Gene Inactivation & Protein-Protein Interactions

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April 6, 2009

Last Time: Knockouts

1) Insertional Mutagenesis
  - Transposon Strategies
  - Insertional Mutations

2) Systematic Knockouts
  - Selectable Marker Replacement

Targeted Knockout Using Zinc Finger Technology

Zn Finger Domain:
Binds 6 bp sequence

Combine Multiple Domains

2 Zn Finger Domain:
Binds 12 bp sequence

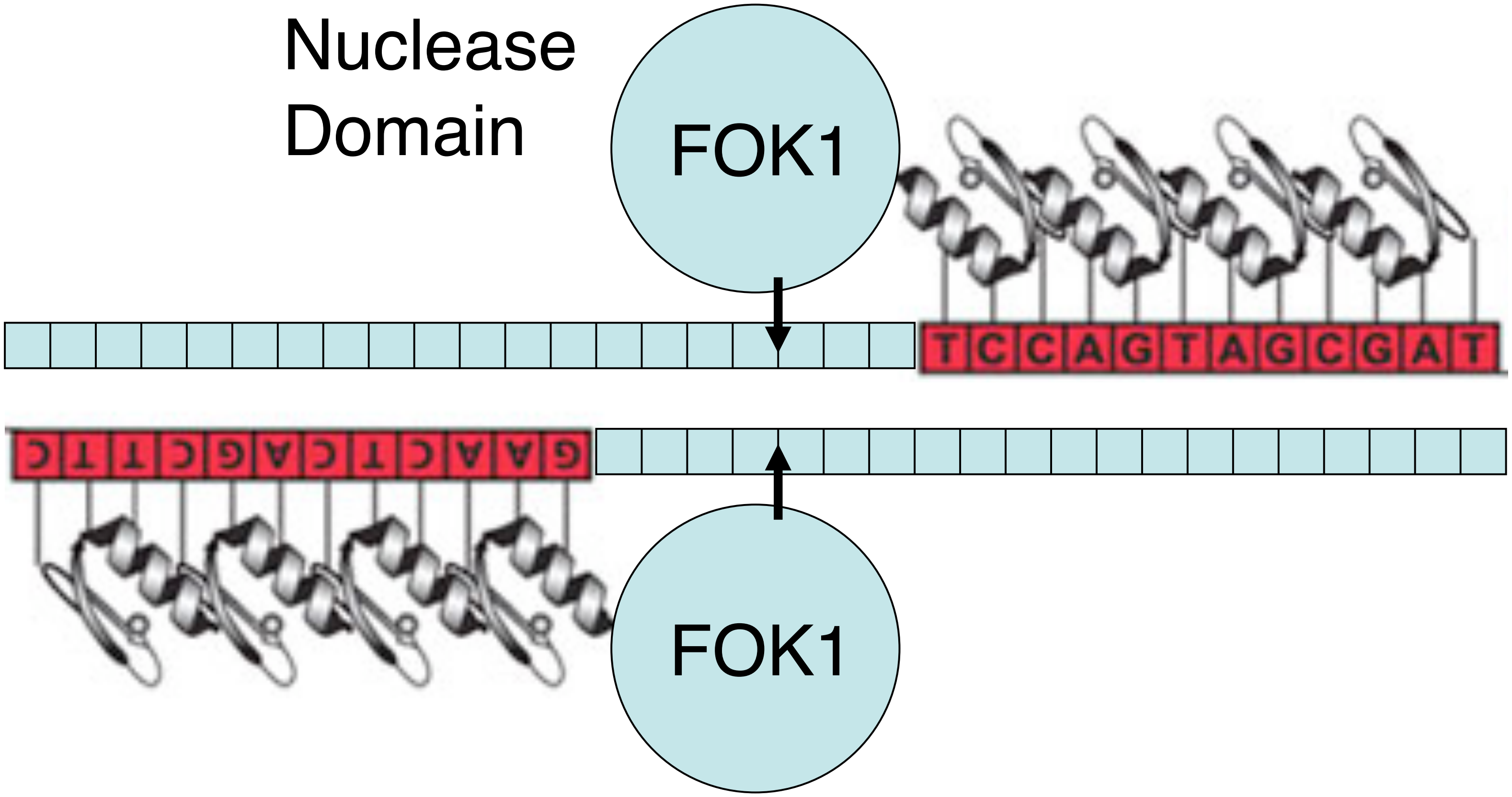
Images from Sigma
Procedure based on Urnov et al., (2005) Nature 435: 646

Targeted Knockout Using Zinc Finger Technology

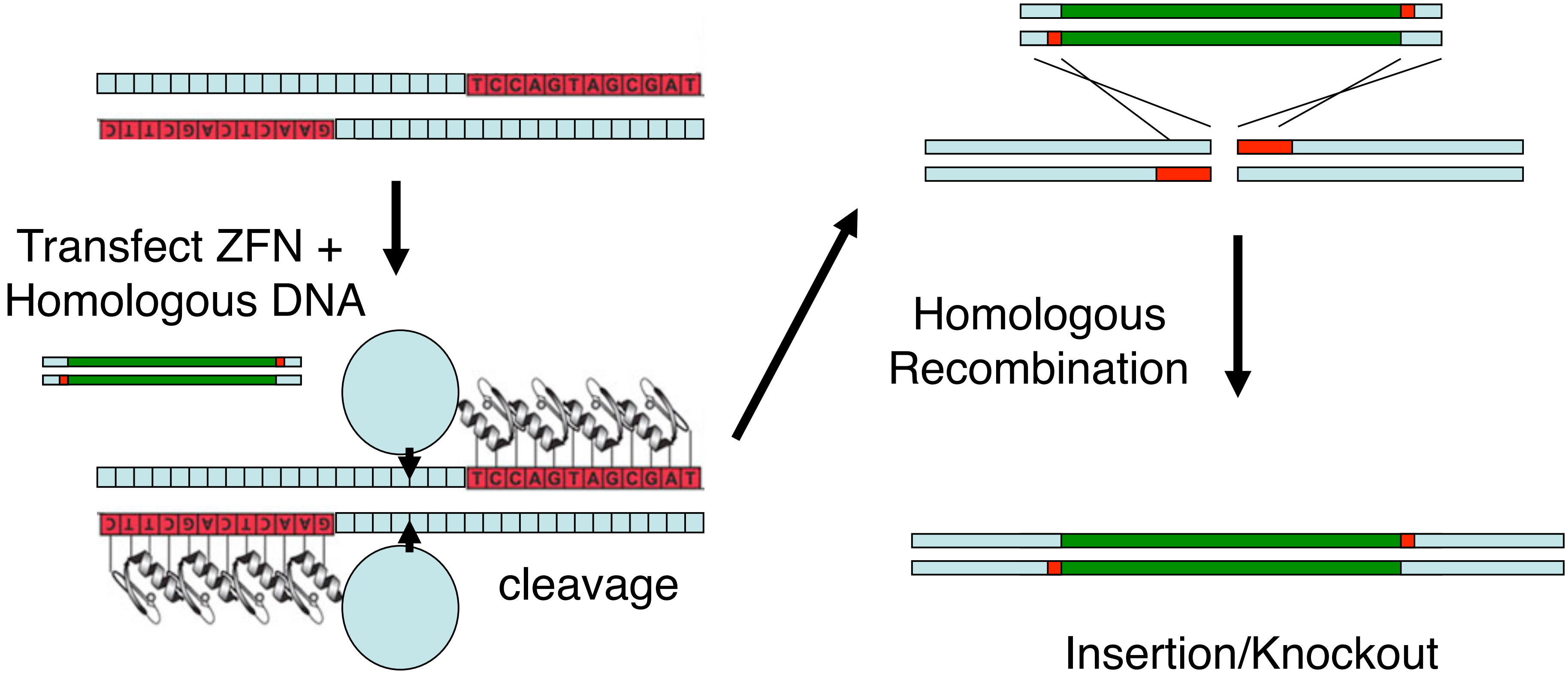
Nuclease Domain

Zn Finger Domain

FOK Cleaves DNA as a Dimer



Gene Knockout/Insertion Using Zn Finger Technology



Transfect ZFN +
Homologous DNA

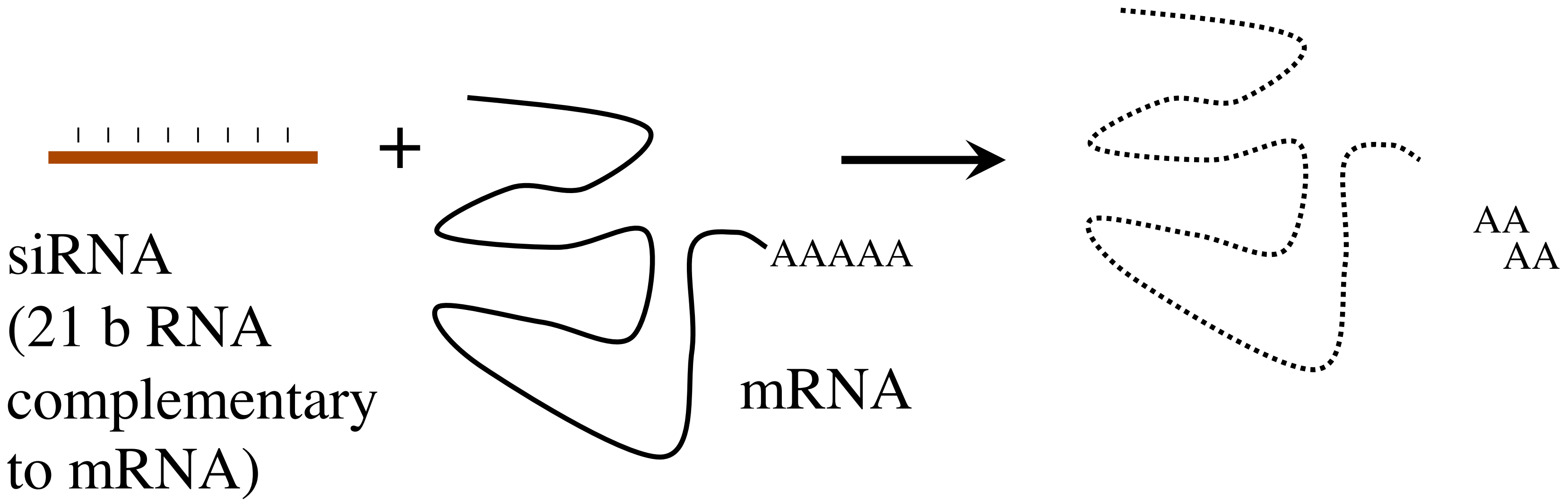
cleavage

Homologous
Recombination

Insertion/Knockout

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

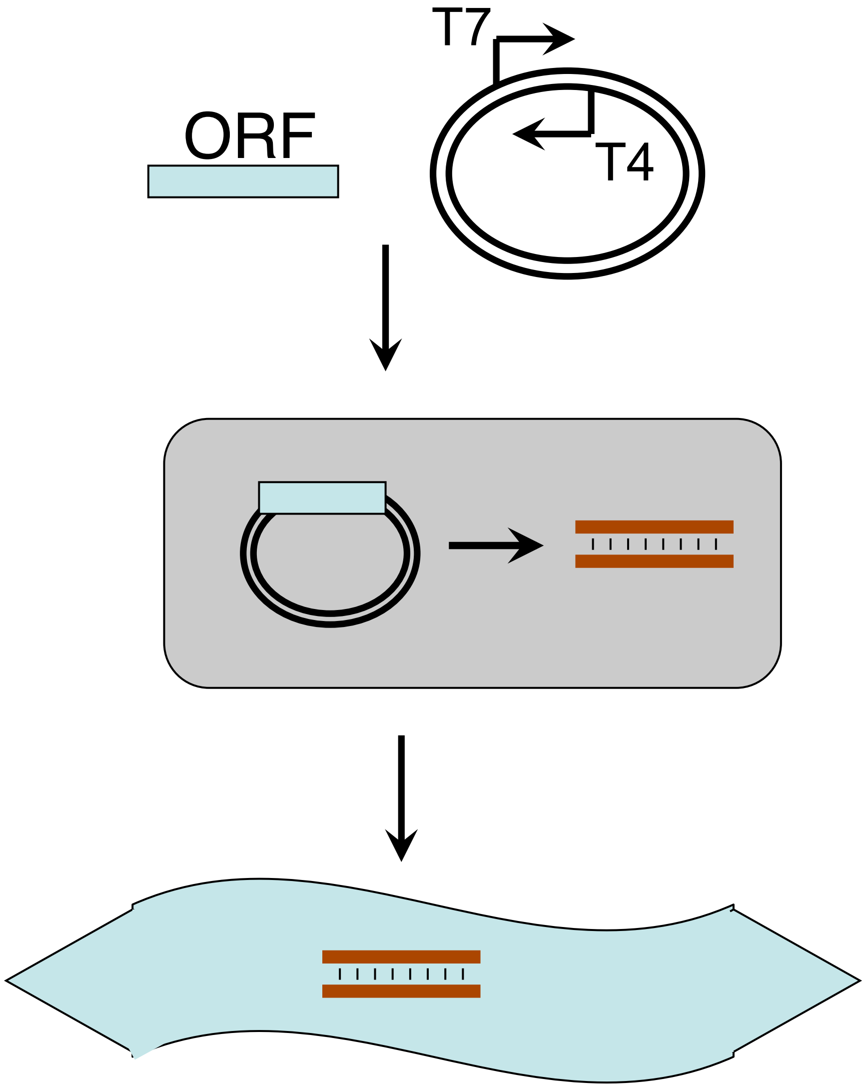
RNAi = RNA interference



siRNA
(21 b RNA
complementary
to mRNA)

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.

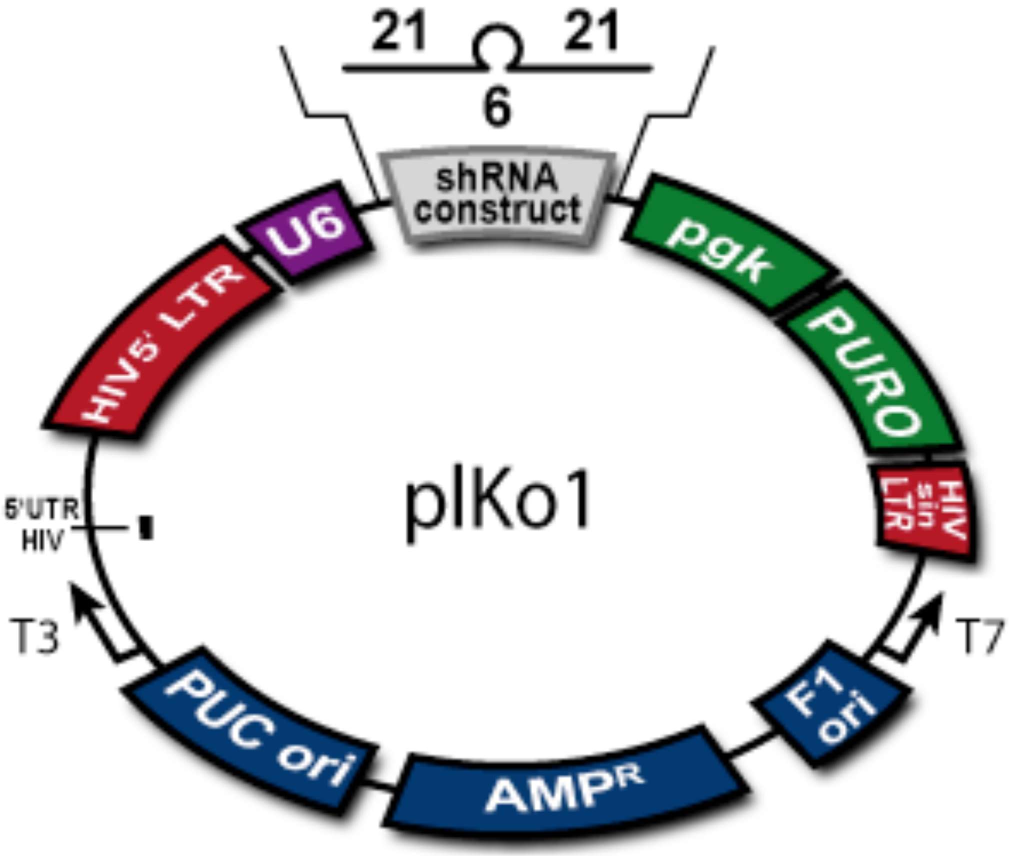
Can be used for many organisms
Drosophila, Mammalian Cells

RNAi Two approaches

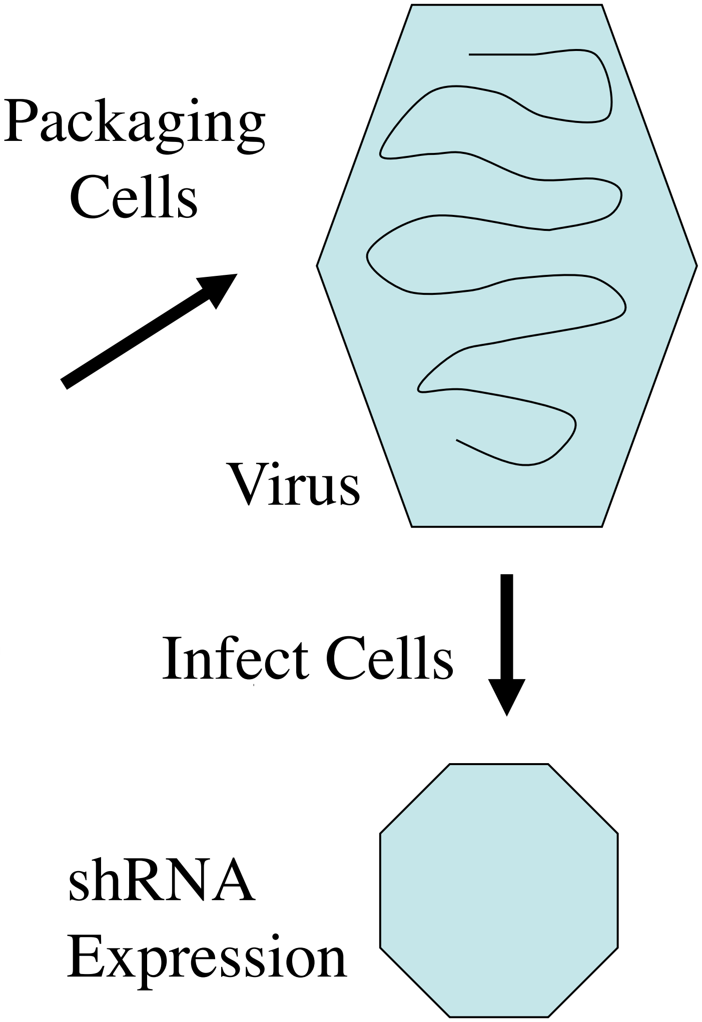
1) siRNA = Transfect 21 bp RNA complementary to mRNA
   (Screened for cellular genes required for HIV infection; involved 21,121 siRNAs)

2) shRNA = short hairpin RNAs
   Expressed from retroviruses

Mammalian RNAi Retrovirus Vector



From RNAi consortium



Packaging Cells

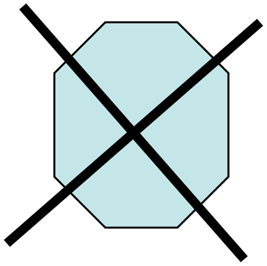
Virus

Infect Cells

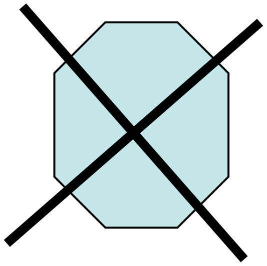
shRNA Expression

Identification of Tumor Suppressors Using RNAi

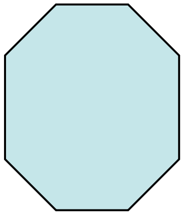
Klofcshoten et al. (2005) Cell 121, 849-858



1° Fibroblasts from humans die

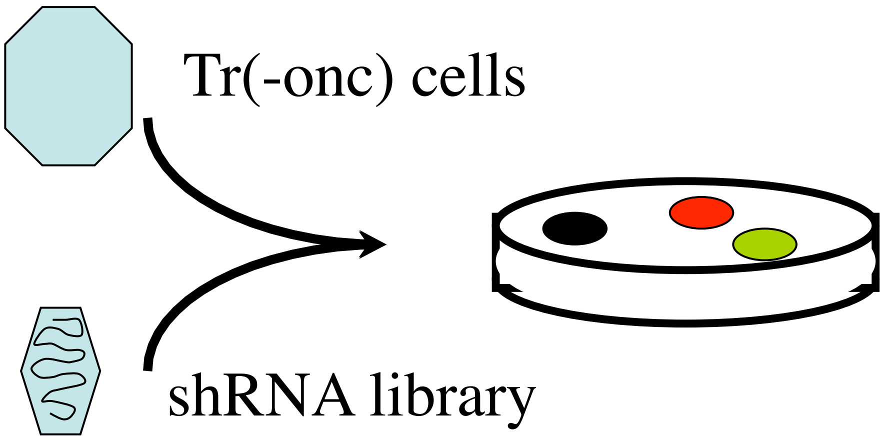


Tr(-onc) Engineered Fibroblasts
(hTERT, small t Antigen, p53-, p16-)
“almost transformed”



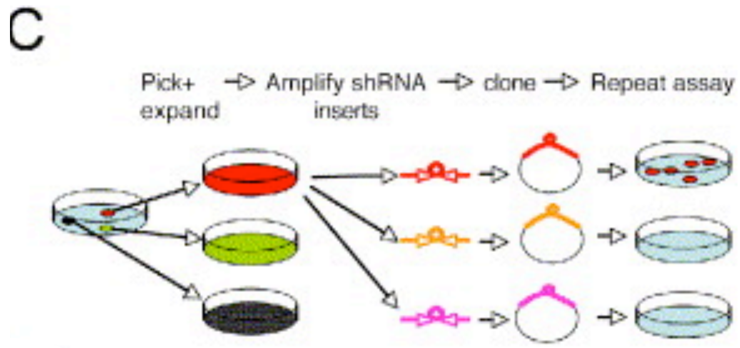
Tr(-onc) Engineered Fibroblasts + RAS^{V12}
Transformed and form colonies

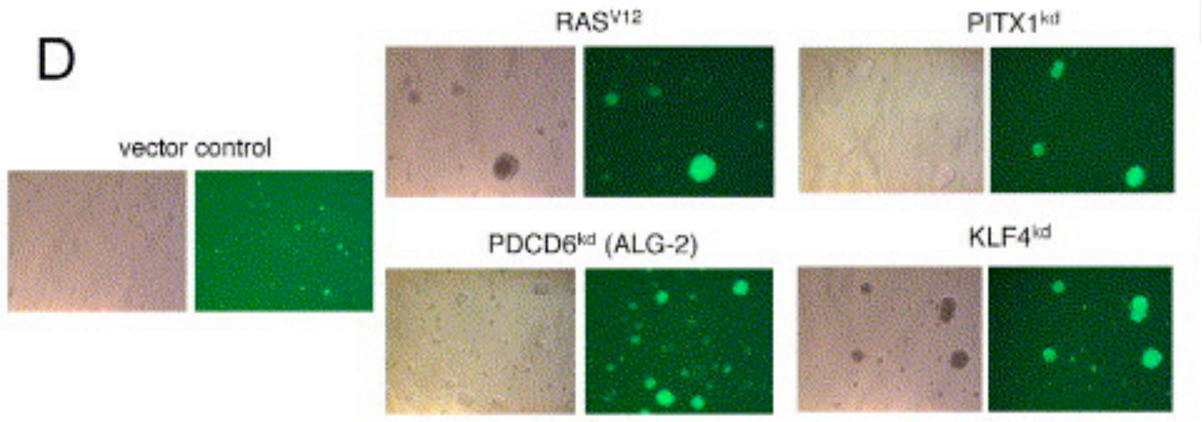
Identification of Tumor Suppressors Using RNAi



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

Global Protein::Protein Interactions

Three Methods:

1) Two Hybrid
2) Complex Analysis: Affinity tagging/Mass Spectrometry
3) Protein Chip

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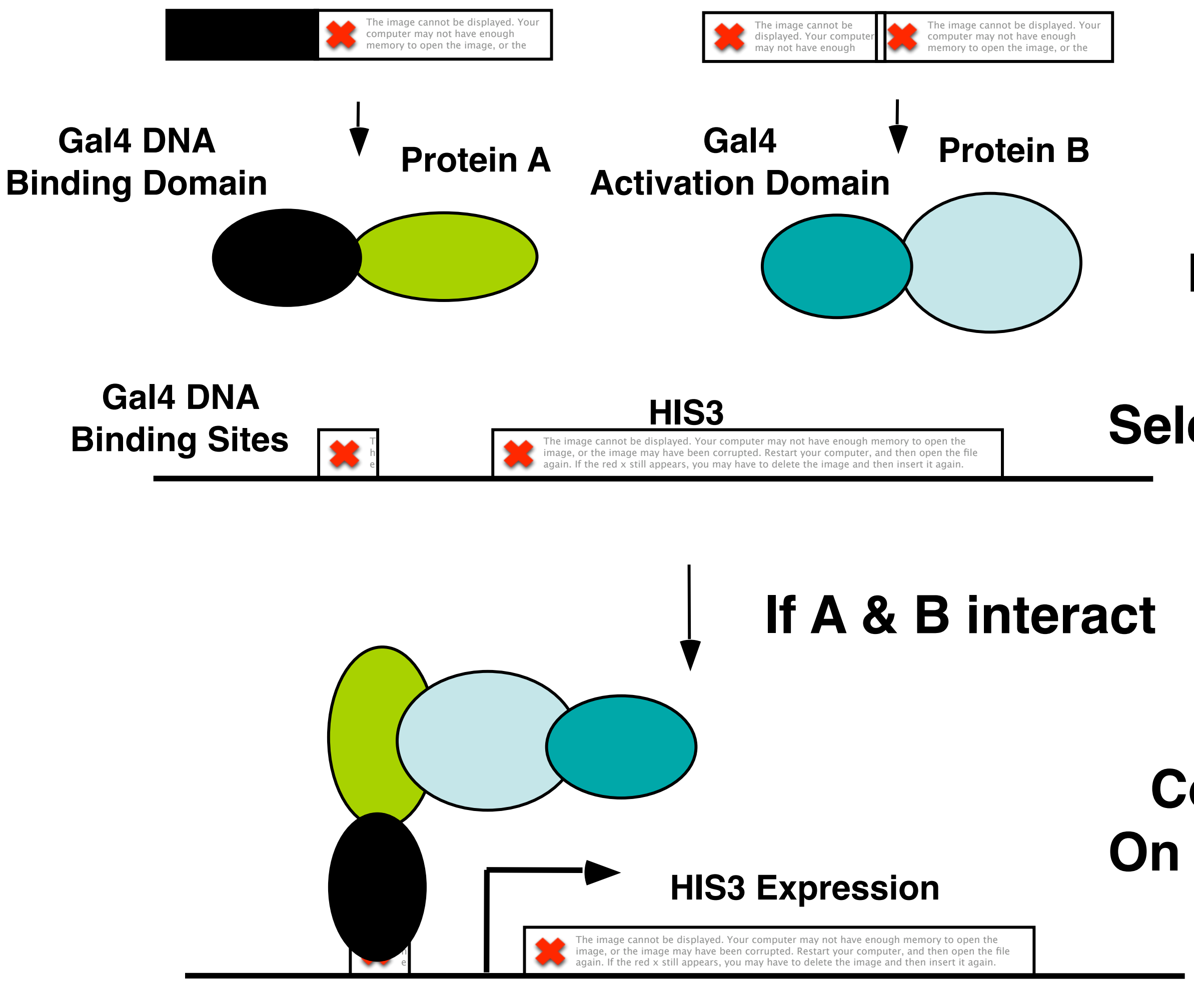
Two-Hybrid System For Detecting Protein-Protein Interaction



Genes

Gal4 DNA
Binding Domain

Protein A

Gal4
Activation Domain

Protein B

Fusion
Proteins

Gal4 DNA
Binding Sites

HIS3

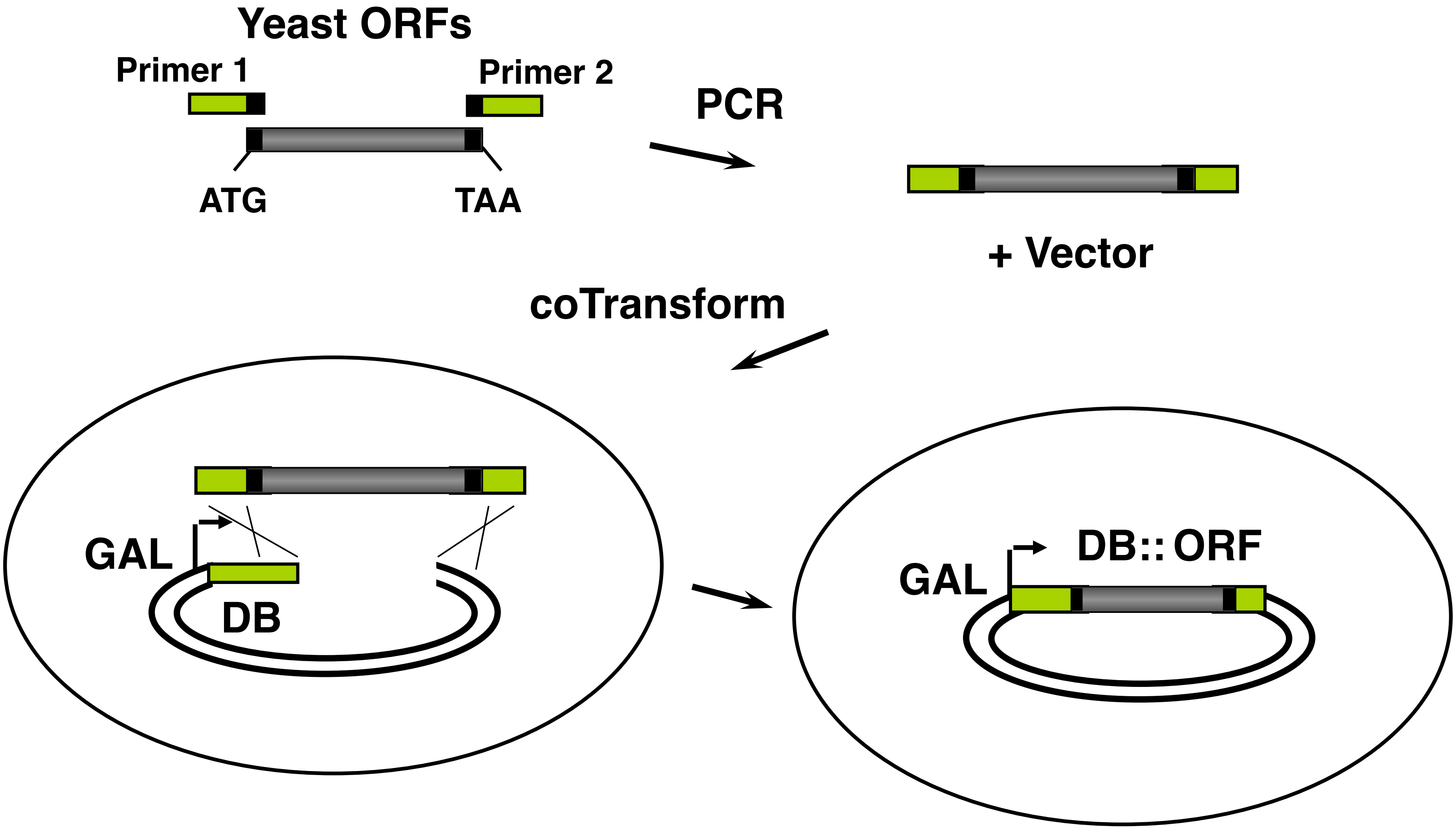
Selectable Marker

If A & B interact

HIS3 Expression

Colonies Grow
On Plates Lacking
Histidine

Cloning Strategy



Yeast ORFs

Primer 1

Primer 2

ATG

TAA

PCR

+ Vector

coTransform

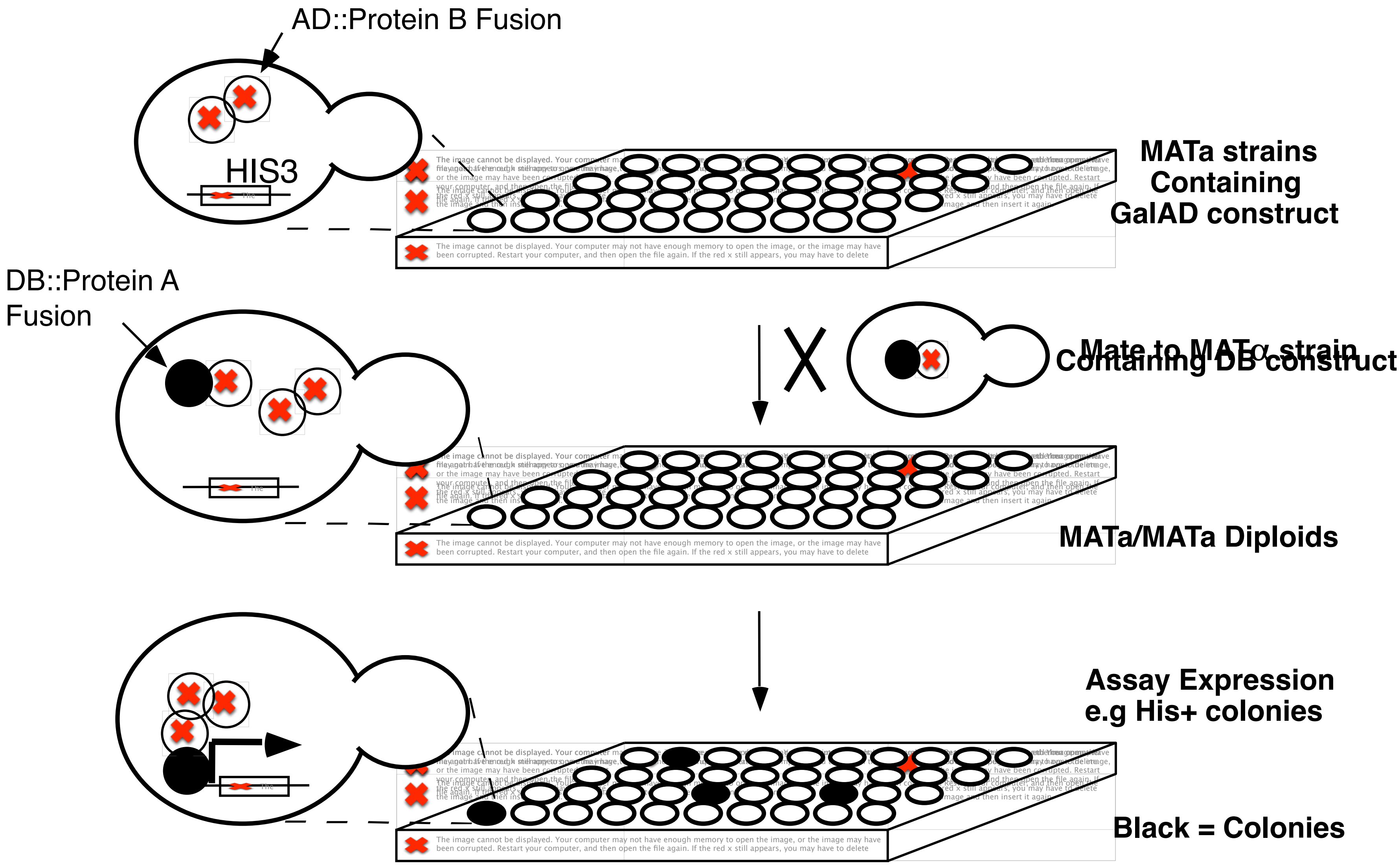
GAL

DB

GAL

DB:: ORF

Screening 6200 X 6200 Interactions



MATa strains Containing GalAD construct

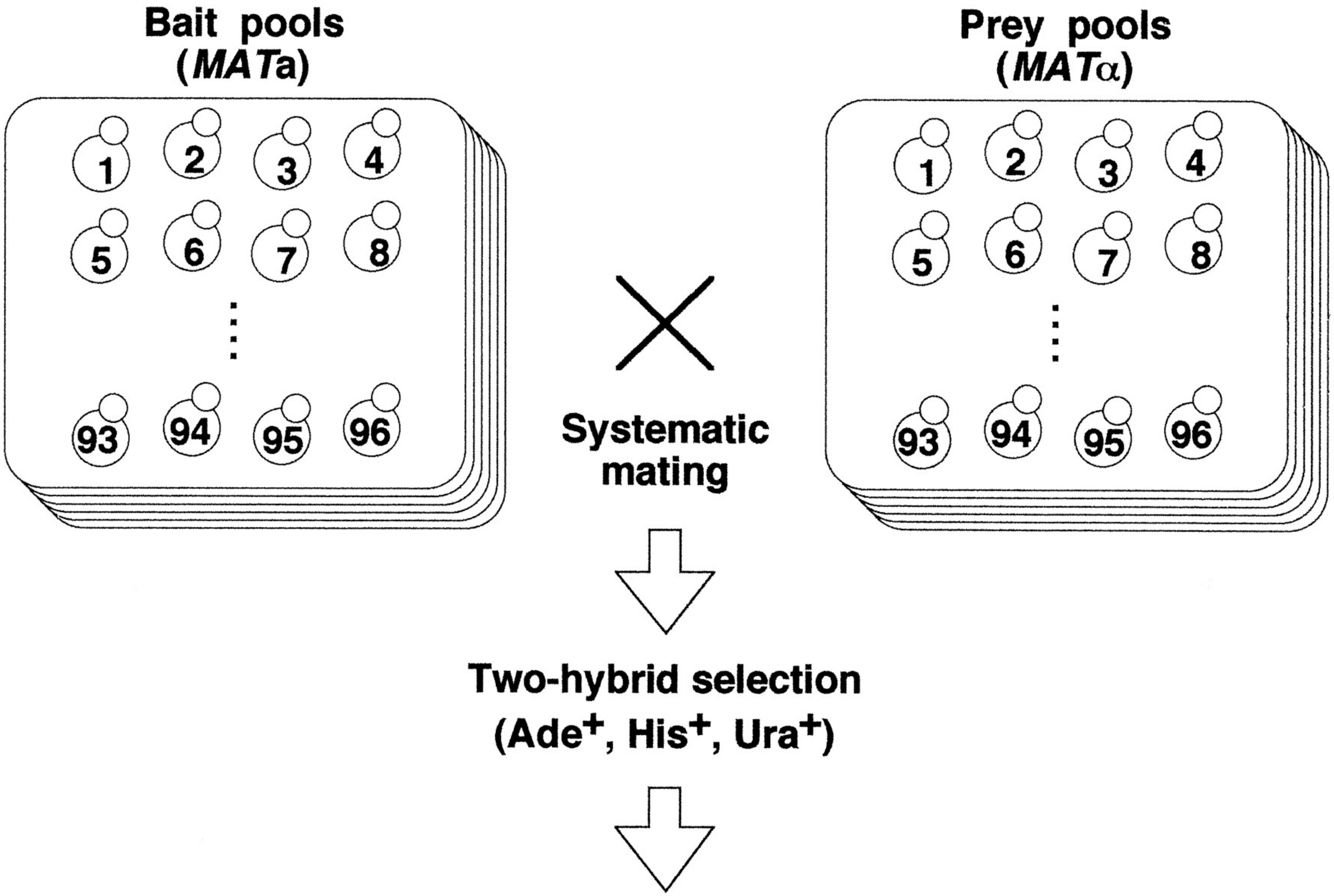
Mate to MATα strain Containing DB construct

MATa/MATa Diploids

Assay Expression e.g His+ colonies

Black = Colonies

Large Scale Two-Hybrid Screening



Bait pools (*MAT*a)

Prey pools (*MAT*α)

Systematic mating

Two-hybrid selection (Ade^+, His^+, Ura^+)

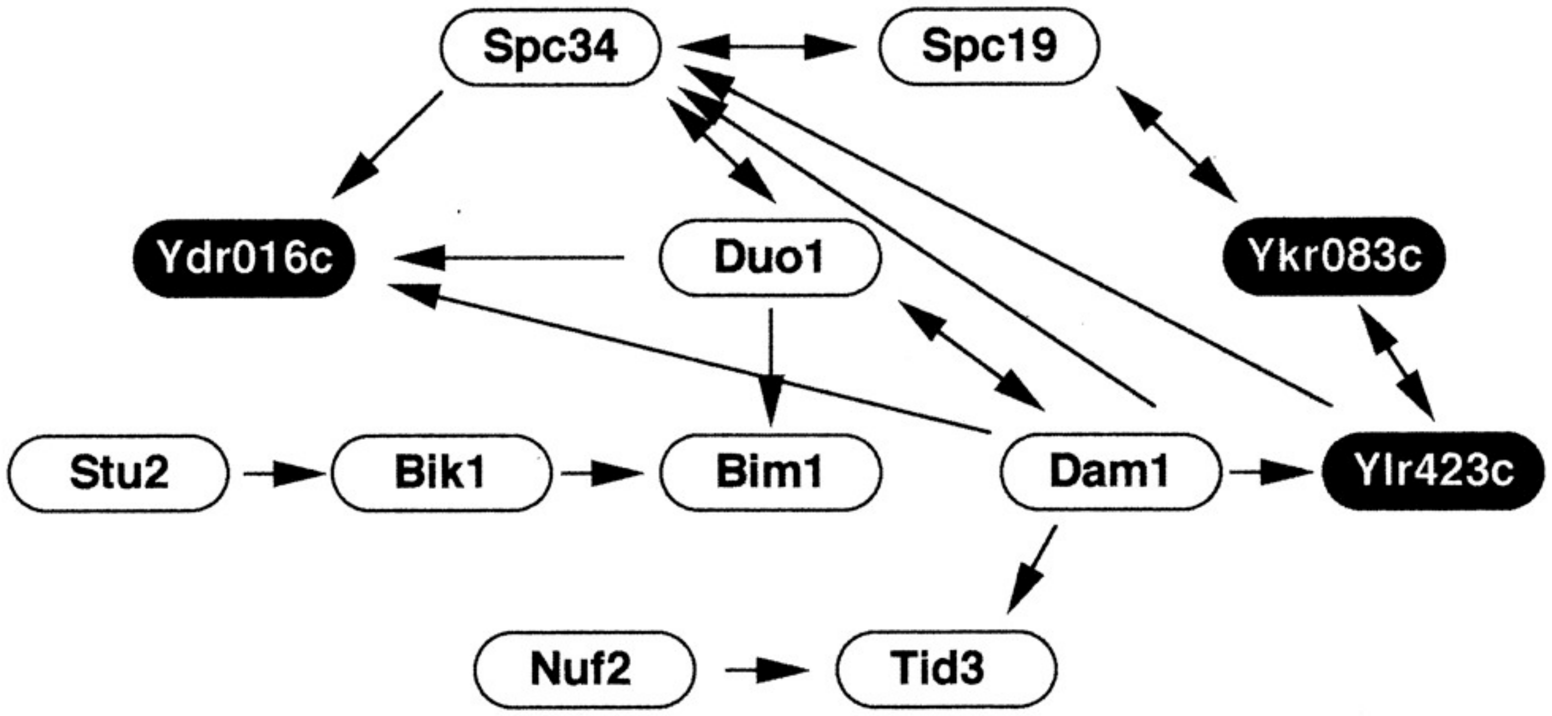
Sequence Adjacent to ORF for each plasmid (Interaction Sequence Tags ISTs)

Results of Two Studies

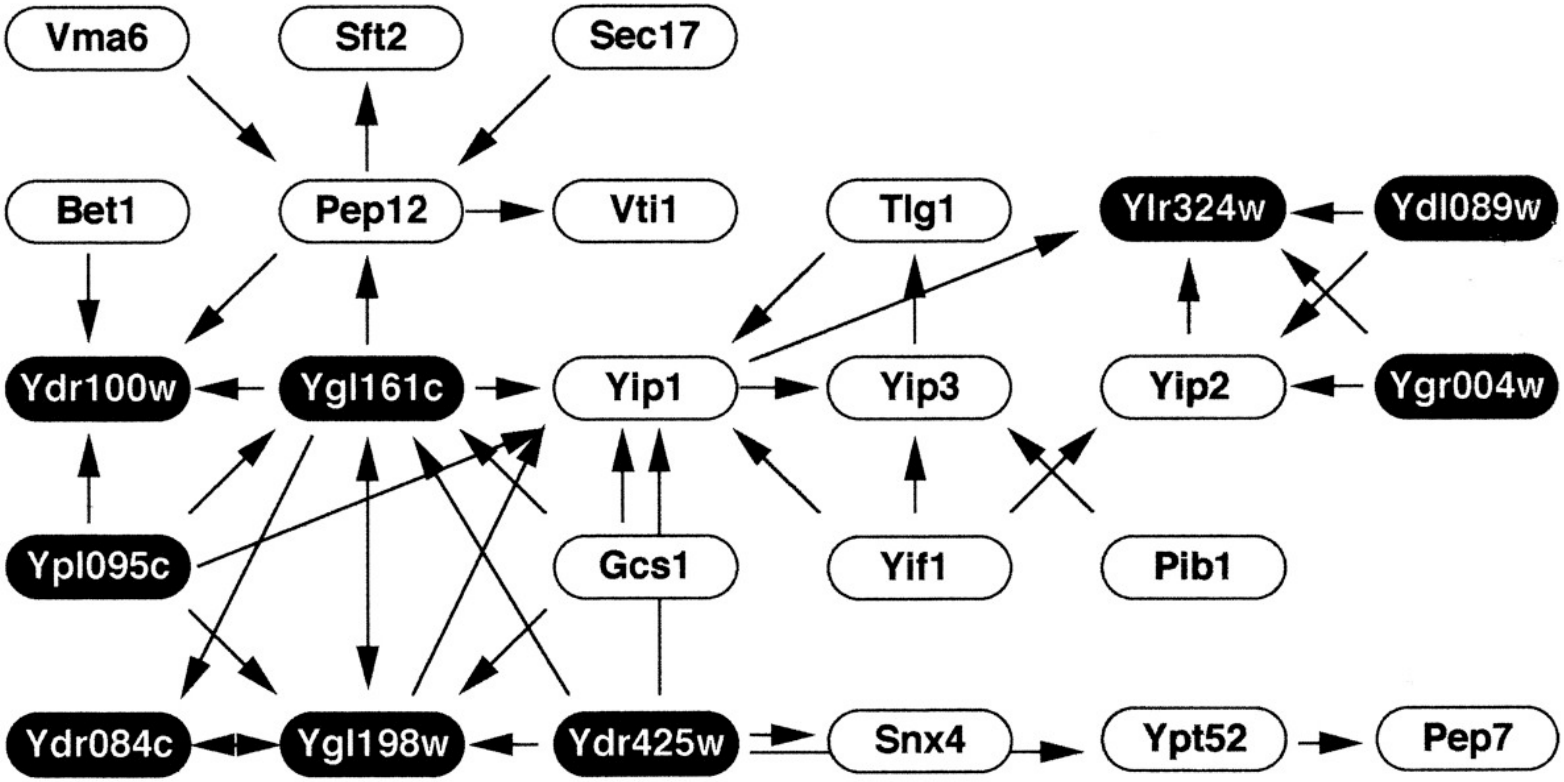
1) 4,549 Interactions Among 3,278 Proteins (Ito et al.)

2) 957 Interactions 1004 proteins (Utez et al.)

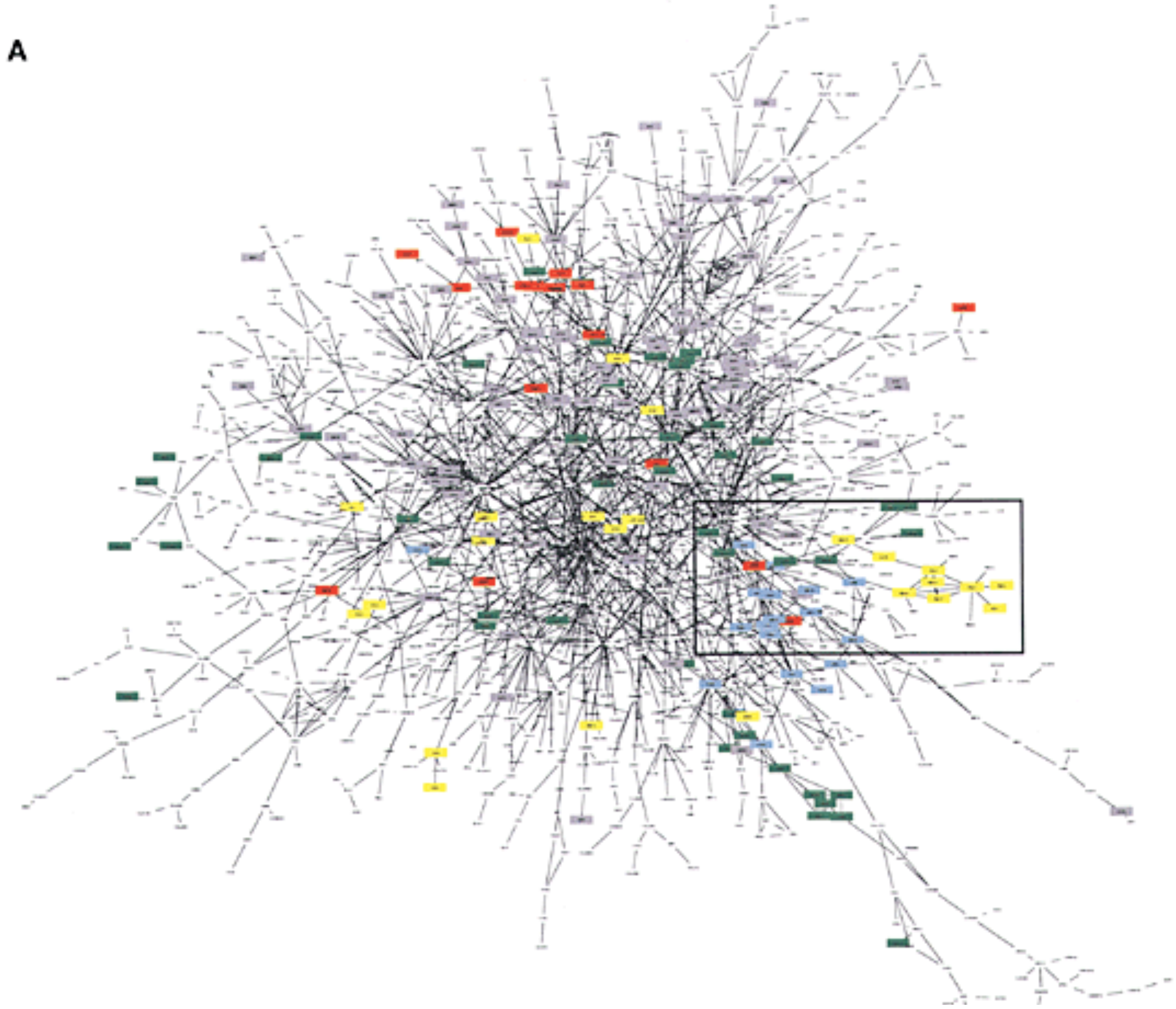
Interaction Map of Spindle Pole Body (MTOC) Proteins



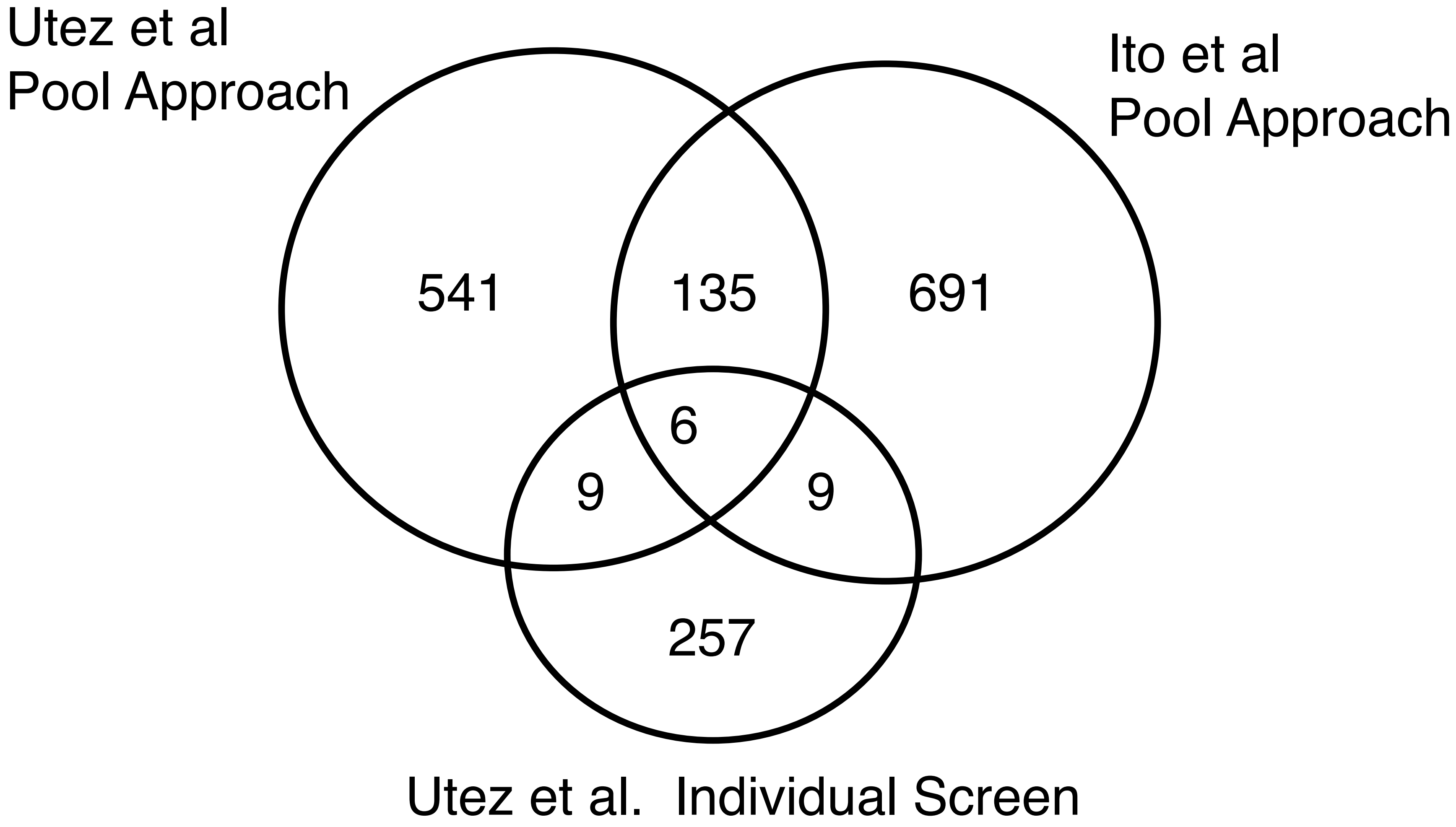
Interaction Map of Vesicular Transport Proteins



A Comprehensive Protein Interaction Map



Overlap of the Two Hybrid Studies



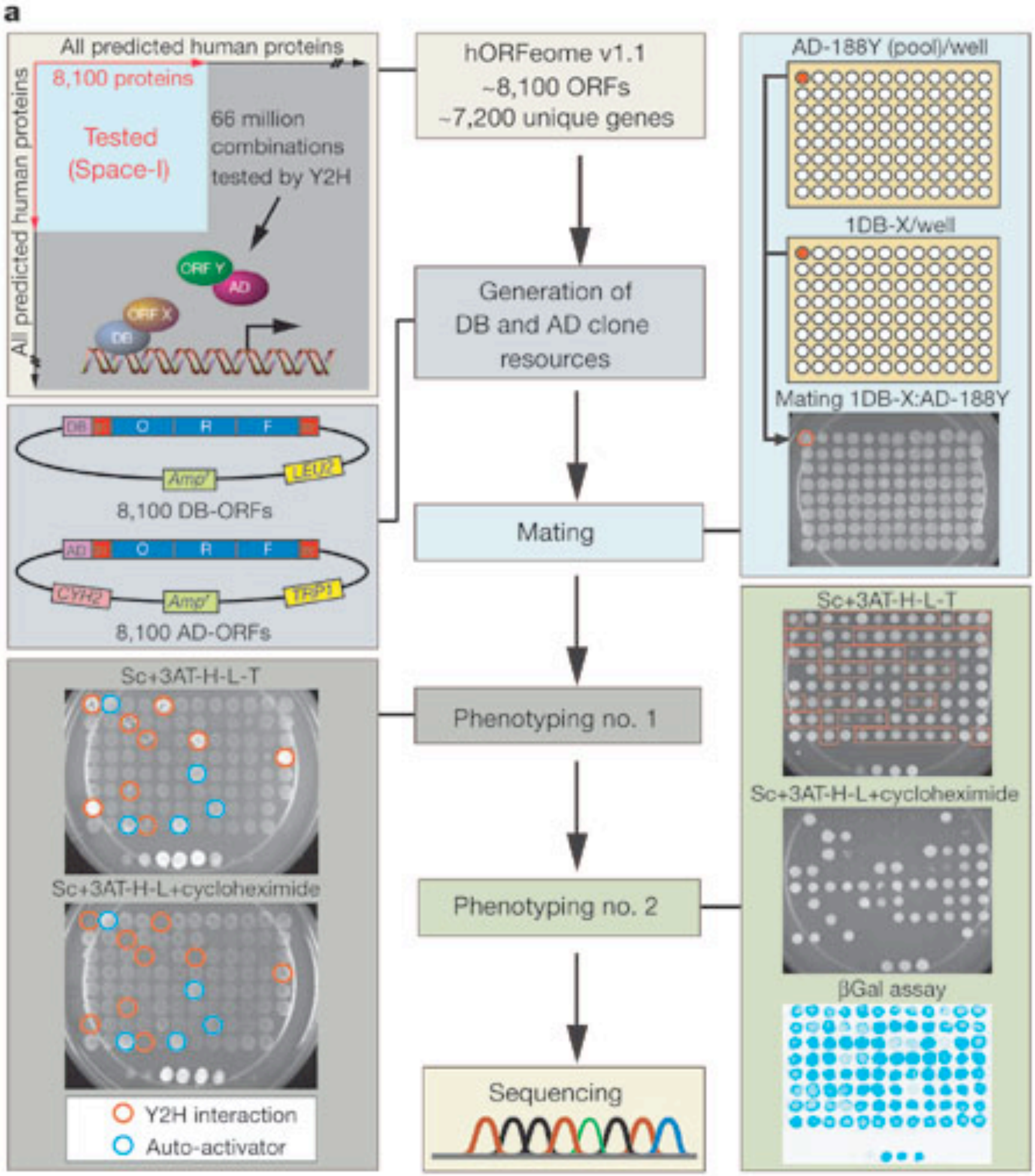
Human Two Hybrid Screen

8,100 X 8,100 ORFs (~7,200 genes)

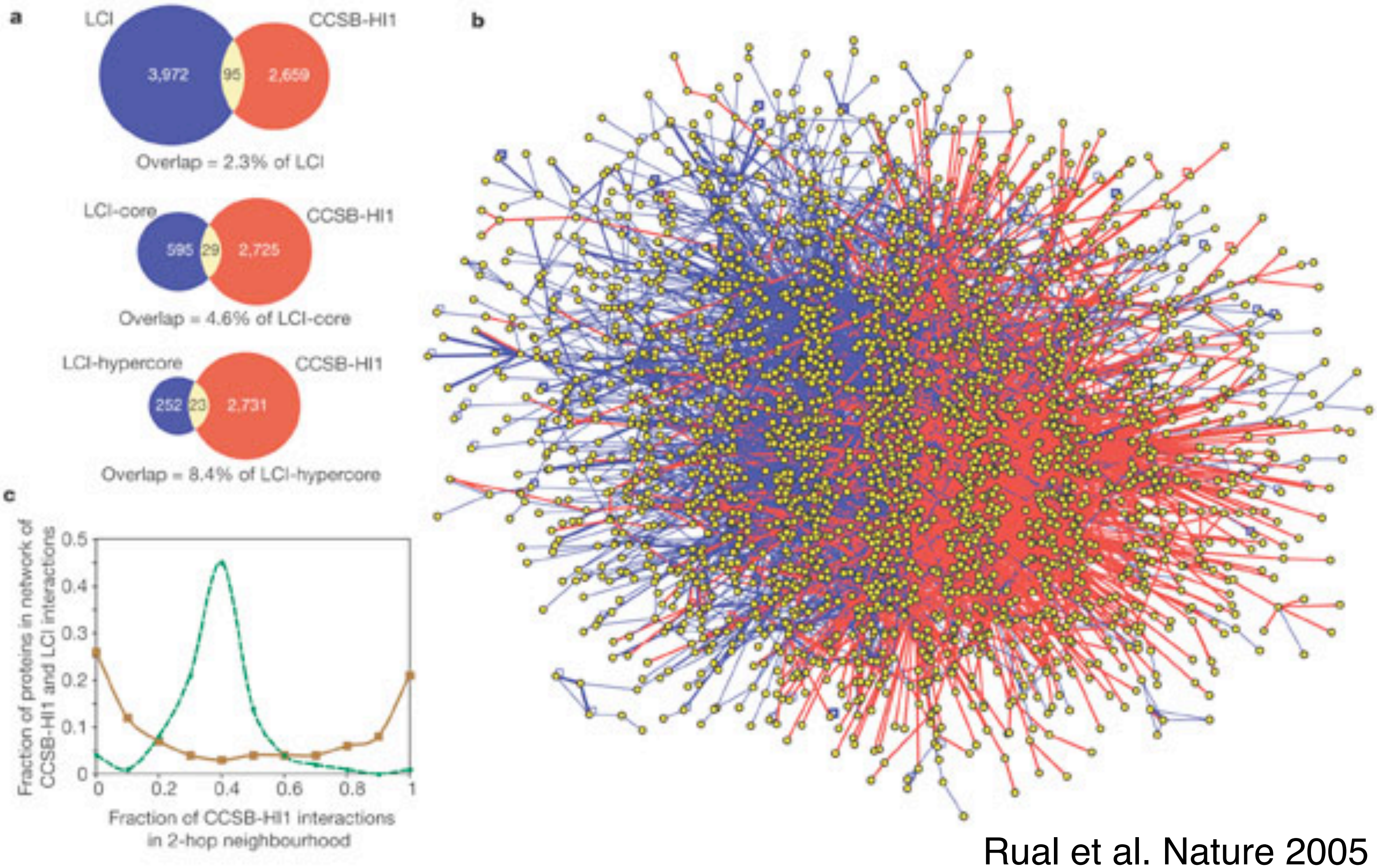
(1 DB clone X pool of 188 AD clones)

10,597 Interactions
2,754 Nonredundant

Rual et al. Nature 2005



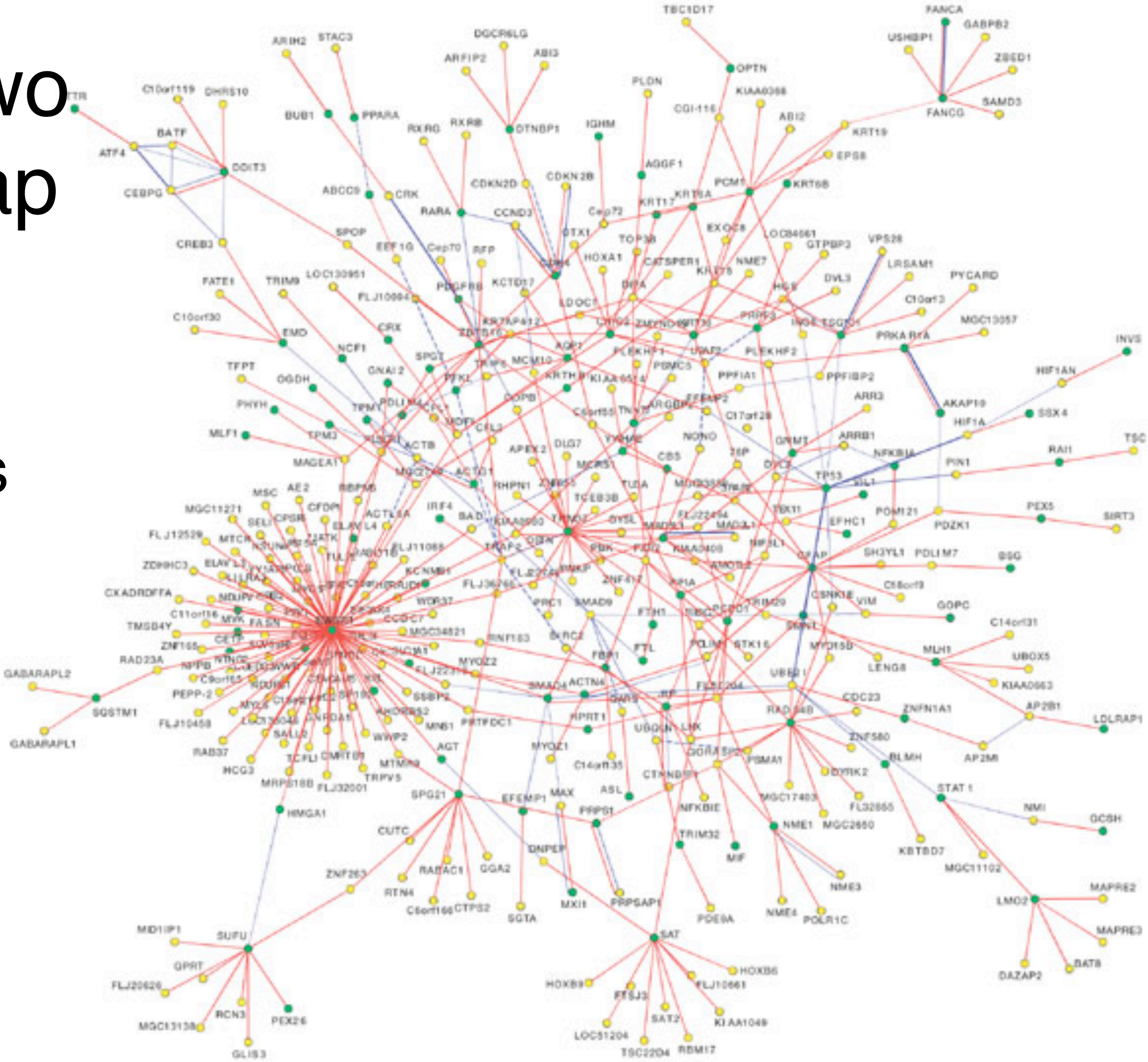
Human Two Hybrid Map



Rual et al. Nature 2005

Human Two Hybrid Map Disease Genes

(121 genes (green))



Rual et al. Nature 2005 Vol 437

Two Hybrid

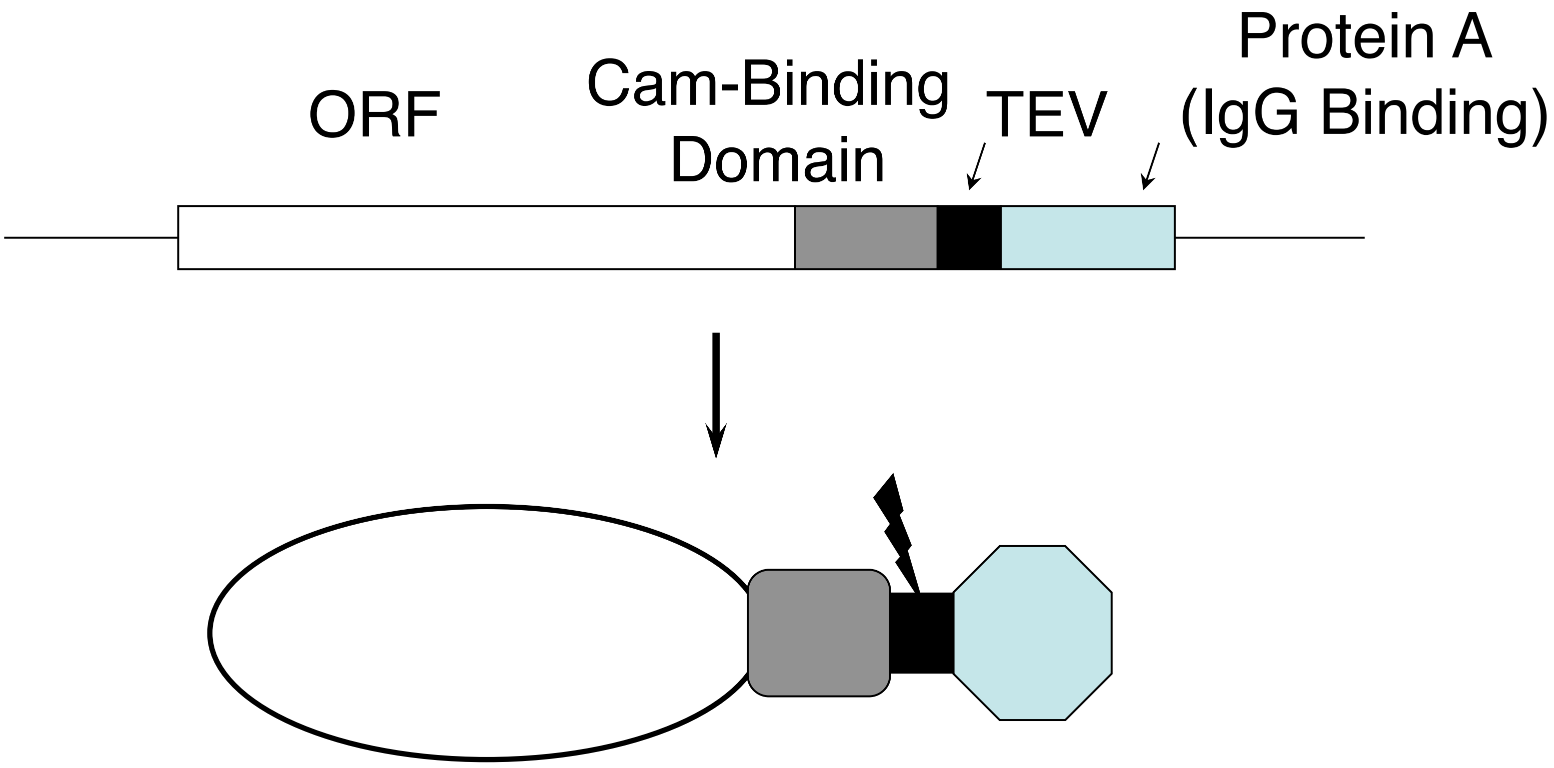
Advantages

- In vivo Assay
- Fairly Simple

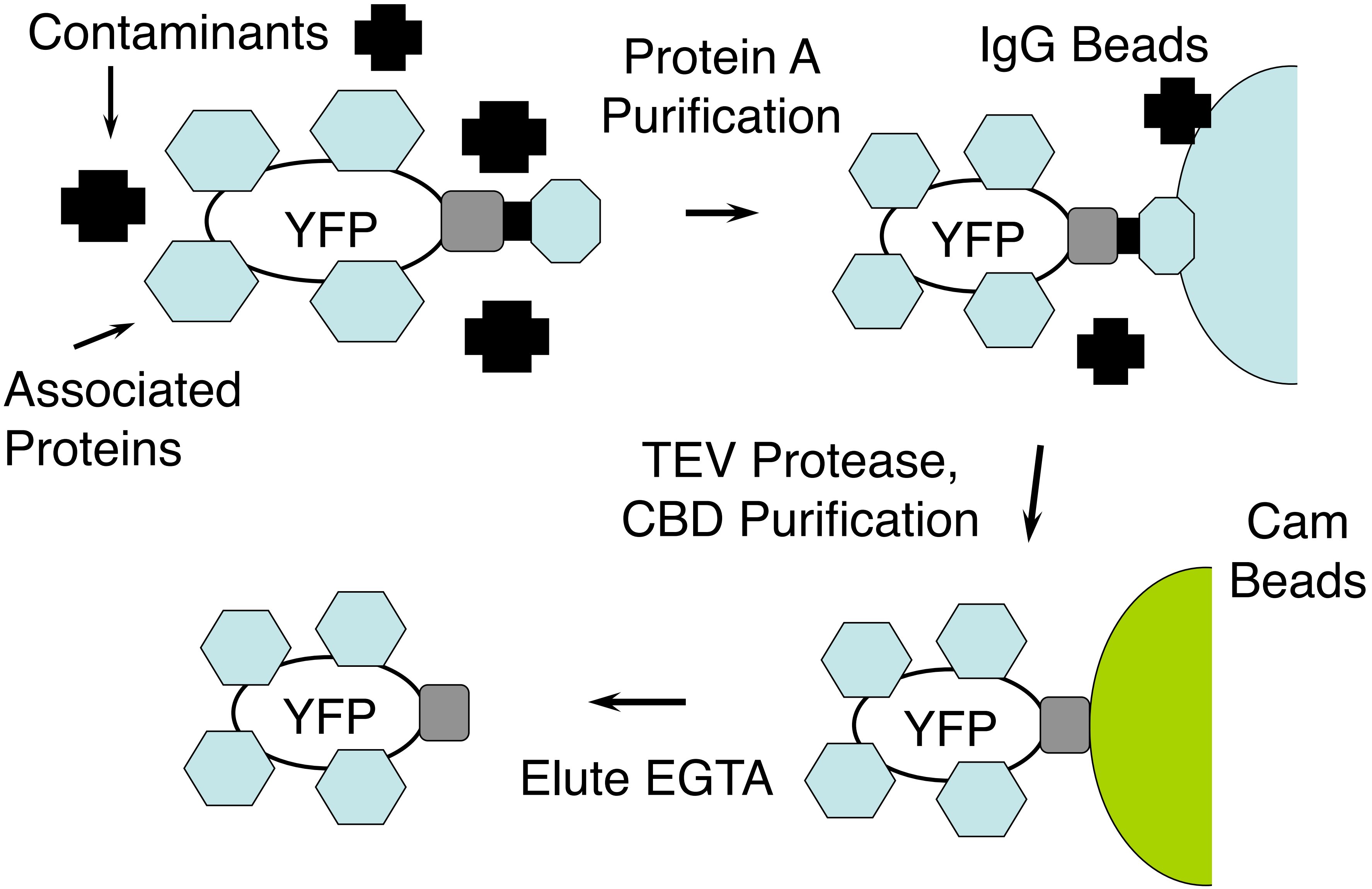
Disadvantages

- Hard to execute on a large scale
- Prone to artifacts 50% False +s
- Interactions mediated in nucleus

Tandem Affinity Purification (TAP) Tagging



TAP Approach



Contaminants

Associated Proteins

Protein A Purification

IgG Beads

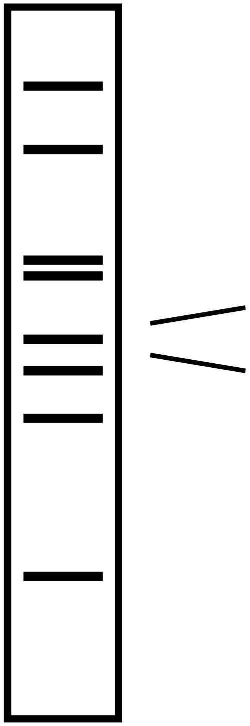
TEV Protease, CBD Purification

Cam Beads

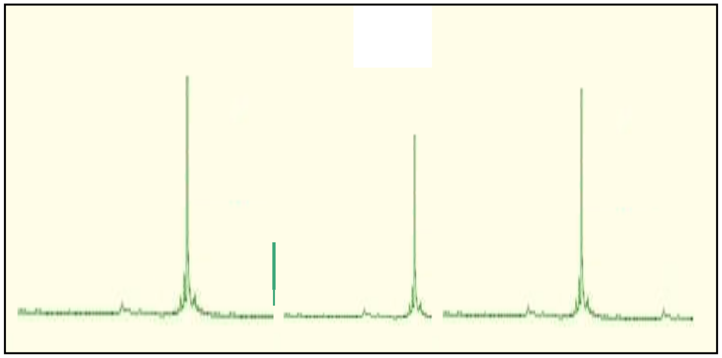
Elute EGTA

Identify Proteins by Mass Spec

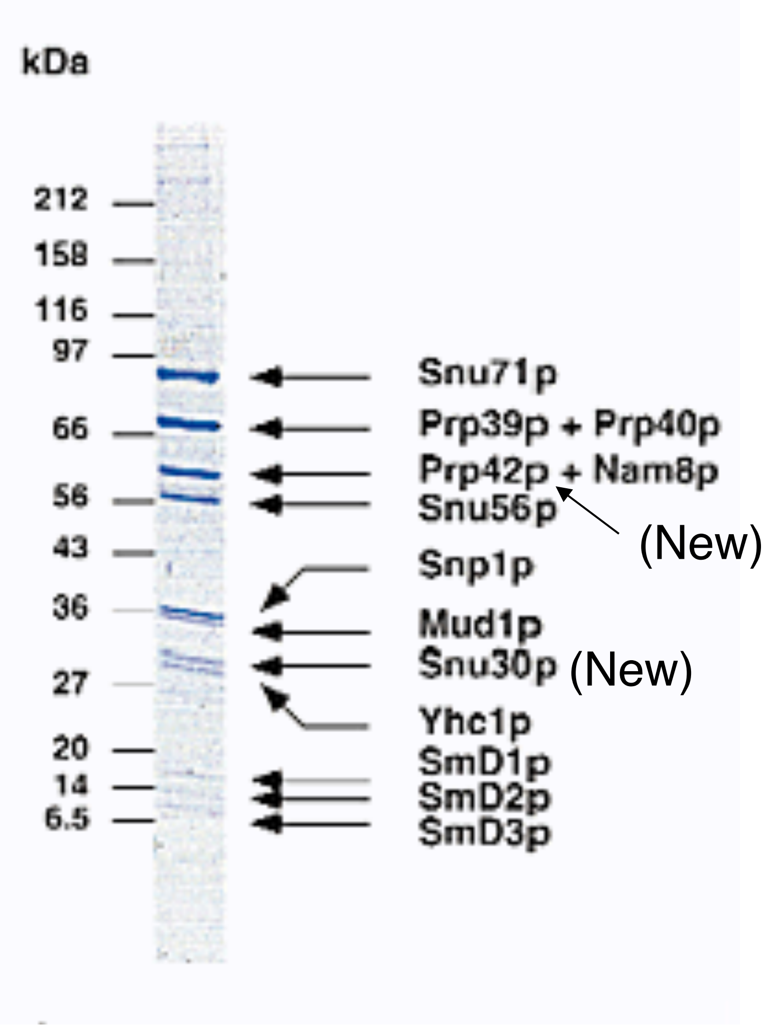
Load on SDS Gel



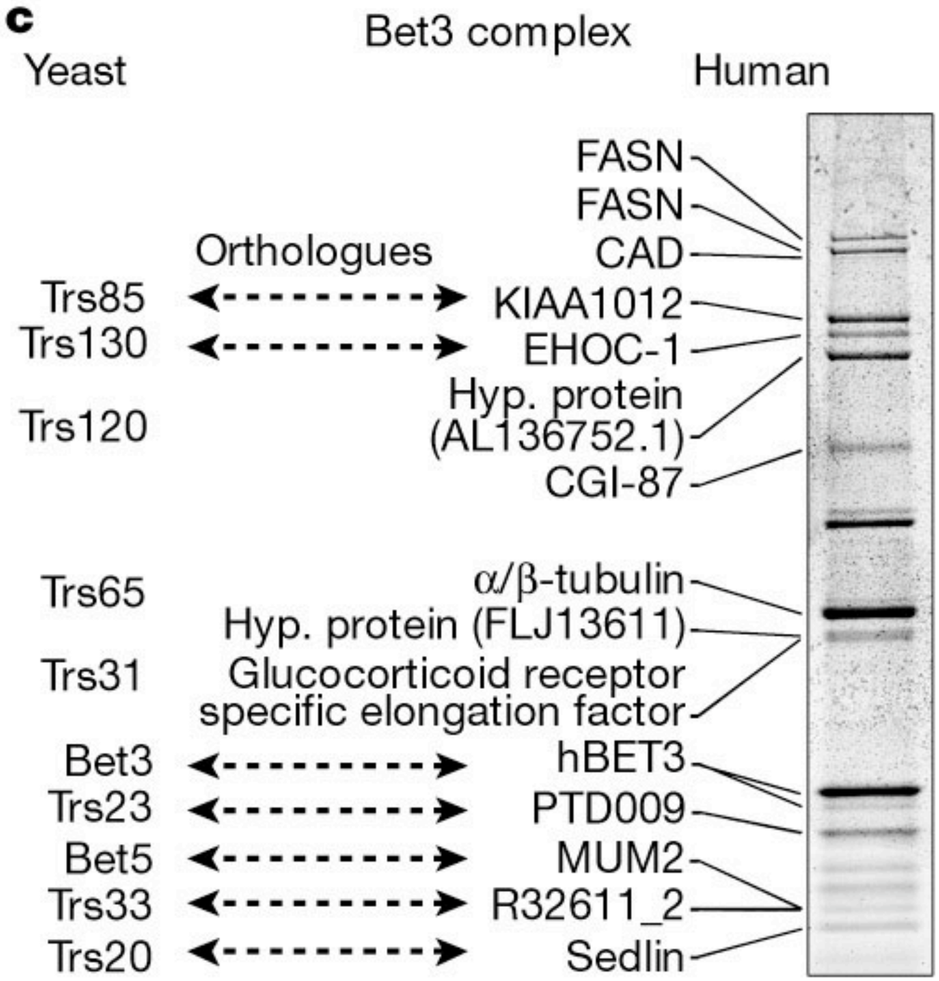
Excise Band;
Digest with Trypsin;
Run Mass Spec



TAP Purification of The U1 Splicing Complex (Snu71p)



Many Complexes Are Conserved



Affinity Purification/Mass Spec Analysis of Complexes - Yeast

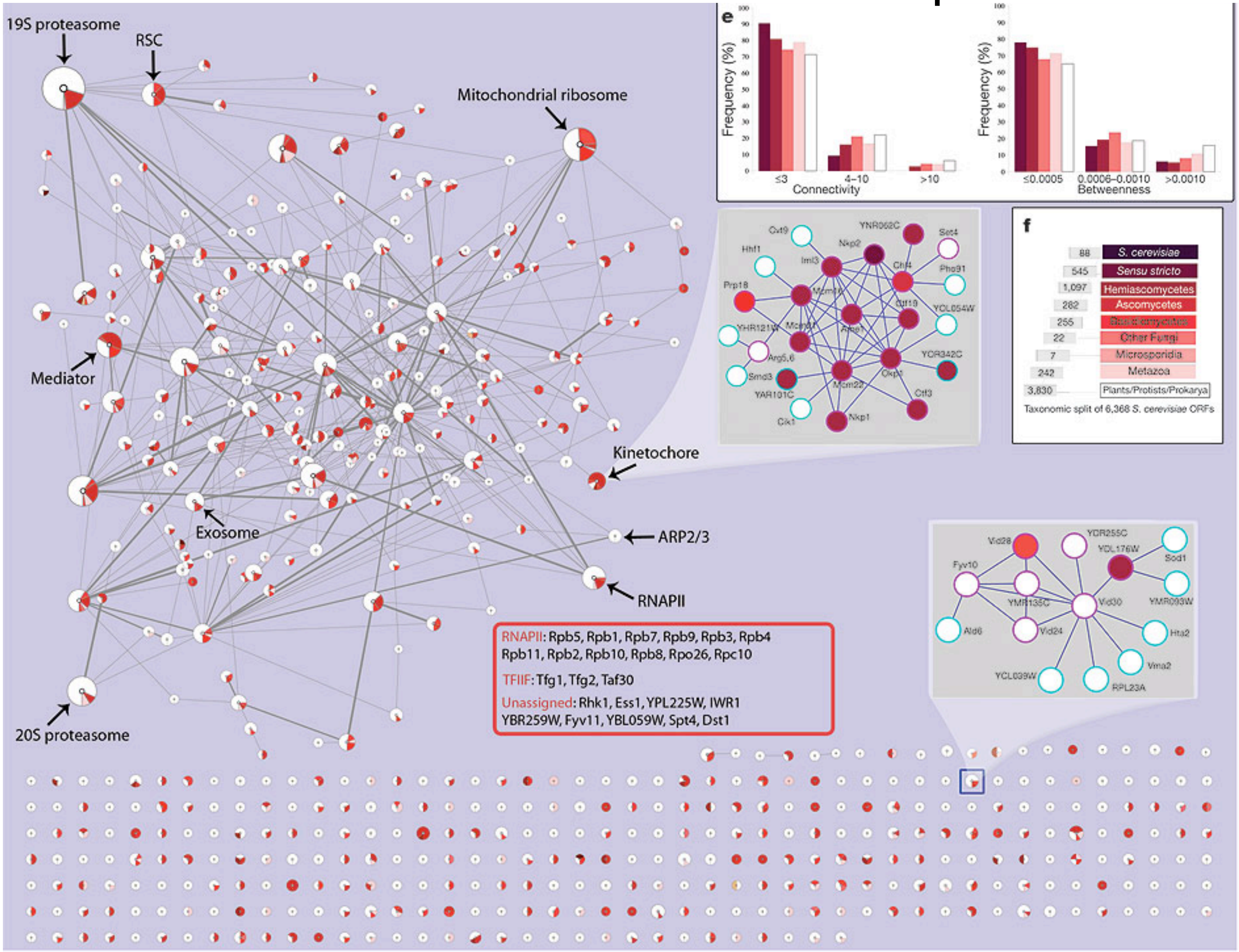
4,562 Purifications (Krogan et al. 2002)
2,357 Successful

4,087 Interacting Proteins
7,123 Core Interactions (2,708 proteins)
14,317 Extended (3,672 proteins)

547 Complexes

Krogan et al. Nature 2006 Vol 440

Size and Conservation of the Complexes



RNAPII: Rpb5, Rpb1, Rpb7, Rpb9, Rpb3, Rpb4 Rpb11, Rpb2, Rpb10, Rpb8, Rpo26, Rpc10

TFIIF: Tfg1, Tfg2, Taf30

Unassigned: Rhk1, Ess1, YPL225W, IWR1 YBR259W, Fyv11, YBL059W, Spt4, Dst1

TAP Tag Approach

Advantages

- In Vivo Assay
- Identifies Entire Complex

Disadvantages

- Interactions may be indirect
- Likely to miss some rare components
- Contaminants may copurify

Summary

- Affinity Purification: ~10,000 High Confidence Interactions Among ~2000 Proteins
- Two Hybrid: >4,549 Interactions Among 3,278 Proteins
- >20,000 Interactions
- Combining Data = More Accuracy