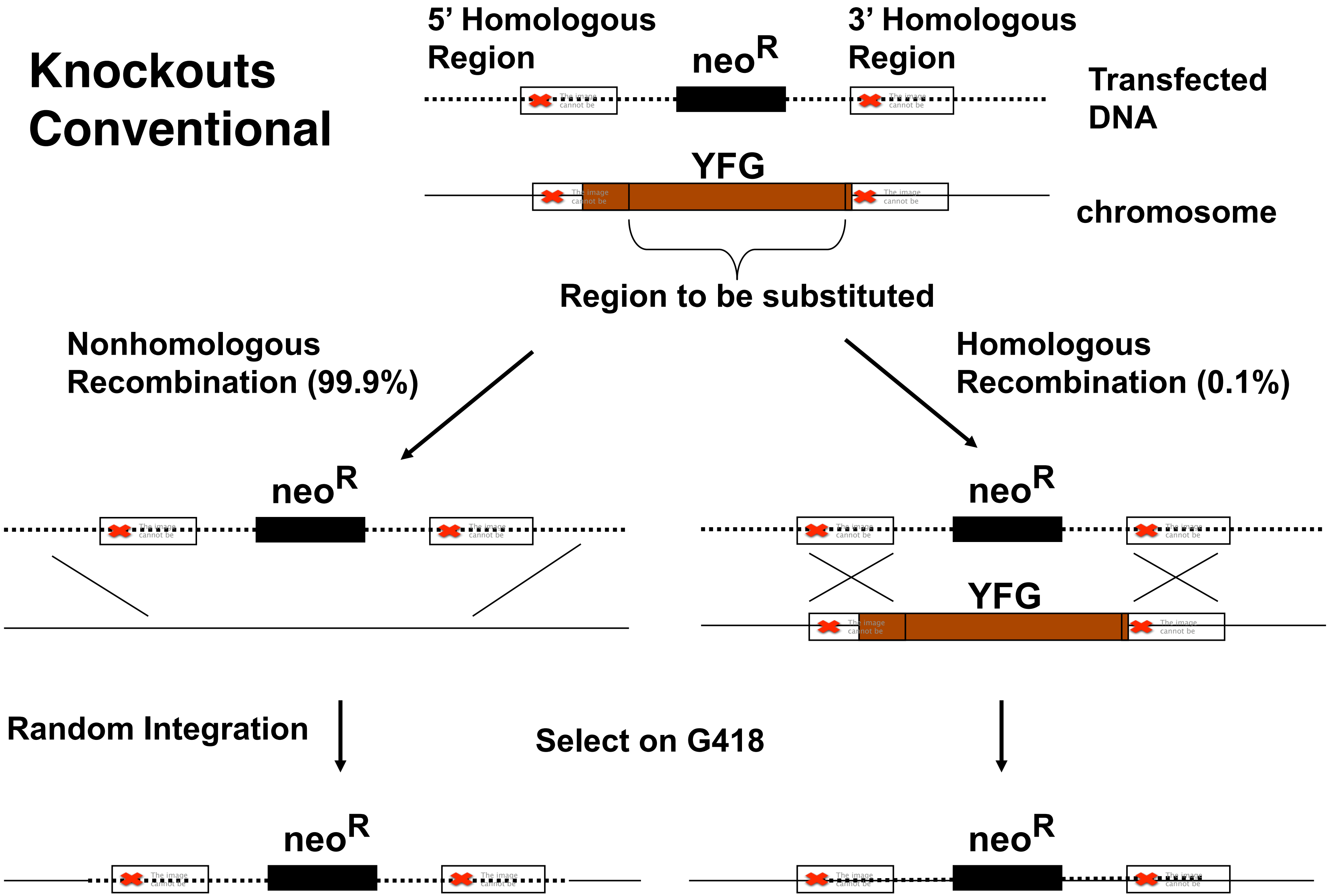


Mouse Knockouts



...when you make your knockouts with Embryonic Stem (ES) cells and Mouse Embryo Fibroblasts (MEF) from Genome Systems, Inc.

Knockouts Conventional



5’ Homologous Region

neo^R

3’ Homologous Region

Transfected DNA

YFG

chromosome

Region to be substituted

Nonhomologous Recombination (99.9%)

Homologous Recombination (0.1%)

neo^R

neo^R

YFG

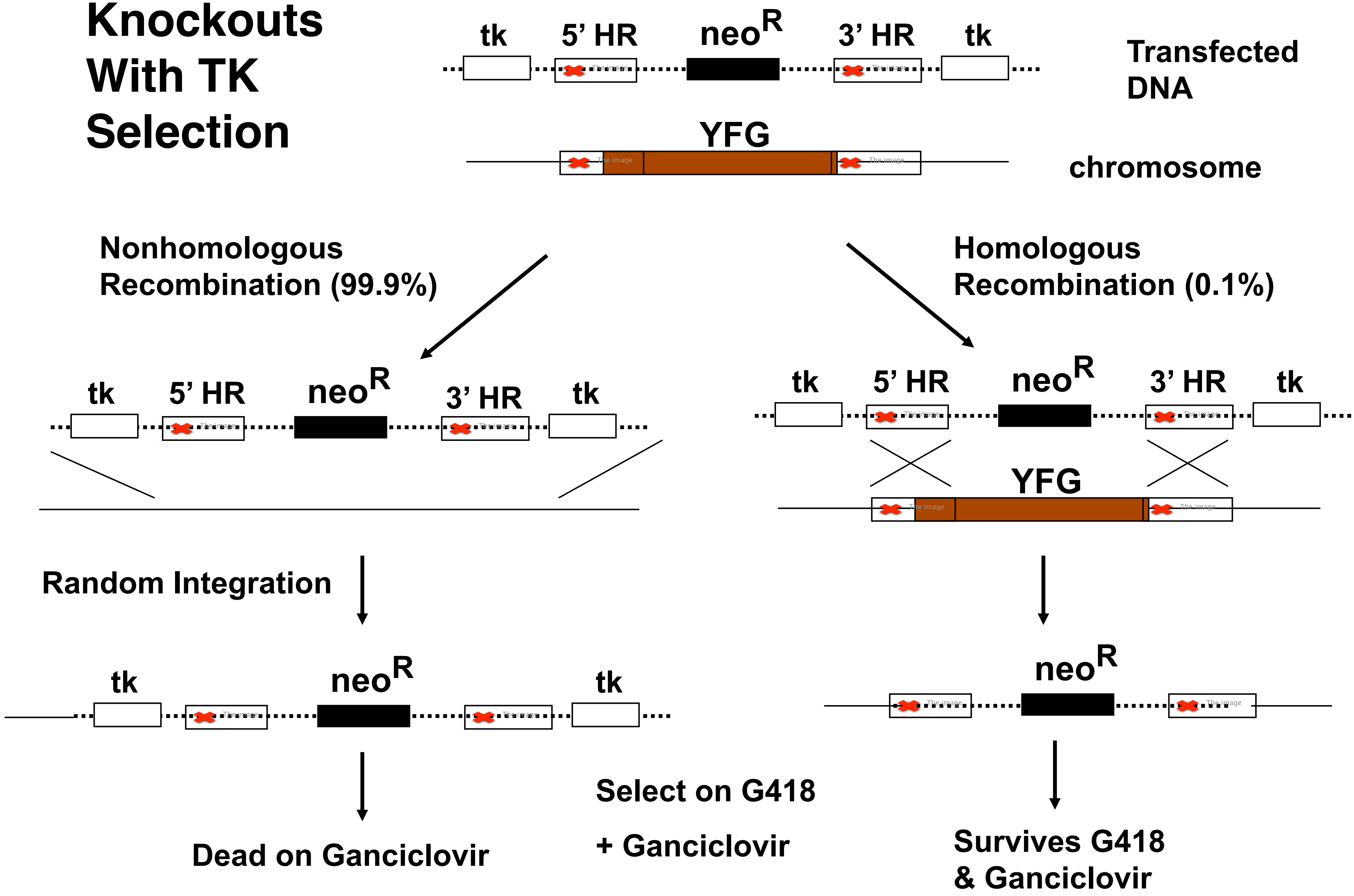
Random Integration

Select on G418

neo^R

neo^R

Knockouts With TK Selection



Transfected DNA

chromosome

Nonhomologous Recombination (99.9%)

Homologous Recombination (0.1%)

Random Integration

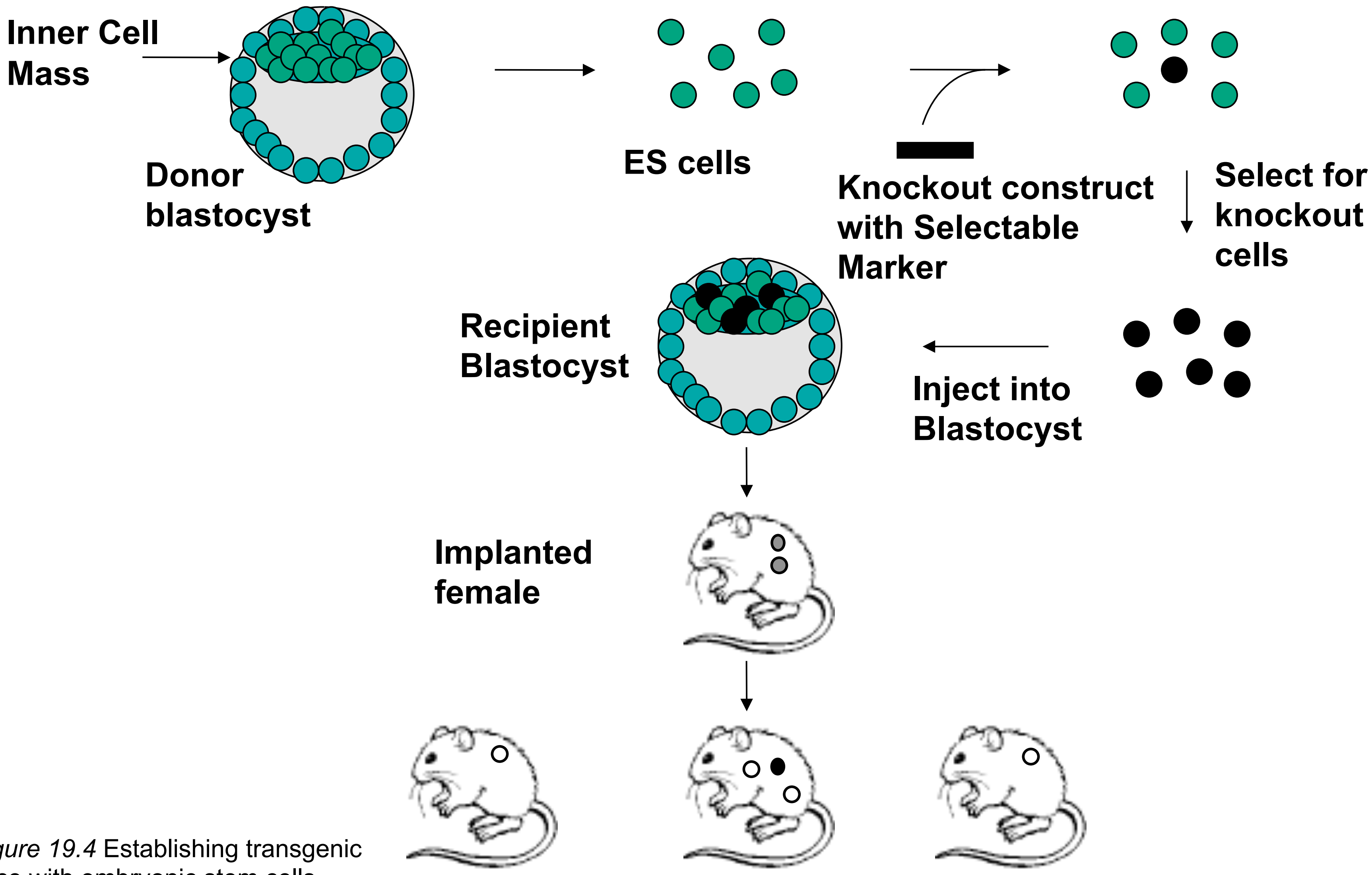
Select on G418

+ Ganciclovir

Dead on Ganciclovir

Survives G418 & Ganciclovir

Knockout Mice - Embryonic Stem Cells



Inner Cell Mass

Donor blastocyst

ES cells

Knockout construct with Selectable Marker

Select for knockout cells

Inject into Blastocyst

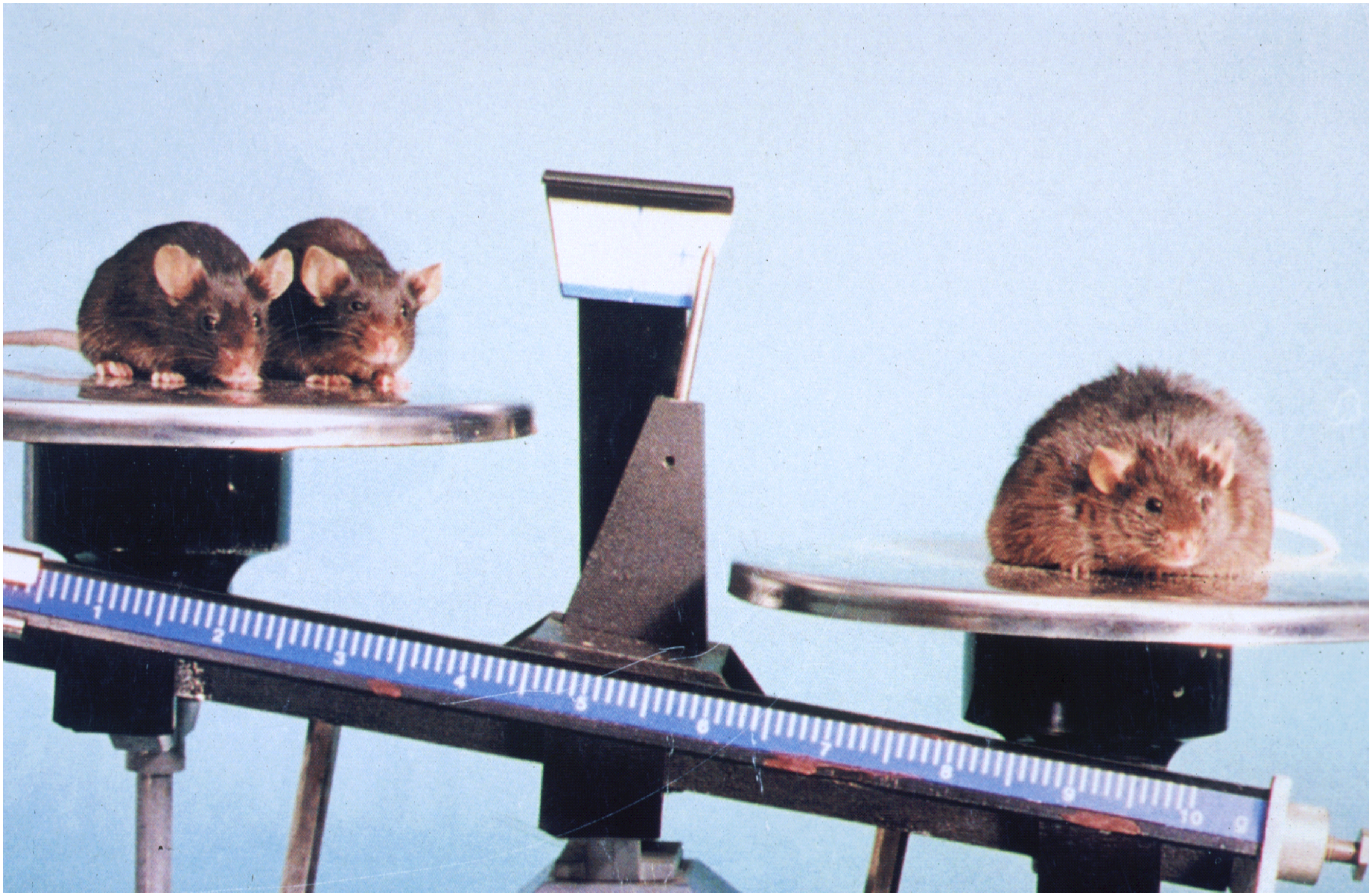
Recipient Blastocyst

Implanted female

Transgenic Mouse - X to WT to Find Germline Knockout

Figure 19.4 Establishing transgenic mice with embryonic stem cells

A Mutation in a Gene Affecting Obseity



Targeted Knockout Using Zinc Finger Technology

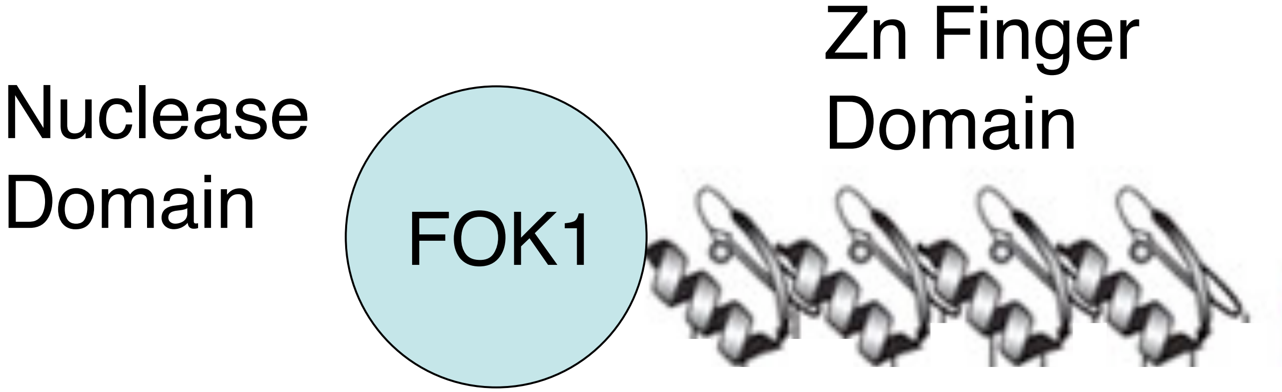
Zn Finger Domain:
Binds 6 bp sequence

Combine Multiple Domains

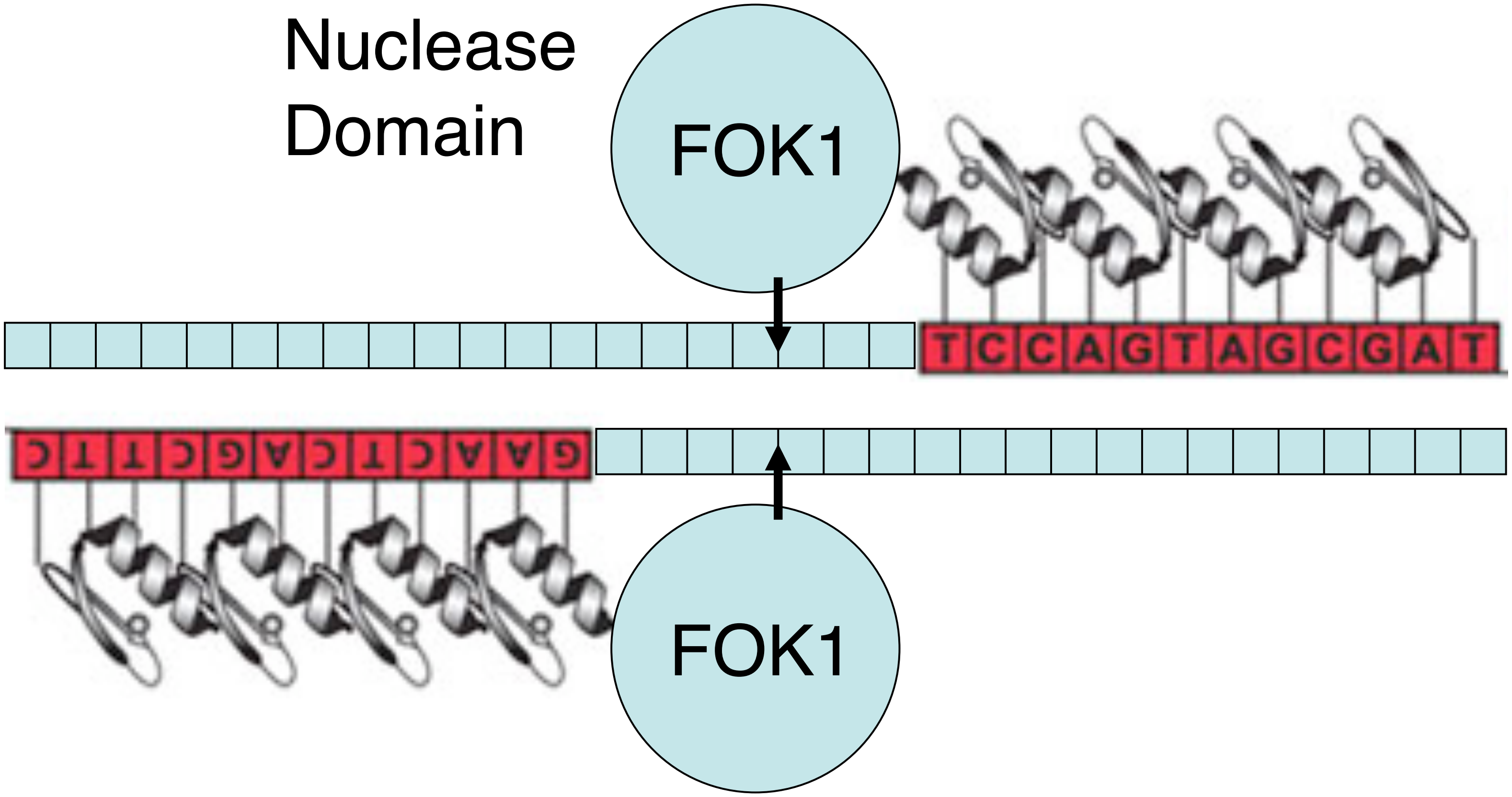
2 Zn Finger Domain:
Binds 12 bp sequence

Images from Sigma
Procedure based on Urnov et al., (2005) Nature 435: 646

Targeted Knockout Using Zinc Finger Technology



FOK Cleaves DNA as a Dimer

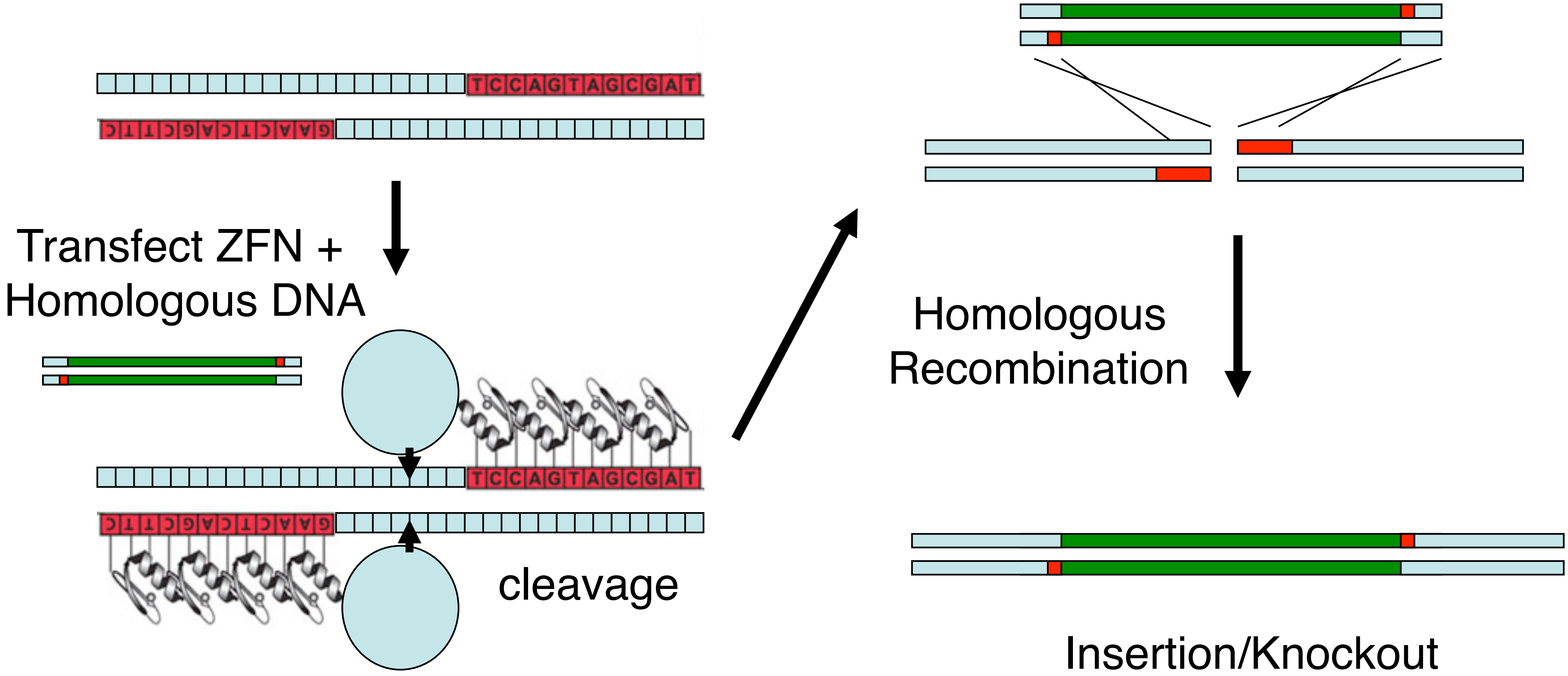


Nuclease Domain

FOK1

FOK1

Gene Knockout/Insertion Using Zn Finger Technology



Transfect ZFN +
Homologous DNA

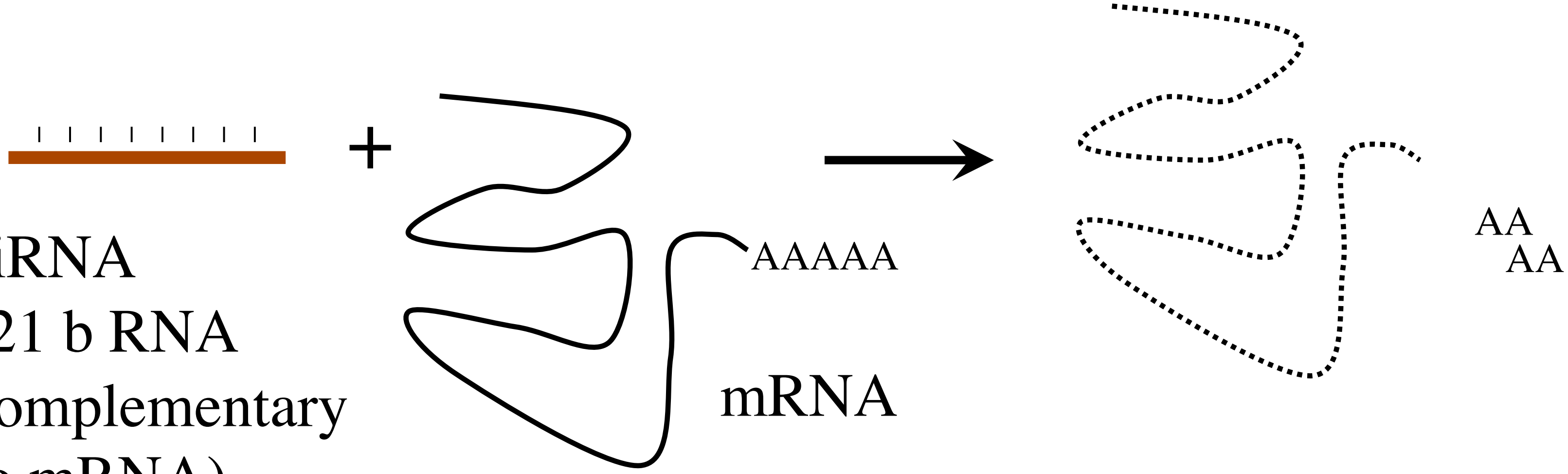
cleavage

Homologous
Recombination

Insertion/Knockout

Very Efficient: 1-20% Insertions without selection
7% of knockouts target both alleles

RNAi = RNA interference



siRNA
(21 b RNA
complementary
to mRNA)

+

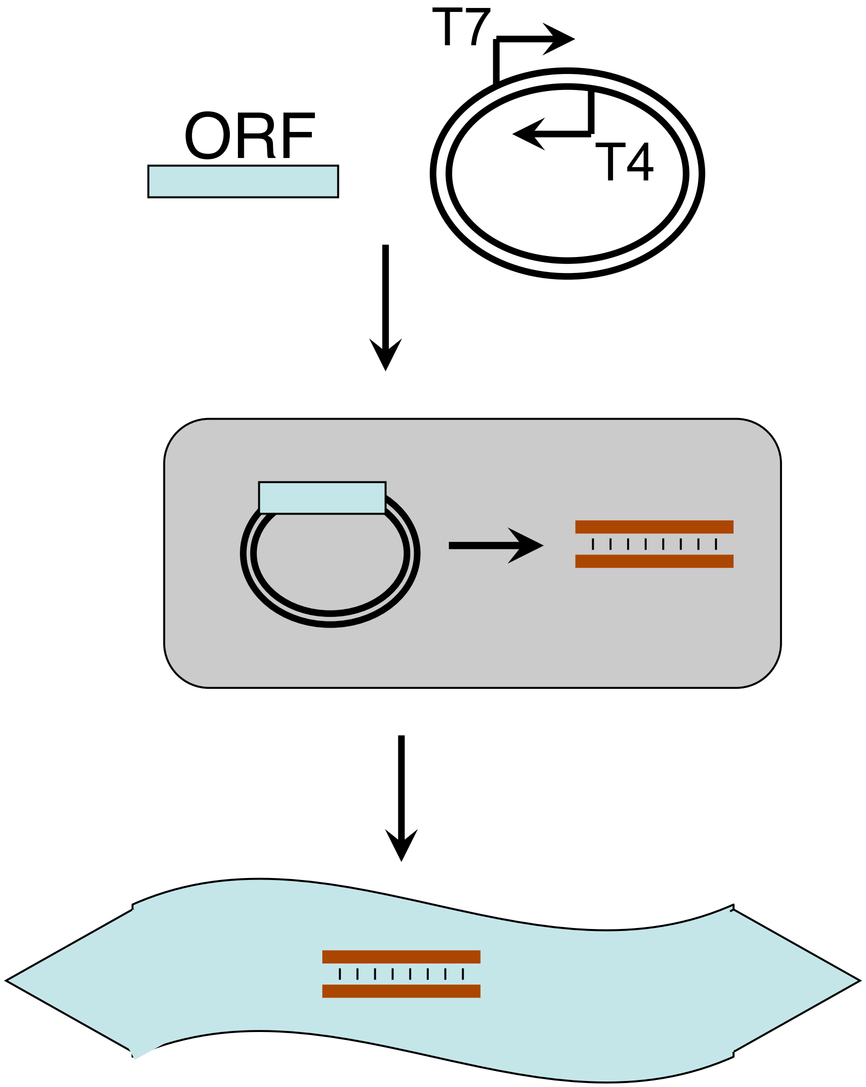
mRNA

siRNA inhibits gene expression

by degrading its complementary mRNA

Genome Wide Approach

Clone genes into E. coli
Expression vector that
makes dsRNA



Feed Worm E. coli;
Score phenotype

RNAi

16,757 (86%) C. elegans Genes RNAied;
1,722 Mutant phenotypes

Ahringer et al., Kohara et al.

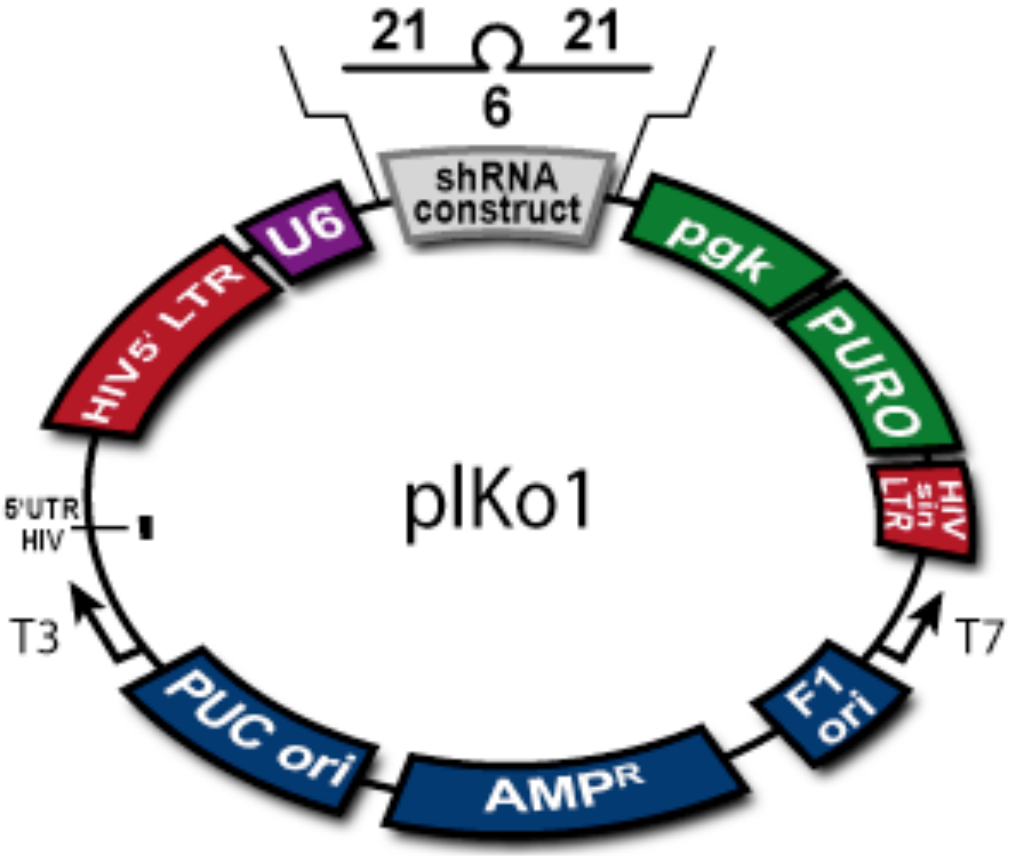
Can be used for many organisms
Drosophila, Mammalian Cells

RNAi Two approaches

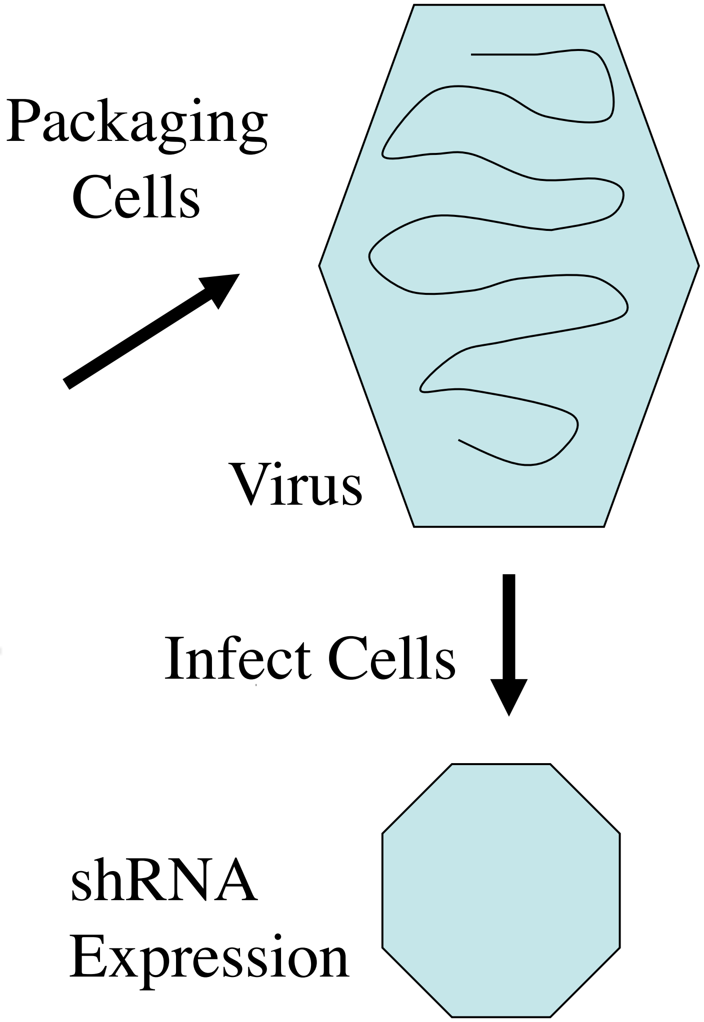
1) siRNA = Transfect 21 bp RNA complementary to mRNA
   (Screened for cellular genes required for HIV infection; involved 21,121 siRNAs)

2) shRNA = short hairpin RNAs
   Expressed from retroviruses

Mammalian RNAi Retrovirus Vector



From RNAi consortium



Packaging Cells

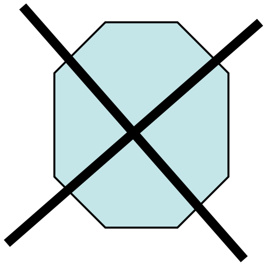
Virus

Infect Cells

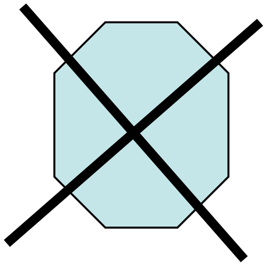
shRNA Expression

Identification of Tumor Suppressors Using RNAi

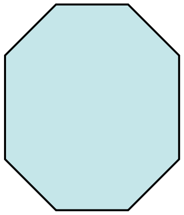
Klofcshoten et al. (2005) Cell 121, 849-858



1° Fibroblasts from humans die

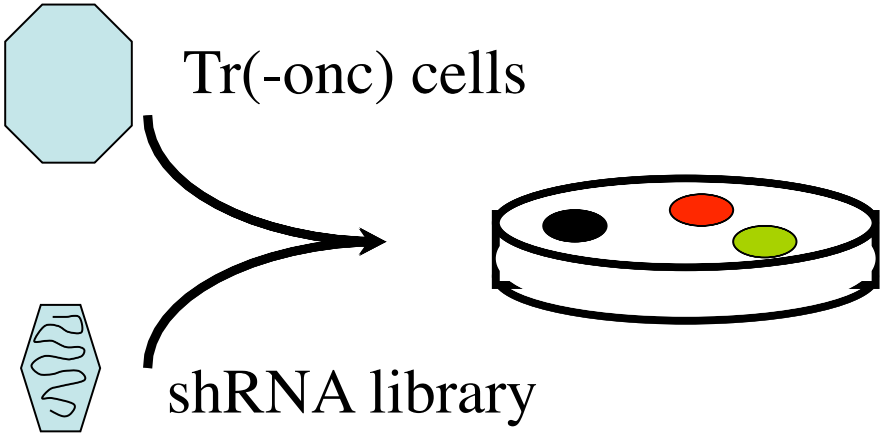


Tr(-onc) Engineered Fibroblasts
(hTERT, small t Antigen, p53-, p16-)
“almost transformed”



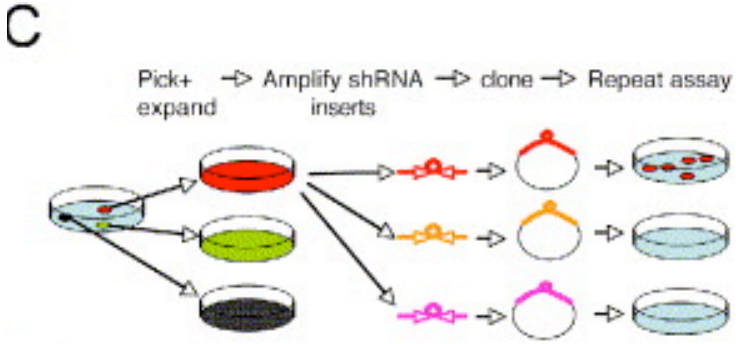
Tr(-onc) Engineered Fibroblasts + RAS^{V12}
Transformed and form colonies

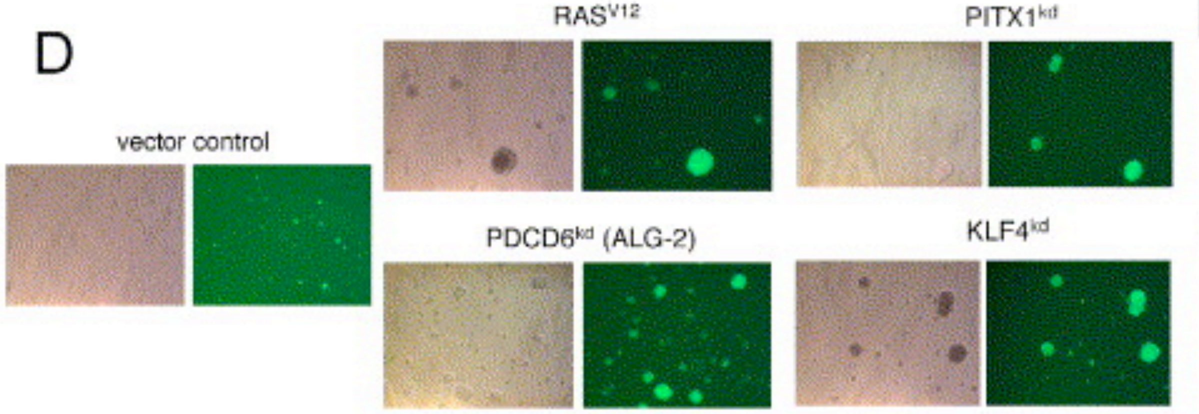
Identification of Tumor Suppressors Using RNAi



Tr(-onc) cells

shRNA library
(against 4000 genes)





New Tumor Suppressor:
PITX1

Klofschoten et al. (2005) Cell 121, 849-858

RNAi

Advantages

- Simple and Inexpensive
- Systematic method--Comprehensive
- Knockout expression of gene families

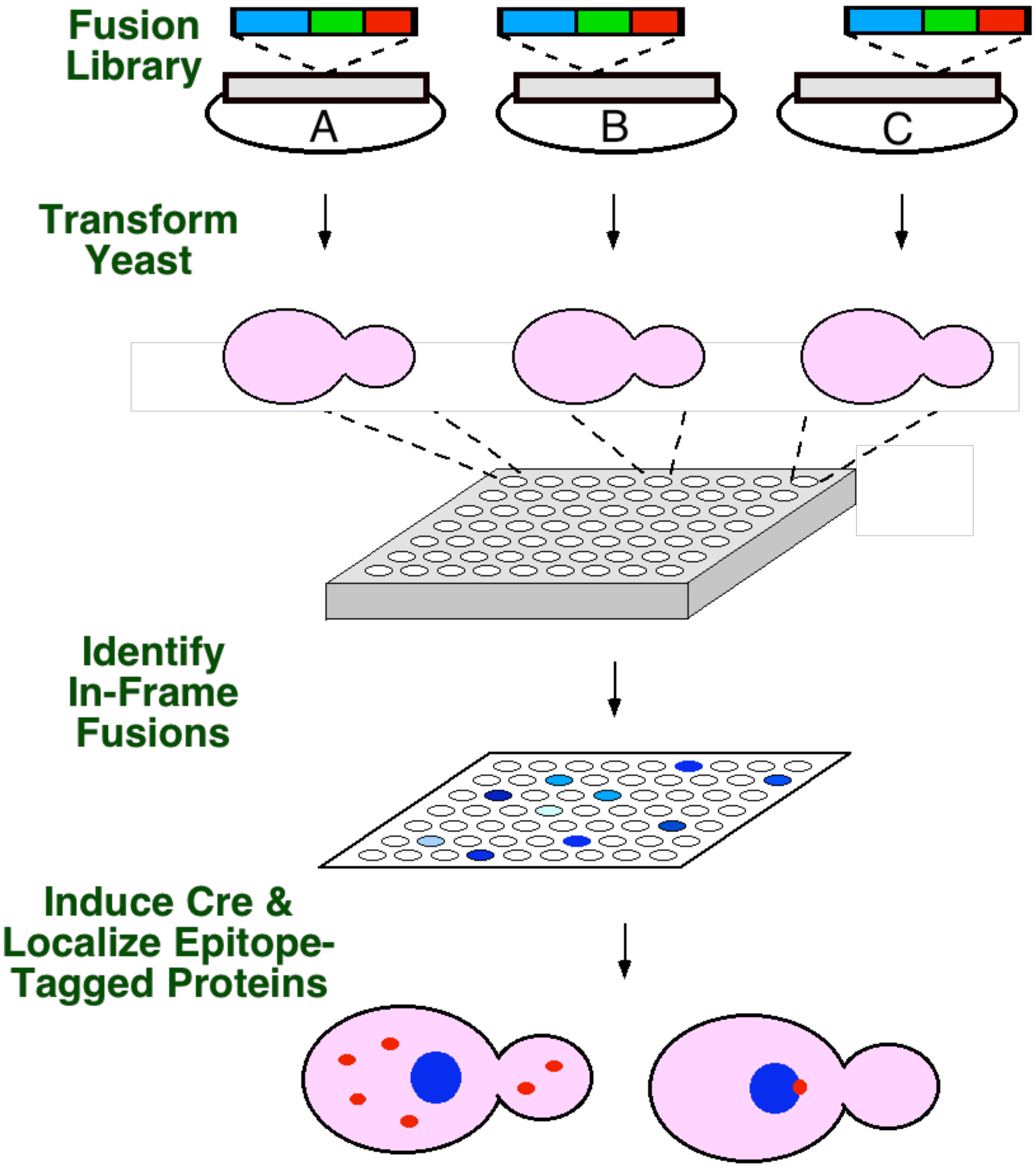
Disadvantages

- Some Genes Not Affected
- Limited alleles
- Off target effects

Uses of Knockouts: Summary

- Score phenotype to understand gene function
- Group different genes together based on phenotype
- Find new interesting genes
- Drug discovery

Screening Approach



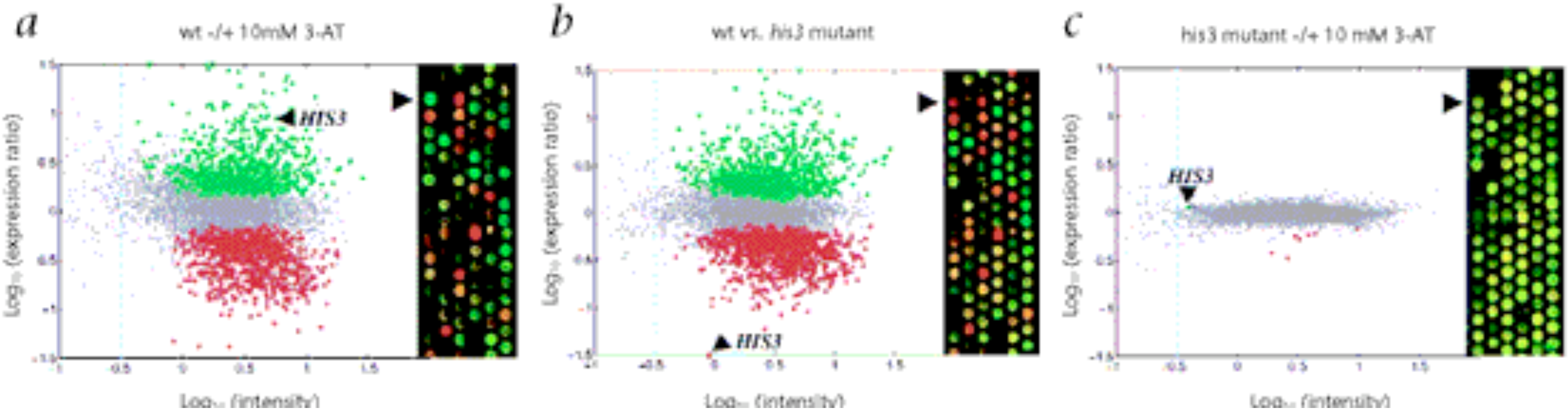
Fusion Library

Transform Yeast

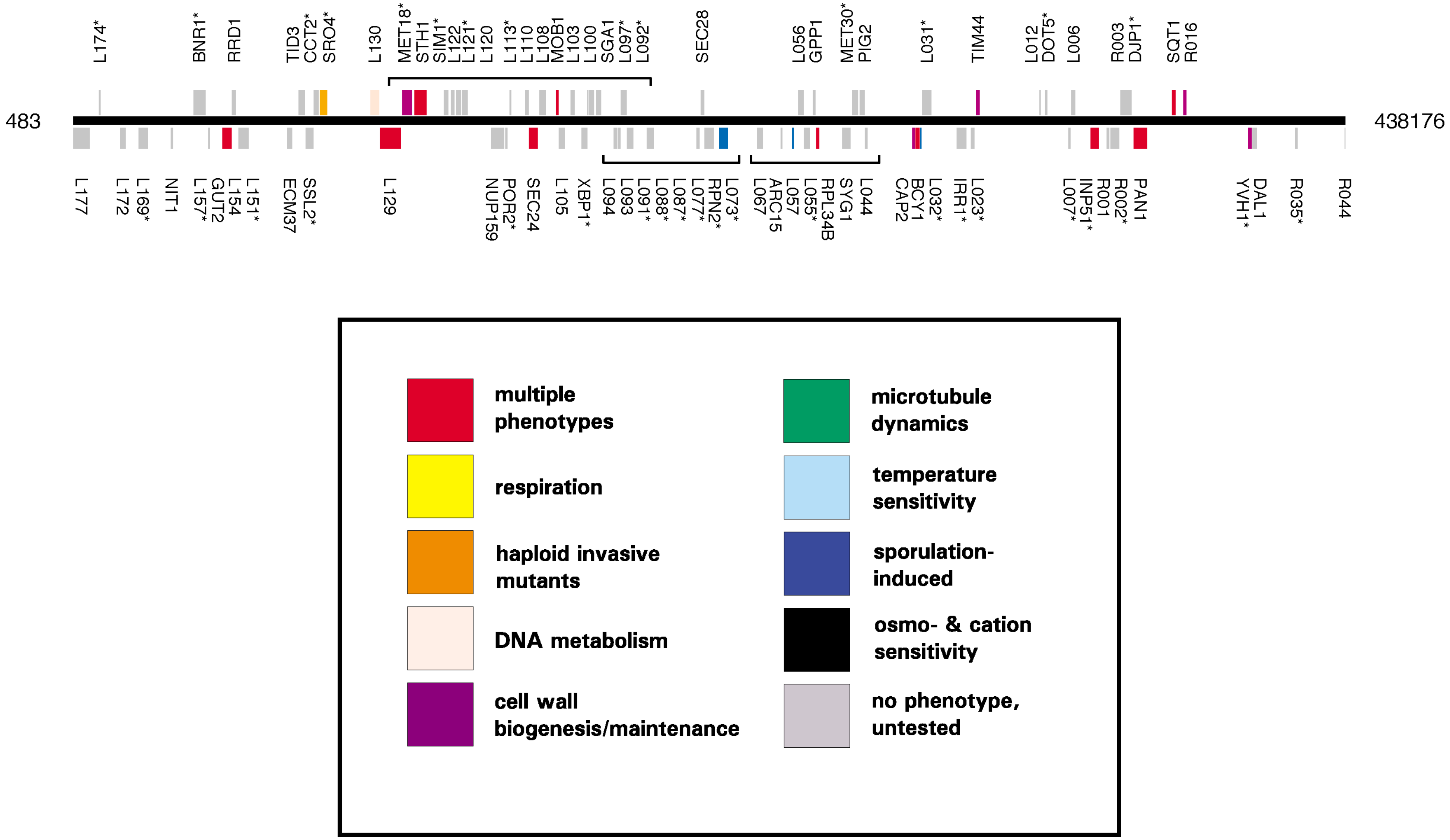
Identify In-Frame Fusions

Induce Cre & Localize Epitope-Tagged Proteins

Confirming a Drug Target



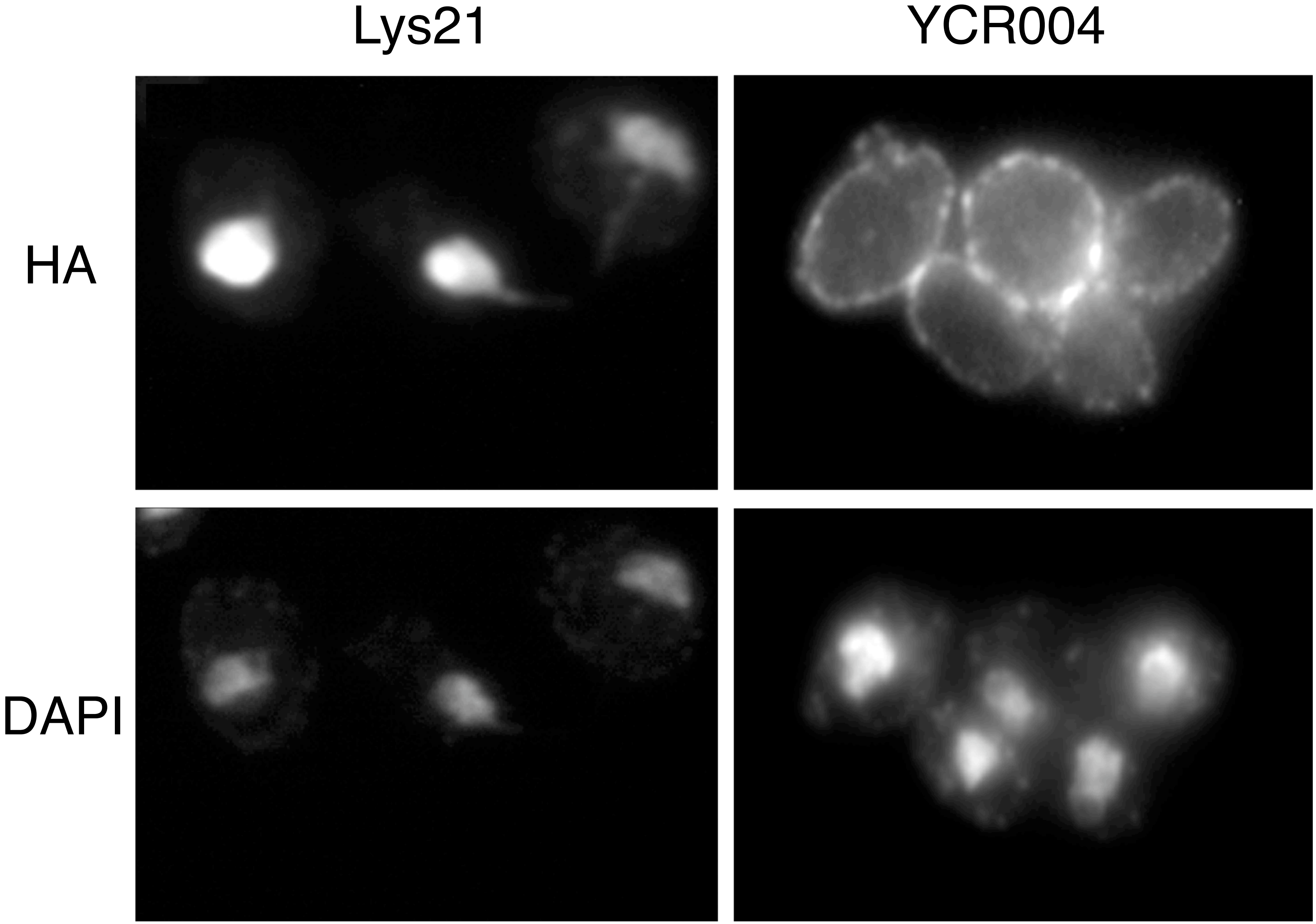
Chromosome IX

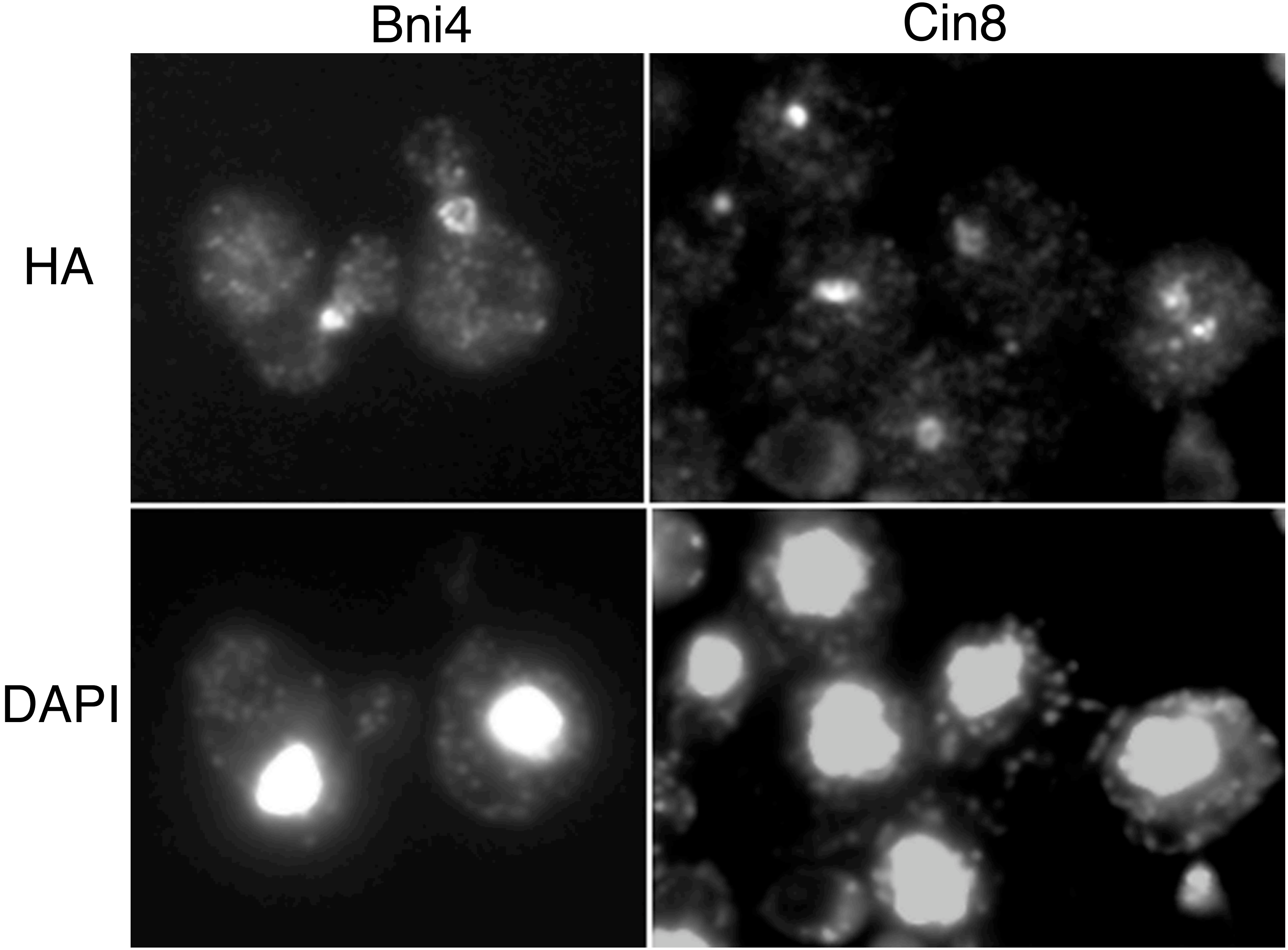


Targeted Gene Knockouts

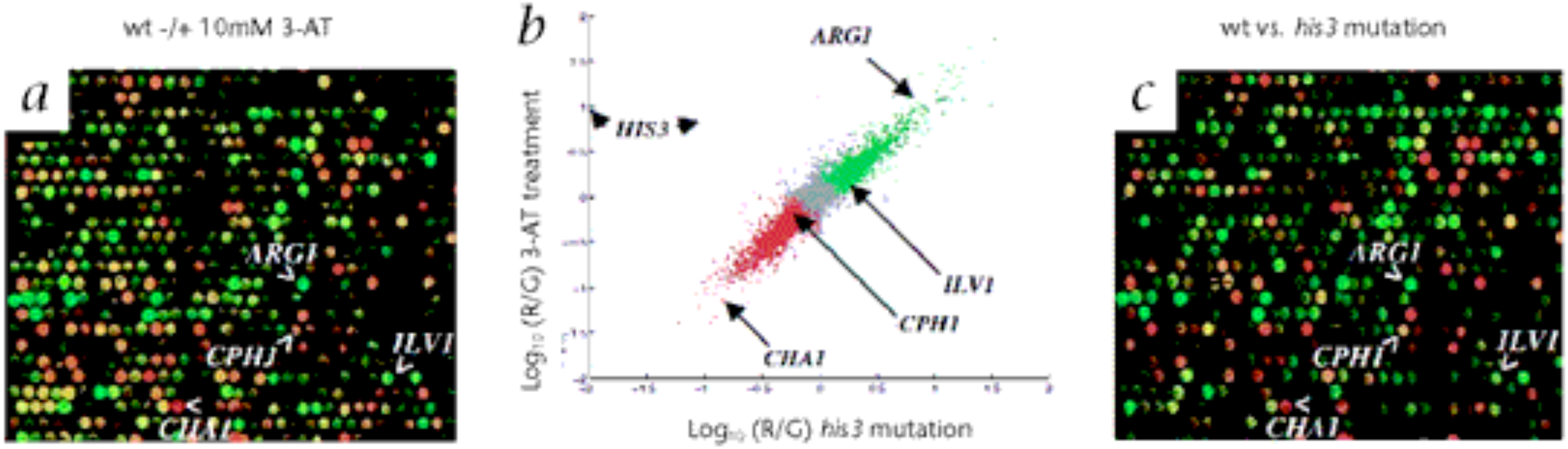
Immunofluorescence Patterns

Nuclear	395
Nucleolar	54
Nuclear rim / ER	93
Mitochondrial	166
Spindle pole body /MTs	6
Cell periphery	46
Cytoplasmic patches / dots	386
Cell neck	10
General cytoplasmic	1,247
TOTAL Strains Screened	6,750


Lys21
YCR004
HA
DAPI

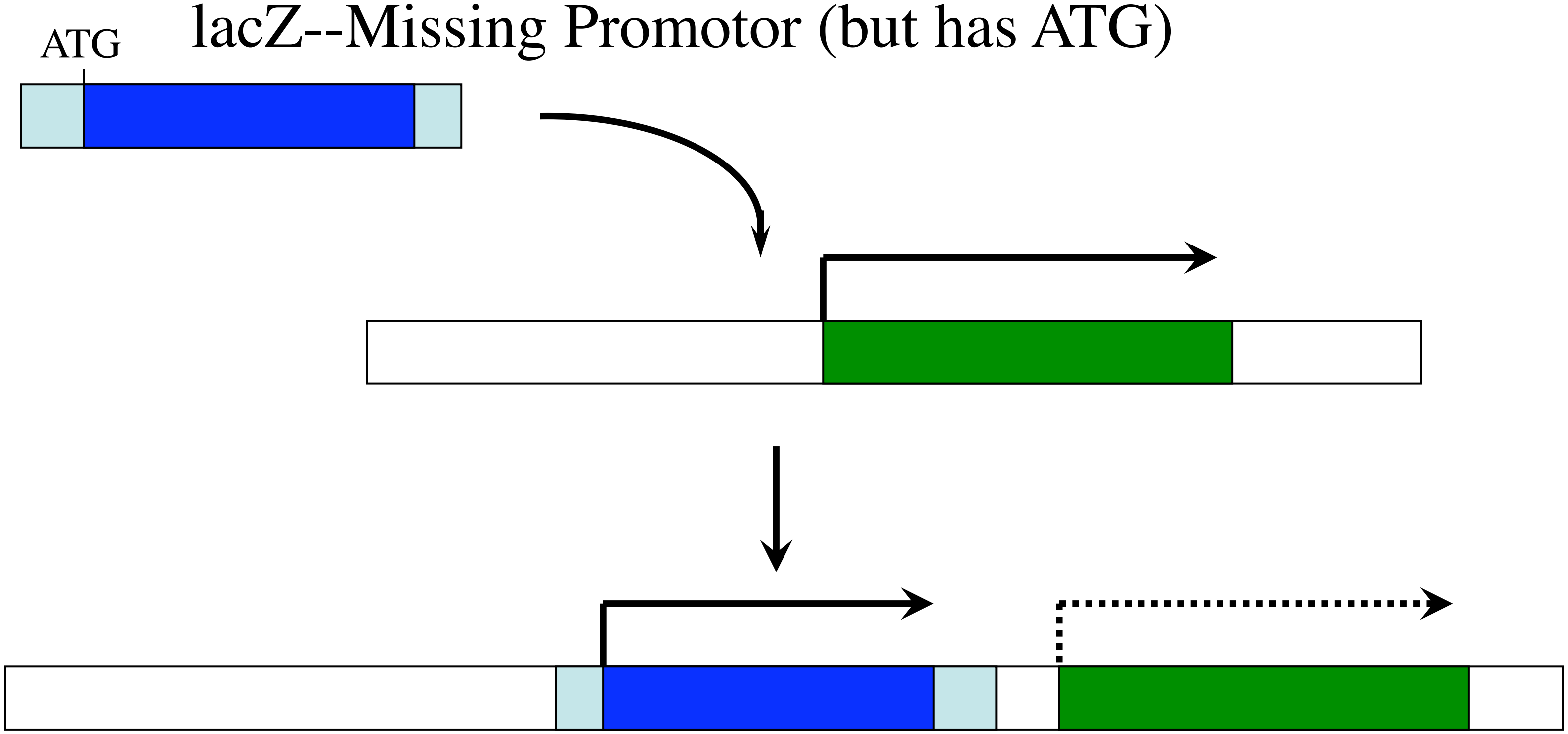

Bni4
Cin8
HA
DAPI

Drug Gives Similar Expression Profile to K/O: HIS3 vs AT



Marton et al. *Nature Med*.
Vol 4, 1293-1301.

Gene Traps: Enhancer Traps



lacZ--Missing Promotor (but has ATG)

May or may not affect Gene Expression

Drosophila melanogaster
13,000 Genes

3900 Starting P element lines

2695 Lines with Single Insertions

1045 Lines in Different Essential Genes

25% of Dm Essential Genes Affected

As of 1999   Rubin Spradling et al.