Protein-Protein Interactions (cont.) & Phosphophorylation

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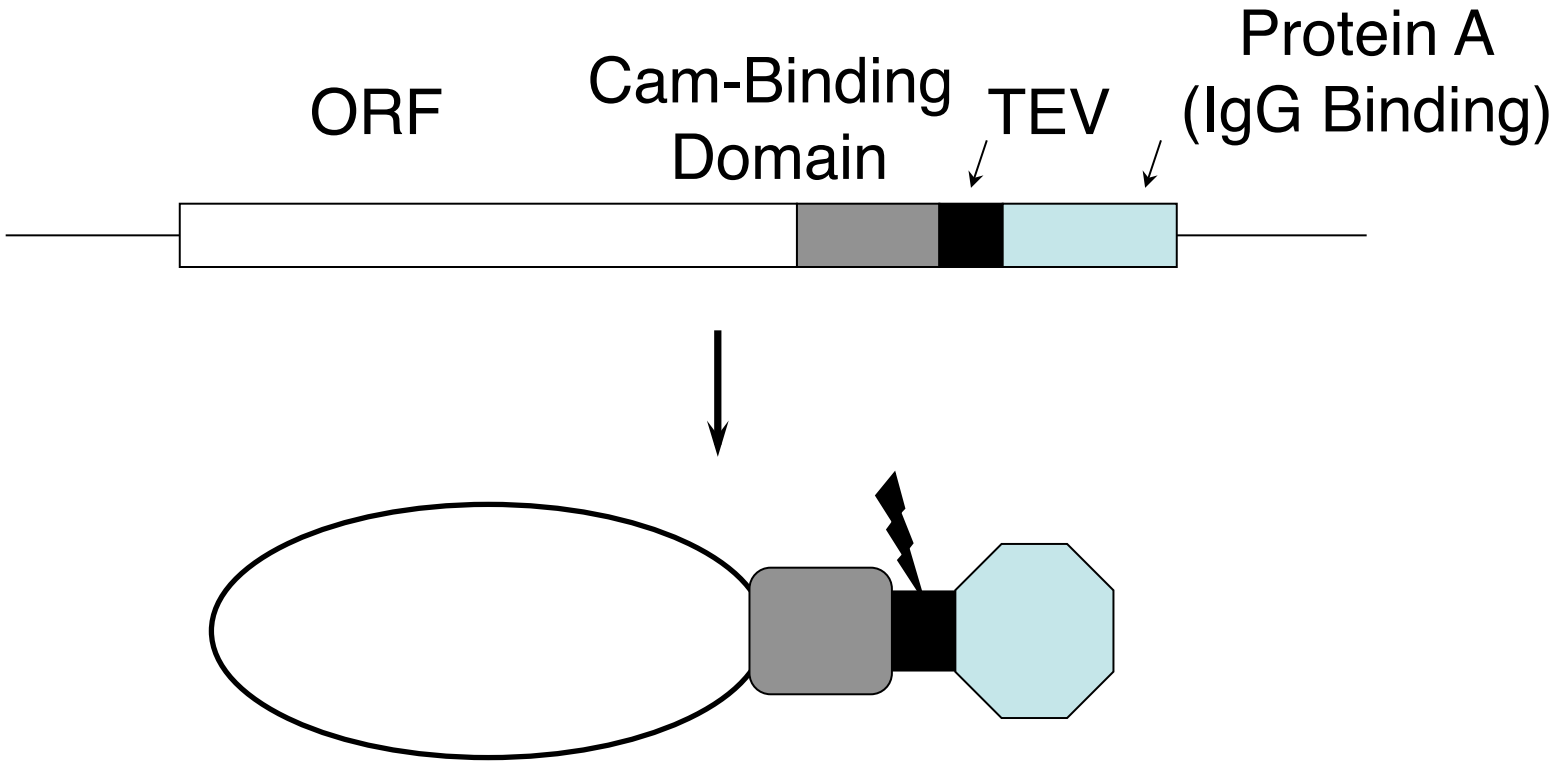
April 8, 2009

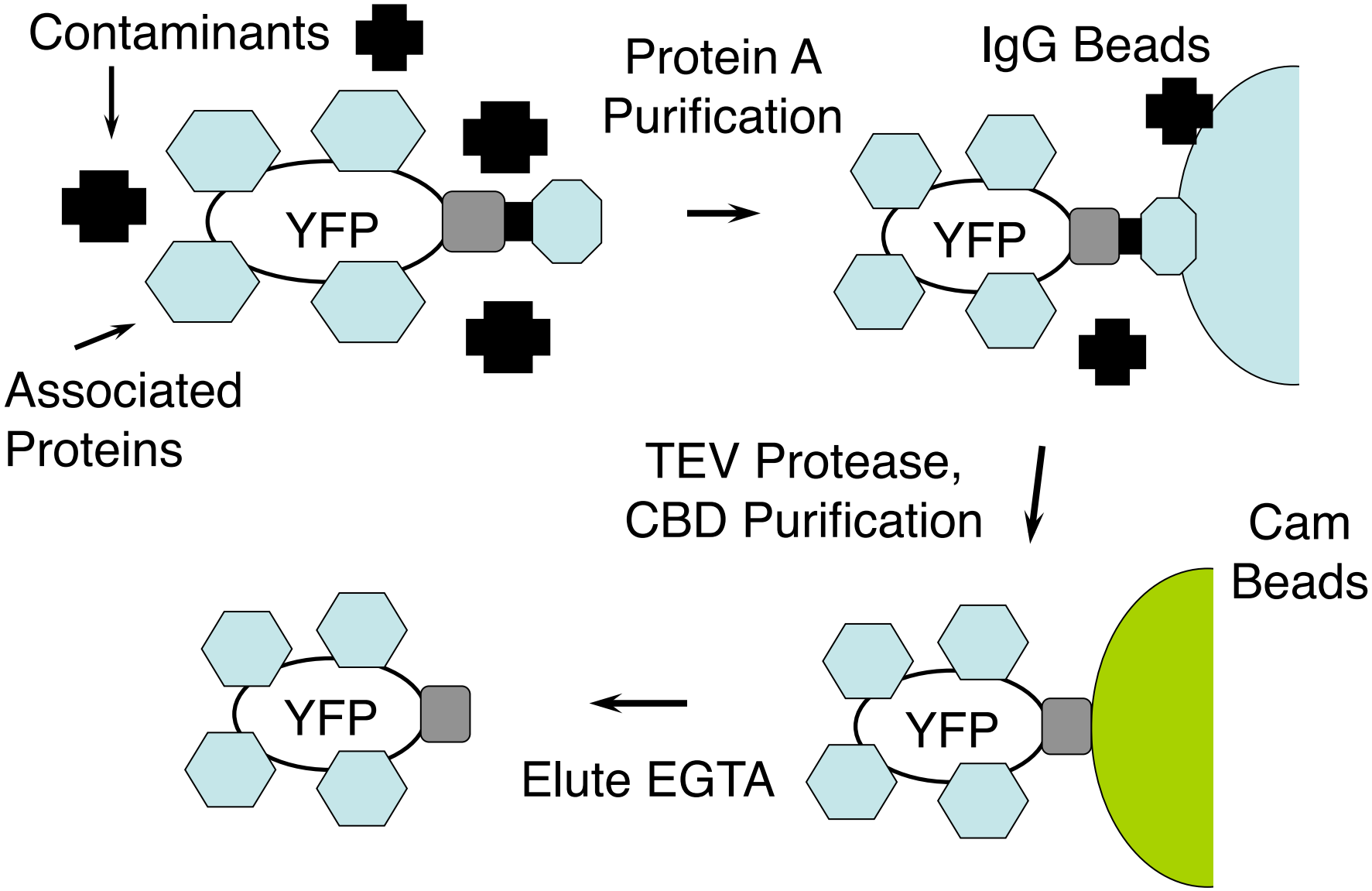
Three Methods:

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

Tandem Affinity Purification (TAP) Tagging

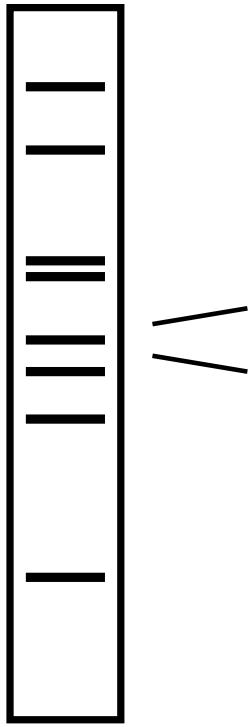


TAP Approach

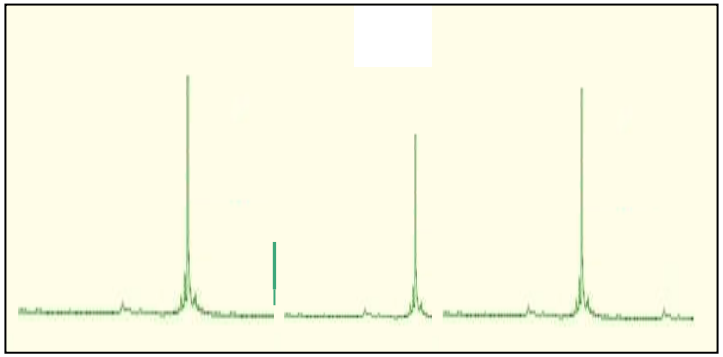


Identify Proteins by Mass Spec

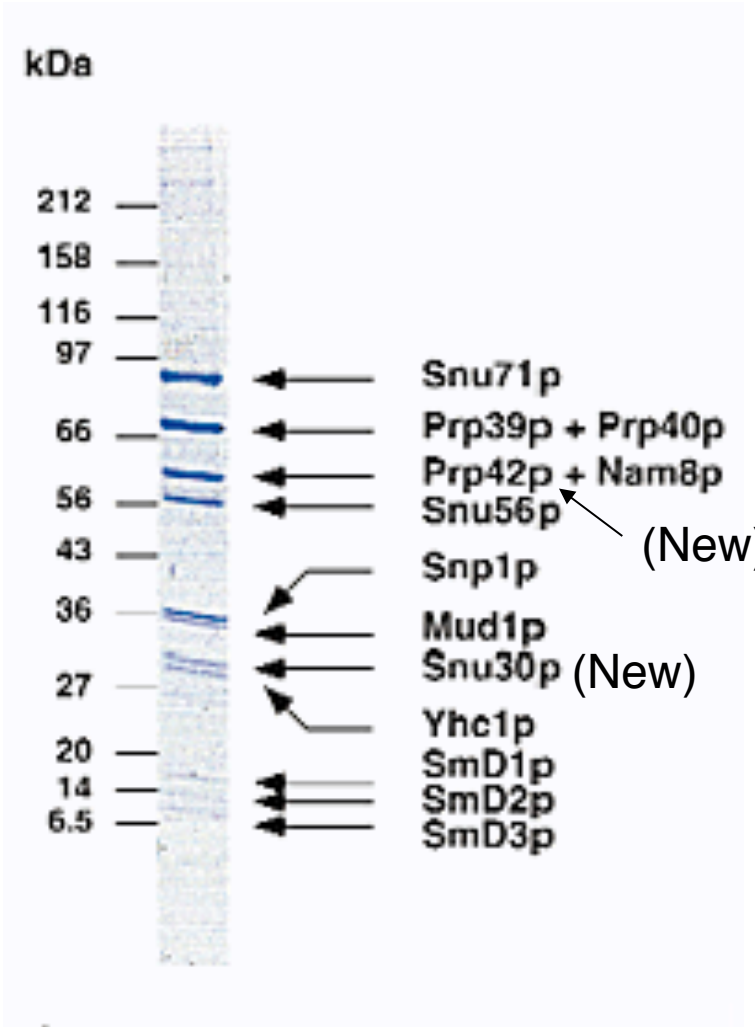
Load on SDS Gel



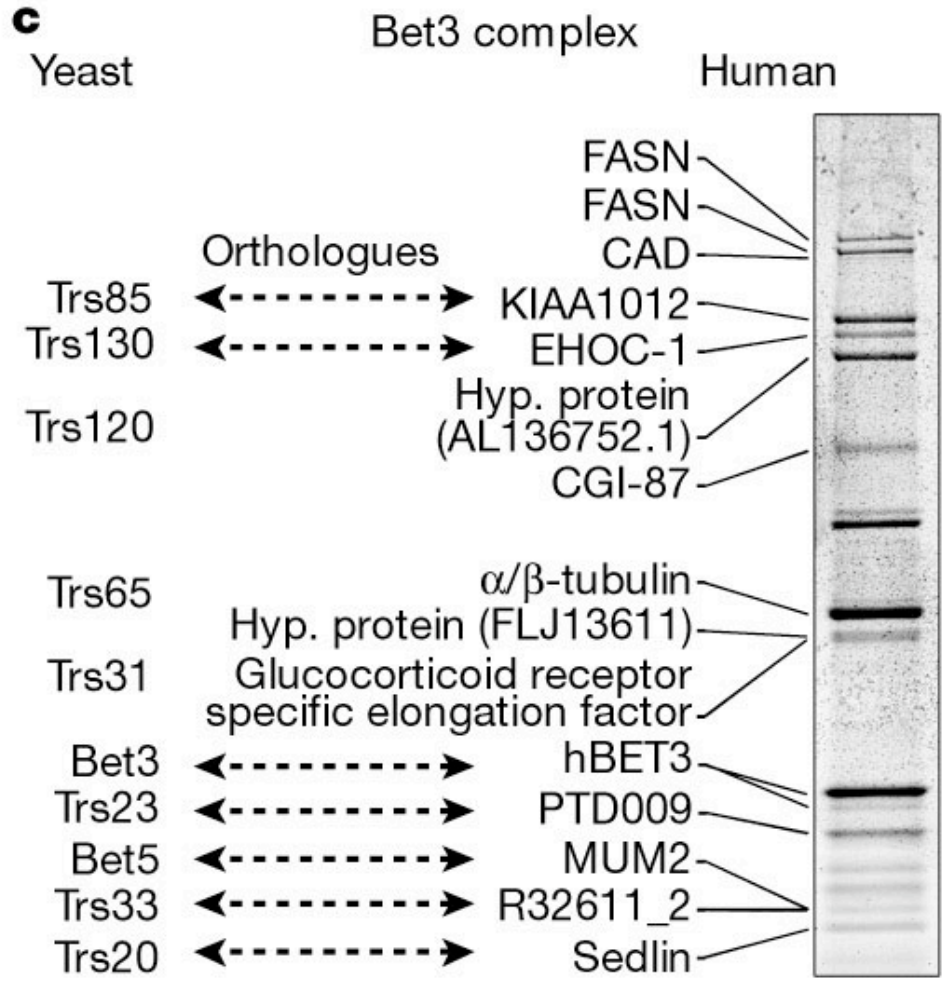
Excise Band;
Digest with Trypsin;
Run Mass Spec



TAP Purification of The U1 Splicing Complex (Snu71p)



Many Complexes Are Conserved



Affinity Purification/Mass Spec Analysis of Complexes - Yeast

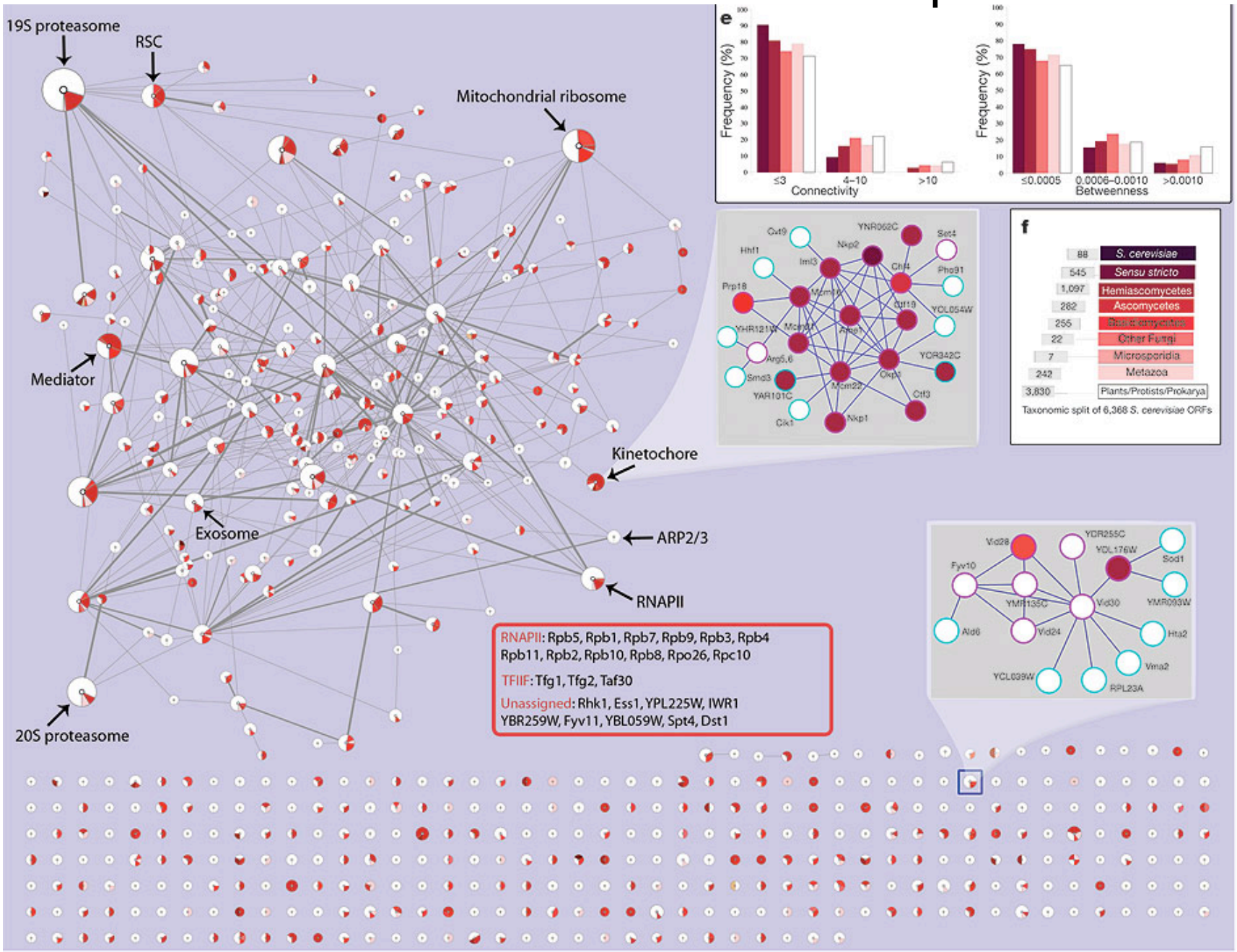
4,562 Purifications (Krogan et al. 2002)
2,357 Successful

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



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

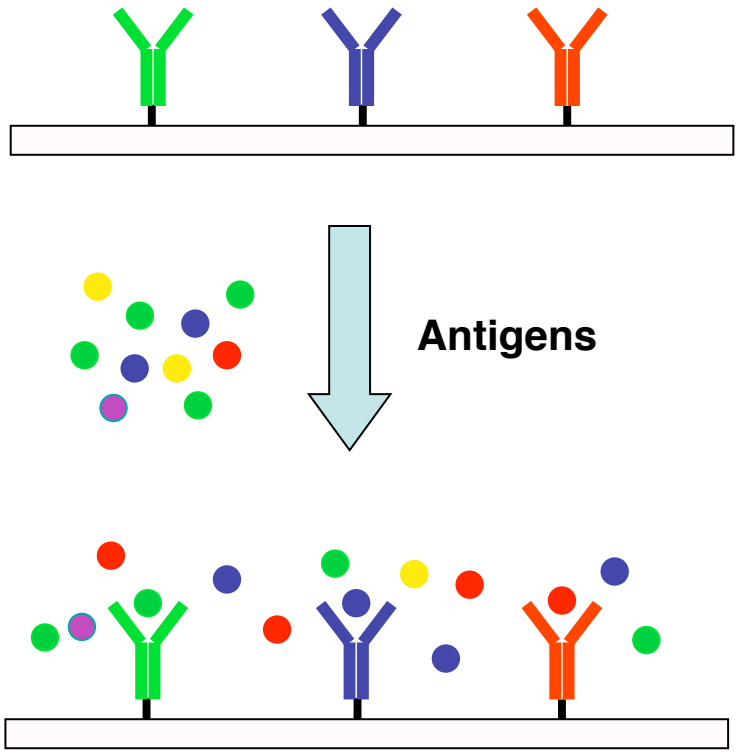
- Affinity Purification: ~10,000 High Confidence Interactions Among ~3-4000 Proteins
- Two Hybrid: >4,549 Interactions Among 3,278 Proteins
- >20,000 Interactions
- Combining Data = More Accuracy

What is a Protein Microarray?

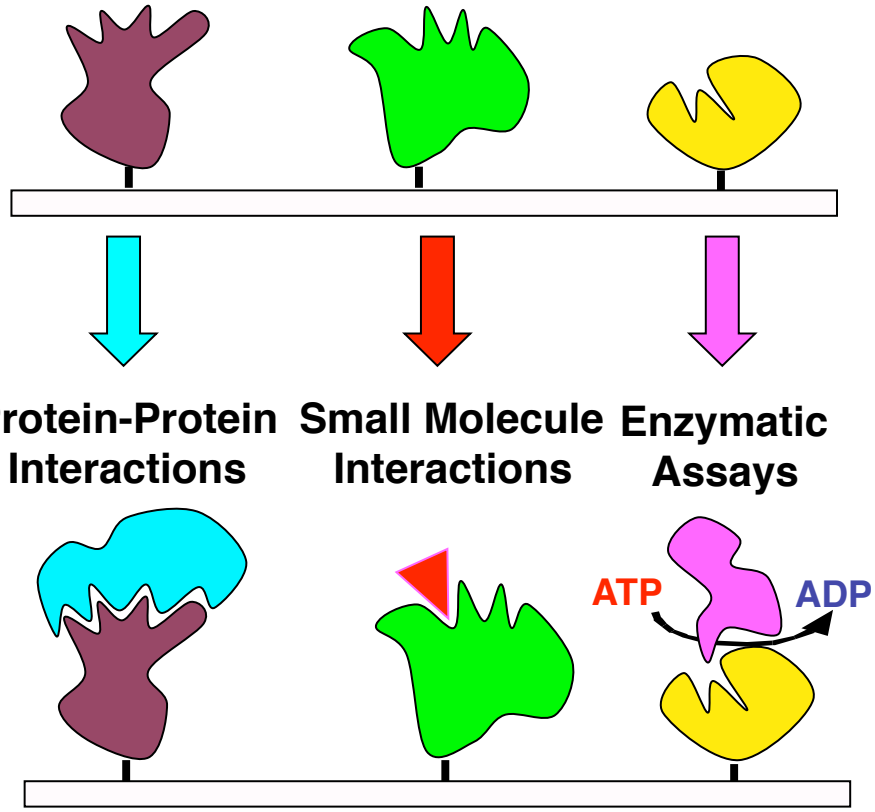
A high density array containing 100s to many thousands of proteins

Two Types of Protein Microarrays

Antibody Microarrays



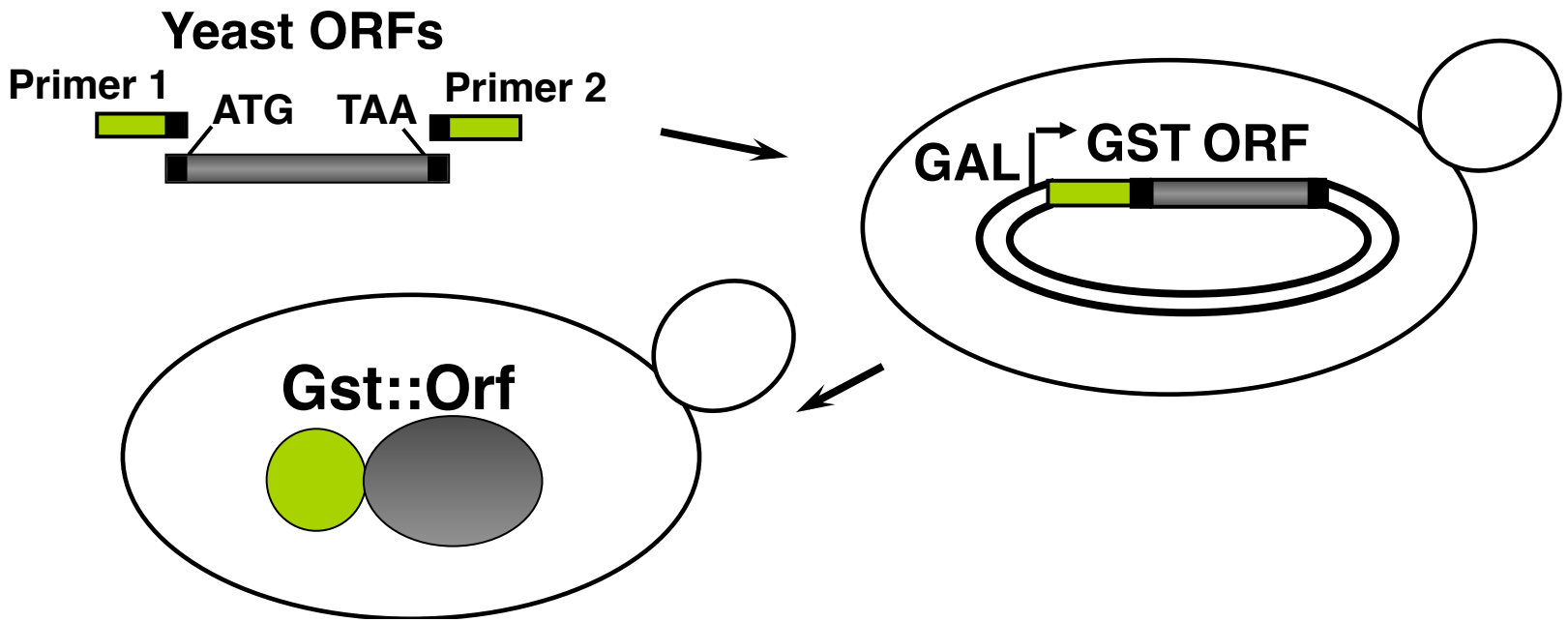
Functional Protein Microarrays

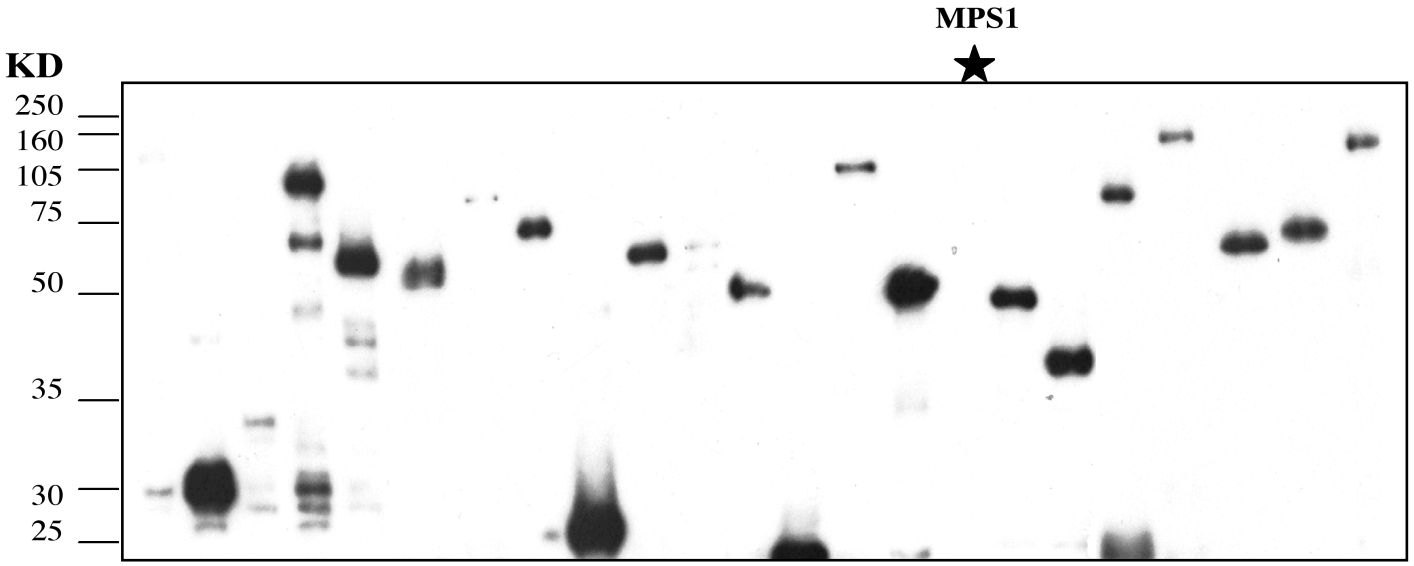


What Is Needed for Preparing and Screening Functional Protein Microarrays?

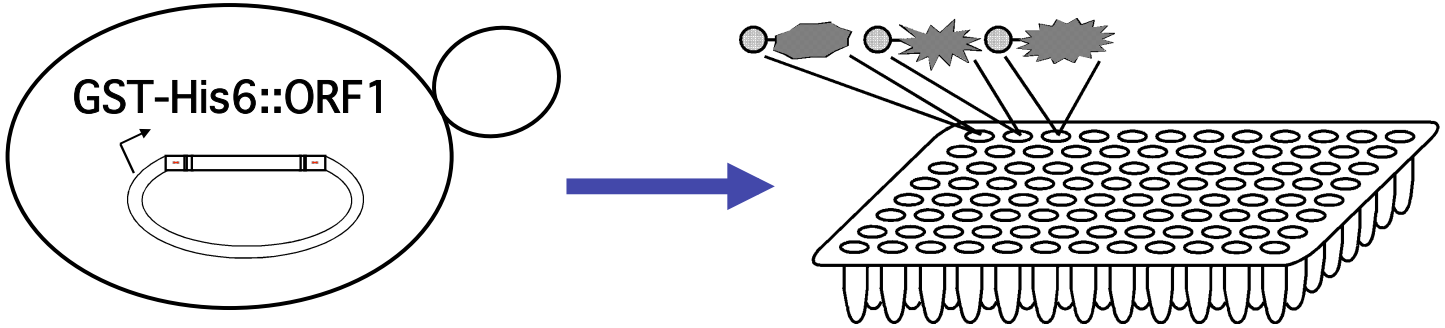
- A high quality expression library
- Methods for preparing large numbers of proteins
- Methods to array the proteins
- Assays for screening

Cloning & Expression Strategy



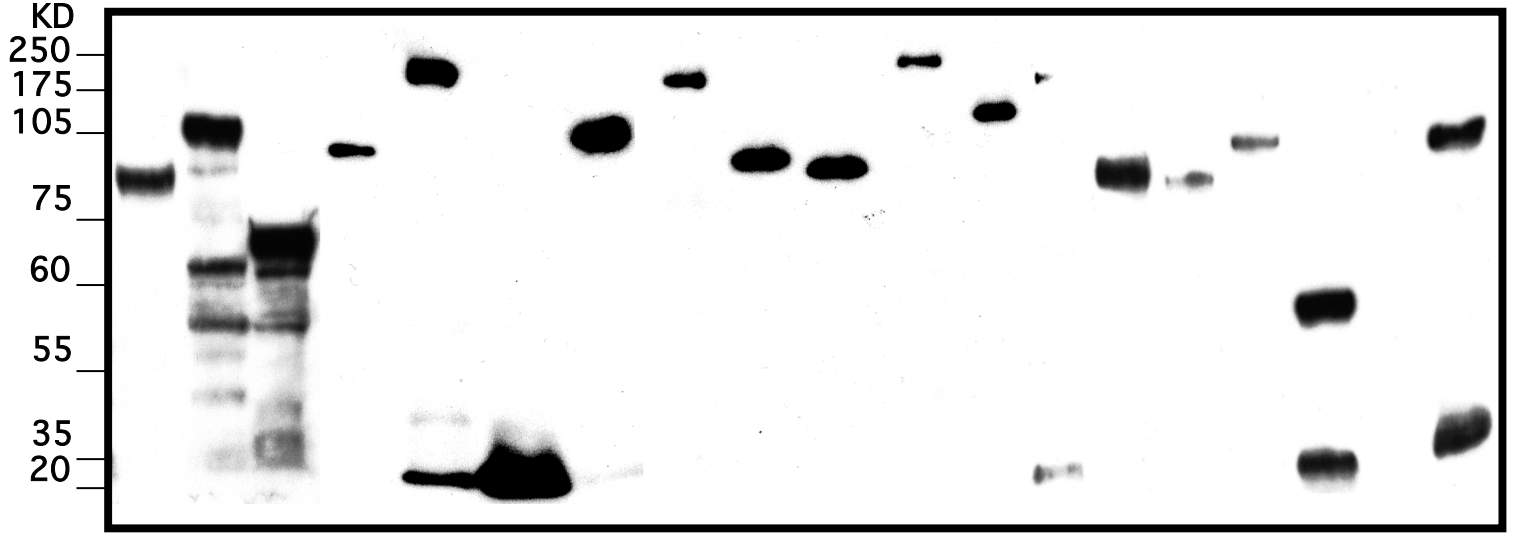


Producing the Yeast Proteome

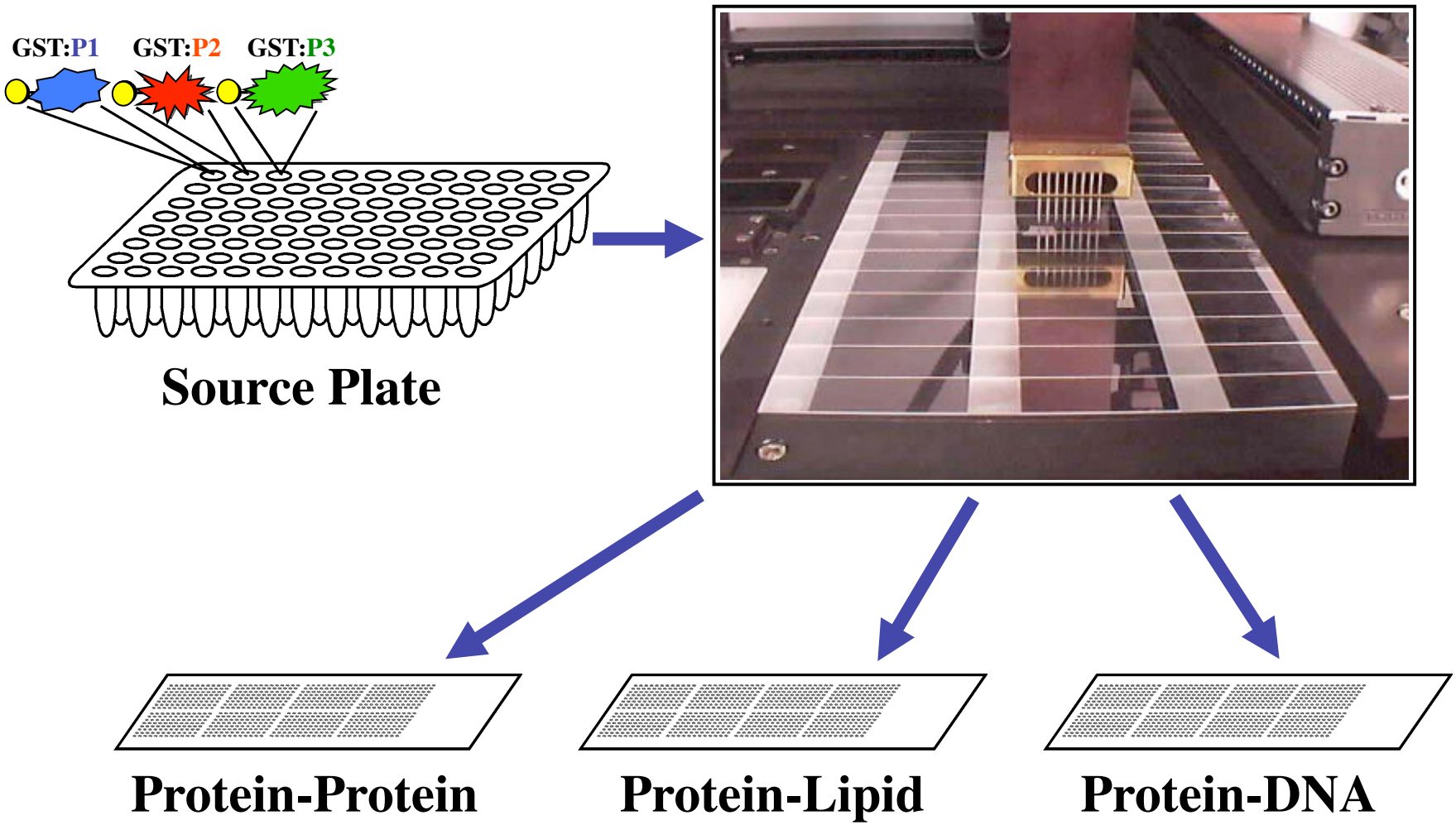


5800 expression clones 93.7%

~80% full-length proteins

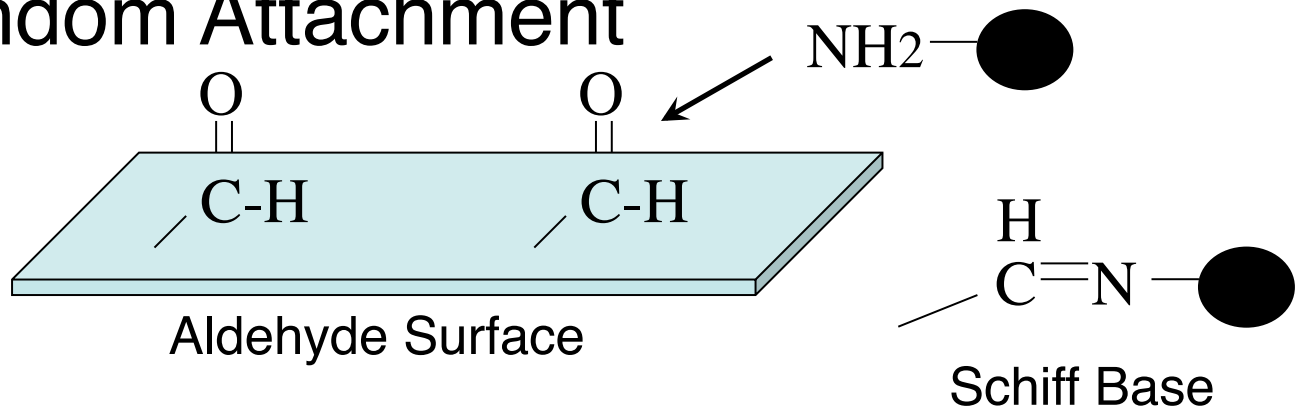


Printing the Yeast Proteome

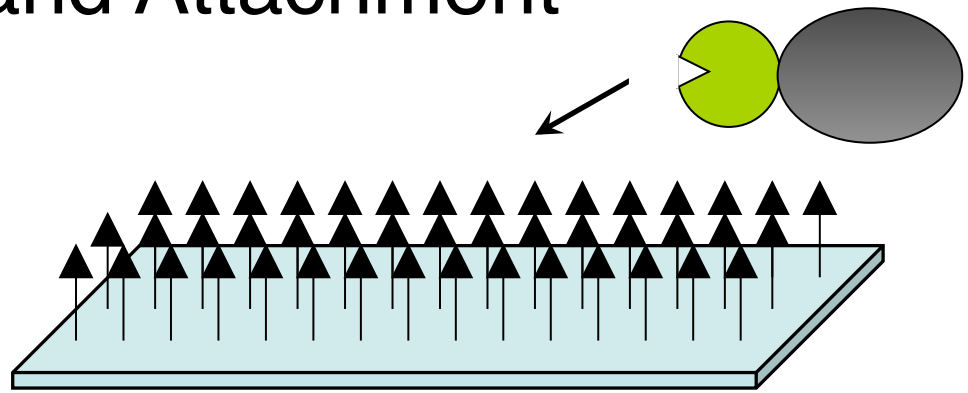


Glass Slides

1) Random Attachment



2) Ligand Attachment



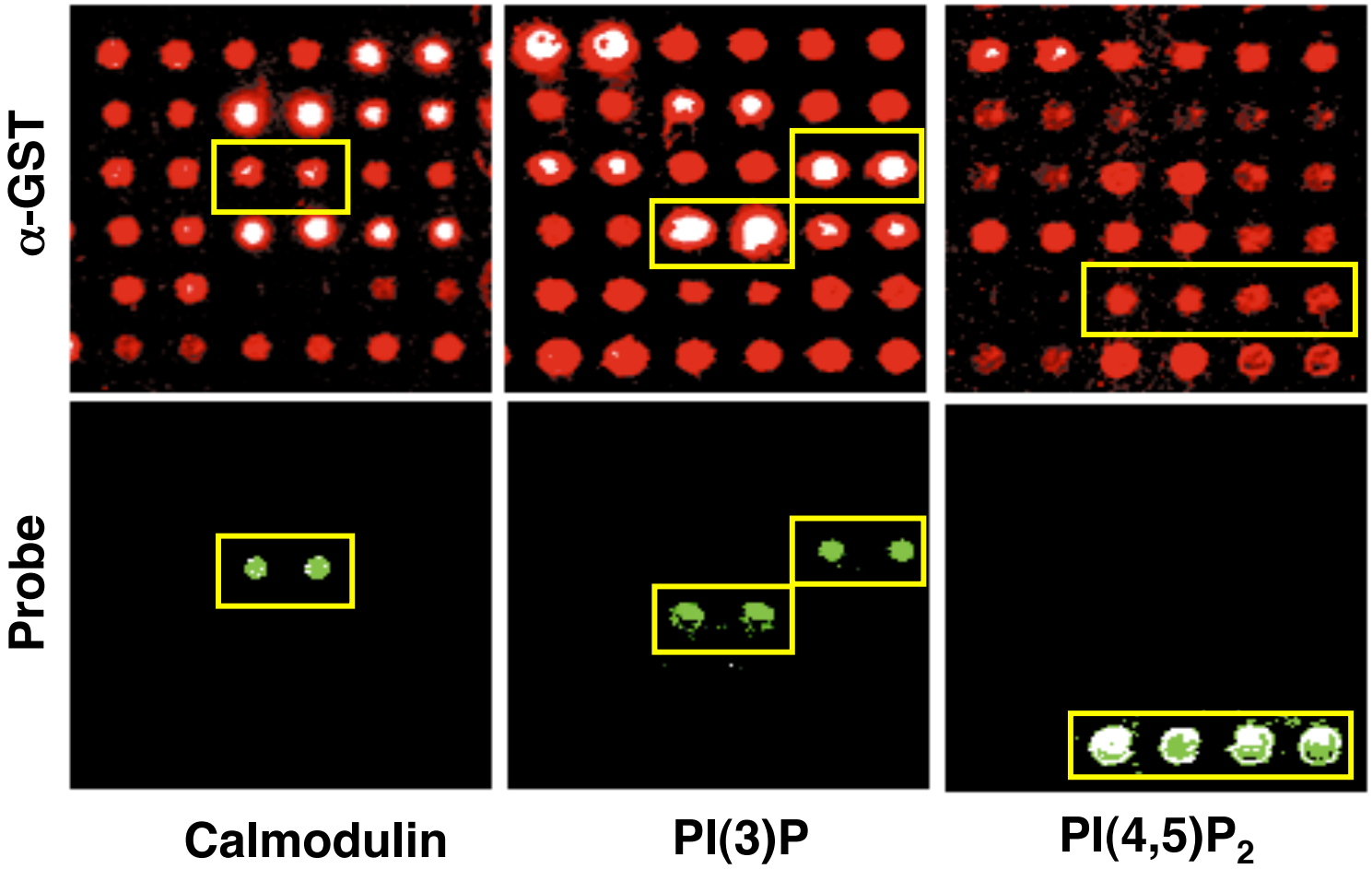
The Yeast Proteome Chip

Probed With
Anti-GST Antibodies

Many Types of Applications

- Binding Assays
  - Protein-Protein Interactions
  - Protein-Lipid Interactions
  - Nucleic Acids (dsDNA, ssDNA, polyA-mRNA)
  - Small Molecule Screens
- Antibody Specificity
- Kinase Substrates and Posttranslational Modifications
- Viral Diagnostics

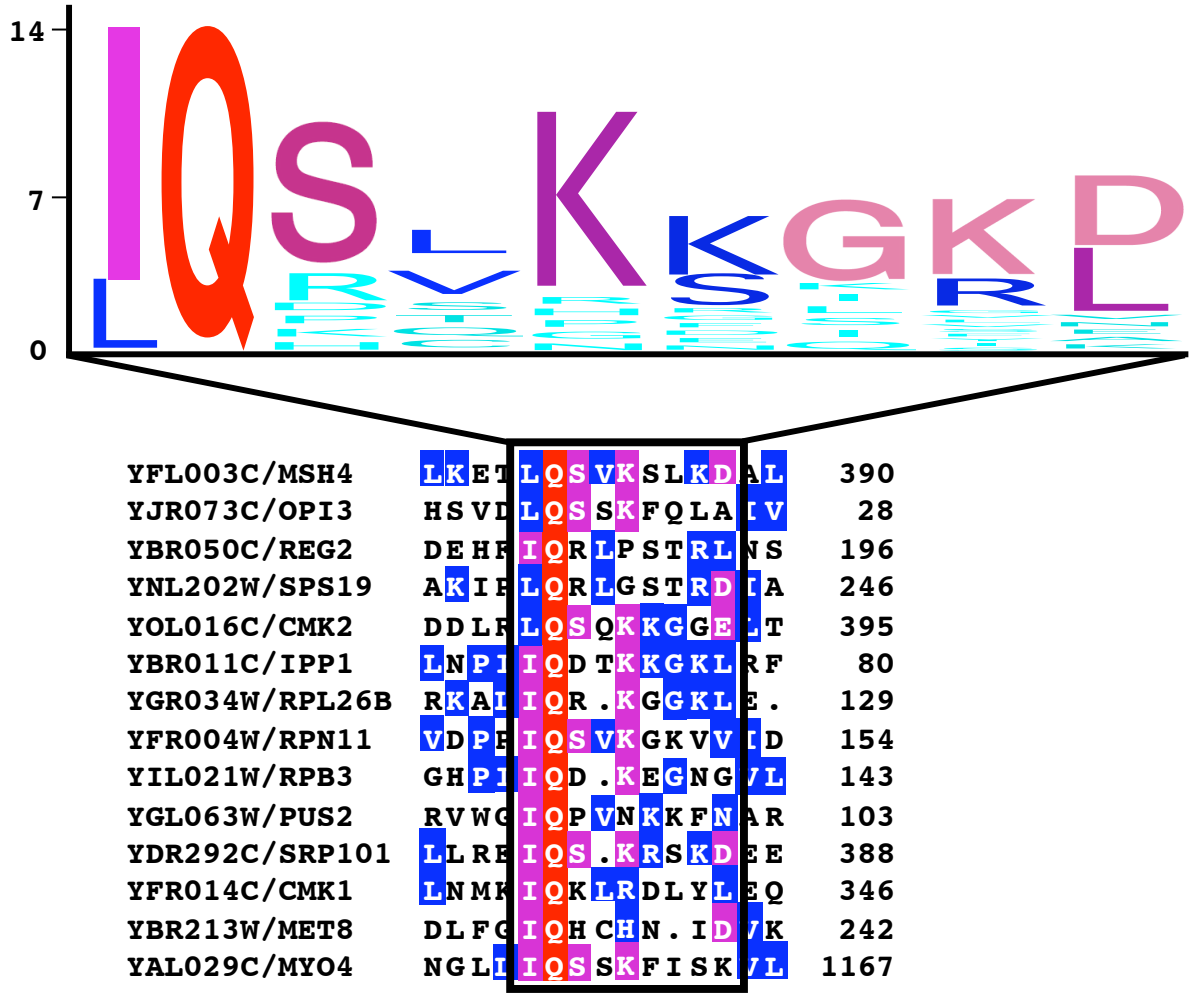
Biochemical Assays on Proteome Chips



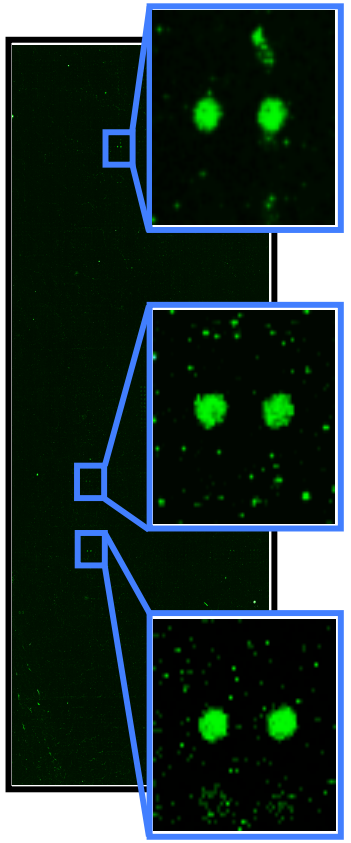
Calmodulin-Binding Proteins

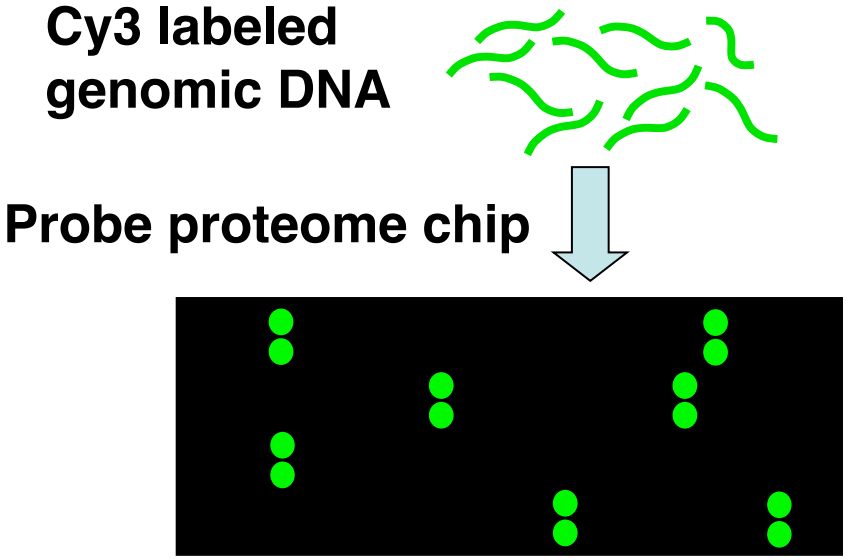
- 12 Known or Suspected Targets
- 33 New Binding Proteins
- Derived New Consensus Binding Site

Potential Calmodulin Binding Motif



Identification of New DNA Binding Activities





- ~200 bound DNA probe

Found New Activity
Arg5,6

Hall et al. (2004) Science

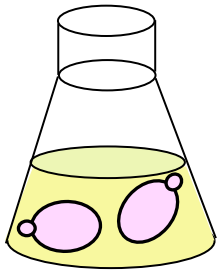
Yeast Phosphorylation

6,000 Proteins

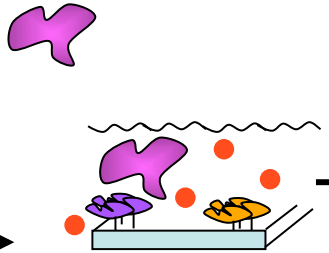
122 Protein Kinase Homologs

- 14 Uncharacterized
- 50% Have no known *in vivo* substrates
- <160 Known kinase-substrate phosphorylations

Global Analysis of Kinase Substrates



Overexpress and Purify Kinase



Proteome Array

Incubate with Kinase and $^{33}P\gamma$-ATP

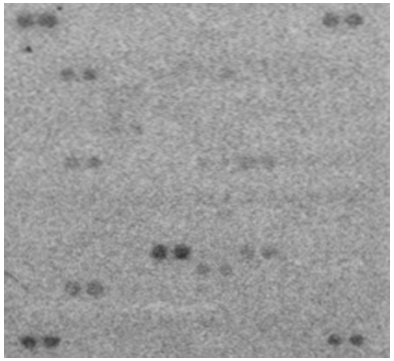
30°C

ATP

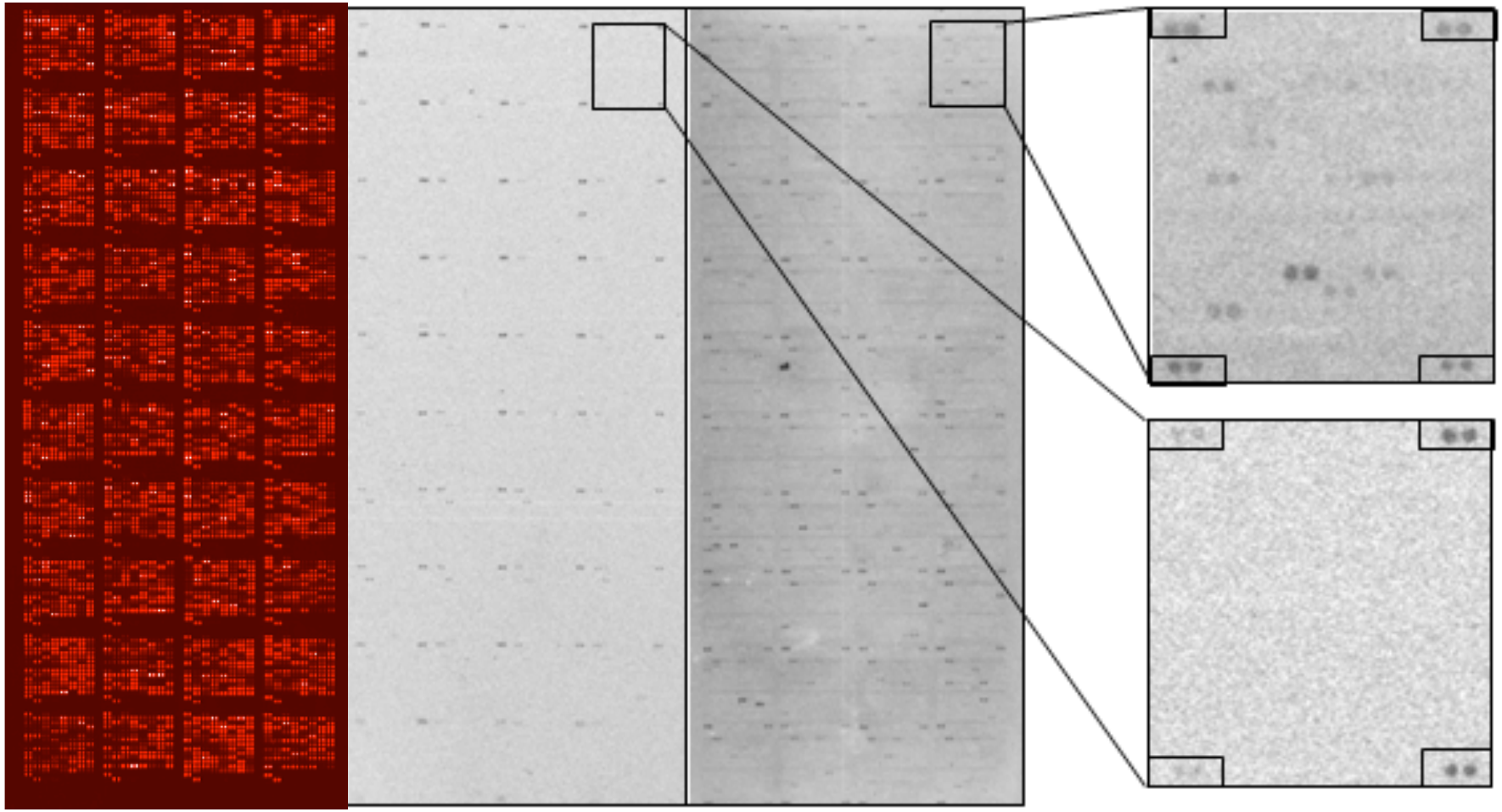
ADP

Wash

Expose to Film



Kinase Assays on Protein Chips



Anti-GST

Auto-Phosphorylation

Tpk1

In Vitro Phosphorylome Summary

AKL1
ARK1
ATG1
BCK1
CDC15
CDC5
CKA1
CLA4
CMK1
CMK2
CTK1
DBF2
DUN1
ELM1
FUN31
FUS3
GIN4
HAL5
HRR25
HSL1
CDC28-CLN2
PHO85-PCL2
RIM15 dead

IKS1
IME2
IPL1
IRE1
KCC4
KIN1
KIN2
KIN28
KIN3
KIN4
KIN82
KNS1
KSP1
KSS1
MCK1
MEK1
MKK1
MPS1
PAK1
PBS2
CDC28-CLB5
PHO85-PCL9
DBF2 dead

PKH2
PRK1
PRR1
PRR2
PTK2
RAD53
RCK1
RCK2
RIM11
RIM15
SAT4
SKM1
SKS1
SKY1
SLT2
SNF1
SPS1
SRB10
SSK22
STE11
PHO85-ALONE
PHO85-PHO80
HSL1 dead

STE20
SWE1
TOS3
TPK1
TPK2
TPK3
VHS1
YAK1
YCK1
YCK2
YCK3
YDR466W
YGL059W
YGR052W
YKL171W
YMR291W
YOL128C
YOR267C
YPL141C
YPL150W
PHO85-PCL1

RAD53 dead

In Vitro Phosphorylome Summary

- 82 unique kinases and several CDKs with different cyclins (Cdc28; Pho85) for 87 specific hit lists

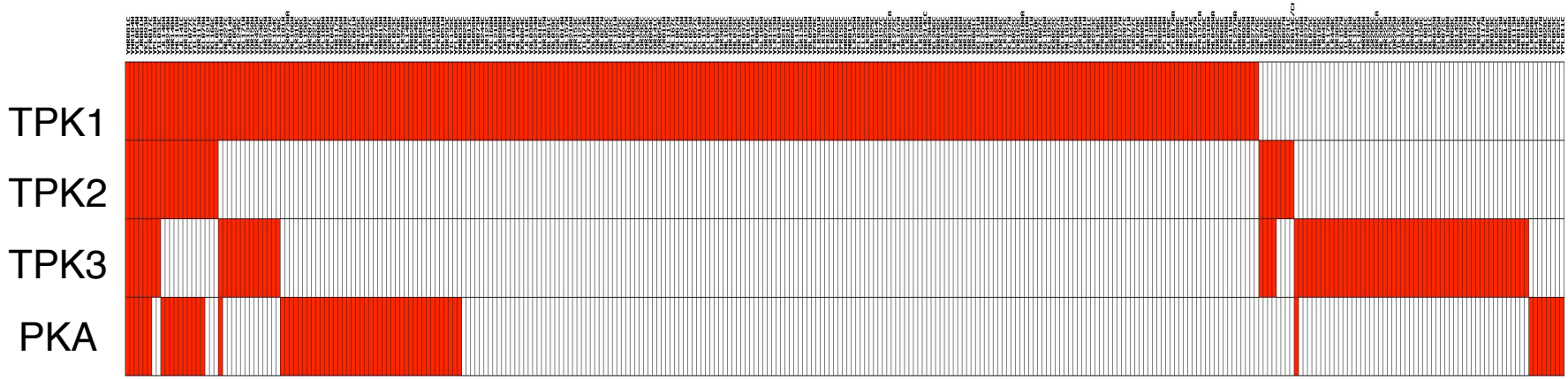
- 4200 total phosphorylation events on 1325 individual targets

- On average kinase phosphorylated 47 proteins on chip (Range 1- 250)

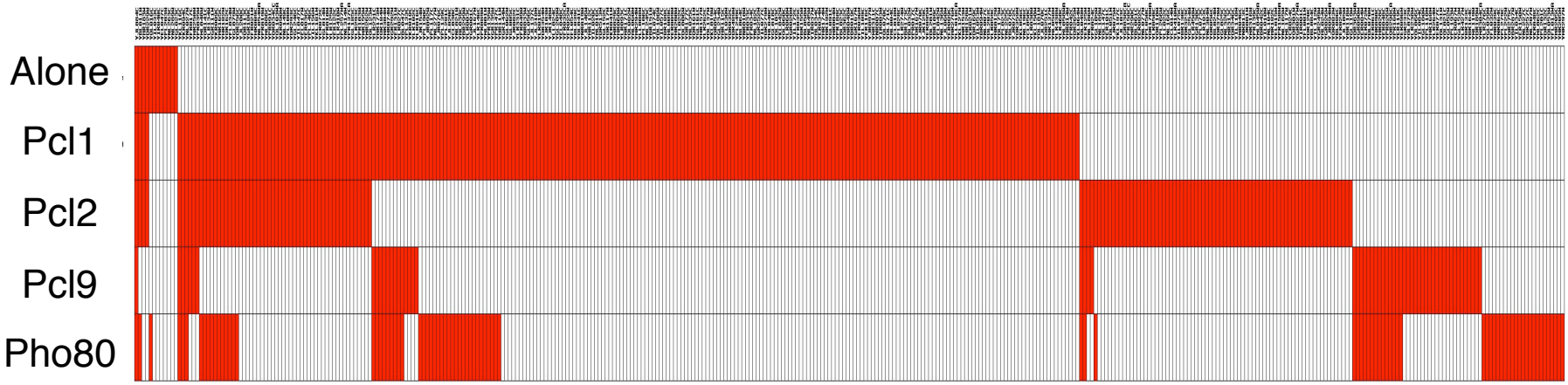
- 50% of substrates were phosphorylated by only one kinase

- Identified at least 21 known kinase-substrate phosphorylations

Substrate List Comparison



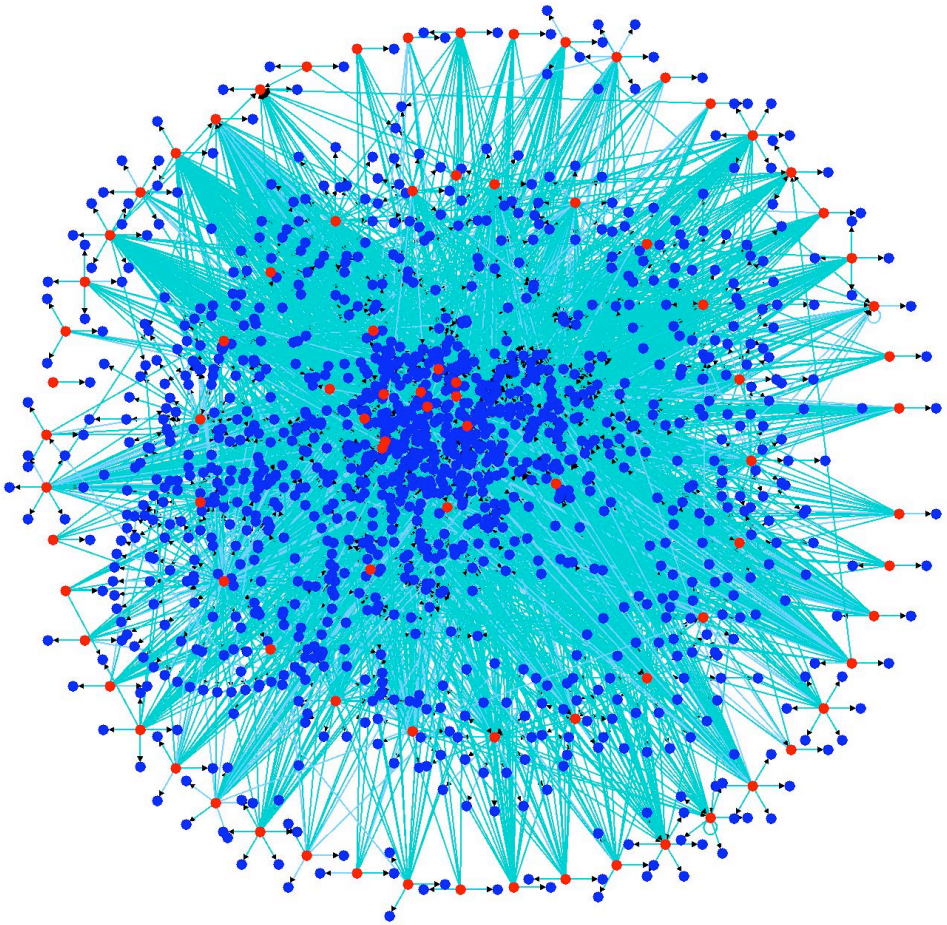
PHO85



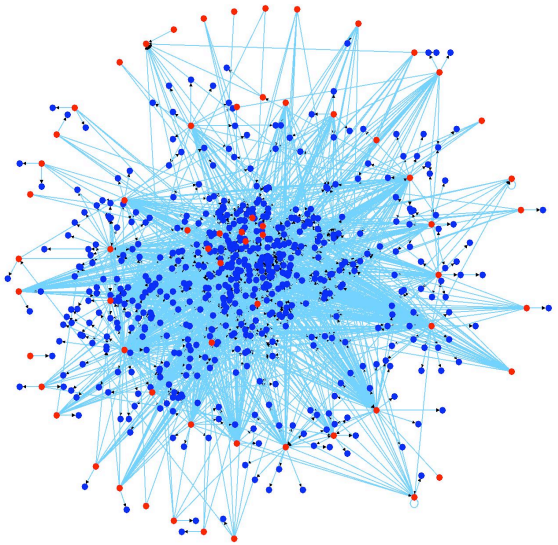
TPK1, PKA overlapped very well

Pcl9, Pho80 overlapped very well

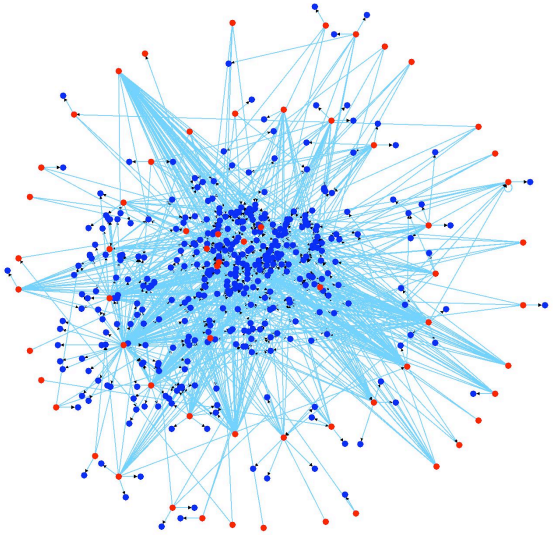
Phosphorylome Network



4200 Phosphorylations, 1325 Proteins

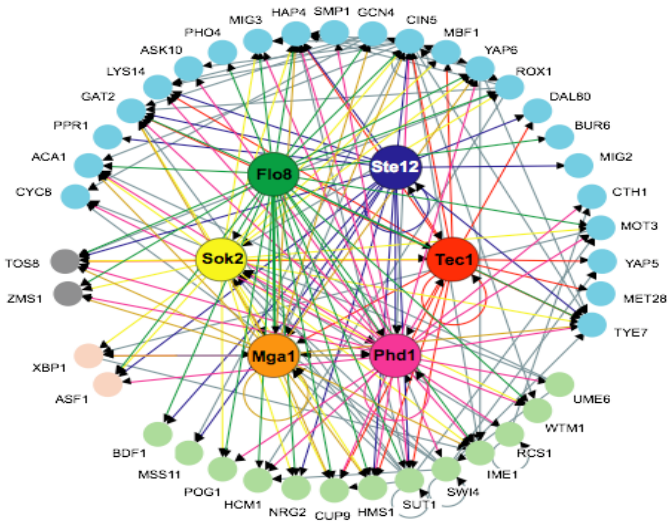


Same localization (33%; p E<-99)

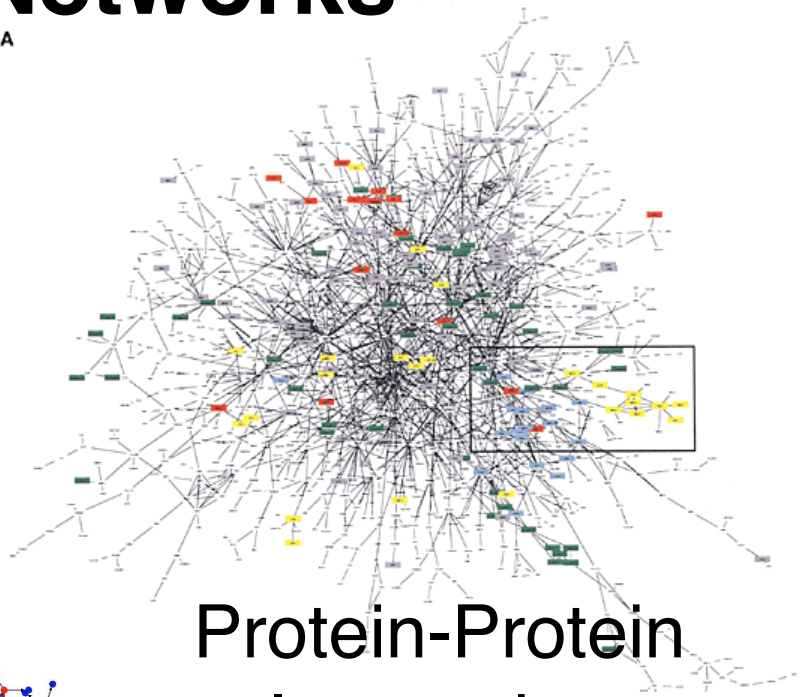


Same function (18.3%; p <E-99)

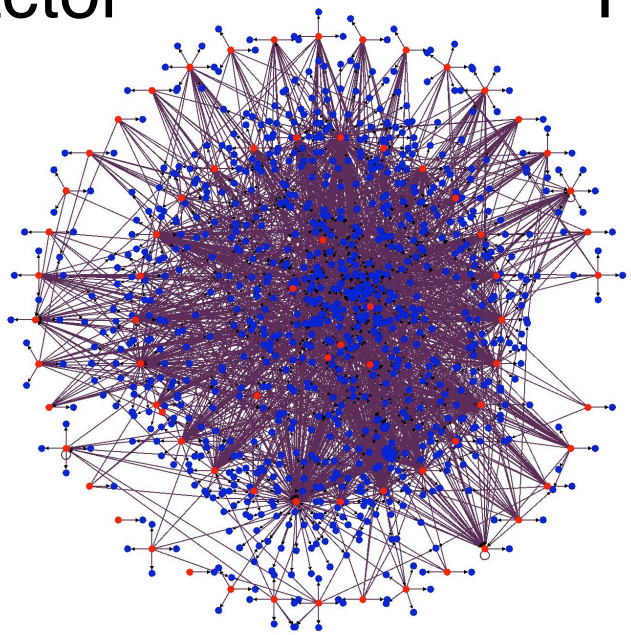
Regulatory Networks



Transcription Factor Binding

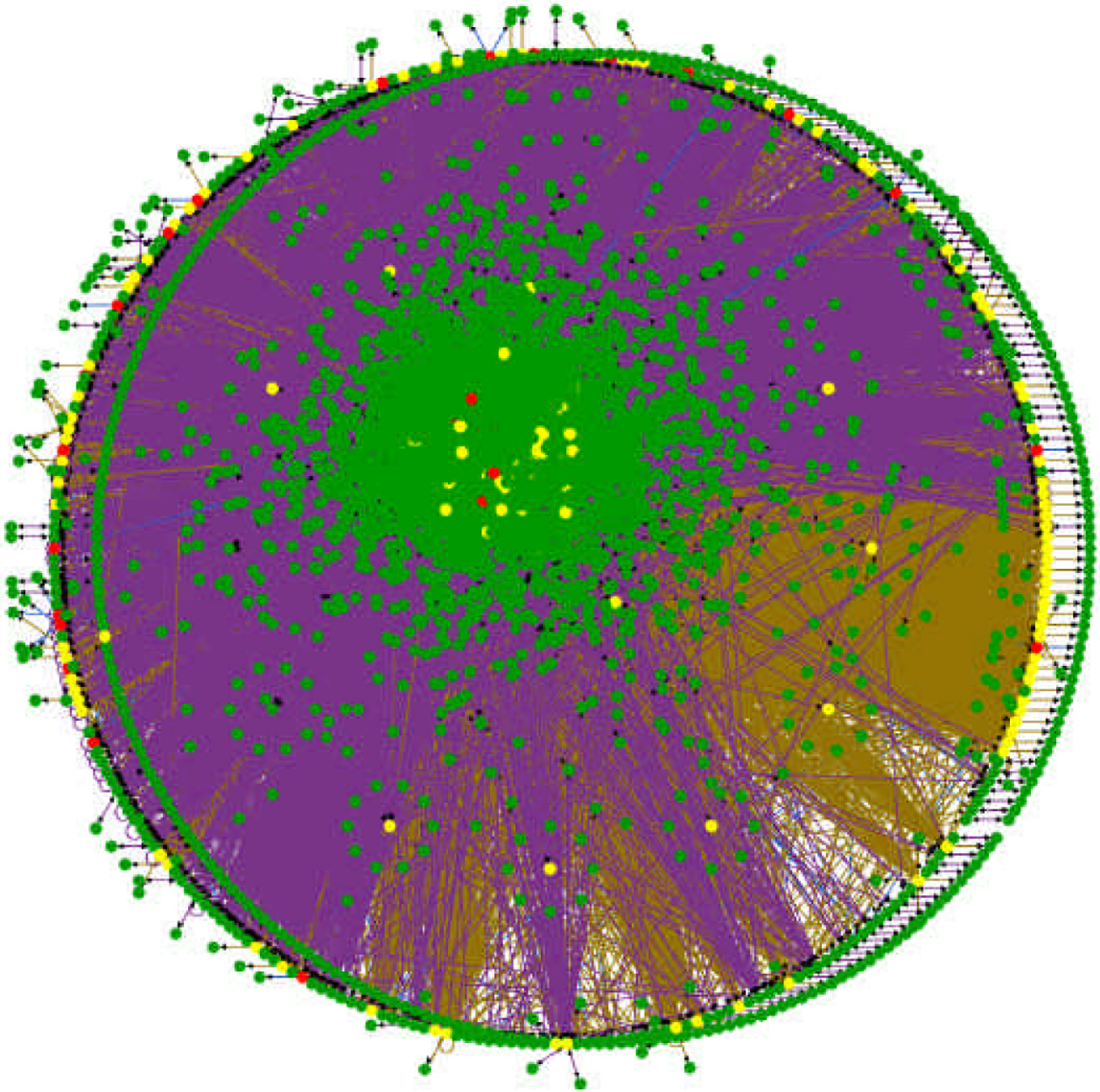


Protein-Protein Interaction

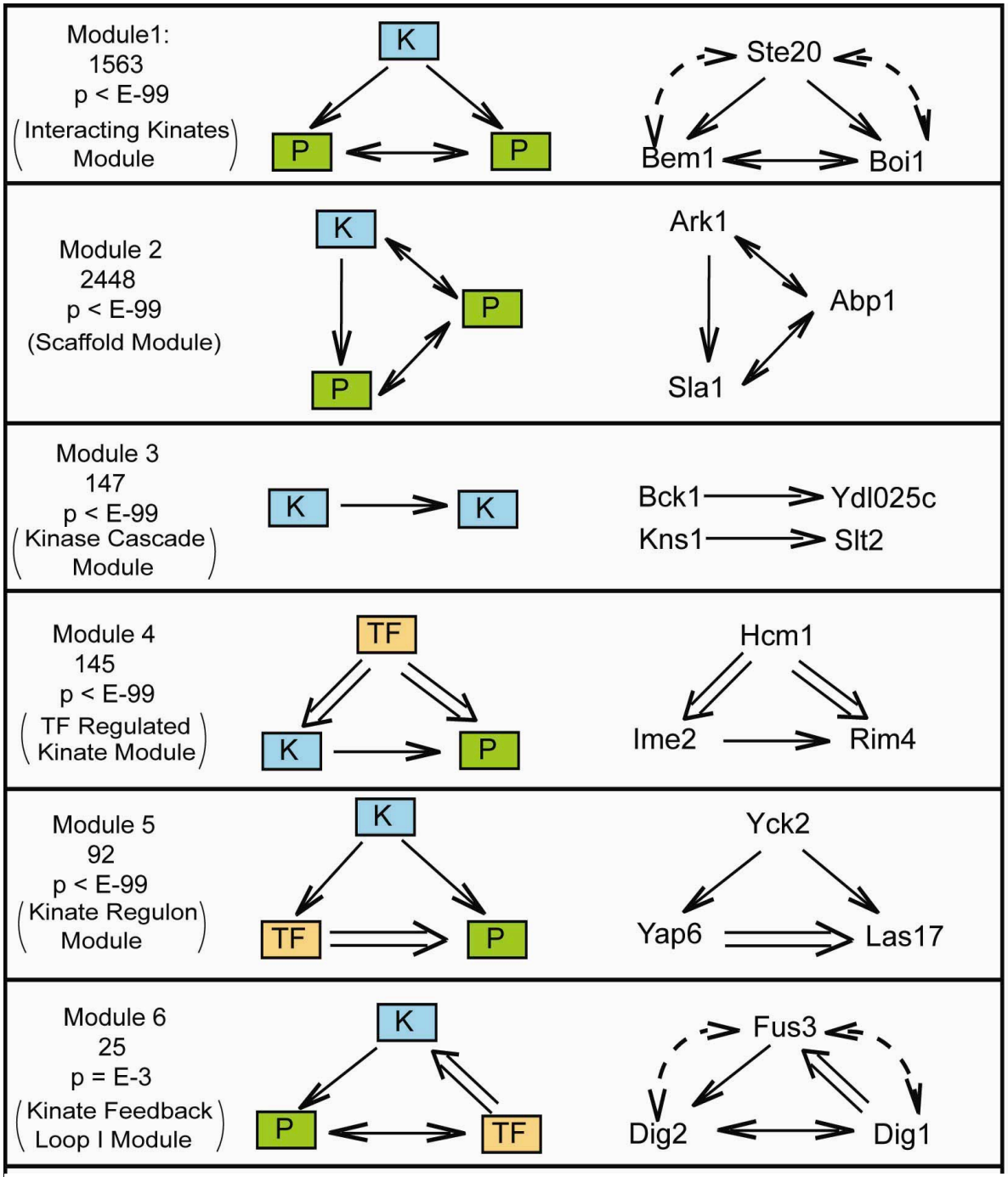


Phosphorylation

Meta Network



Regulatory Modules



Advantages of Protein Chips

- Can screen many proteins simultaneously
- Small amounts of proteins and reagents
- High throughput
- Diverse applications-biochemical assays, posttranslational modifications, small molecule screening

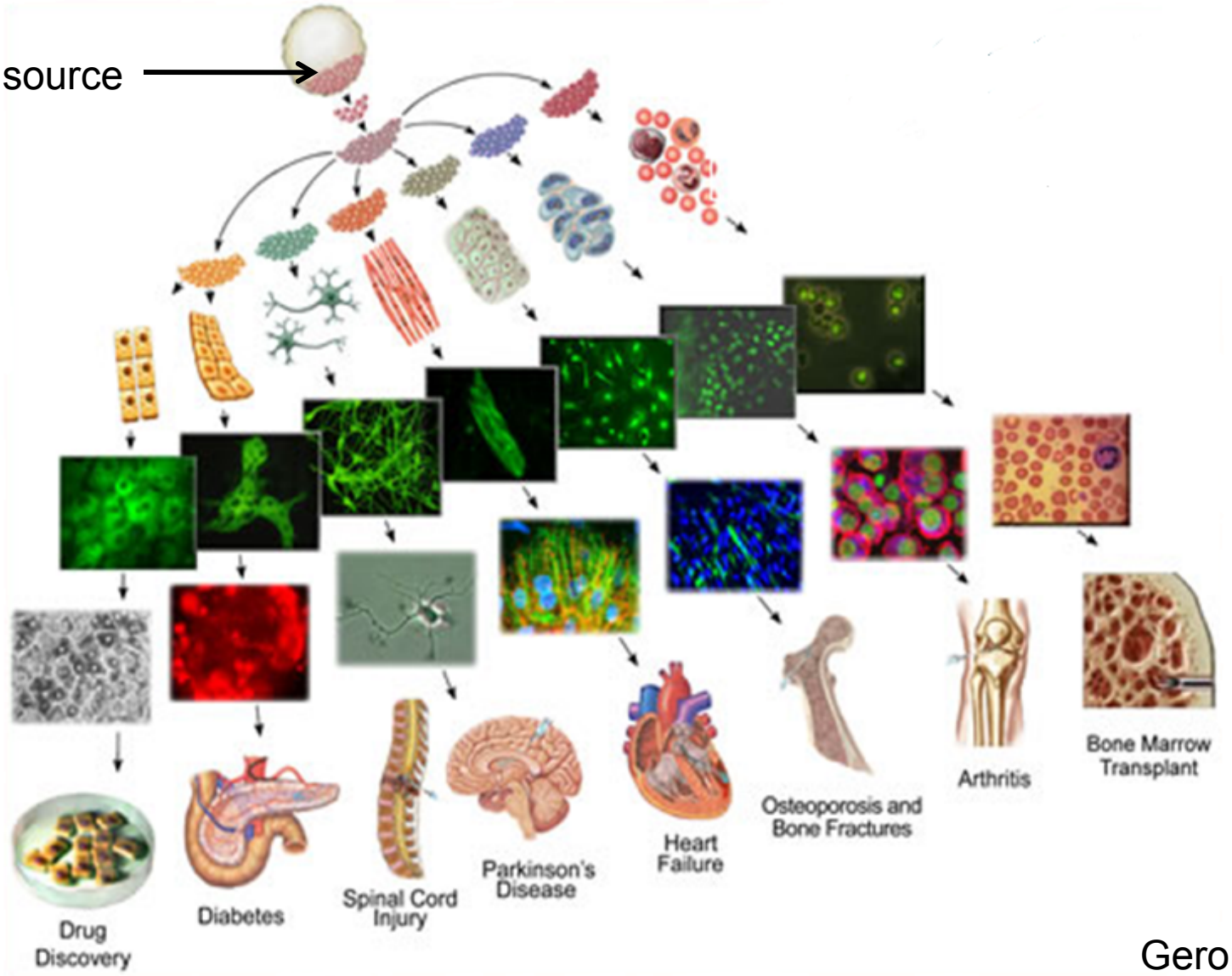
Disadvantage

- In Vitro Assay - Must validate In vivo

Many Transcription Factors Remain Uncharacterized

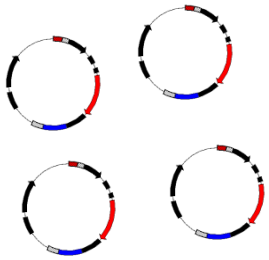
- 1400-1700 human transcription factors
- Methods of studying TF function in differentiation
  - Map binding sites
  - Tissue/cell localization
  - Knock down
  - Ectopic expression
- Ectopically expressed TFs with the ability to activate differentiation programs
  - MyoD (muscle), PPARgamma (fat), Sox 5, 6, 9 (cartilage)

Embryonic Stem Cells are a 'Blank Slate' for Studying Gene Function

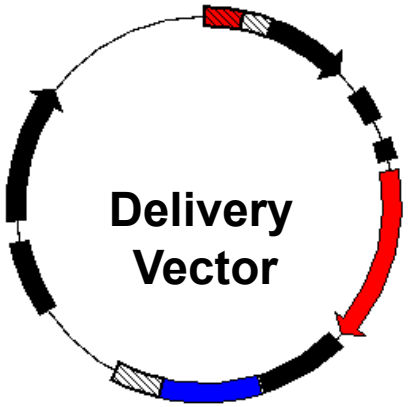


Geron Corp.

Screen 700 Human TFs in mES Cells

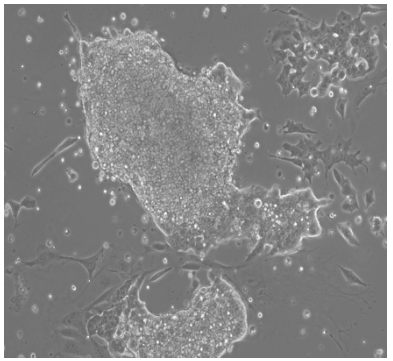


TF ORFs



Transfect

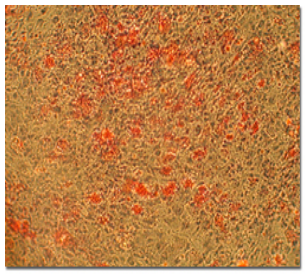
mES Cells



Express cDNA as VP16 fusions
Using tet repressible system

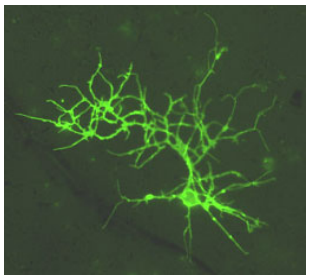
Integrate at the
ROSA locus

Adipocytes



Oil Red O

Neurons

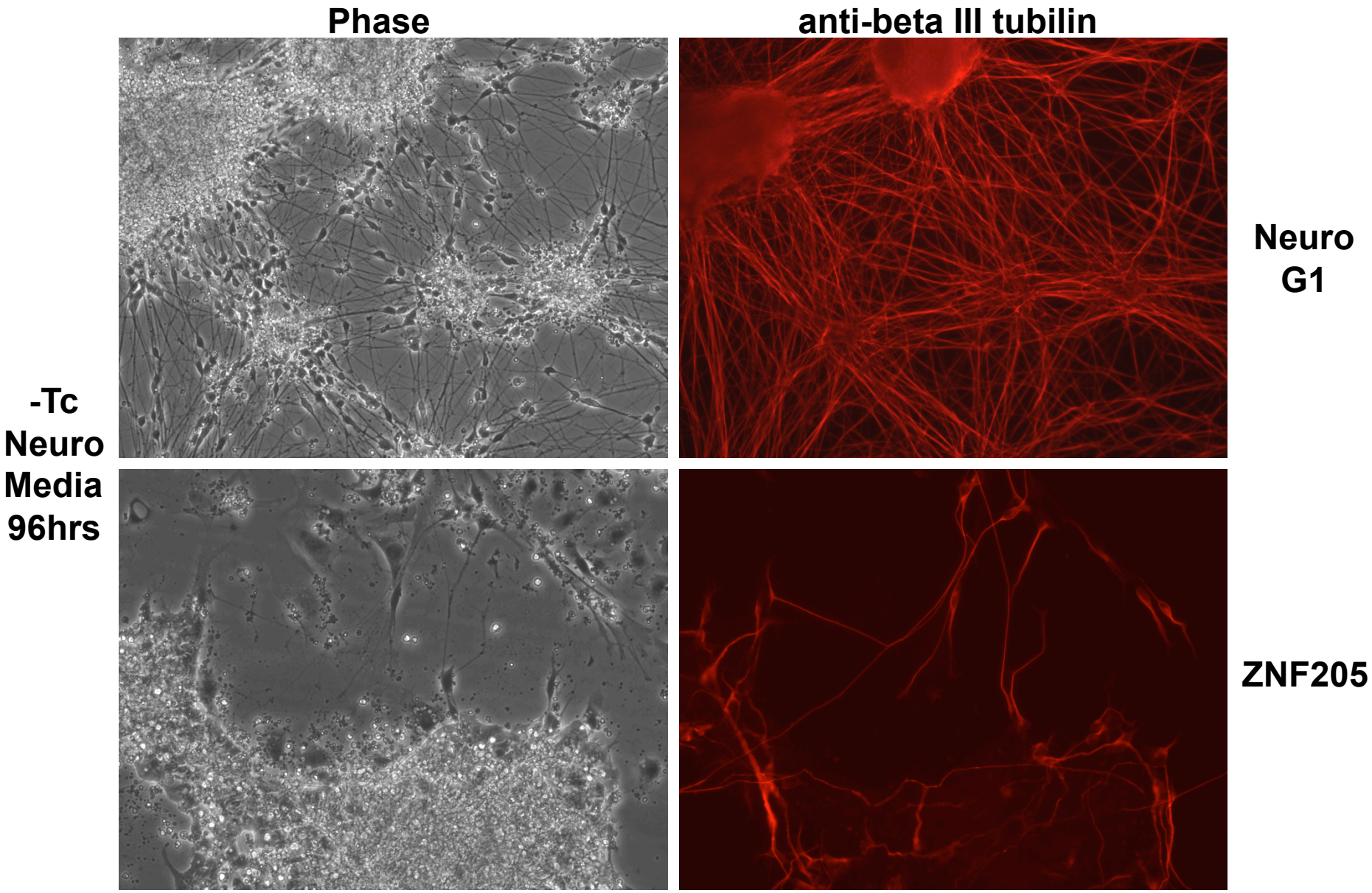


Ab staining
GFP reporter

Screen
Individually

+ tet = Off - tet = On

24 TFs Induce Differentiation



24 TFs Induce Neural Differentiation

Hits with VP16

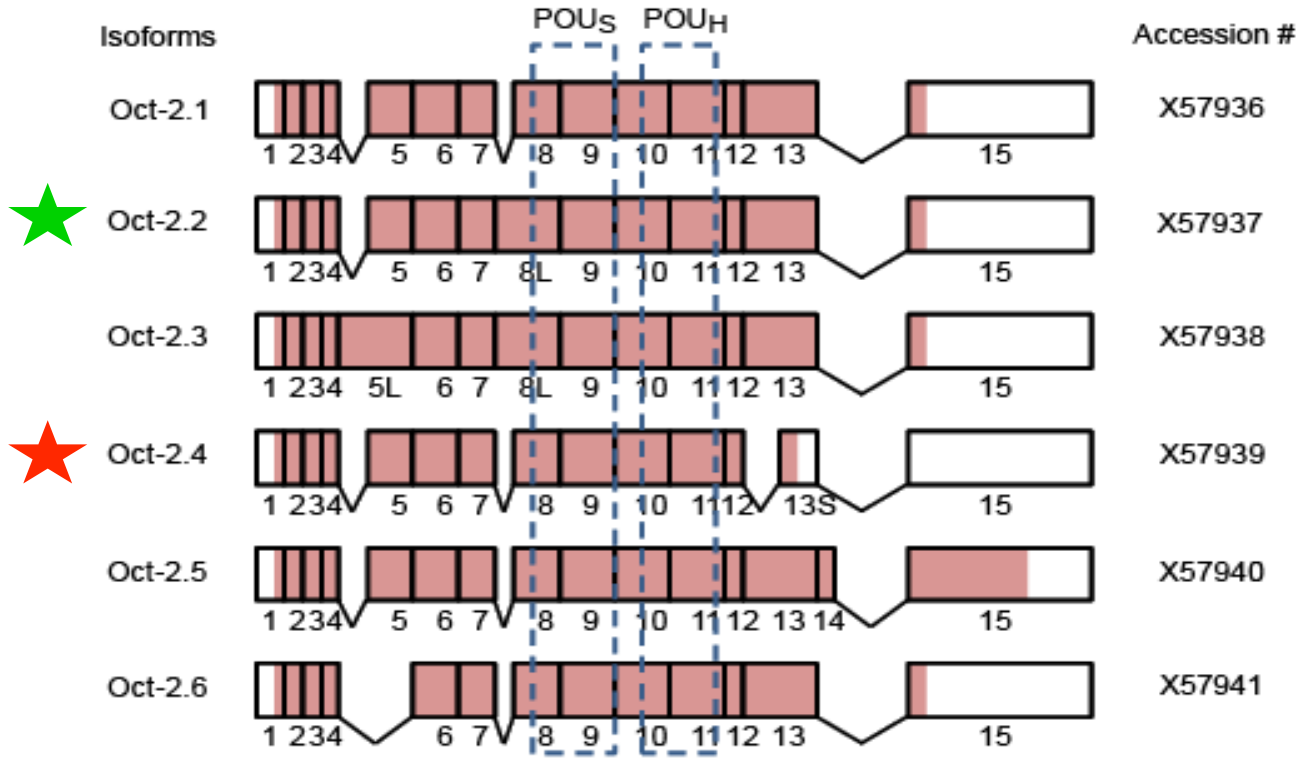
NeuroD1	KLF15
NeuroD6	Braf35
NeuroG1	Meox2
Nkx2.5	Oct-2 *
Tead2	KLF12
Tcf4	ZNF43
MTGR1	ZNF205
Six1	ZNF37A
DMRTC2	ZFP64
Hoxd3	ZNF435
Hoxa3	ZNF408
SATB1	WDR34

Hits without VP16

Nkx2.5
NeuroD1
Six1
NeuroD6
Hoxd3
Hoxa3
ZNF205
NeuroG1
KLF15

+ Control
Implicated
Repress
Novel

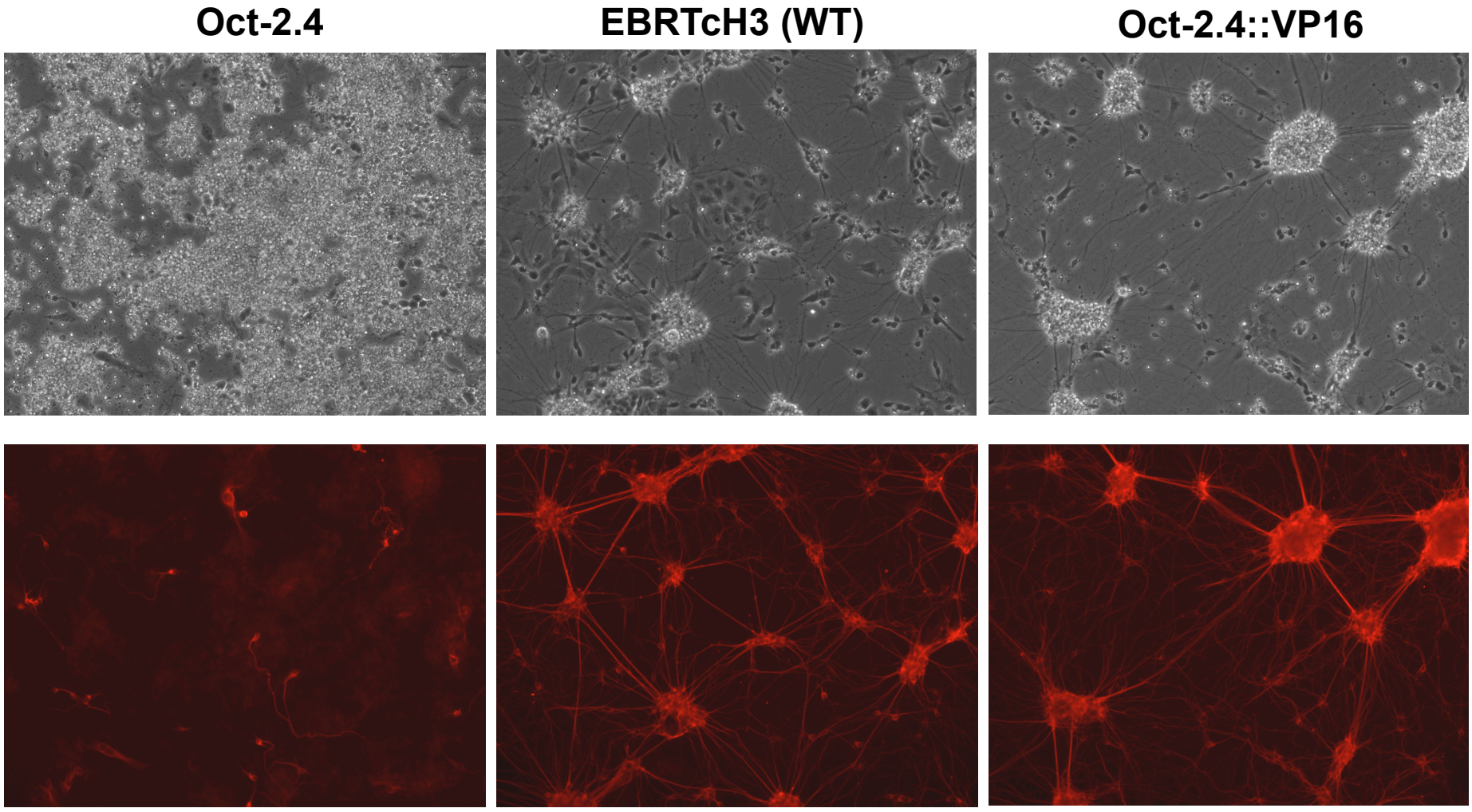
Oct-2 Many Isoforms



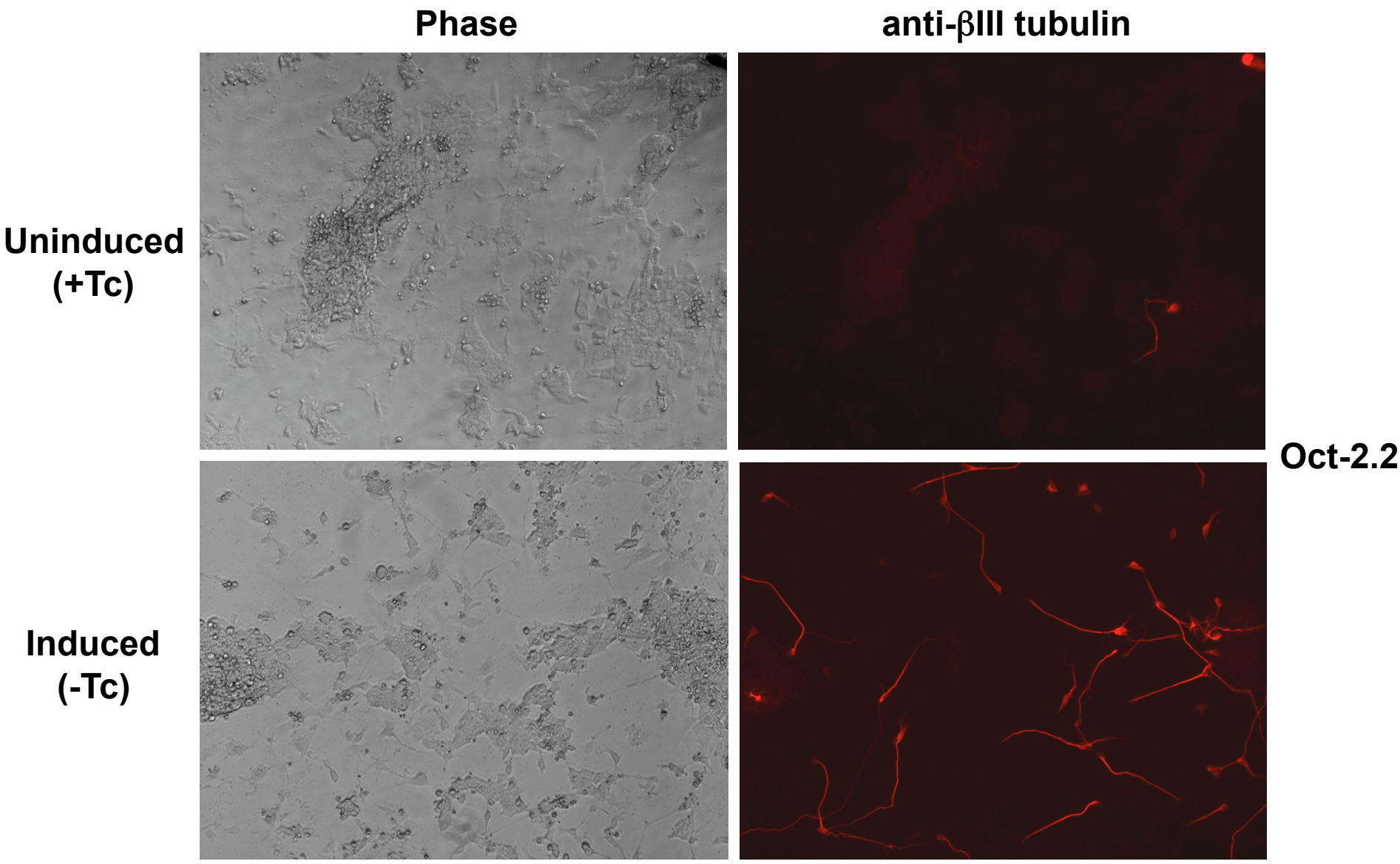
Adapted from Dong et al., 2007

Oct-2.4 Inhibits Neuronal Differentiation

EBs dissociated and plated onto poly-ornithine/laminin coated plates



Oct-2.2 Isoform Induces Differentiation



Oct-2 Differentiation Model