Quantitative proteomics for elucidating protein-protein interactions and pathways

Akhilesh Pandey, M.D., Ph.D.

McKusick-Nathans Institute of Genetic Medicine, Biological Chemistry, Pathology & Oncology

Lab URL: http://pandeylab.igm.jhmi.edu/
Outline

• SILAC for quantitative proteomics
• Characterizing protein complex association with an adapter protein, Odin, in EGFR signaling
• Signaling pathways activated by TSLPR, a receptor involved in the pathogenesis of asthma and acute lymphoblastic leukemias
SILAC for *in vivo* labeling

- Simple
- Does not require any extra processing steps
- All proteins are uniformly labeled
- Complete and predictable incorporation
- Choice of labeled amino acids
- De novo sequencing can be performed

www.silac.org
5-state SILAC experiment to study proteome dynamics

Label 1
Label 2
Label 3
Label 4
Label 5

Start (Day 0)
Day 1
Day 3
Day 5
Day 7
mix

Differentiated-adipocytes

Analyze by Mass spectrometry
A Mass Spectrum From a 5-Plex Experiment

Day 0
- 502.3112

Day 1
- 504.3033

Day 3
- 505.3150

Day 5
- 507.3127
- 507.8133

Day 6
- 507.8191

Day 7
- 510.8355
- 511.3394
Protein Dynamics of >100 Proteins Measured by SILAC

Time [Days]

Fold change

extracellular matrix protein 1
biglycan
PAS domain containing serine/threonine kinase
ectonucleotide pyrophosphatase/phosphodiesterase 2
orosomucoid 1
heat shock prot. 8
lamin A
Function of Odin in Growth Factor Signaling
Odin is a negative regulator of growth factor signaling

NIH3T3 fibroblasts

Thymidine incorporation assay

Primary mouse embryo fibroblasts treated with or without 20ng/ml PDGF-BB for 8h

Pandey et al., Oncogene, 2002
Kristiansen et al., DNA Research 2004
Odin undergoes tyrosine phosphorylation in receptor tyrosine kinase signaling
Identifying Odin Interactors Using SILAC

Transfection:
-EGFR + Empty Vector

Light ($^{12}$C$_6$-Lys/$^{12}$C$_6$-Arg)

IP with anti-FLAG antibodies

Wash and mix

EGFR + Odin

Heavy ($^{13}$C$_6$-Lys/$^{13}$C$_6$-Arg)

IP with anti-FLAG antibodies

Elute with FLAG peptide

In-gel digestion using trypsin

LC-MS/MS

Relative intensity

m/z

Quantitation

Heavy

Light
Odin is identified from Transfected Cells

A

Relative intensity

631 632 633 634 635 636

m/z

631.30 633.78 634.28 634.78 635.28 635.78

B

Relative intensity

y7 771.37

y6 694.35

y5 570.32

y4 410.27

y3 323.24

y2 210.15

b2 217.08

b3 88 266.10

b4 98 399.20

b5 98 486.22

SEpSLSNC SIGK^*

m/z

C

Relative intensity

803 804 805 806 807 808

m/z

802.89 803.40 806.40 806.90 807.40 807.90

D

Relative intensity

802.89 803.40 806.40 806.90 807.40 807.90

m/z

b2 125.10

b5 98 254.13

b7 825.46

b6 994.53

b5 112.62

SPpSFASEWDEIEK^*

m/z
Specific and non-specific Odin Interactors

A

CD2-associated protein

B

Ribosomal protein S7

C

D

SVDFDSLTVR

AIIIIFVPVQLK
## Specific Odin Interactors

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<tr>
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<th>Gene Symbol</th>
<th>Protein</th>
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<tr>
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<td>YWHAG</td>
<td>Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide (14-3-3 gamma)</td>
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<tr>
<td>2</td>
<td>YWHAZ</td>
<td>Tyrosine 3/tryptophan 5-monooxygenase activation protein, zeta polypeptide (14-3-3 theta)</td>
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<tr>
<td>3</td>
<td>YWHAB</td>
<td>Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, beta polypeptide (14-3-3 beta)</td>
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<td>4</td>
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<tr>
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<td>6</td>
<td>SFN</td>
<td>Stratifin (14-3-3 sigma)</td>
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<td>7</td>
<td>SH3KBP1</td>
<td>SH3-domain kinase binding protein 1</td>
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<tr>
<td>8</td>
<td>CD2AP</td>
<td>CD2-associated protein (CMS)</td>
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<td>9</td>
<td>RASAL2</td>
<td>RAS protein activator like 2</td>
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<tr>
<td>10</td>
<td>YWHAQ</td>
<td>Tyrosine 3/tryptophan 5-monooxygenase activation protein, theta polypeptide (14-3-3 theta)</td>
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<tr>
<td>11</td>
<td>CAPZB</td>
<td>F-actin capping protein beta subunit</td>
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<tr>
<td>12</td>
<td>DAB2IP</td>
<td>DAB2 interacting protein</td>
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<tr>
<td>13</td>
<td>TLN2</td>
<td>Talin 2</td>
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<tr>
<td>14</td>
<td>GART</td>
<td>Phosphoribosylglycinamide formyltransferase, phosphoribosylglycinamide synthetase, phosphoribosylaminomimidazole synthetase</td>
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<tr>
<td>15</td>
<td>VAPA</td>
<td>Vesicle-associated membrane protein (VAMP)-associated protein A</td>
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<td>16</td>
<td>ARHGAP10</td>
<td>Rho GTPase activating protein 10</td>
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<td>17</td>
<td>HSPA9</td>
<td>Heat shock 70 kDa protein 9 (mortalin)</td>
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<td>18</td>
<td>UACA</td>
<td>Uveal autoantigen</td>
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Validation of protein interactions

<table>
<thead>
<tr>
<th>Lysates</th>
<th>IP: anti-FLAG</th>
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<tr>
<td>FL Odin</td>
<td>FL Odin</td>
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<tr>
<td>WB: Odin</td>
<td>WB: Odin</td>
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<tr>
<td>WB: Talin2</td>
<td>WB: Talin2</td>
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<tr>
<td>WB: SH3KBP1</td>
<td>WB: SH3KBP1</td>
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<tr>
<td>WB: CD2AP</td>
<td>WB: CD2AP</td>
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<tr>
<td>WB: Mortalin</td>
<td>WB: Mortalin</td>
</tr>
<tr>
<td>WB: 14-3-3ε</td>
<td>WB: 14-3-3ε</td>
</tr>
<tr>
<td>WB: 14-3-3ζ</td>
<td>WB: 14-3-3ζ</td>
</tr>
<tr>
<td>WB: 14-3-3γ</td>
<td>WB: 14-3-3γ</td>
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</table>
Known Interaction Network between Odin and its Interactors

- Odin
- RASAL2
- 14-3-3β
- 14-3-3θ
- 14-3-3ζ
- 14-3-3η
- 14-3-3ε
- 14-3-3γ
Summary

• It is possible to derive ‘clues’ about the function of a protein from protein complex studies
• Odin is possibly involved in endocytosis pathway (guilt by association) but additional directed experiments need to be carried out
Not all protein complexes are stable...
Thymic Stromal Lymphopoietin (TSLP)

- A novel IL-7-like cytokine
- Originally identified from a murine thymic stromal cell line
- Important in the pathogenesis of asthma
- Mutations recently identified in certain types of leukemias
Biological Functions of TSLP

Epithelia

- TSLP
  - Dendritic cells
    - Survival
    - Maturation
    - TH2 cytokine / chemokine production
  - T cells
    - Treg development
    - TH2 differentiation
    - CD4+ T cell homeostasis
    - CD8+ T cell homeostasis
  - Mast cells
    - TH2 cytokine / chemokine production

Keratinocytes

B cells

Development

Allergic inflammation (i.e. asthma and atopic dermatitis)
TSLP receptor complex

• Consists of two receptor subunits
  • Interleukin 7 receptor alpha chain (shared with another cytokine, IL-7)
  • A unique receptor designated TSLPR
TSLP Signaling

- JAK family kinases shown not to be activated based on Western blotting data (lack of increased tyrosine phosphorylation in phosphotyrosine Western blots)
- Scientists in the field trying to identify the ‘elusive’ kinase(s)
TSLP signaling – a Western blot perspective

Cell Lysates

<table>
<thead>
<tr>
<th>TSLP</th>
<th>—</th>
<th>+</th>
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<tbody>
<tr>
<td>kD</td>
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<tr>
<td>17</td>
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</table>

WB: anti-pTyr

IP: anti-Stat5

Reprobe: anti-Stat5a
How do we start to map the detailed circuitry in signaling pathways?

- Experimentally identify components of signaling pathways
- Determine the ordering of the components by biochemical experiments
- Determine the interactions among the components
- Mapping of enzymes and their substrates in signaling is a key step
Profiling of activated kinases: Identifying direct kinase substrates is difficult

- Kinase-substrate interactions are transient
- Stoichiometry of phosphorylation is low
- Analytical methods to identify phosphorylation sites are difficult and not comprehensive
- Establishing a protein as a direct substrate of a kinase is not trivial
TSLP requires IL-7R and TSLPR to transmit signals
TSLP requires at least one cytoplasmic tyrosine residue to transmit proliferative signals.
SILAC-based pTyr and pSer/pThr proteome profiling
TSLP induces tyrosine phosphorylation of signaling molecules

<table>
<thead>
<tr>
<th>Protein</th>
<th>Phosphopeptides with sites</th>
<th>Fold upregulation</th>
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<tbody>
<tr>
<td>STAT5A</td>
<td>AKAVDGPYVKPQIK</td>
<td>3.2</td>
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<tr>
<td>STAT5B</td>
<td>AKAADGPYVKPQIK</td>
<td>10.0</td>
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<tr>
<td>TEC</td>
<td>YVLDDQPYTSSSGA</td>
<td>1.4</td>
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<tr>
<td>BTK</td>
<td>HYVCSTPQSQPYYLAEK</td>
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<tr>
<td>SHIP1</td>
<td>LPYDFVKTERDESSGMK</td>
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<td>JAK2</td>
<td>EVGDpYGQLHKTEVLLK</td>
<td>1.4</td>
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<tr>
<td></td>
<td>REVGDPYGQLHK</td>
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<tr>
<td>FcRγ</td>
<td>SQETpYETLK</td>
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<tr>
<td>FYB</td>
<td>TTAVEIDpYDSLK</td>
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<td>SHP2</td>
<td>IQNTGDPYYDLYGGEK</td>
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<tr>
<td>LYN</td>
<td>EKAERPTFDYLQSVLDDFYTATEGqPYYQQpQP</td>
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<td>SLDNGGpYYISPR</td>
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<tr>
<td>ERK1</td>
<td>IADPEHDHTGFLTEpYYVATR</td>
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<tr>
<td>CDK3</td>
<td>VEKIGEGTPYGVVpYK</td>
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<tr>
<td>GSK3A</td>
<td>GEPNVSPYICSR</td>
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Phosphorylation changes in Lyn reflect activation

SLDNGGpYYISPR
(Y193)

EKAERPTFDYLQSVLDFFYTATEGQpYQQQP
(Y508)
Src family kinase inhibitor, SU6656, inhibits TSLP-induced cell proliferation
Bruton’s tyrosine kinase

HYVVCSTPQSQQpYYLAEK

(Y344)

Relative Abundance

m/z

1077 1078 1079 1080 1081 1082

1076.97 1077.48 1077.98 1079.98 1080.49 1080.99

γ

PH BTK SH3 SH2 TyrK
BTK kinase inhibitor, LFM-A13, inhibits TSLP-mediated proliferation
JAK2 inhibitor inhibits TSLP-mediated proliferation
JAK2 inhibitor inhibits TSLP-induced Stat5 phosphorylation
A partial list of serine/threonine phosphorylated peptides identified from SCX fractionation experiments

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Phosphopeptides</th>
<th>Fold upregulation with TSLP treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pumilio homolog 2</td>
<td>pTPGSROApSPTEVVER</td>
<td>4.3</td>
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<tr>
<td>Programmed cell death protein 5</td>
<td>KVMDpSDEDDADY</td>
<td>10.0</td>
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<tr>
<td>Protein transport protein Sec61 subunit beta</td>
<td>PGPTPSGTNVPSSGRpSPSK</td>
<td>6.6</td>
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<tr>
<td>60S ribosomal protein L8</td>
<td>GAGpSVFR</td>
<td>4.5</td>
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<tr>
<td>Plectin1</td>
<td>SSpSVGSSSSYPISSAGPR</td>
<td>5.5</td>
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<tr>
<td>Heterogeneous nuclear ribonucleoprotein A3</td>
<td>SSGSPYGGGYGpSGGGSGGYGSR</td>
<td>5.2</td>
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<tr>
<td>Programmed cell death protein 4</td>
<td>FVpSEGDDGGR</td>
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<tr>
<td>Rho guanine nucleotide exchange factor 1</td>
<td>pSESLRVpSDR</td>
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<tr>
<td>FcRγ</td>
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<tr>
<td>Nucleolar RNA helicase 2</td>
<td>SNSSDAPGEEpSSpSETEKEIPVEQK</td>
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<tr>
<td>Eukaryotic translation initiation factor 4E transporter</td>
<td>SSpSPVGLAK</td>
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TSLP Signaling: BEFORE

TSLP

IL-7Rα

TSLPR

IL-7Rα

TSLPR

STAT5A

STAT5B

ERK1

AKT1

Transcription
TSLP Signaling: AFTER
Lessons Learnt

• Exercise caution in interpreting negative data – ‘not detectable’ is not the same as ‘not occurring in cells’

• It is possible to take unbiased discovery approaches to dissect signaling pathways in a global fashion using quantitative proteomics

• Activated kinases identified using this proteomic approach are candidate therapeutic targets in asthma and leukemias
News

PhosphoMotif Finder, published in Nature Biotechnology
Comparison of Protein-Protein Interaction Databases, published in BMC Bioinformatics

Highlights

PhosphoMotif Finder
Allows you to check if your protein contains any phosphorylation motif described in the literature

Pathways
A set of 20 curated signaling pathways are available as part of a new pathway resource that we have developed called 'NetPath.'

HPRD Release 7 New
The latest Release 7 is available for download. Click here...

Statistics

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Vimentin – tissue expression from Human Proteinpedia
Acknowledgments

- Jun Zhong
- Raghothama Chaerkady, Kumaran Kandasamy
- Harsha Gowda, Xinyan Wu, Yi Yang, Min-Sik Kim
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