HIGH THROUGHPUT
BIOLOGY

Protein Chip Development and Applications

Heng Zhu

Dept. of Pharmacology & the HiT Center
Johns Hopkins University School of Medicine

- Background in Proteomics
- Protein Chip Development
- Application in Basic Research
- Applications in Clinical Research

❖ Background in Proteomics

Progresses in Proteomics

Protein profiling
  2D-MS, analytical protein chip
High-throughput protein localization
  Transposon vs GFT
Biochemical Genomics
  Pooling strategy
Large-scale protein interaction mapping
  Y2H and protein complex coupled w/ MS
Transcription factor-DNA interaction
  ChIP-chip
High throughput biochemistry assays
  Functional protein chips

Why Microarrays?

Higher Sensitivity

Much higher throughput

More flexibility

Less sample consumption

Quantitation

Direct target detection

Protein Microarrays

- Protein-Protein Interactions
- Protein Modification and Regulation
- Serum Profiling
- Signaling Pathways
- Drug Discovery

Comparison of Interaction Proteomics

Approach	Application	Advantage	Disadvantage
Yeast two-hybrid	Protein-protein interactions Protein-DNA interactions	High-throughput and systematic to reveal protein interactions	No control over interaction condition; Interactions are usually in the nucleus
Affinity tagging/MS	Dissecting protein complexes	In vivo interactions that involve multiple partners	May miss transient or weak interactions, hard to identify false positives
Antibody array	Protein profiling, protein detection, clinical diagnostics	Very sensitive and low sample consumption, great potential in biomarker and drug development	Highly restricted by the quantity and quality of available antibodies; semi-quantitative protein detection
Functional protein array	Diverse, e. g. protein-protein, protein-lipid, protein-small molecule, enzyme-substrate interactions as well as drug discovery and posttranslational modifications	Great potentials for analyzing biochemical activities of proteins and high-throughput drug and drug target screening	In vitro assays
Peptide array	Enzyme-substrate interaction and drug discovery	Sensitive and straightforward to identify epitopes	Expensive to fabricate; in vitro assays
Carbohydrate array	Carbohydrate-mediated molecular recognition and anti-infection response	A new and sensitive way to study carbohydrate-mediated molecular events	In vitro arrays; tough to acquire carbohydrate molecules in pure forms
Small molecule array	Protein-small molecule interaction, drug discovery, enzyme specificity profiling	Minimum small molecule consumption and high sensitivity	In vitro assays; necessary to improve throughput to cover 10^6 molecules in a normal combinatorial chemistry library

❖ Protein Chip Development

Protein Chip Fabrication

High Quality clone collection

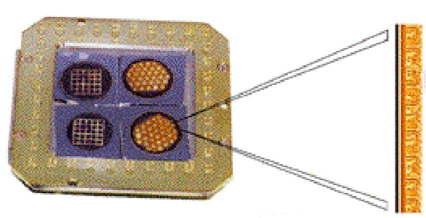
High throughput protein production

Surface structure on chips

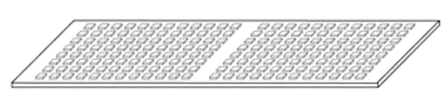
Surface chemistry

Storage

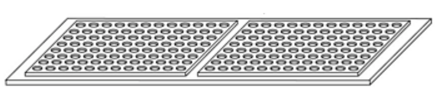
Surface Structure



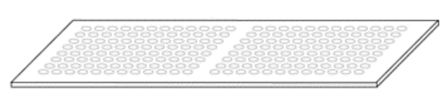
Porous Surface



3-D Surface Structure

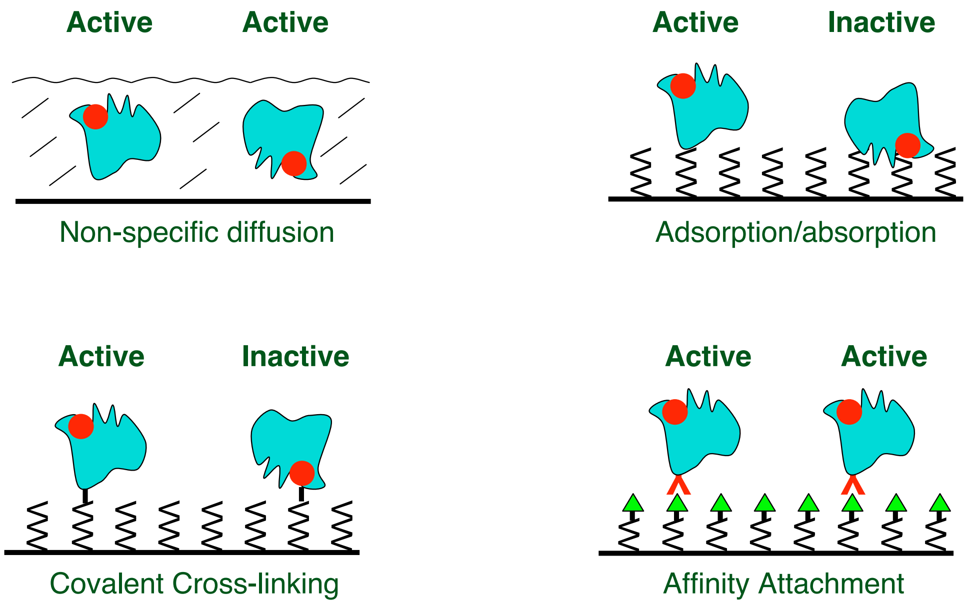


Nanowell

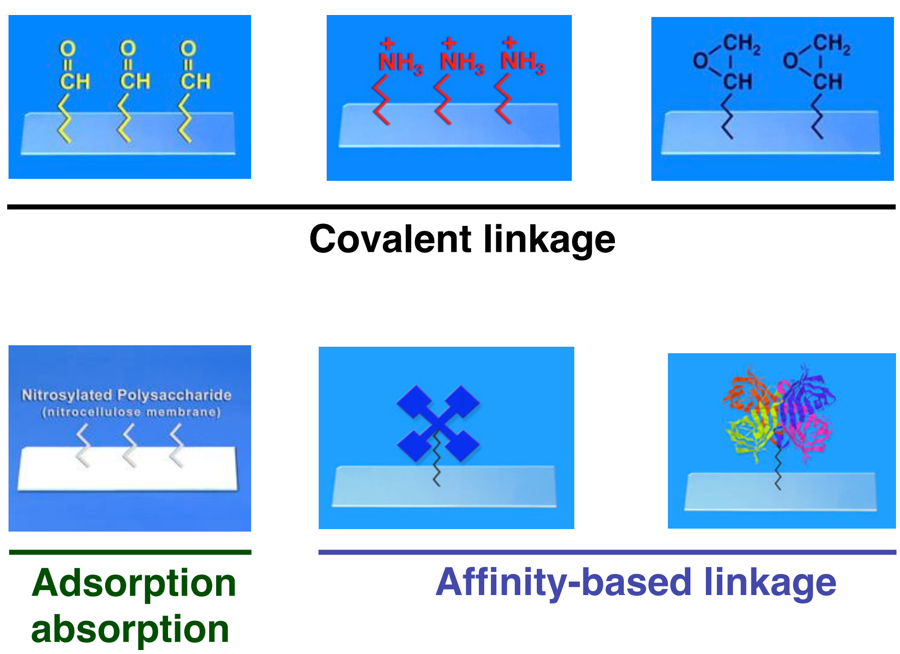


Plain Glass Surface

Chemically Modified Surface



Common Surfaces to Immobilize Proteins



TeleChem.com

Comparison of Surface Chemistry

Surface	Attachment	Advantage	Disadvantage
PVDF	Adsorption and Absorption	No protein modification requirement, high protein binding capacity	Non-specific protein attachment in random orientation
Nitrocellulose	Adsorption and Absorption	No protein modification requirement, high protein binding capacity	Non-specific binding, high background. Low-density arrays
Poly-lysine coated	Adsorption	No protein modification requirement	Non-specific adsorption
Aldehyde-activated	Covalent cross-linking	High-density and strong protein attachment. High-resolution detection methods available	Random orientation of surface attached proteins
Epoxy-activated	Covalent cross-linking	High-density and strong protein attachment. High-resolution detection methods available	Random orientation of surface attached proteins
Avidin coated	Affinity binding	Strong, specific and high- density protein attachment, low-background	Proteins have to be biotinylated
Ni-NTA coated	Affinity binding	Strong, specific and high-density protein attachment, low-background, uniform orientation of surface attached proteins	Proteins have to be Hisx6 tagged
Gold-coated silicon	Covalent cross-linking	Strong and high-density protein attachment, low-background. Can be easily coupled with SPR and Mass-spectrometry	Random orientation of surface attached proteins, tough to fabricate, not commercially available
PDMS nanowell	Covalent cross-linking	Strong and high-density protein attachment, well suited for sophisticated biochemical analyses	Random orientation of surface attached proteins
3-D gel pad and agarose thin film	Diffusion	High protein binding capacity, no protein modification requirement.	Tough to fabricate, not commercially available
DNA/RNA coated	Hybridization.	Strong, specific and high-density protein attachment, low-background, uniform orientation of surface attached proteins.	Sophisticated in vitro production of labeled proteins

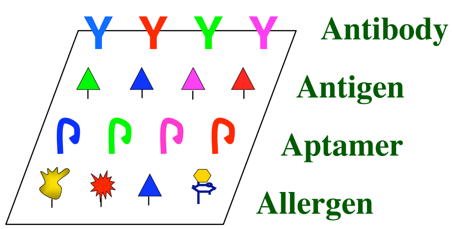
Comparison of Detection Methods

Detection	Probe labeling	Data acquirement	Real time	Resolution
ELISA	Enzyme-linked antibodies	CCD imaging	No	Low
Isotropic labeling	Radio isotope-labeled analyte	X-ray film or phosphaimager	No	High
Sandwich immunoassay	Fluorescently-labeled antibodies	Laser scanning	No	High
SPR	Not necessary	Refractive index change	Yes	Low
Non-contact AFM	Not necessary	Surface topological change	No	High
Planar waveguide	Fluorescently-labeled antibodies	CCD imaging	Yes	High
Silicon biosensor	Fluorescently-labeled antibodies	CCD imaging	Yes	High
SELDI	Not necessary	Mass spectrometry	No	Low
Electro-chemical	Metal-coupled analyte.	Conductivity measurement	Yes	Medium

❖ Application in Basic Research

Protein Microarrays Are of Two Types

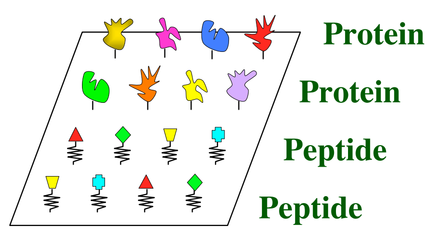
Analytical



Serum probes
Cell lysates
Living cells

Protein expression level
Protein profiling
Diagnostics

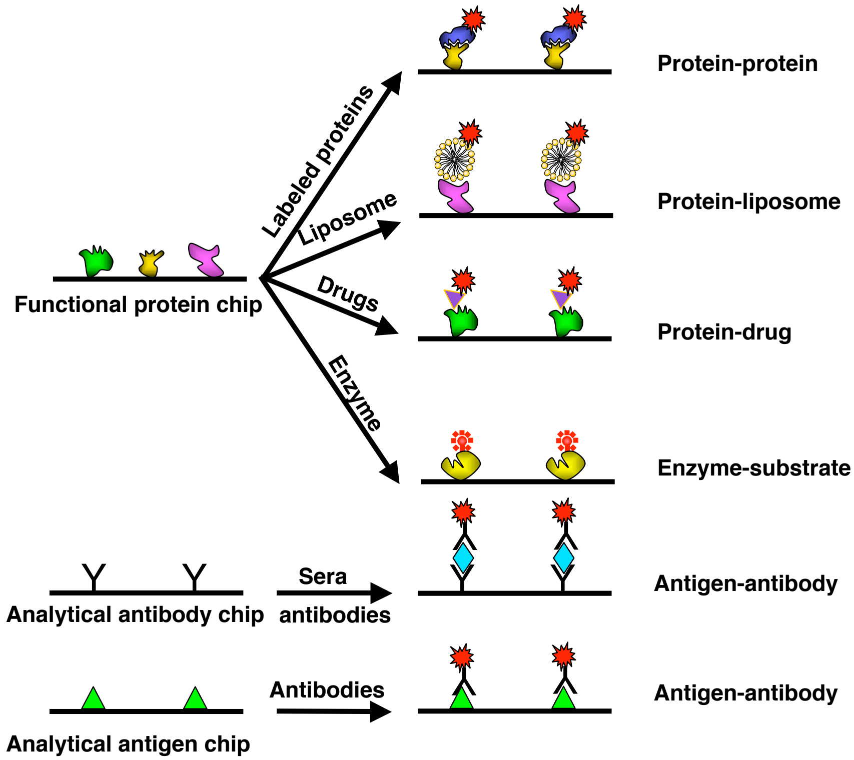
Functional



Protein probes
Nucleic acid probes
Drug probes
Enzymes

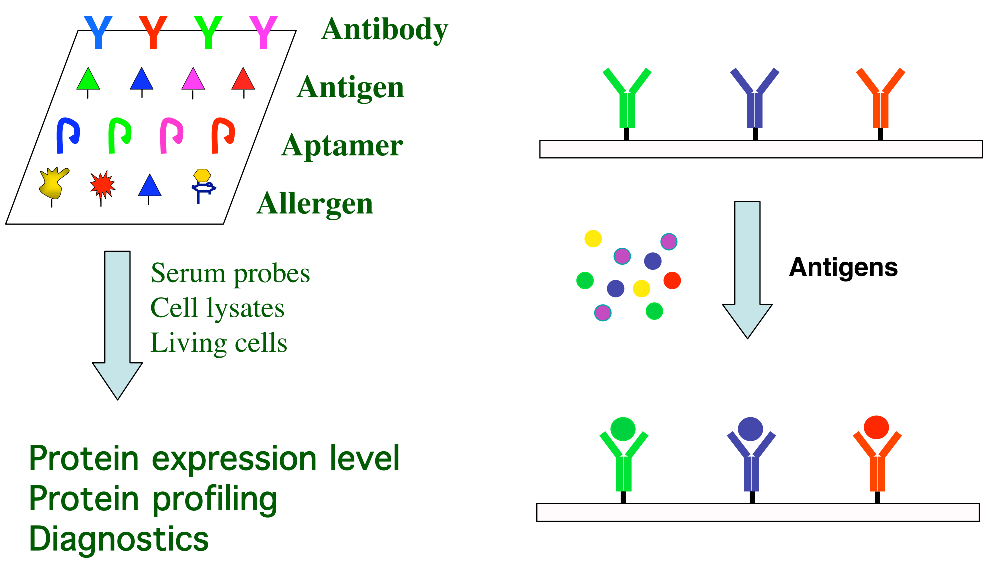
Protein binding properties
Pathway building
Posttranslational modification
Drug discovery

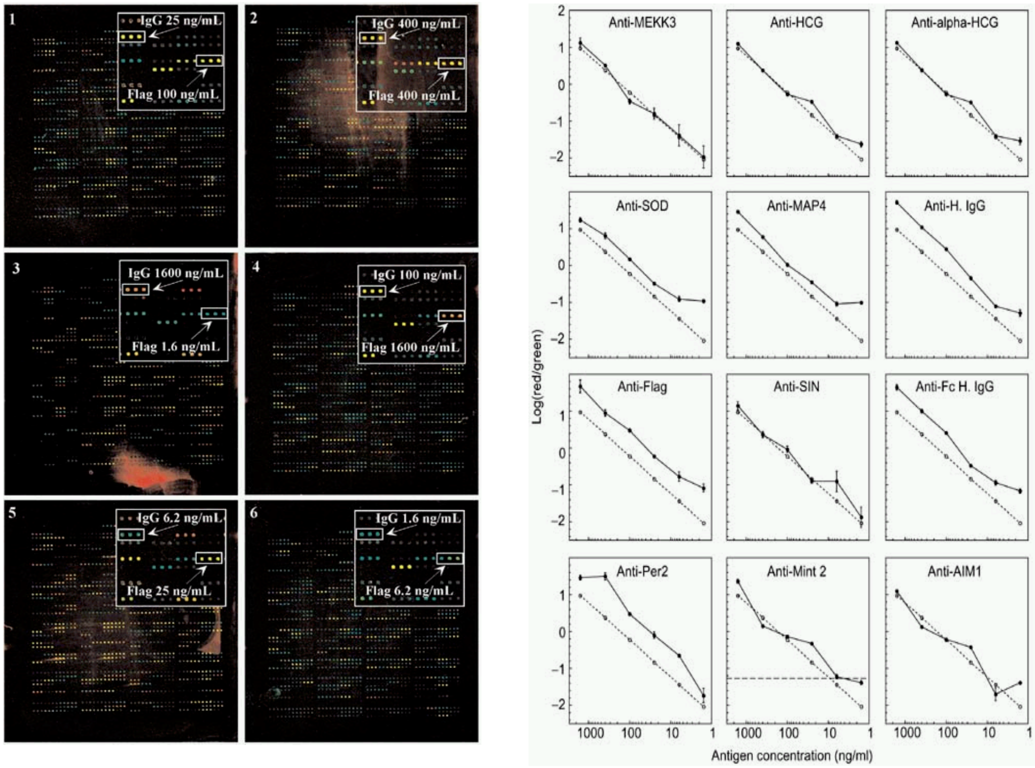
Summary of Protein Chip Applications



Application of Analytical Microarrays

Analytical Antibody Chip

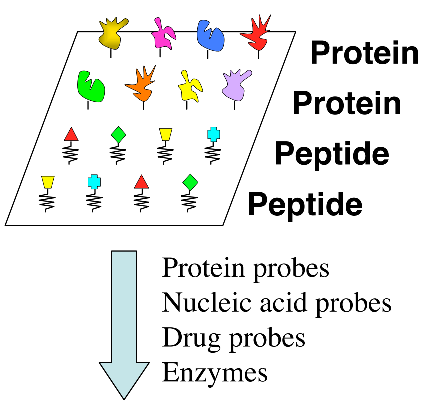




Haab BB et al. *Genome Biology* 2001, 2:research0004.1-0004.13

Protein Microarrays Are of Two Types

Functional



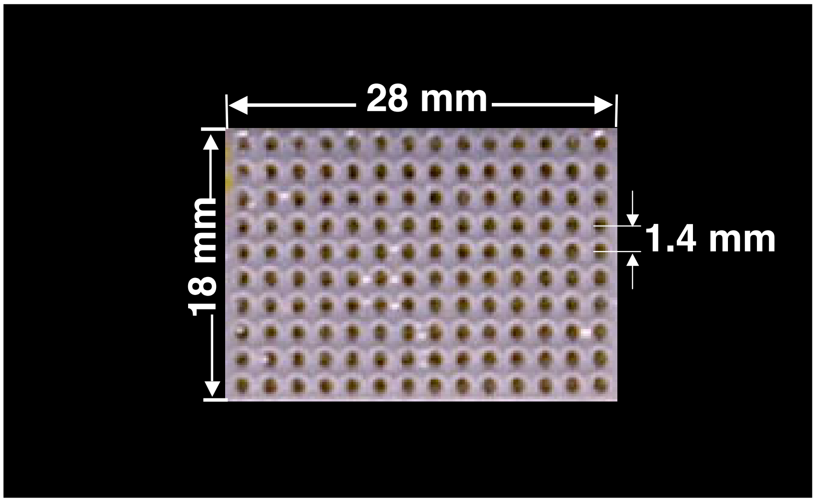
Protein binding properties
Pathway building
Posttranslational modification
Drug discovery

Key points

Clone collection
- **Cloning strategy**
- **Yeast, *C. elegans*, humans**

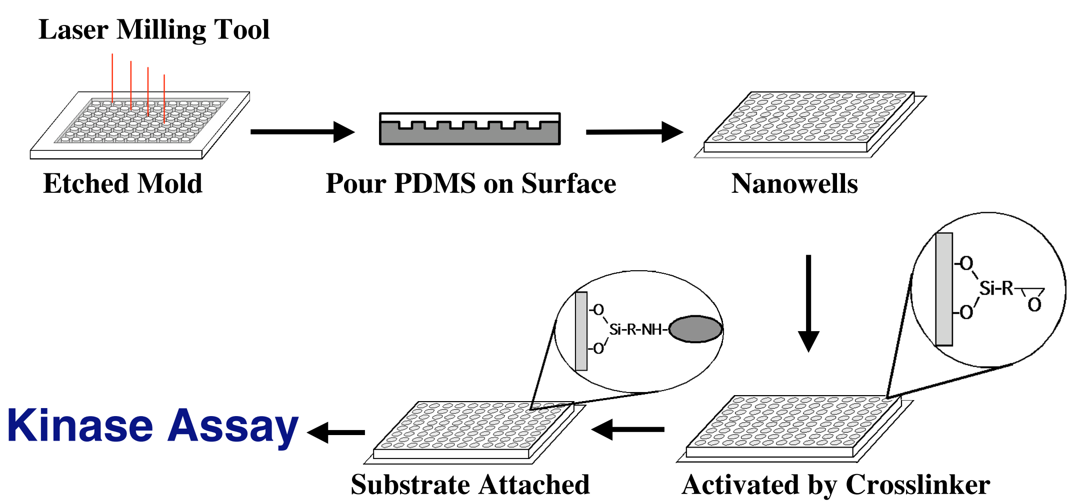
Protein production
- **Hosts for making proteins**
- **Affinity tags**
- **In vitro system**

Nanowell Chip

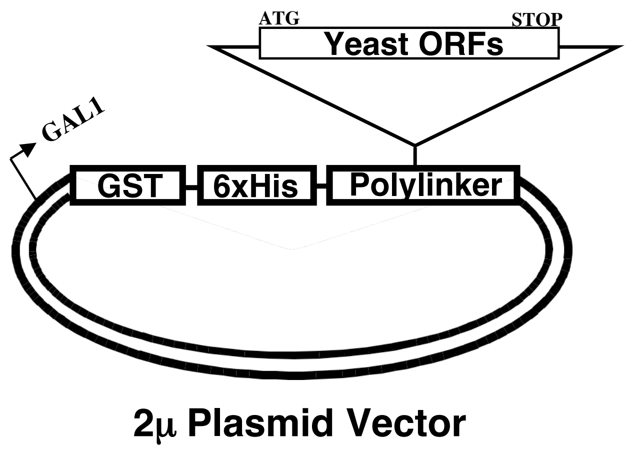


- Round shape wells
- 1.4 mm diameter
- 300 micron depth
- 2.0 mm pitch
- 300 nl volume

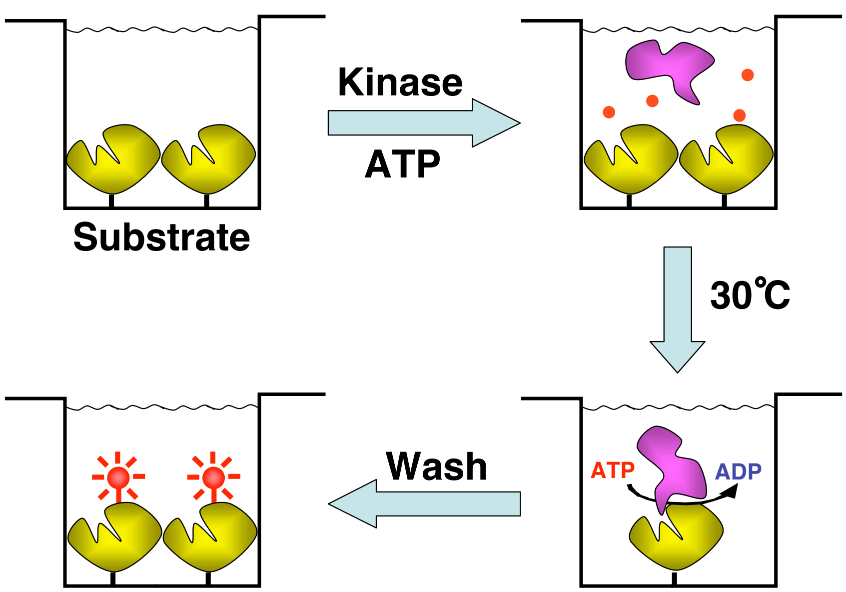
Nanowell Chips and Protein Attachment



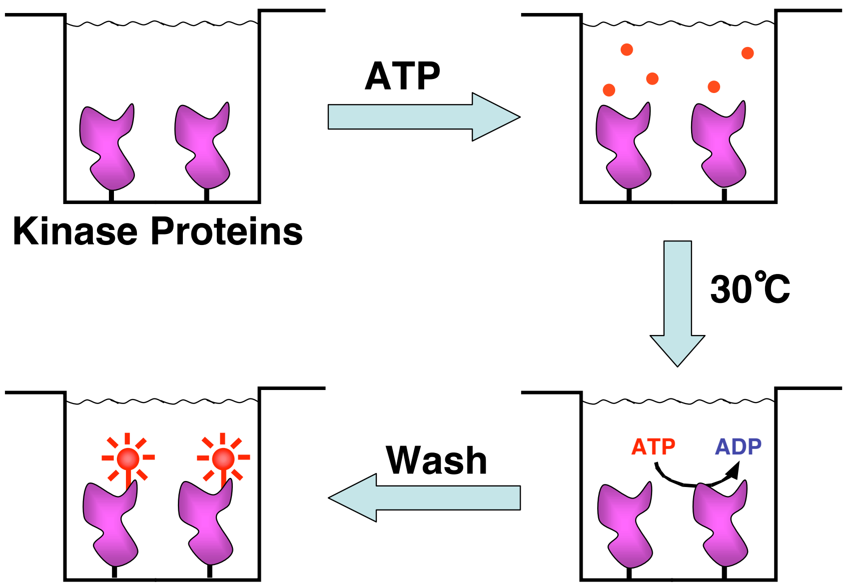
Modified GST Expression Vector pEGH



Kinase-Substrate Assays on Nanowell Chips

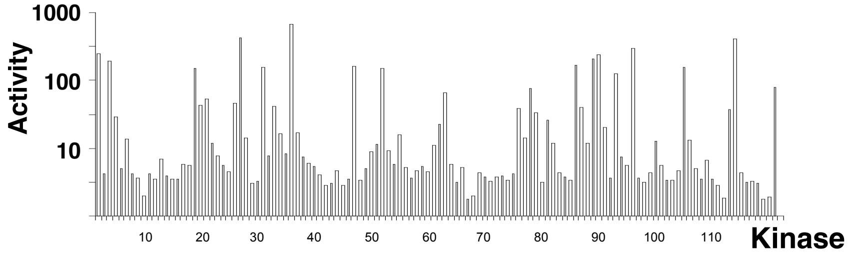


Autophosphorylation Assays on Nanowell Chips

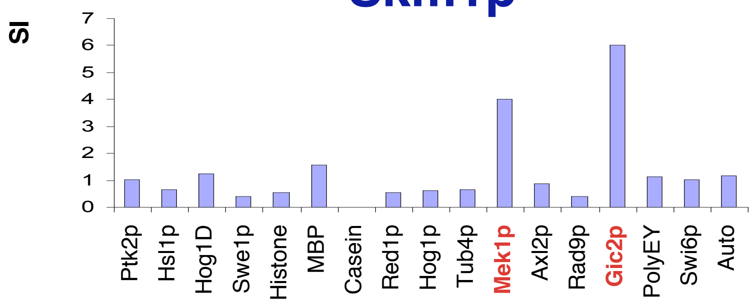


Kinase Assays Using Protein Chips

Swi6p as a Substrate



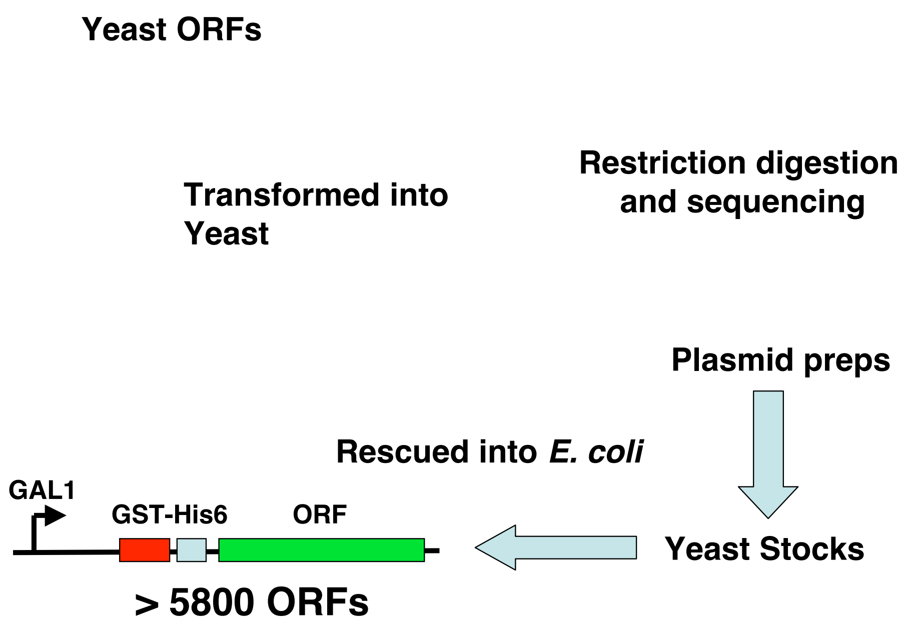
Skm1p



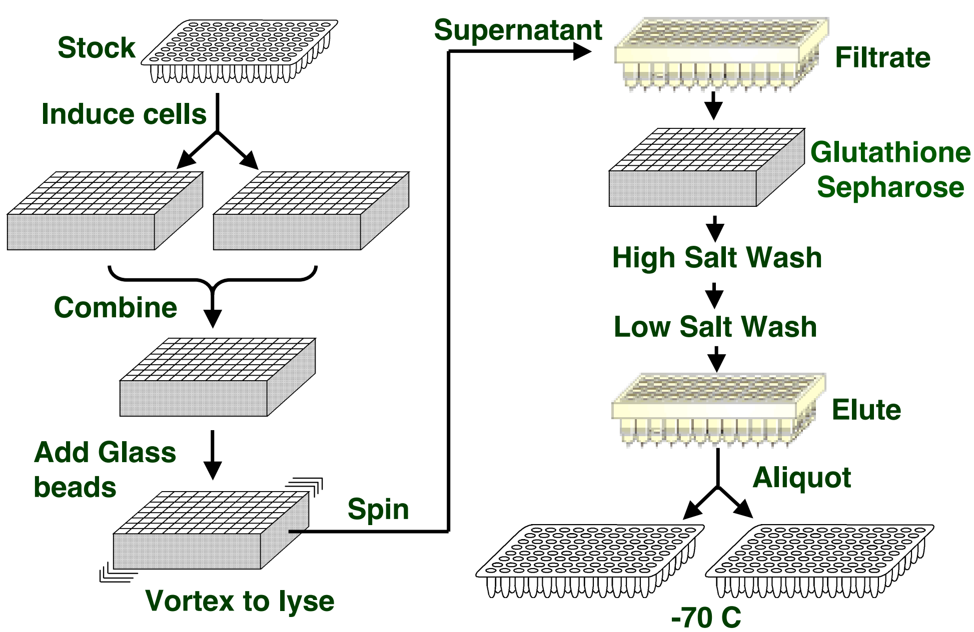
❖ Yeast Proteome

- 6282 Protein Coding Genes
- 4042 Characterized
- 2244 Uncharacterized
  - 334 Homologs
  - 1910 Unique

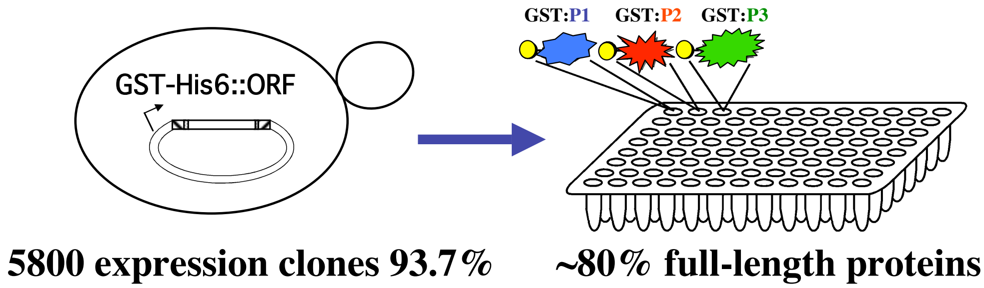
Cloning Strategy

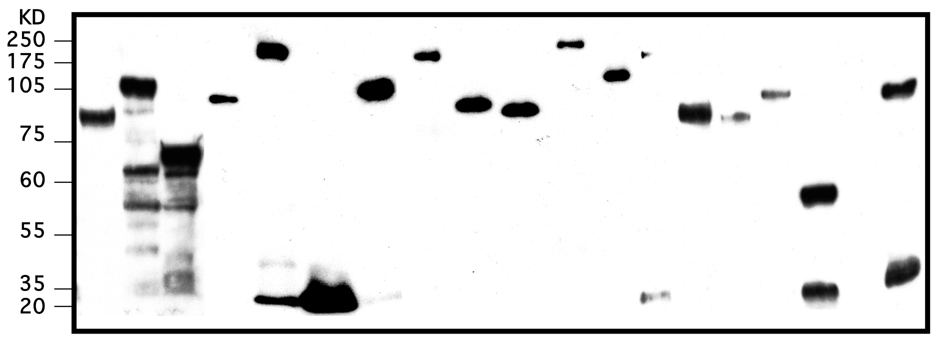


96-Well Yeast Protein Purification

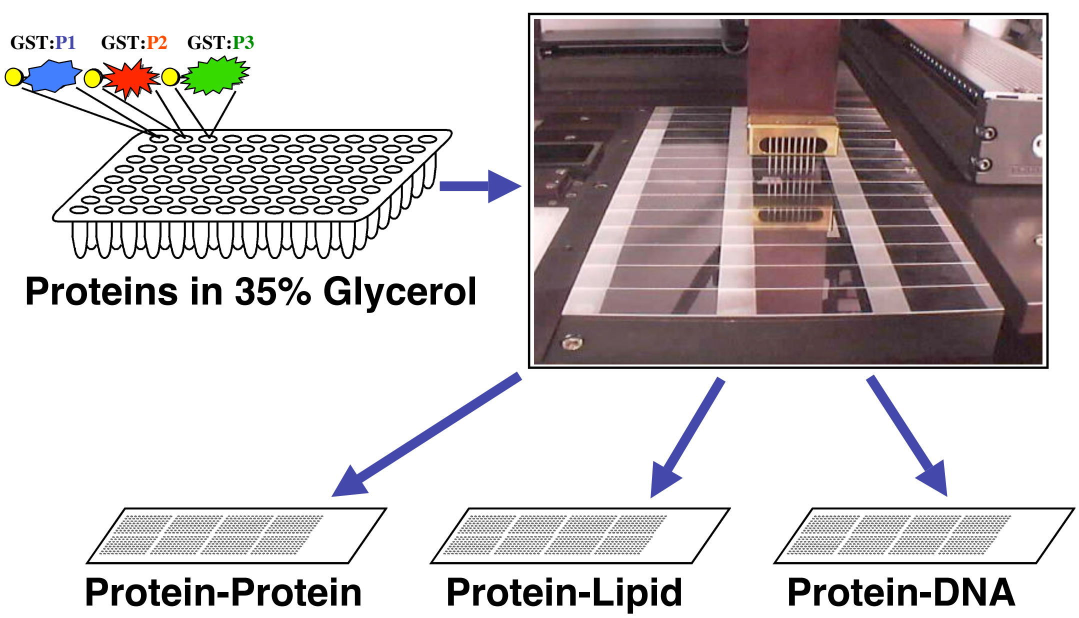


Producing the Yeast Proteome

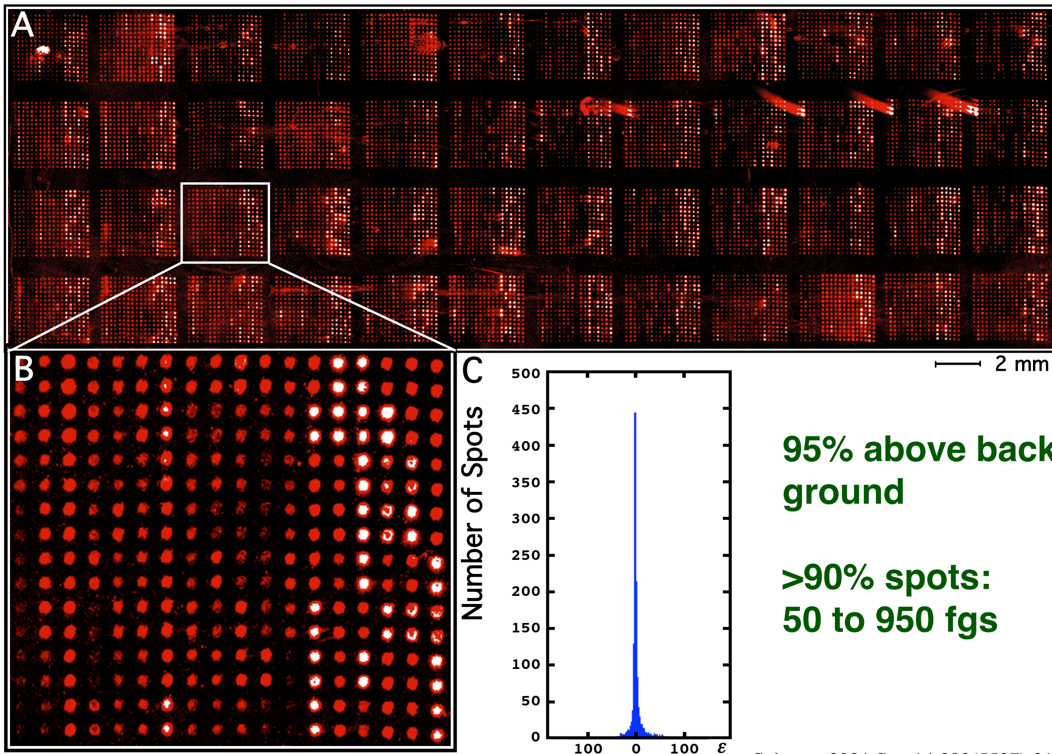




Printing the Yeast Proteome



The Yeast Proteome Chip



95% above background

>90% spots: 50 to 950 fgs

Science. 2001 Sep 14;293(5537):2101-5.

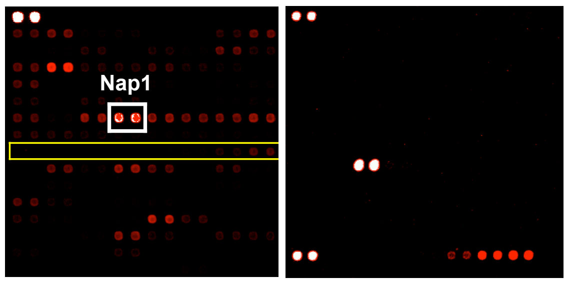
Types of Assays Developed

- Protein-protein
- Protein-antibody
- Protein-lipid
- Protein-DNA/RNA
- Protein-drug
- Protein-small molecule
- Phosphorylation
- Acetylation
- Ubiquitinylation
- Glycosylation

Antibody Probing of the Yeast Proteome Microarray

	Antibody	# of +s
Monoclonal (3 Yeast + 3 Control)	α-Sed3, α-Cox4	1
	α-Pep12	4
Anti-Peptide Polyclonal (6)	α-Hda1	8
	α-Mad2	1
Anti-FL Protein Polyclonal (2)	α-Nap1	1770
	α-Cdc11	7

Anti-Nap1



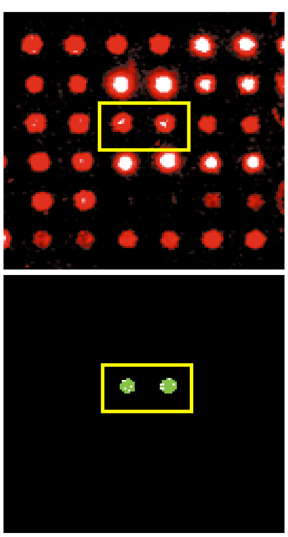
Anti-Sed3p

Nat Biotechnol. 2003 Dec;21(12):1509-12.

Calmodulin-Binding Proteins

α-GST

Probe



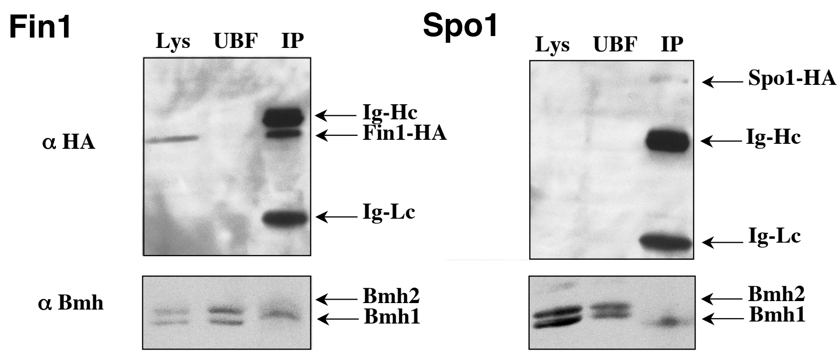
Calmodulin

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

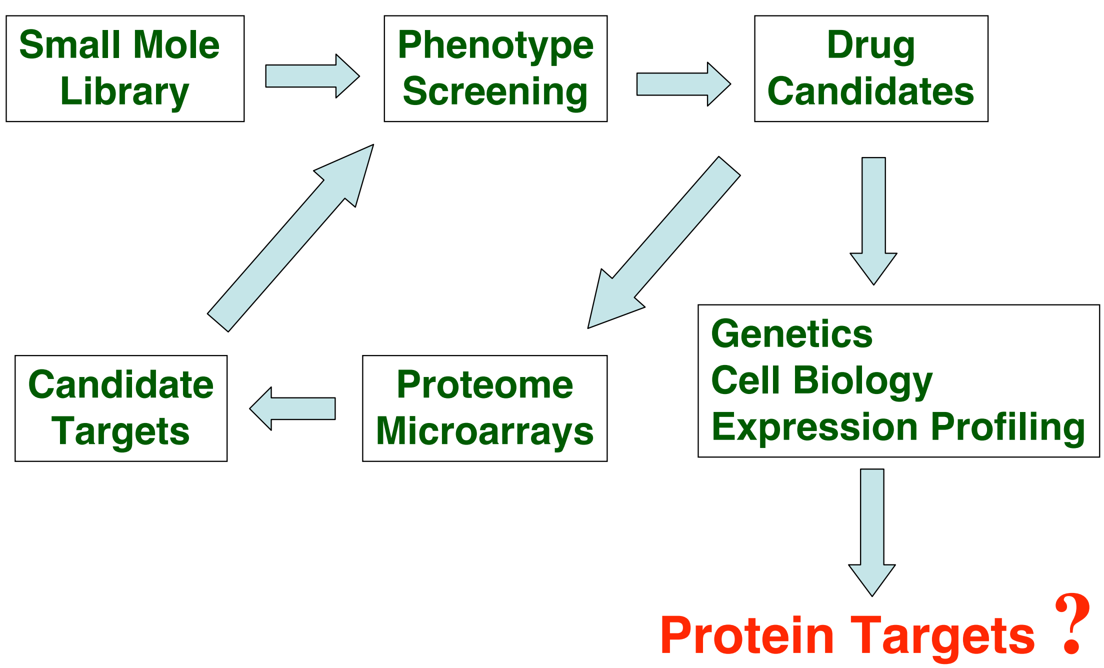
Science. 2001 Sep 14;293(5537):2101-5.

Validation of Bmh1,2 Targets

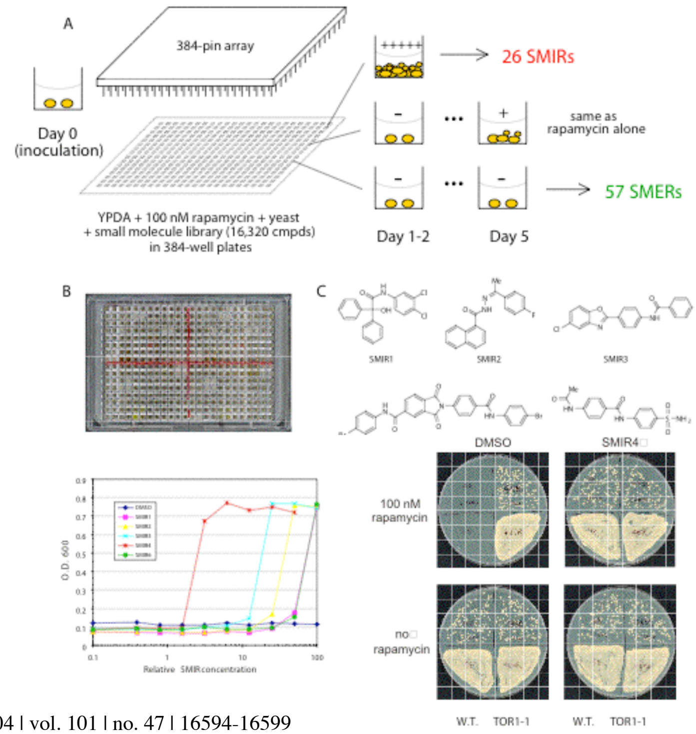
- ~140 *in vitro* targets
- 4 of 5 targets verified co-IP.
- All 4 IP preferentially with Bmh1



Drug Discovery and Target Validation

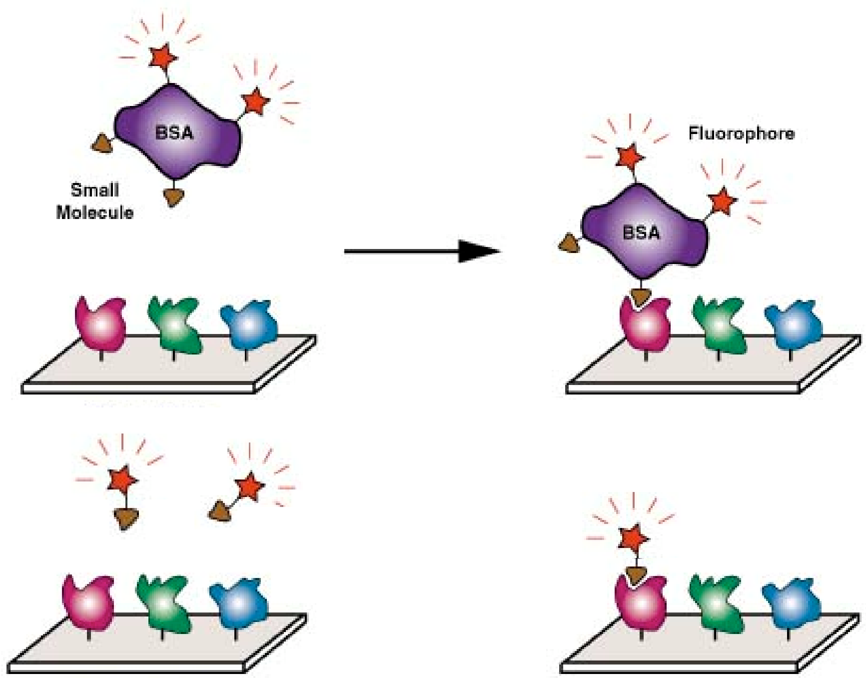


SMIR3 & 4 Function in Tor1/2 Pathway



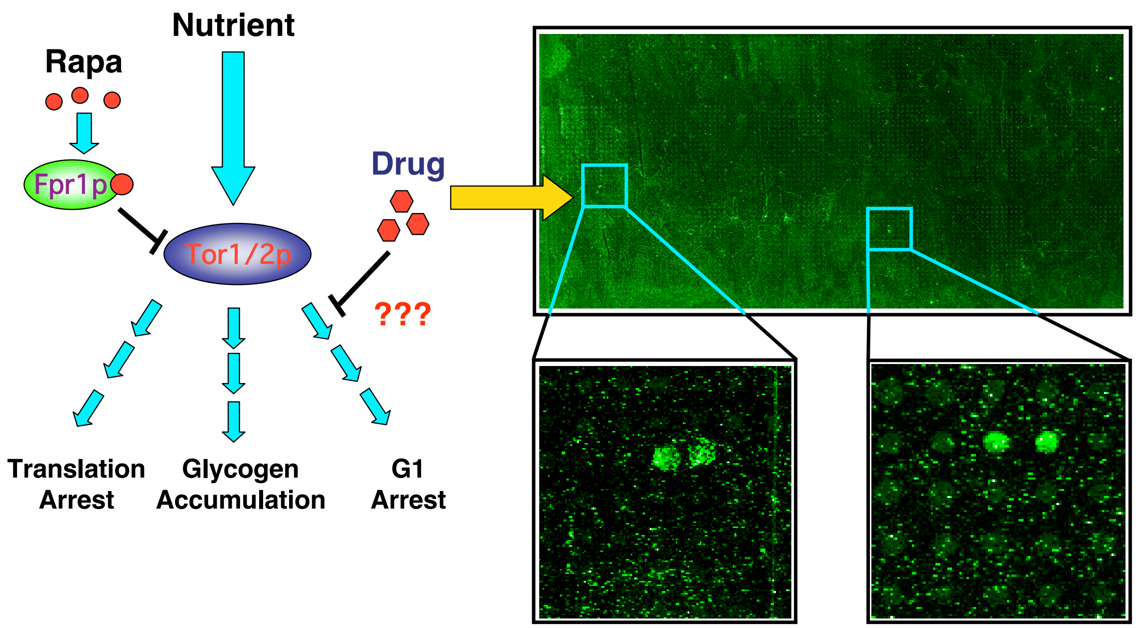
PNAS | November 23, 2004 | vol. 101 | no. 47 | 16594-16599

Protein Chips in Drug Discovery

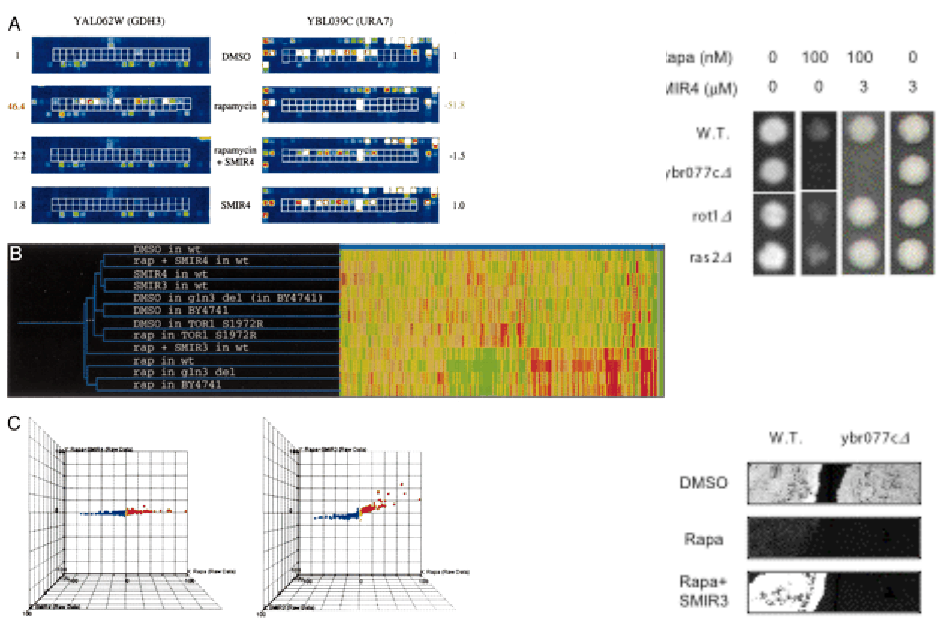


MacBeath et al.

Identification of Drug Targets



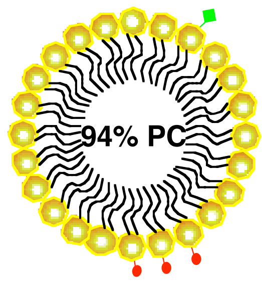
Identification of Drug Targets



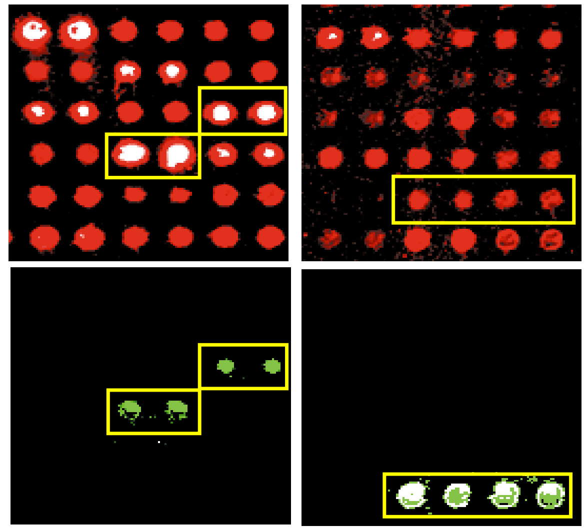
PNAS | November 23, 2004 | vol. 101 | no. 47 | 16594-16599

Probed with PC & 5 PIs, PI(3)P, PI(4)P, $PI(3,4)P_2$, $PI(4,5)P_2$, $PI(3,4,5)P_3$

1% Biotinylated DHPE



5% Phosphotidyl-inositide



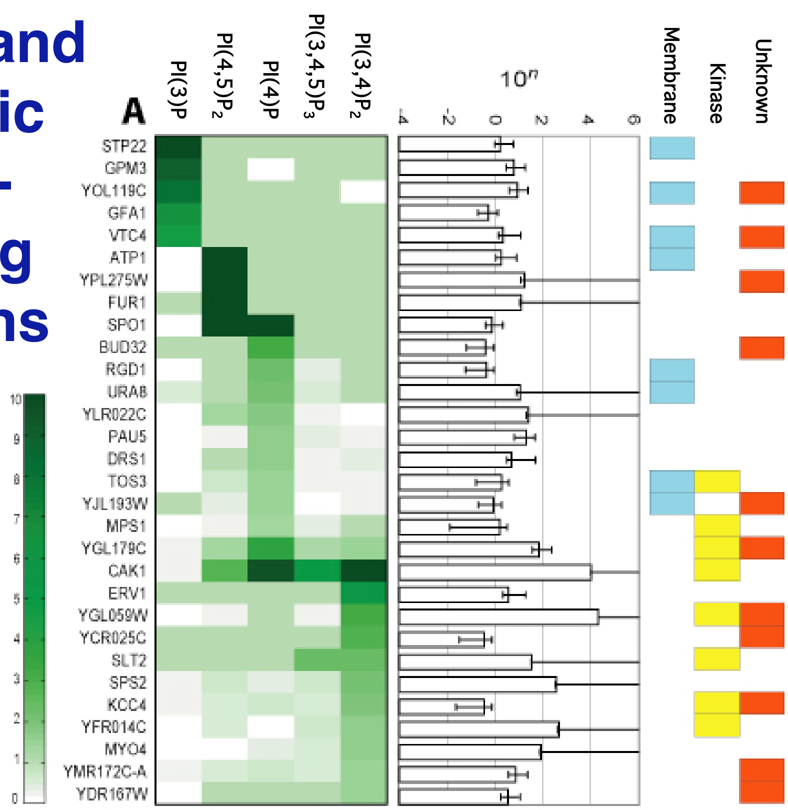
PI(3)P | $PI(4,5)P_2$

150 lipid binding proteins
52 Uncharacterized

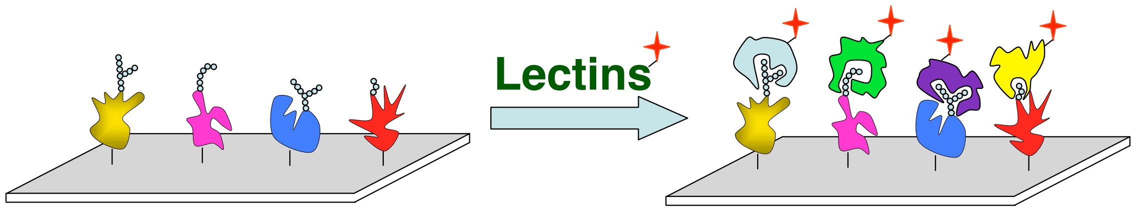
98 Known function
45 Membrane, 8 lipid metabolism

Science. 2001 Sep 14;293(5537):2101-5.

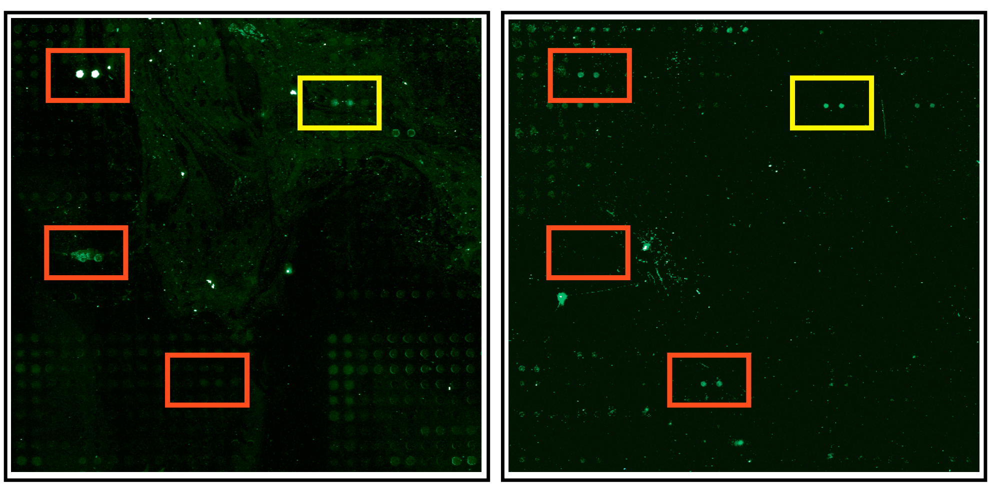
Strong and Specific Lipid-Binding Proteins



Detection of Posttranslational Modification



Detection of Sugar Modifications at the Proteome Level

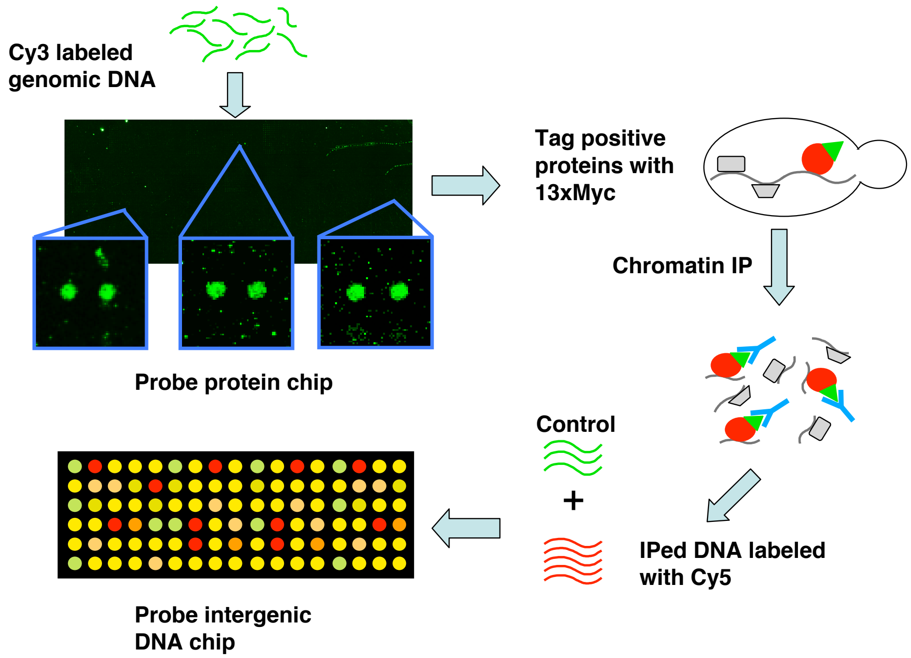


PSA Probing
Mannose and Glucose

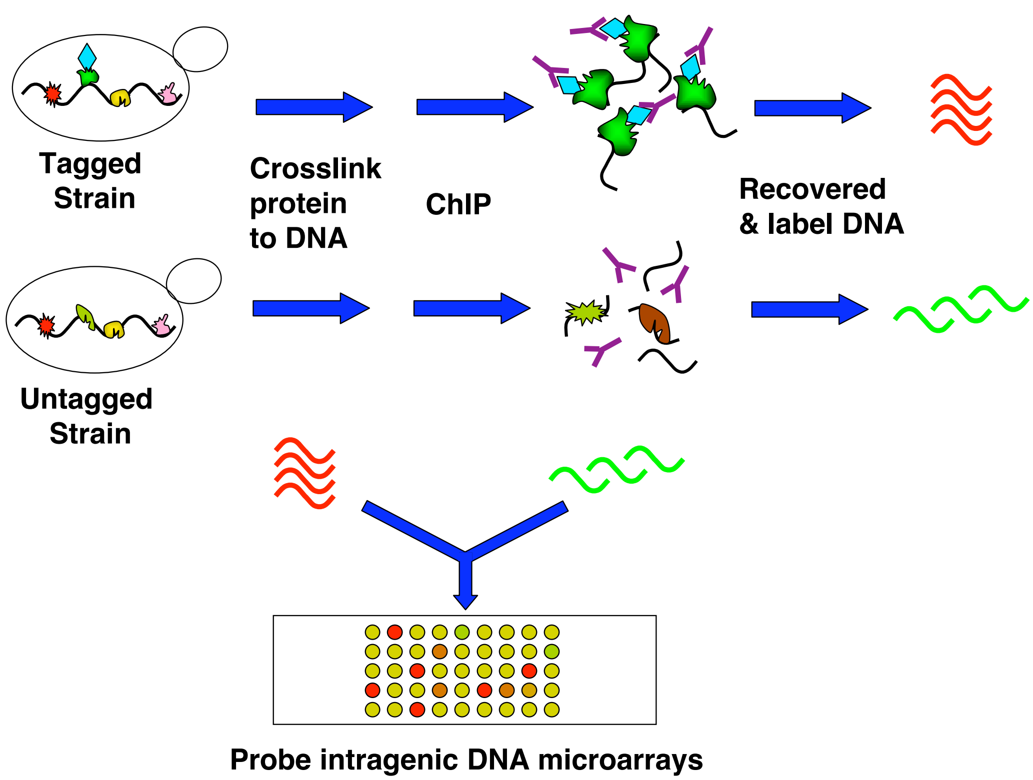
WGA Probing
N-Acetyl Glucosamine

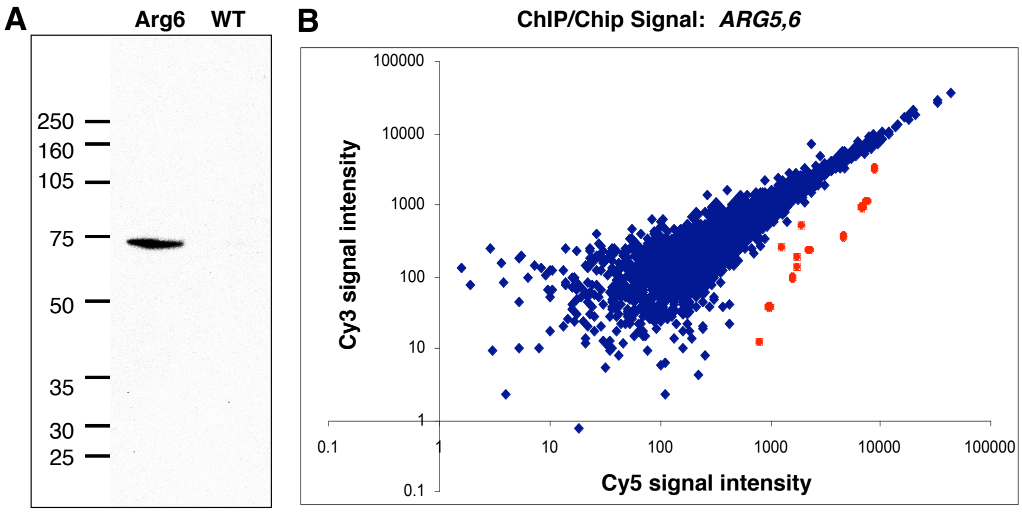
Zhu et al. unpublished

Identification of DNA Binding Proteins



Science. 2004 Oct 15;306(5695):482-4

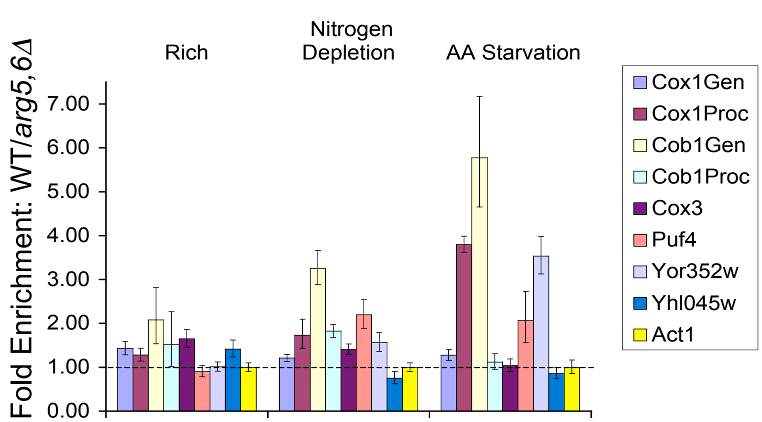




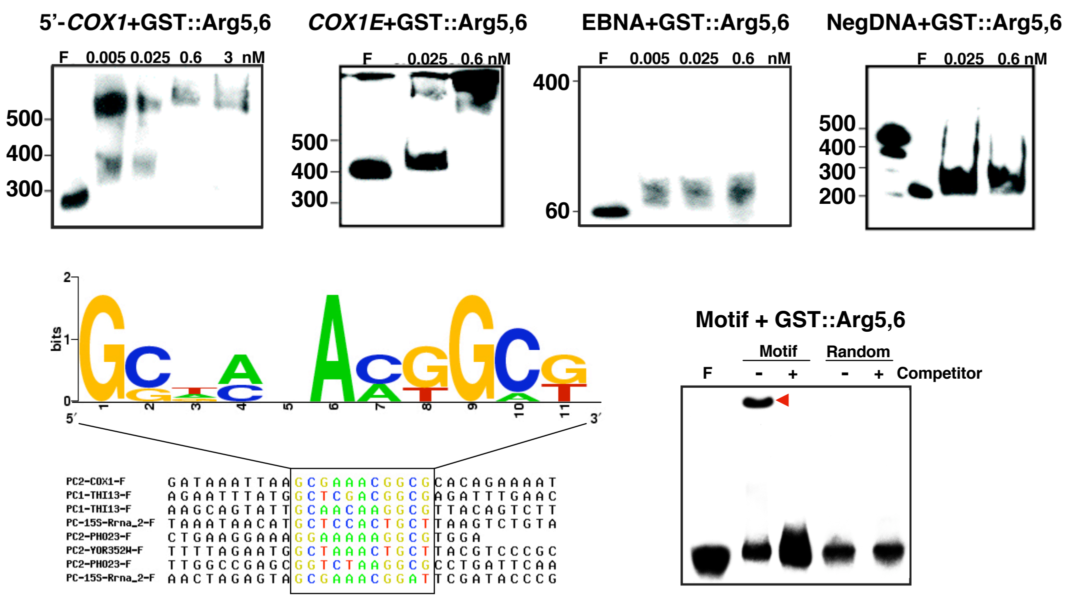
C

Fragment	*15S rRNA2*	*PC1 COB*	*PC2 COX1*	*PC4 COX1*	*PC6 COX1*	*PC8 COX1*	*PC2 YOR352W*	*PC2 PHO23*	*PC1 THI13*	*PC YHL045W*	*PC PUF4*	Negative Control
Fold Enrichment	10.1±8.6	3.9±2.0	22.0±7.9	3.7±2.2	13.8±12.0	4.5±0.2	8.5±3.6	8.4±4.1	>30	2.0±0.4	3.8±2.3	1.0±0.1

COX1 Expression Is Regulated by Arg5,6



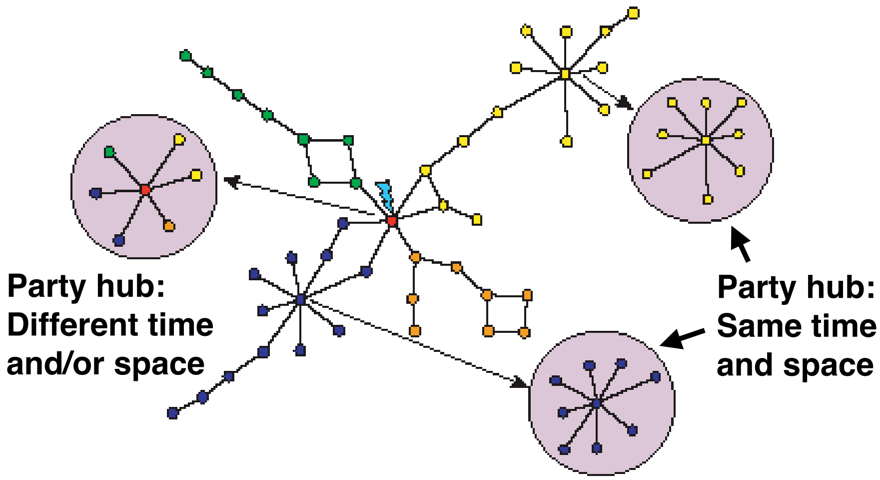
Arg5,6 Binds Mitochondrial DNA



Posttranslational Modification

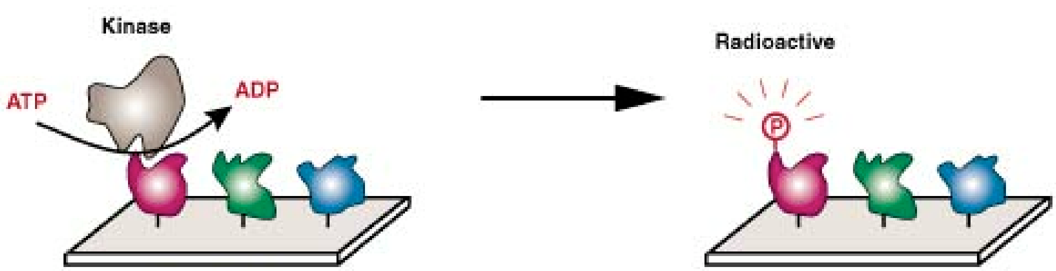
- Phosphorylation
- Dephosphorylation
- Acetylation
- Ubiquitinylation
- Glycosylation

Network Biology



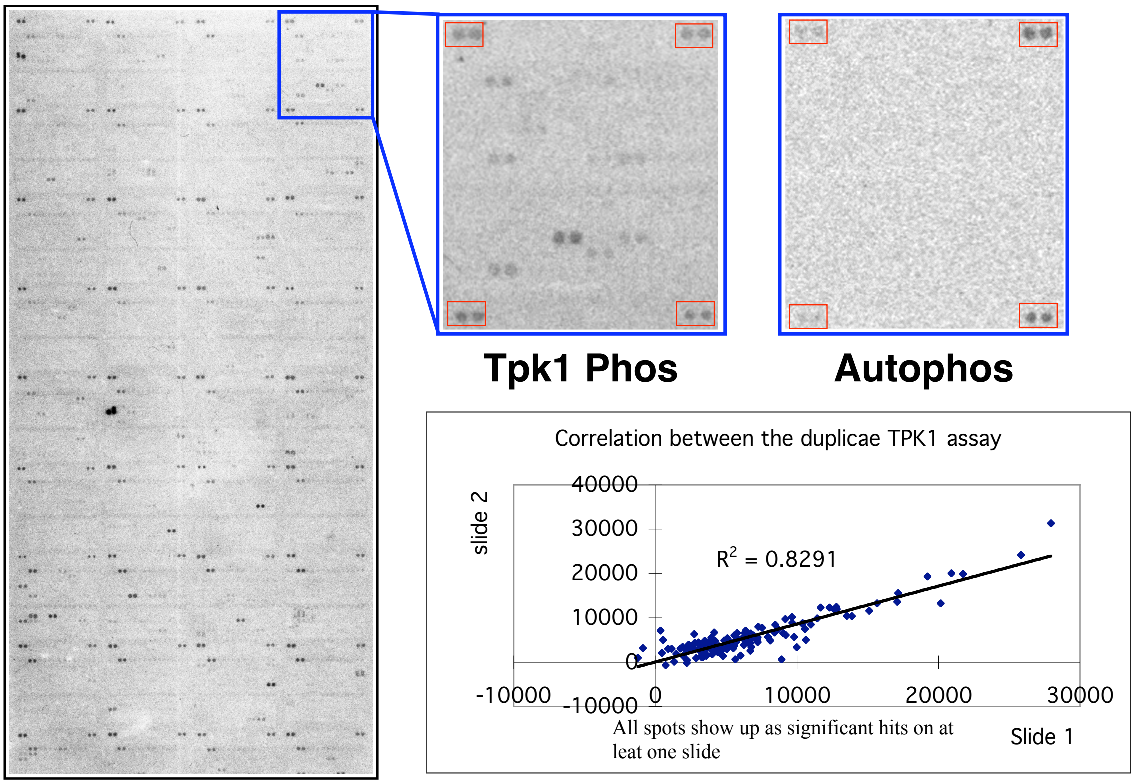
Han et al., Nature 430:88-93, 2004

Kinase Assays on Protein Chips

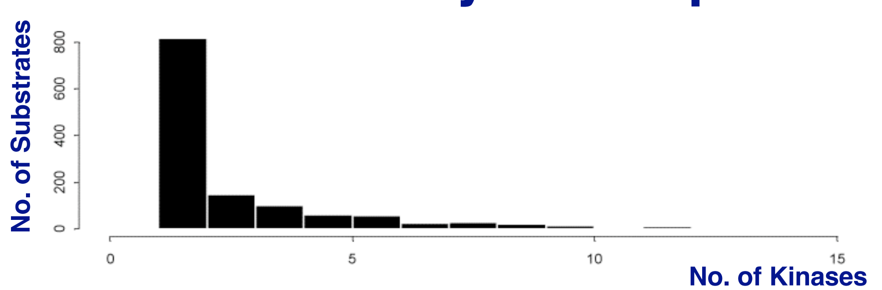


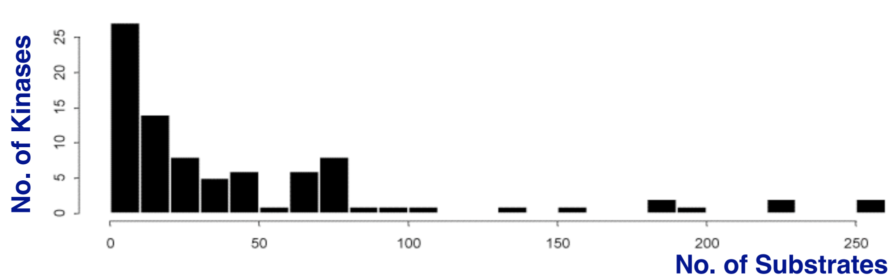
MacBeath et al.

Identification of Kinase Substrates



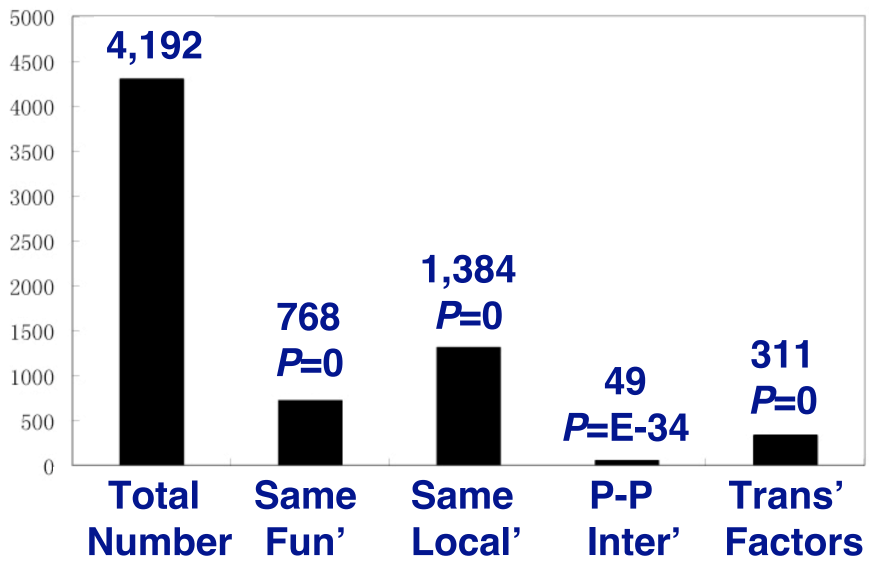
Kinase Assays Are Specific



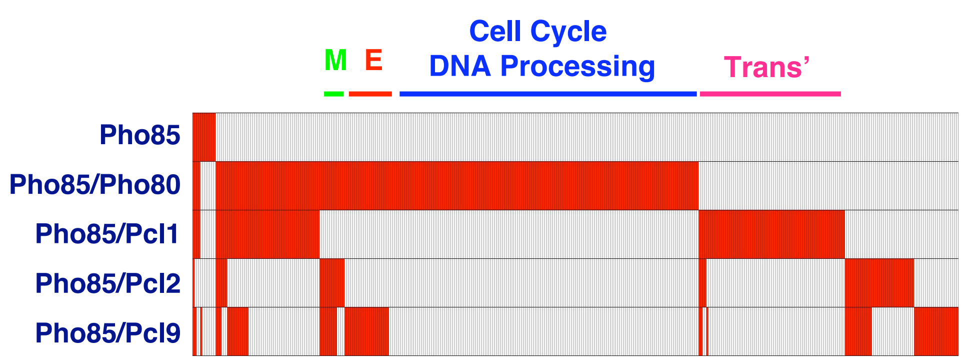


Average No. = 47, ranging from 1 to 256
73% substrates were recognized by fewer than 3 kinases

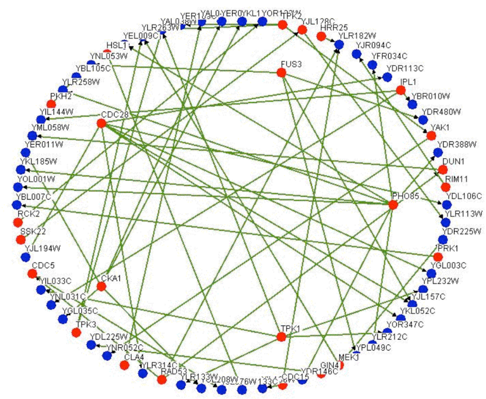
Kinases Often Recognize Functional Classes of Protein Substrates



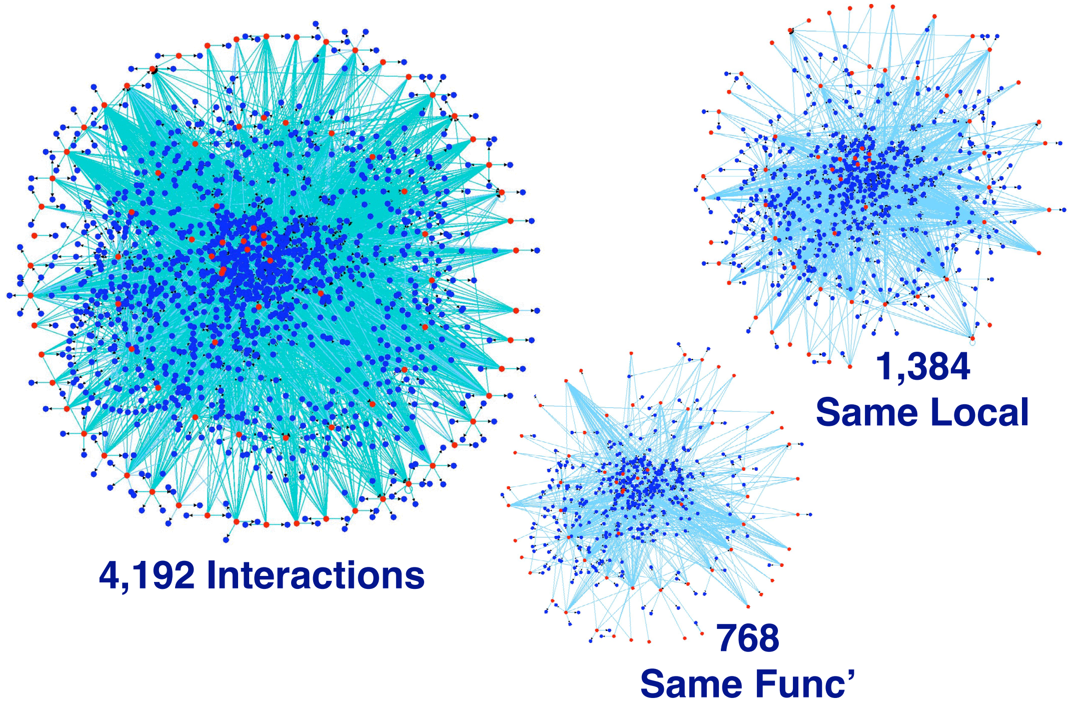
Related Kinases Recognize Different Substrates



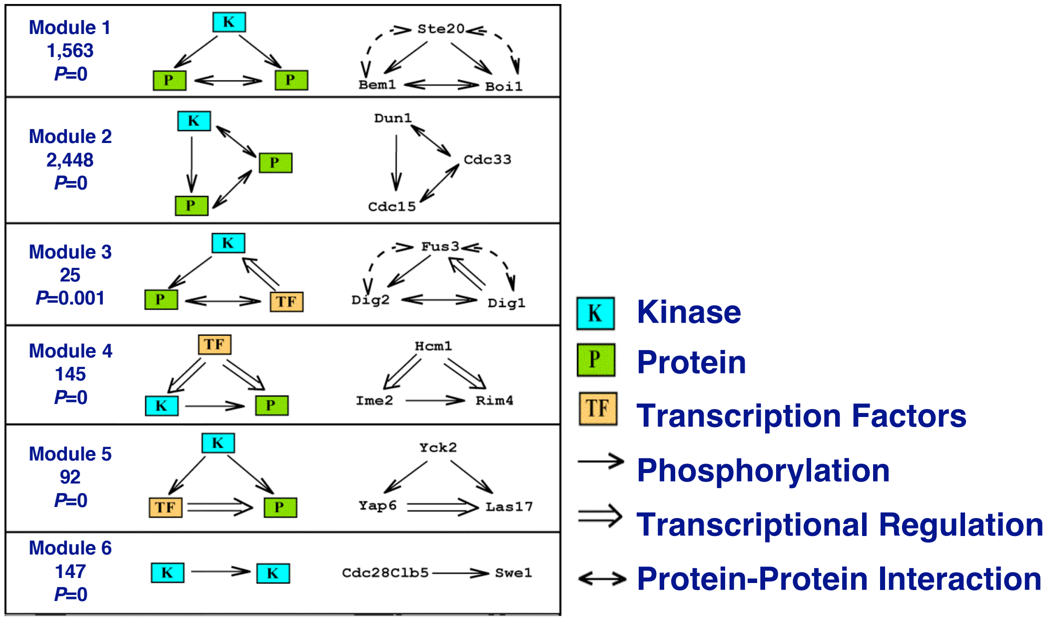
Previously Known Kinase-Substrate Interactions in Yeast



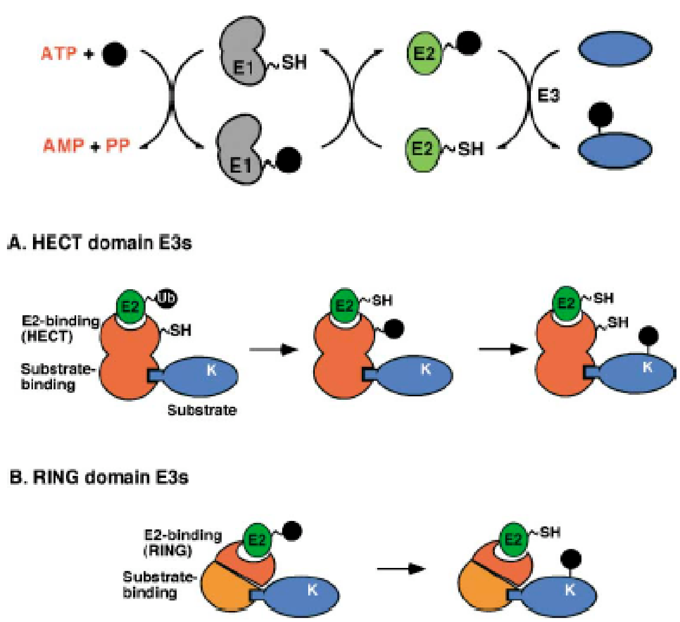
Phosphorylation Network in Yeast



New Regulatory Modules Are Revealed

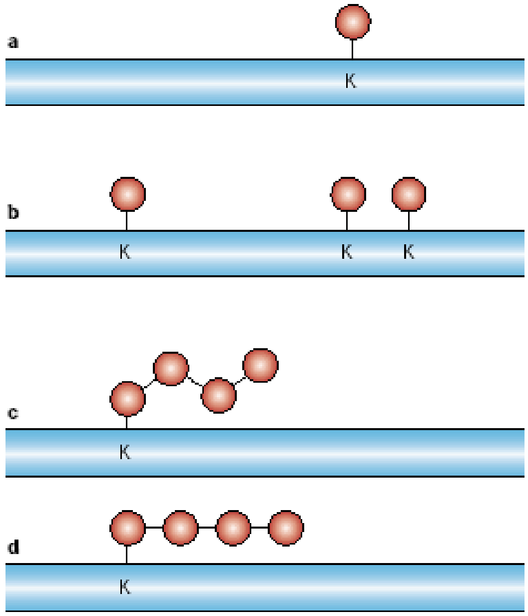


Ubiquitinylation Requires Three Enzymes Sequentially



Cecile Pickart, Cell 116:181-90, 2004

Versatile Ubiquitin – Different Functions For Different Length And Position

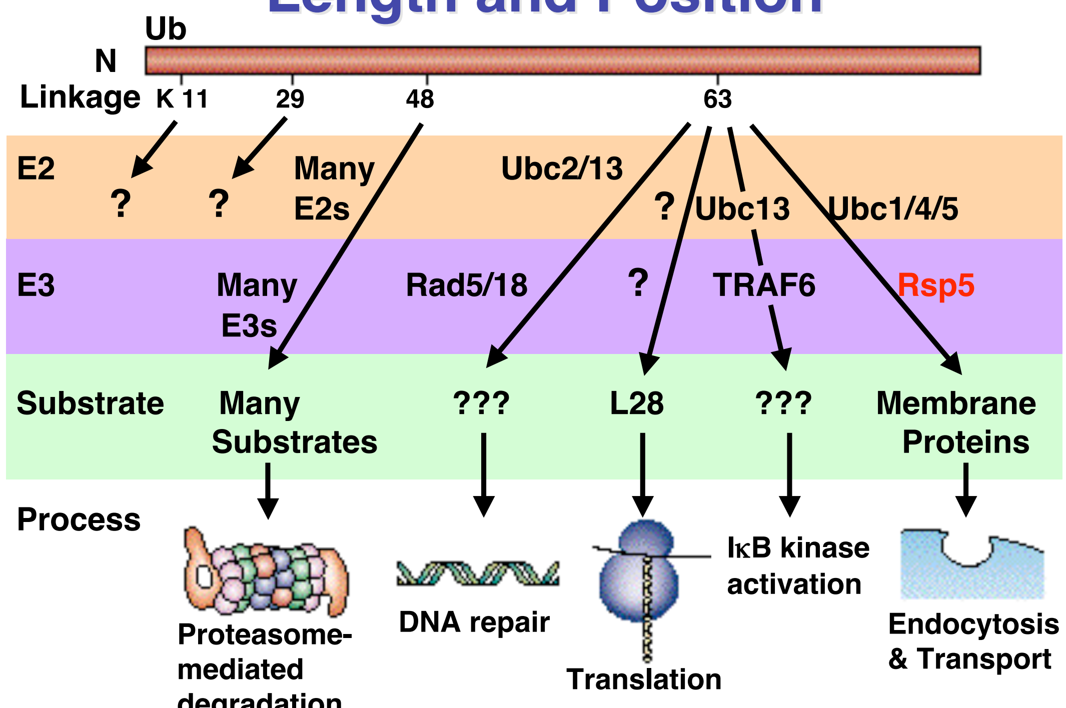


Mono-Ub:
- protein sorting
- protein-protein interaction
- virus budding

Poly-Ub:
- K48: protein degradation (26S proteasome)
- K63: IKK activation
  - protein sorting
  - DNA repair
- K29: protein degradation (26S proteasome)

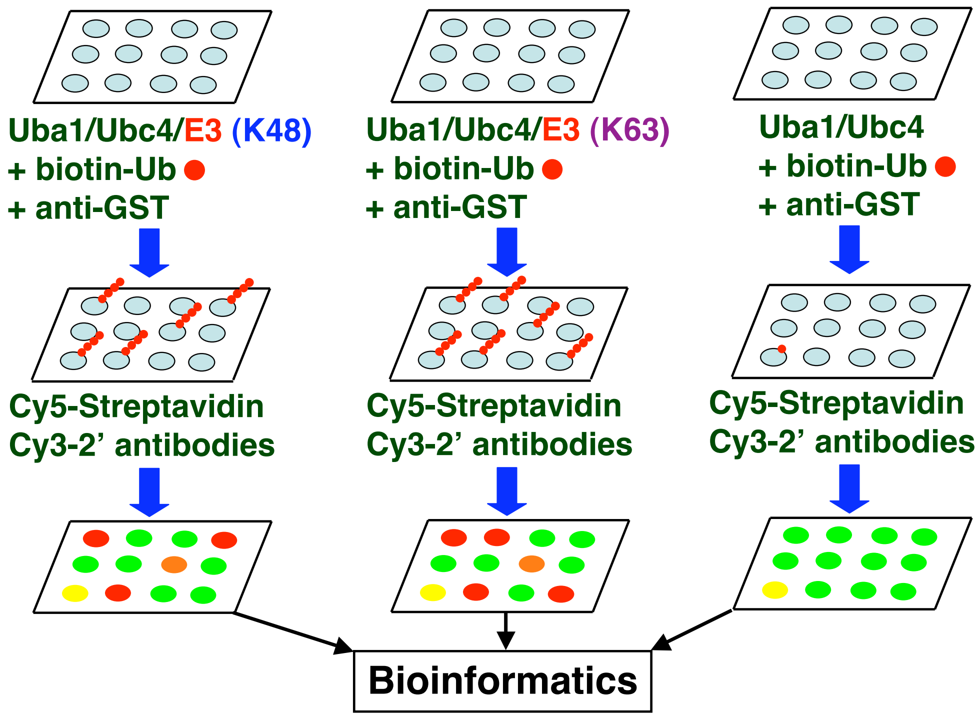
Linda Hicke, Nat. Rev. Mol. Cell Bio. 2:195-201, 2001

Different Functions for Different Length and Position

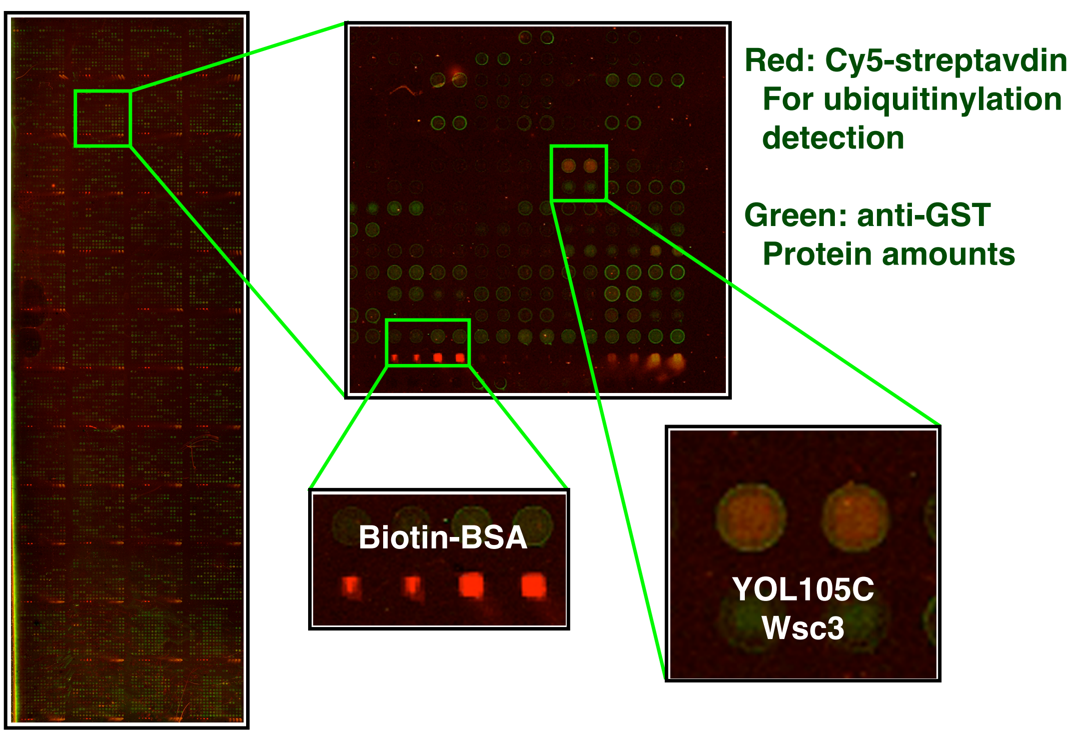


Allen Weissman, Nat. Rev. Mol. Cell Bio. 2:169-78, 2001

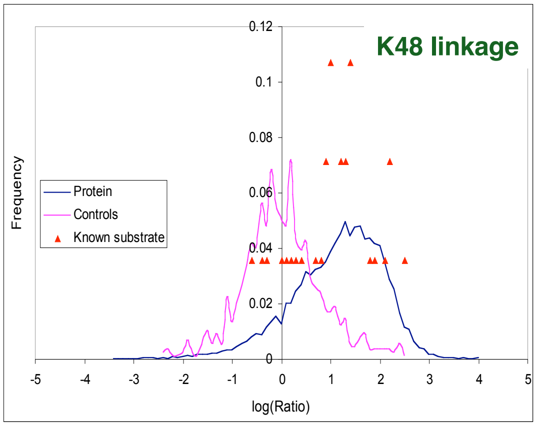
Strategy to Identify HECT substrates



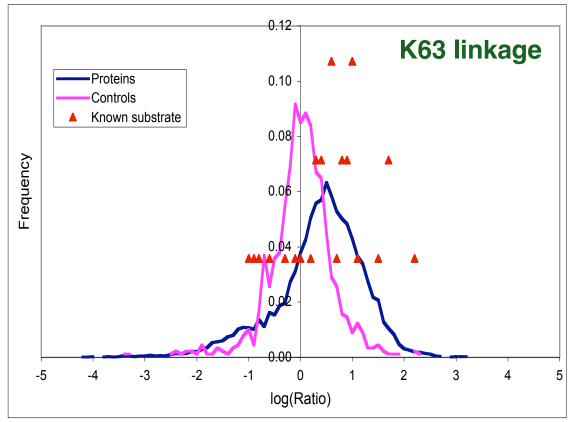
Ubiquitinylation by Rsp5 + K63 Ub



Histogram



Rsp5+Ub K48/Ub K48 control

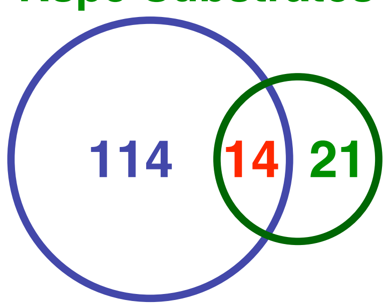


Rsp5+Ub K63/Ub K63 control

Top Substrates of Rsp5 and Hul5

Rsp5 Substrates

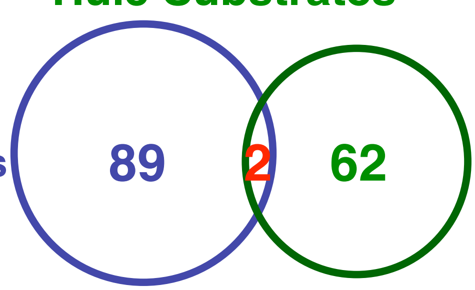
K63 linkages



K48 linkages

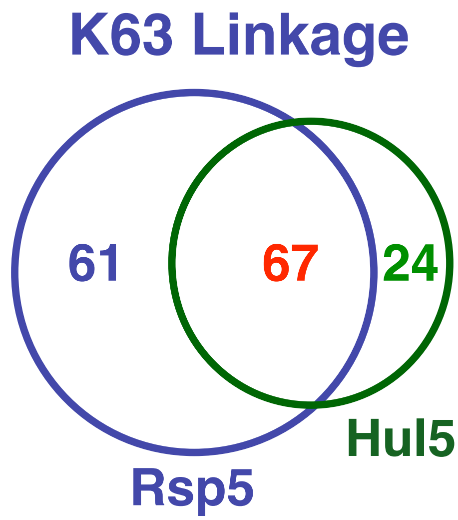
Hul5 Substrates

K63 linkages

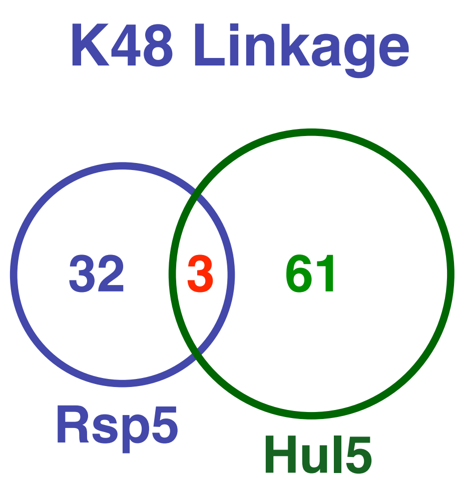


K48 linkages

Many Substrates of Rsp5 and Hul5 Are Shared in K63 Linkage



Few Substrates of Rsp5 and Hul5 Are Shared in K48 Linkage



Top Candidate Substrates – Rsp5 With Both Forms of Ubiquitin

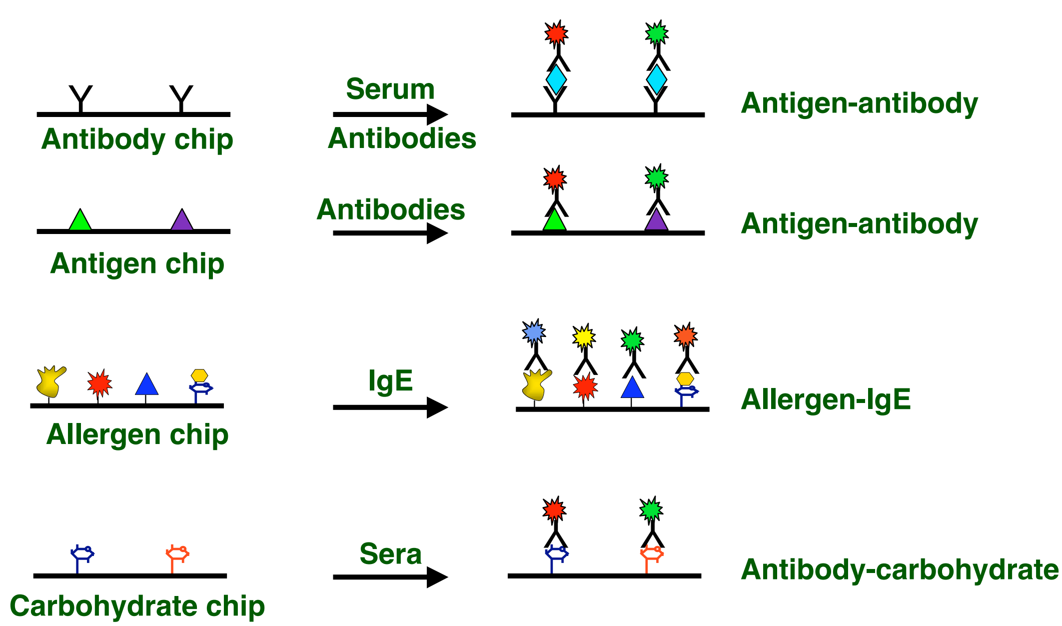
Gene	Protein	Domain	Function	Binding with Rsp5
YDL070W	Bdf2	bromo-domain	transcription factor (predicted)	
YHR097C			PIP3 binding	
YJL084C		Arrestin	unknown	
YMR275C	Bul1		Ub-dependent protein degradation	yes
YMR316W	Dia1		unknown	yes
YOR042W	Cue5	Cue	monoubiquitin binding	
YPR030W	Csr2		Galactose transport?	

Top Candidate Substrates – Rsp5 With Ub K63 Only (Specific?)

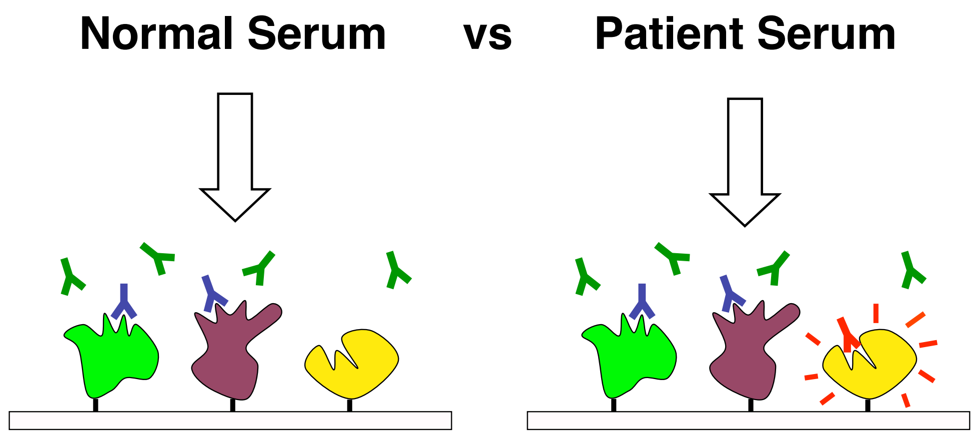
Gene	Protein	Domain	Function	Binding with Rsp5
YJL031C	Bet4		CAAX-protein GG-transferase	
YML013W	Sel1	UBX	protein sorting, Ub-dependent protein degradation	
YMR140W	Sip5		unknown	
YNL094W	App1		actin cytoskeleton assembly	no, but bind to Rvs167
YPR154W	Pin3	SH3	actin cytoskeleton assembly	

❖ Applications in Clinical Research

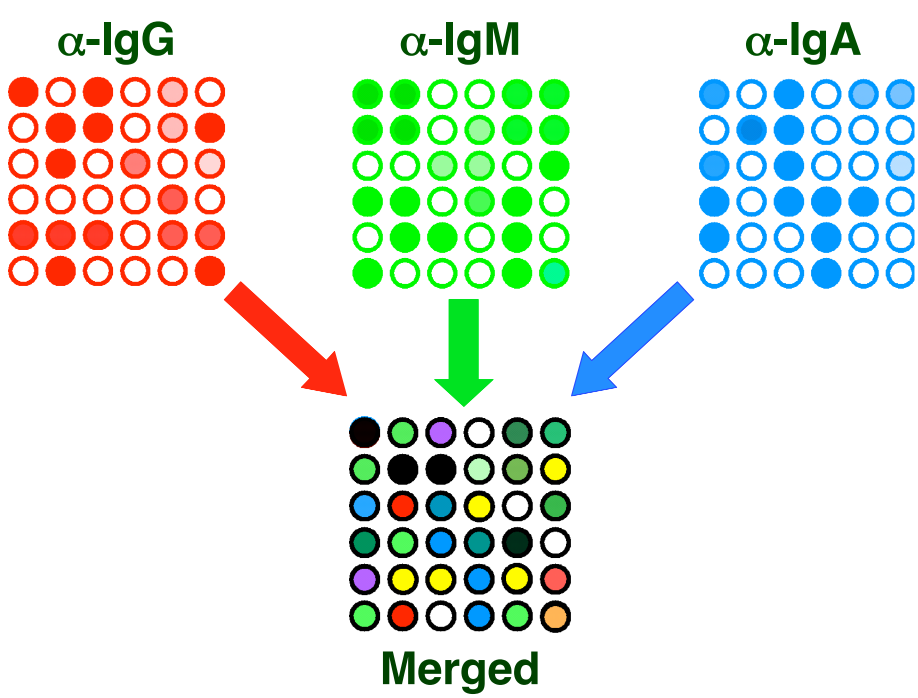
Applications in Clinical Diagnostics



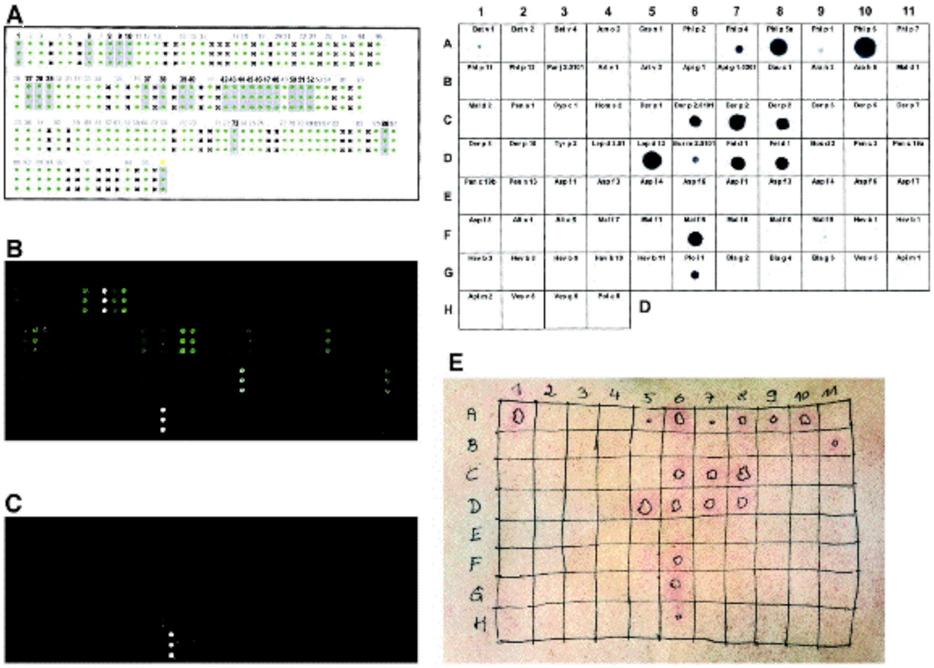
Serum Profiling



Serum Profiling

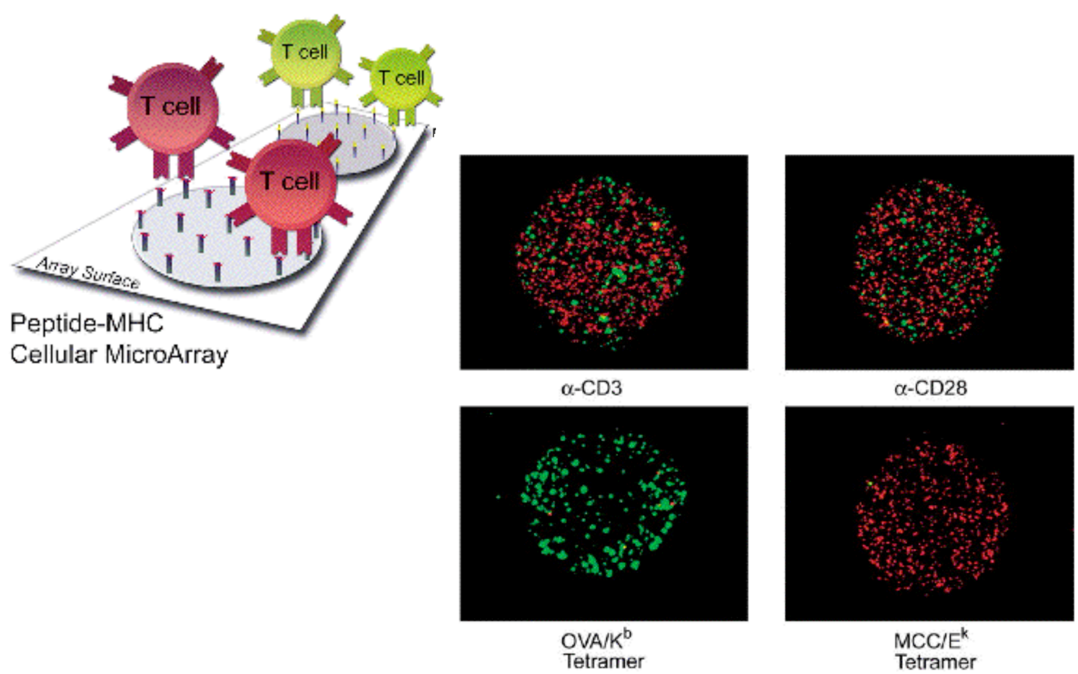


Allergen Microarray



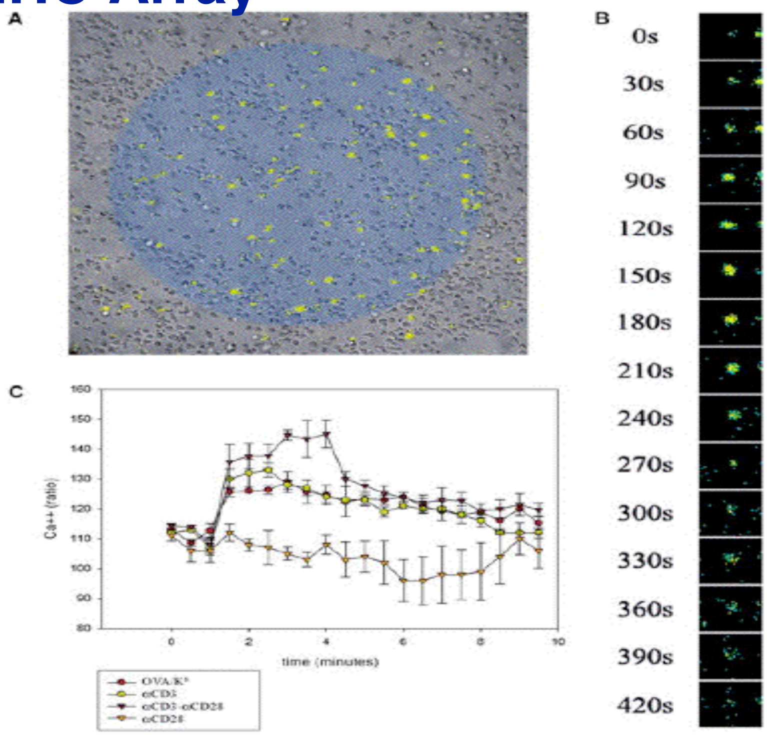
Hiller et al. FASEB J. 2002 Mar;16(3):414-6.

MHC Chips to Profile T Cells

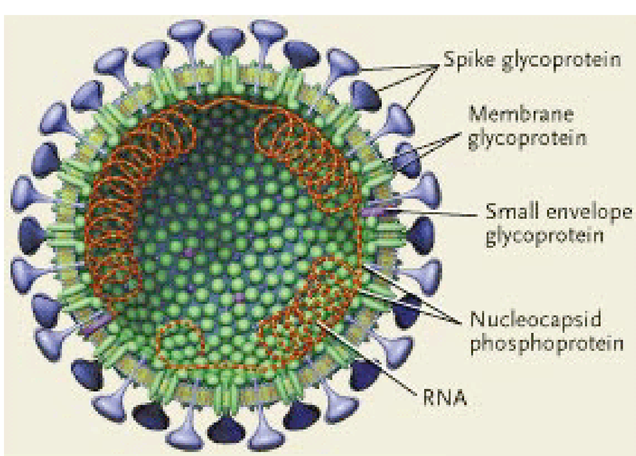


Soen et al. PLoS Biol. 2003 Dec;1(3):E65.

Activation of OT-1 Lymphocytes on an MHC Array



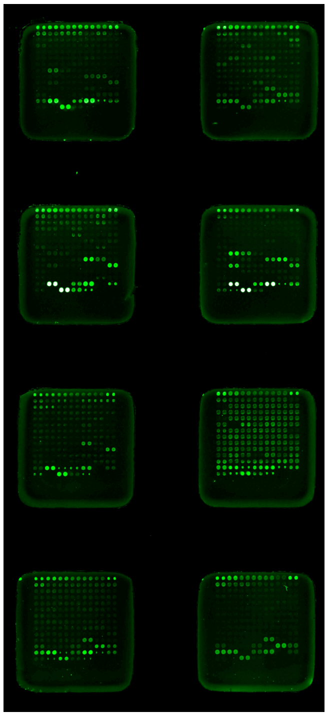
SARS Coronavirus



Virol J. 2005 Apr 15;2(1):35.

- RNA virus
- spherical in shape
- club-shaped peplomers
- HCV:
  - SARS
  - 229E
  - OC43

Experimental design



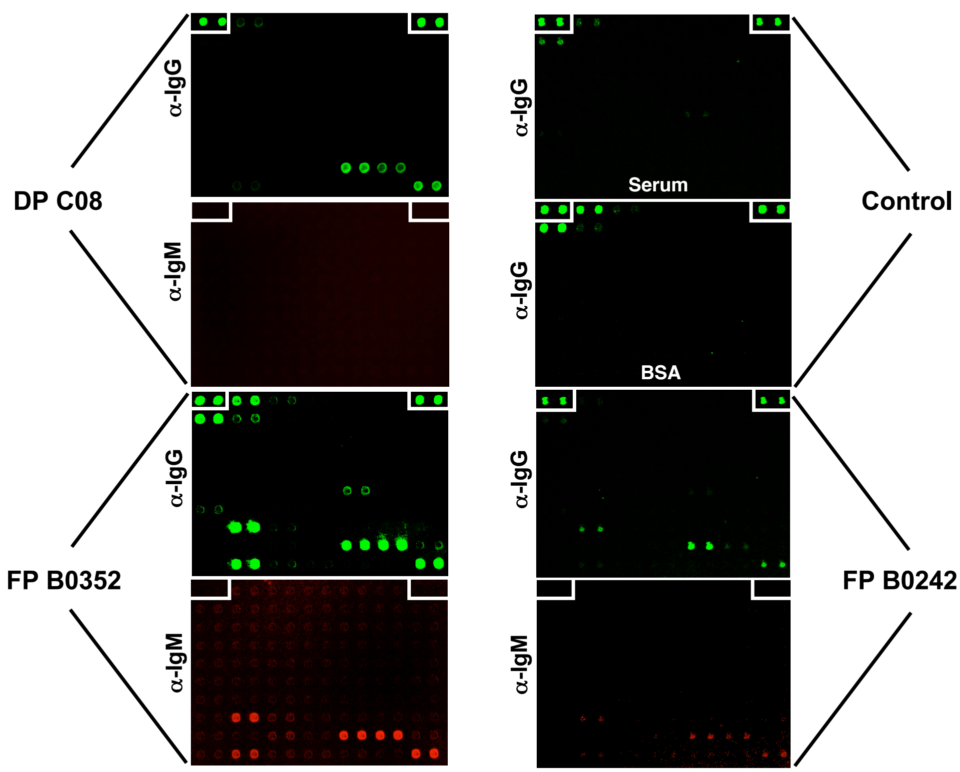
- **Cloning:**

SARS-CoV:	Human
229E :	Human
OC43:	Human
FIPV:	Cats
MHVA59:	Mouse

- **Expression:**

  Yeast and *E. coli.*

Serum Probing on Coronaviral Chips



SARS Patients Tested

Three Datasets:

- China I (**56**): Sera from recovered patients
- China II (**150**): Fever patients
- Toronto (**350**): Fever patients

Toronto Dataset:

- 521 probings
  - 493 good
    - 262 normal
    - 231 SARS
  - 28 bad – will be repeated

-65 unique features
 protein fragments from 5 viruses

Hierarchical Clustering