

BMG 744 Proteomics-Mass Spectrometry

Qualitative burrowing of the proteome

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1/25/13

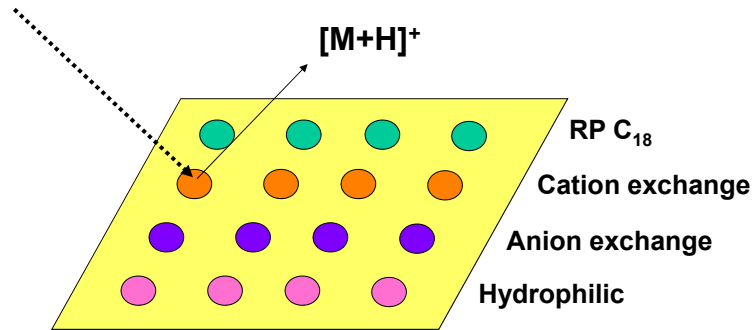
Topics

Qualitative changes in the predicted proteome

- Digging deeper - SELDI/MALDI
- Posttranslational modifications
 - Isolation and characterization
 - Making use of the chemistry of the modification
 - Ubiquitination/SUMOylation
 - Global discovery

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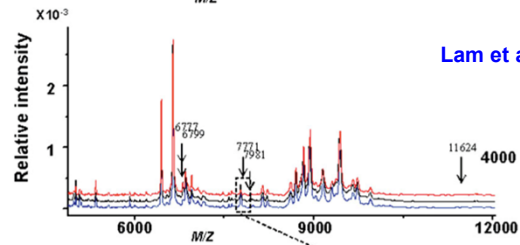
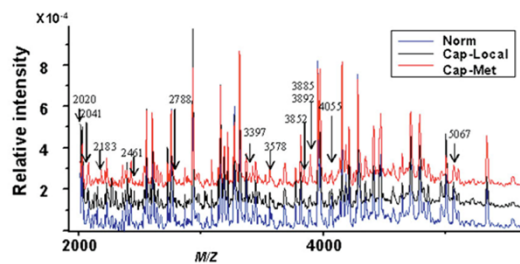
Surface enhanced laser desorption ionization (SELDI)



SELDI chip (plate) with local chemistry on surface causing selective binding of peptides

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MALDI-TOF analysis of peptides recovered by C₁₈ extraction of sera from prostate patients, with and without metastases, and controls

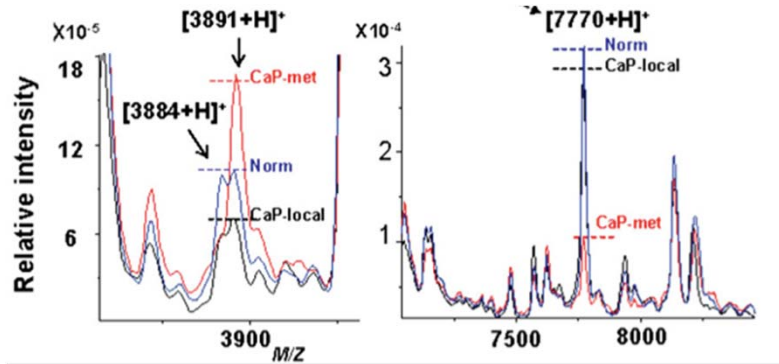


Lam et al., Proteomics 5, 2927

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Identification of a significantly altered peptide in men with metastatic prostate cancer

Lam et al., Proteomics 5, 2927

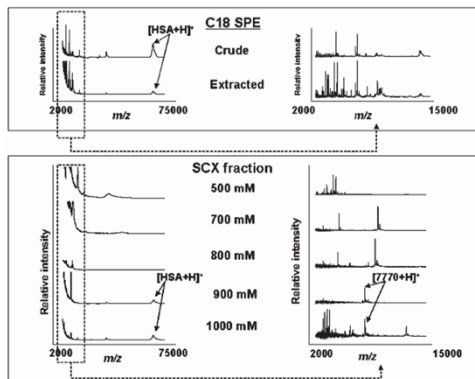


But what is it?

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Purification of prostate peptide by SPE extraction and strong cation exchange

Lam et al., Proteomics 5, 2927



Serum was first extracted with a C₁₈ SPE cartridge

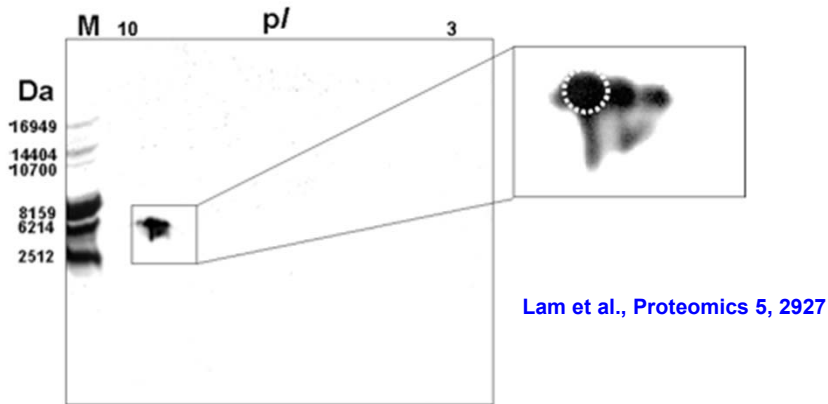
SPE fraction was passed over a [H⁺]-form cation exchange resin. Bound peptides were eluted with increasing step gradients of NaCl

Analyses of fractions were performed by MALDI-TOF MS

The most enriched fraction was contaminated with albumin

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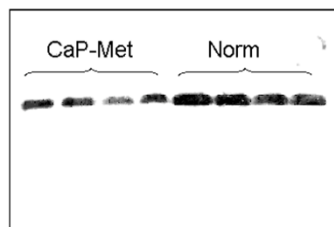
Purification of prostate peptide needed 2D-electrophoresis



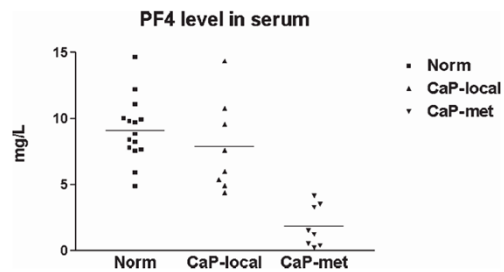
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Non-mass spec verification of prostate peptide

Lam et al., Proteomics 5, 2927



Western blot analysis for PF4



ELISA analysis for PF4

**Moral: proteomics is a serious business that requires
multiple dimensions of separation - glib methods don't work**

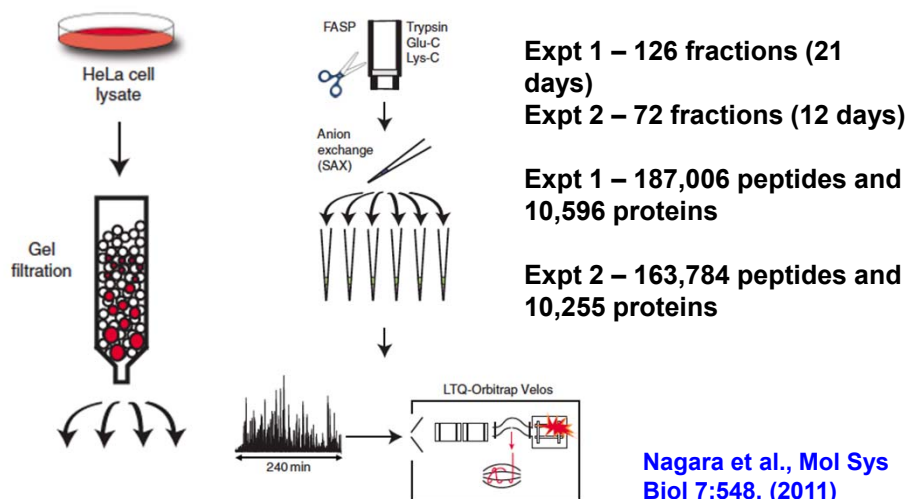
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Challenges in proteomics

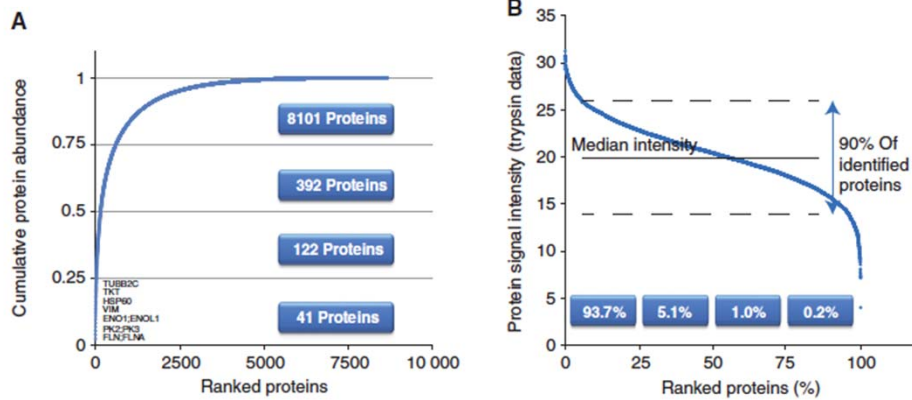
- RNA can be amplified by PCR and converted to DNA (cDNA) - then it is used to hybridize to DNA sequences on a microarray
 - Proteomics doesn't have an amplification system
- RNA-Seq sequences rather than hybridizes and dispenses with predetermined microarrays
 - Discovers new messages - signals not predicted by conventional gene annotations

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How deep can we go?



Description of protein abundances

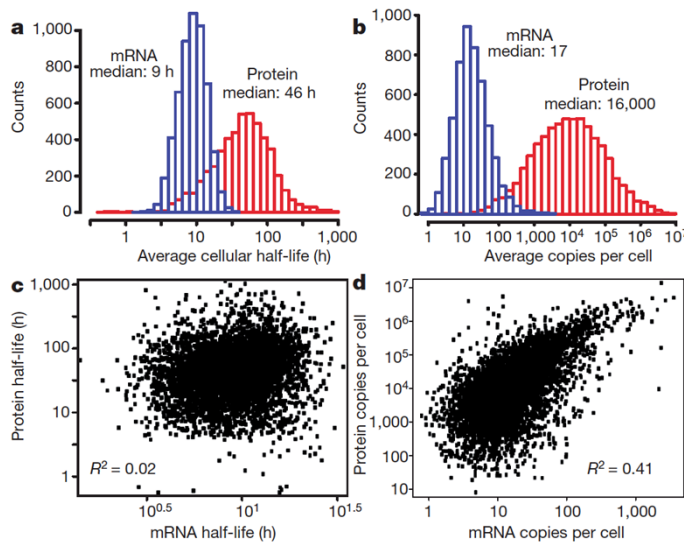


Just 41 proteins accounted for 25% of the combined tryptic peptide ion intensities – 122 accounted for 50% and 392 for 75%.

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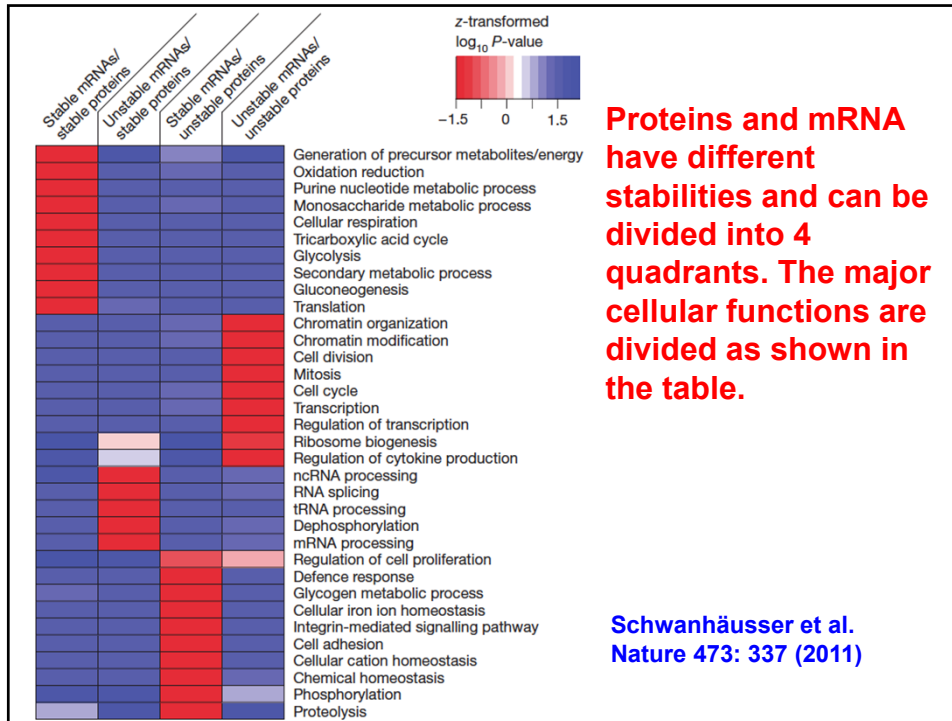
Nagara et al., Mol Sys Biol 7:548, (2011)

Protein – mRNA correlations



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Schwanhäusser et al. Nature 473: 337 (2011)



Moral about protein discovery in 2013

- It is now possible to detect more proteins than there are separate forms of message
- However, such discovery is still “heroic”
- Proteomics does not have the equivalent of the PCR reaction
 - However, a cell does amplification of message
- Detecting the minor components requires being able to start with enough sample
 - 10 attomole is 6.023 million molecules
 - If present as 10 copies per cell, then minimum # of cells needed is 0.6×10^5

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How to dig deeper

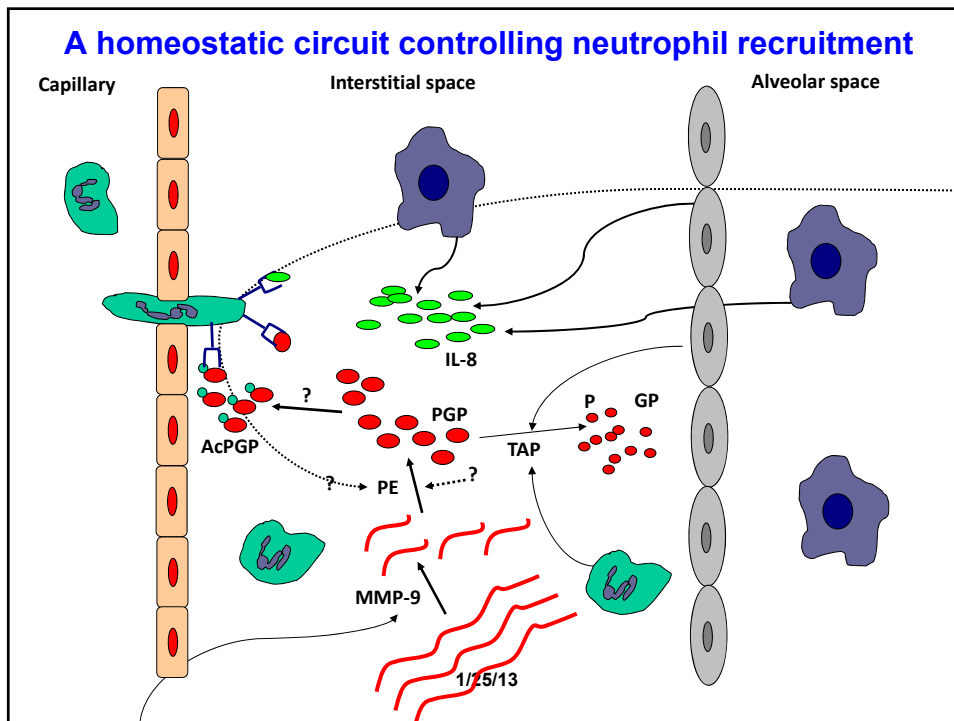
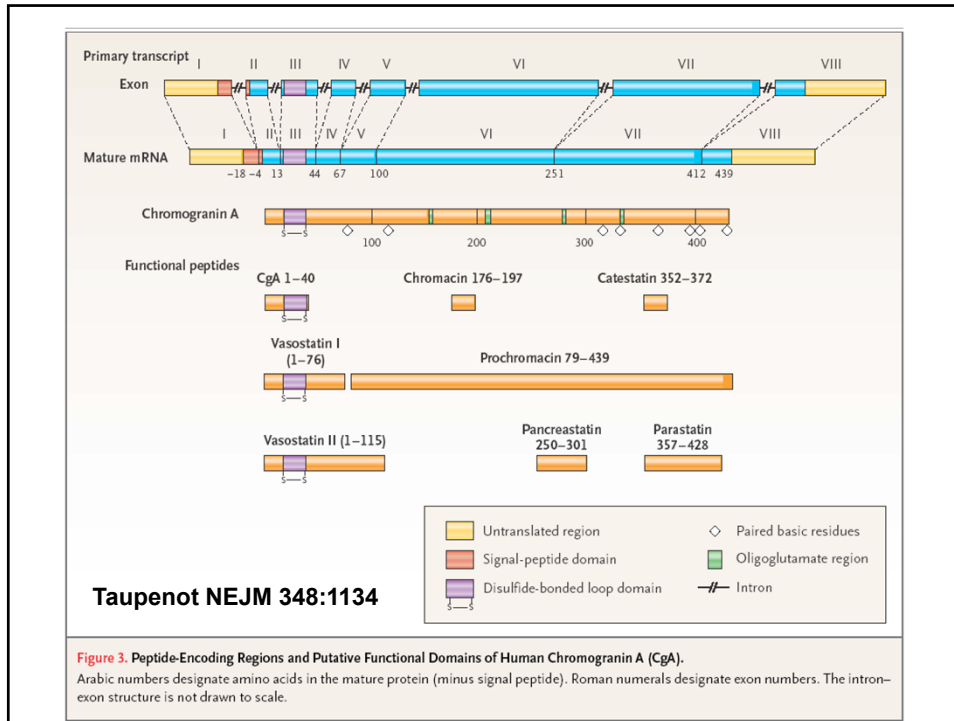
- **Sub-fractionate the sample being analyzed to enrich for the lower abundance components**
 - Choose the right biofluid/tissue
 - Organelle isolation
 - Affinity chromatography
 - Antibody pulldowns
 - Abundant protein(s) depletion

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General classes of modification

- **Biochemical events involving peptide processing**
- **Biochemical events stimulated by enzymes**
- **Chemical events driven by reactive species**
- **Chemical events determined by investigator**

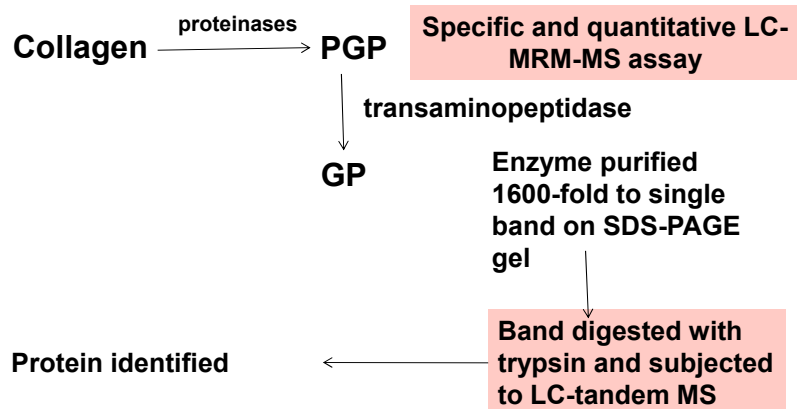
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PGP is a common peptide in human collagen

MFSFVDLRLLLLLAATALLTHGQEEGQVEGQDEDEDIPPITCVQNGRLRYHDRDVKPEPCRI
 CVCDNGKVLCDVVICDETKNCPGAEVPEGECCPVCPDGSESPDQETTGVVEGPKGDTGPR
 GPRGPAGPPGRDGI PQPGLPGPPGPPGPPGPPGLGGNFAPQLSYGYDEKSTGGISVPGP
 MGPSGPRGLPGPPGAPGPPQGFQPPGEPGEPGASGPMGPRGP PGPPGKNGDDGEAGKPGR
 PGERGP PGPPQGARGLPGTAGLPGMKGHRGFSGLDGAKGDAGPAGPKGEPGSPGENGAPGQ
 MGPRGLPGERGRPGAPGPPAGARGNDGATGAAGP PGPTGPAGPPGFPFPAVGAKEAGPQGP
 RGSEGPQGV RGE PGPPGPPAGAAGPAGNPGADGQPGAKGANGAPGIAGAPGFPGARGPSG
 QGPPGPPGPPKGNSEFPAGPSKGD TGAKGEPGPPVGVQGP PGPPAGEEGKR GARGE PGPTGL
 PGPPGERGGP GSRGFPGADGVAGPKGPAGERGS PGPPAGPKGSPGEAGRPGEAGLP GAKGL
 TGSPGS PGPPDGKTGP PGPPAGQDGR PGPPGPPGARGQAGVMGF PGPPKGAAGEPGKAGERV
 PGPPGAVGPAGKDEAGA QGP PGPPAGPAGERGEQGPAGSPGFQGL PGPPAGPPGEAGKPGE
 QGV PDLGA PGPPSGARGERGFPPGERGVQGP PGPPAGFRGANGAPGNDGAKGDAGAPGAPGS
 QGAPLQGMPPGERGAAGL PGPPKDRGDAGPKGADGSPGKDGVRGLTGPIGPPGAPAGPD
 KGESGSPGPAGPTGARGAPDRGE PGPPGPPAGFAGPPGADGQPGAKGEPGDAGAKGDAGP
 PGPPAGPAGP PGPPIGNV GAPGAKGARG SAGPPGATGFPGAAGR VGP PGPPSGNAGP PGPPG
 AGKEGKGRGETGPAGRPGEVGP PGPPGPPAGEKGS PGADGPAGAPGT PGPPQGIAGQRGV
 VGLPGQRGERGF PGL PGPPSGEPGKQGPSGASGERGF PGPPMGPPGLAGPPGESGREGAPGA
 EGSPGRDGS PGAKDRGETGPAGPPGAPGAPGA PGPPVGPAGKSGDRGETGPAGPAGPVGP
 VGARGPAGPQGP RDKGETGEQDRGIKGRGFSGLQGP PGPPGSPGEEQGPSGASGPAGP
 RGP G SAGAPGKDGLNGL PGPPIGP PGPPRGRITGDAGPVGP PGPPGPPGPPGPPPSAGFDFSF
 LPQPPQEKAHDGGRYRADDANVVRDRDLEVDTTLKSLSQQIENIRSPGSRKNPARTCR
 DLKMCHSDWKS GEYWIDPNQGCNLDAIKVFCNMETGETCVYPTQPSVAQKNWYISKNPKD
 KRHVWFGESMTDGFQFEYGGQSDPADVAIQLTFLRLMSTEASQNTITYHCKNSVAYMDQQ
 TGNLKKALLLQGSNEIEIRAEGNSRFTYSVTVDGCTSHTGAWGKTVIEYKTTKTSRLPII
 DVAPLDV GADPQEF GFDVGPVCFL

Mass spec and PGP



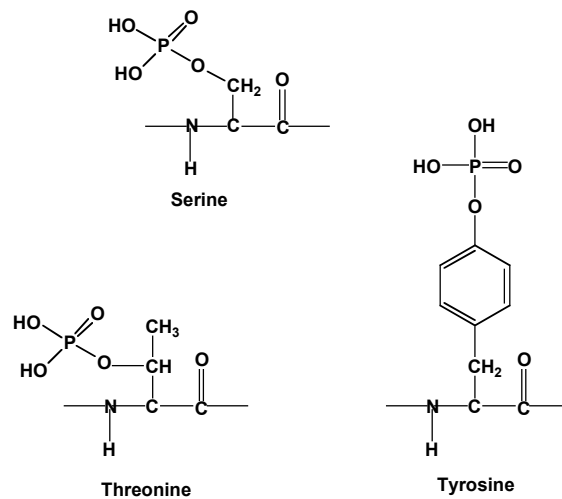
(Robert Snelgrove et al., Science 2010)

Enzymatic modifications

- **Phosphorylation/dephosphorylation**
 - On serine, threonine, tyrosine (also histidine)
- **Glycosylation**
 - N-glycosylation (asparagine-linked)
 - O-glycosylation (serine-, threonine-linked)
- **N-Acetylation/deacetylation**
 - On lysines – also higher acyl groups
- **N-Methylation/demethylation**
 - On lysines - mono-, di- and trimethylation

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Chemistry of phosphorylation



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Mass spectrometry of phosphorylated proteins

- Adds H_3PO_4 (+98)
- Eliminates water (-18)
- Net change +80
- if the phosphate ion is released (i.e., in negative ion spectra), it is seen as m/z 79 and/or m/z 63
- Phosphate can be confused with sulfate and bromide (sulfate, 79.9568 Da; phosphate, 79.9663 Da; and ^{79}Br , 79.9083 Da)

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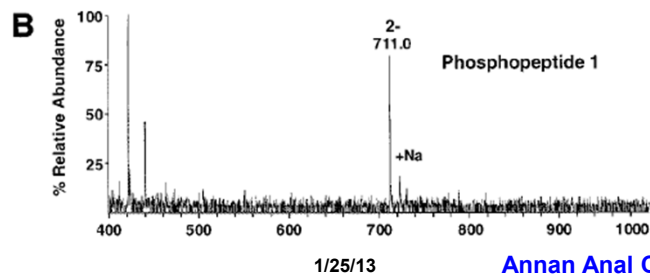
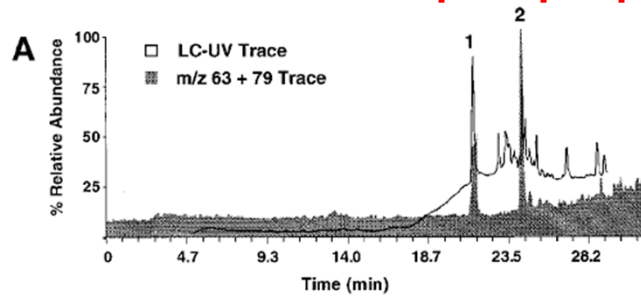
Finding a phosphate group

Several methods are in current use for detection of phosphopeptides

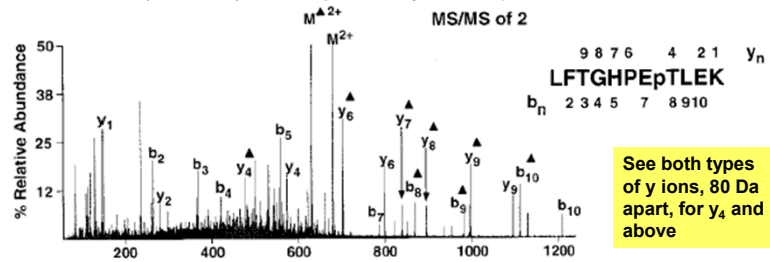
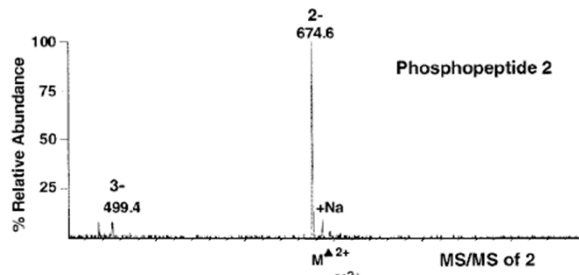
- use of parent ion or neutral loss scanning
- phosphatase sensitivity
- affinity methods for enrichment of phosphopeptides
 - anti-phospho-Ser/Thr/Tyr antibodies
 - metal ion affinity
 - chemical reaction/biotin affinity

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Parent ion scan to detect phosphopeptide



Phosphopeptide 2



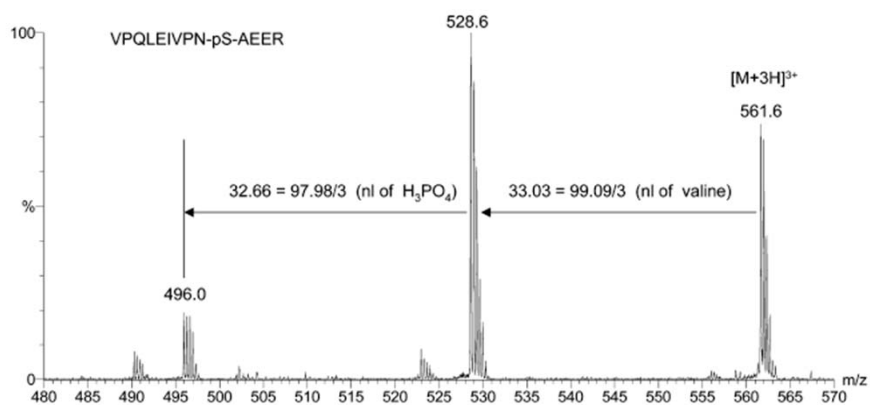
Annan Anal Chem 73:393

Neutral loss for phosphopeptides

- Based on the loss of phosphoric acid (H_3PO_4) - 97.98 Da
- If the peptide is doubly charged, then it's m/z 48.88
- If it's triply charged, then it's m/z 32.66
- Problems can occur if the peptide contains an N-terminal proline (97 Da) or valine (99 Da) and a low resolution instrument is used for the analysis

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Casein peptide - mono- or diphosphate?



Lehmann et al., J Prot Res 7-2866

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Problems of low mass resolution for parent ion scanning for phosphate

Lehmann et al., J Prot Res 7-2866

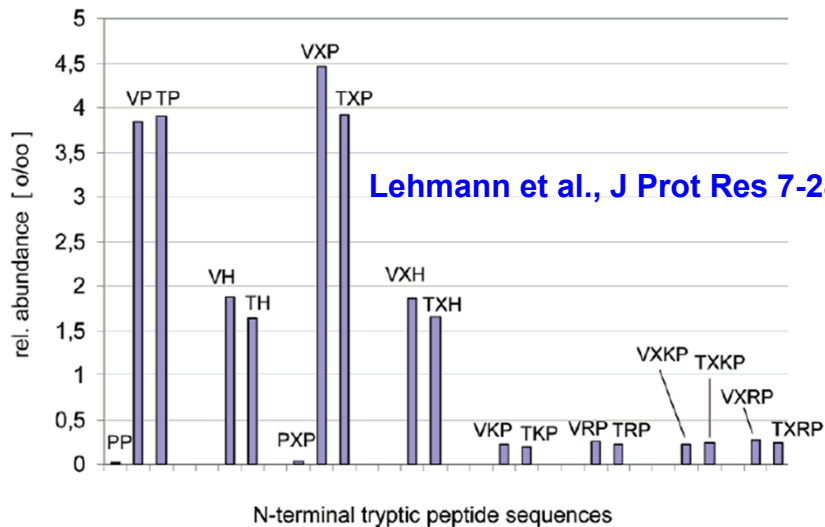
Table 1. Peptide Modifications Showing an Abundant 'Close-to-98/z' Neutral Loss Compared to the Specific Loss of H₃PO₄ from pSer/pThr Phosphopeptides^a

structure	neutral loss	1+	2+	3+	4+
phosphoS/phosphoT	H ₃ PO ₄	97.9769	48.9884	32.6590	24.4942
P -P... (N-term)	Pro	97.0528	48.5264	32.3509	24.2632
C-sulfo	SO ₃ + H ₂ O	97.9674	48.9837	32.6558	24.4918
V -P... (N-term)	Val	99.0684	49.5342	33.0228	24.7671
T -P... (N-term)	Thr	101.0477	50.5238	33.6826	25.2619
...-dhBA (C-term)	dhBA	101.0477	50.5238	33.6862	25.2619
C -P... (N-term)	Cys	103.0092	51.5046	34.3364	25.7523
...-S (C-term)	Ser + H ₂ O	105.0426	52.5213	35.0142	26.2606
M-acetamido	MTA	105.0248	52.5124	35.0083	26.2562

^a Neutral loss *m/z* values for the charge states +1 to +4 are listed (dhBA, dehydrobutyric acid; MTA, 2-(methylthio)acetamide; all amino acid symbols refer to the amino acid mass minus water).

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Residues in the proteome with phosphate-like neutral loss



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Recovery and enhancement of phosphopeptides

The biggest problem in the detection of phosphopeptides is how to convert the initial sample matrix into a form suitable for mass spectrometry analysis.

- how to handle minute samples with minimal losses
- how to recover and detect all the phosphopeptides
- how to recover and detect the non-phosphorylated proteins to determine the extent of phosphorylation at individual sites

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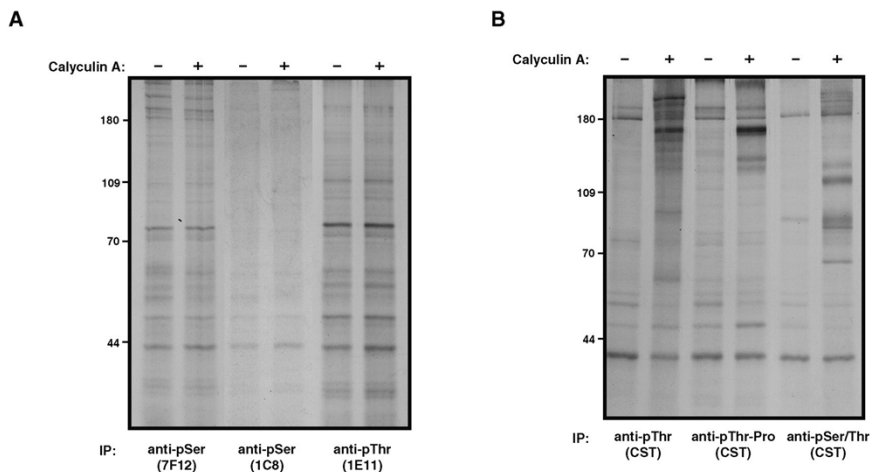
Antibodies and phosphopeptides

- In this approach, both the phosphorylated and non-phosphorylated forms of a protein may be recovered from the sample matrix
- This can most easily be achieved by immunoprecipitation of the protein with an antibody that recognizes epitope(s) that is(are) in common with both forms

NOTE *that as with all immunoprecipitation methods, the best results will be achieved if the antibody is coupled to agarose beads. This allows selective immuno-absorption of the antigen, washing free of contaminating proteins, AND following elution, minimization of the amount of antibody that is in the eluate and therefore would be analyzed by mass spectrometry*

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Variability of anti-phosphoserine and anti-phosphothreonine antibodies



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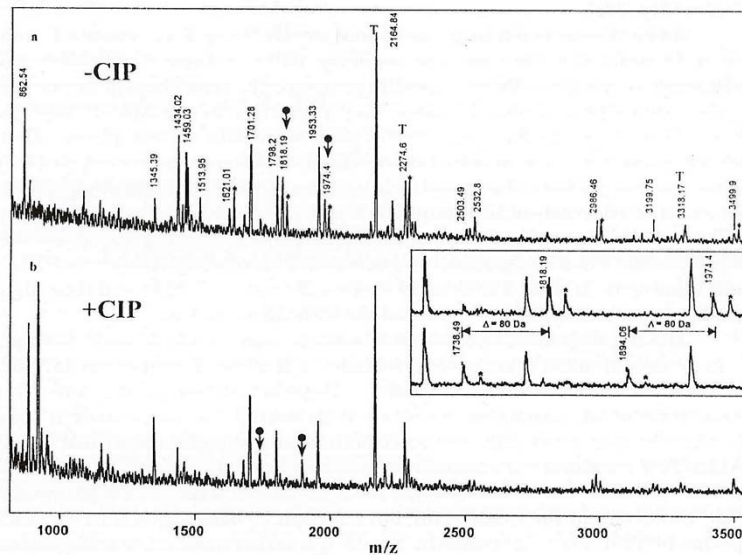
Gronborg et al., 2002

Detection of phosphopeptides based on their sensitivity to phosphatase

- An alternative source of potentially phosphorylated proteins are individual spots on 2D-IEF/SDS gels. The protein preparation so isolated is either hydrolyzed by trypsin in solution (or in the gel piece) or using solid-phase trypsin
- One portion of the resulting tryptic peptides (in 50% acetonitrile:water) is analyzed by MALDI-TOF-MS. A second portion is diluted into 50 mM NH_4HCO_3 buffer and reacted with 0.5 U calf intestinal alkaline phosphatase at 37°C for 30 min. Sample is dried with a SpeedVac, redissolved in 50% acetonitrile:water, and re-analyzed by MALDI-TOF-MS

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Detecting a phosphopeptide with alkaline phosphatase



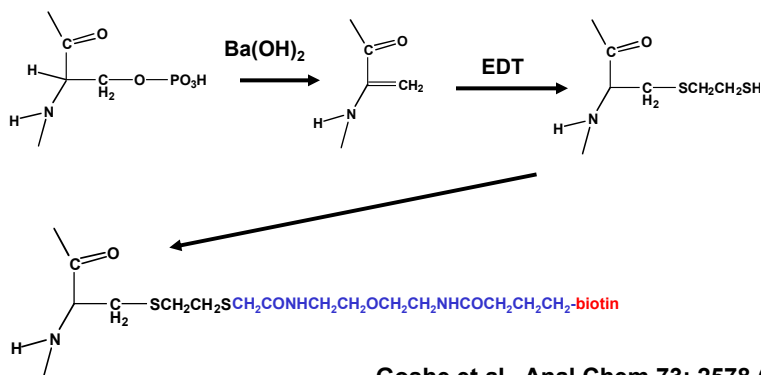
Zhang et al., 2000
MS in Biology & Medicine

Selective enhancement of phosphopeptides in tryptic digests

- Immobilized metal affinity chromatography (IMAC). Similar to Ni-affinity resins used in the purification of 6xHis-tagged proteins. The affinity phase can be charged with different metal ions (as their chlorides)
- Fe(III) and Ga(III), and to a lesser extent Zr(IV), were the most effective for the recovery of two synthetic phosphopeptides
- A tryptic digest containing both phosphorylated and non-phosphorylated peptides is passed over the IMAC column at acid pH (pH 2.5-3). The column is washed with 0.1 M acetic acid to remove unbound peptides. Elute with sodium phosphate (have to desalt) or with NH_4OH
- Esterification may prevent Asp- or Glu-containing peptides from binding
- TiO_2 is now being used with success

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Selective biotinylation of phospho-groups



Goshe et al., Anal Chem 73: 2578 (2001)
Adamcyk et al., Rapid Commun Mass Spec 15: 1481 (2001)

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How to identify phosphorylated peaks by searching databases

- The databases you have used earlier in this class have some ability to predict the expected masses for a limited number of posttranslational modifications
 - MASCOT (<http://www.matrixscience.com>)
 - PROTEIN PROSPECTOR (<http://prospector.ucsf.edu/>)
 - Phosphosite is more focused <http://www.phosphosite.org/staticAboutPhosphosite.do>

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How to identify posttranslational modifications

FindMod at

<http://www.expasy.org/tools/findmod/>

It examines mass fingerprinting data for mass differences between empirical and theoretical peptides. If the mass difference corresponds to a known modification, it also makes intelligent guesses as to the site of modification.

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Unimod

- As Matt Renfrow described on January 18th, Unimod
– (http://www.unimod.org/modifications_list.php)
provides a more detailed and more accurate list of possible modifications

Unimod Logged as Guest Log out Change password Advanced search

Search for: Details found: 956 Page 1 of 48 Records Per Page: 20

	Accession #	PSI-MS Name	Interim name	Description	Monoisotopic mass	Average mass	Composition
View	1287		Arg-loss	Loss of arginine due to transpeptidation	-156.101111	-156.1857	H(-12) C(-6) N(-4) O(-1)
View	765		Met-loss	Removal of initiator methionine from protein N-terminus	-131.040485	-131.1961	H(-9) C(-5) N(-1) O(-1) S(-1)
View	676		Trp->Gly	Trp->Gly substitution	-129.057849	-129.1586	H(-7) C(-9) N(-1)
View	313	Lys-loss	-K	Loss of C-terminal K from Heavy Chain of MAb	-128.094963	-128.1723	H(-12) C(-6) N(-2) O(-1)

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FindMod

<http://web.expasy.org/findmod/>

Some of the modifications considered are:

acetylation	amidation	biotinylation
carboxymethyl cysteine	carboxyamidomethyl cysteine	
C-mannosylation	deamidation	flavinilylation
farnesylation	formylation	geranyl-geranylation
γ -carboxyglutamic acid	hydroxylation	lipoylation
methylation	myristoylation	N-acyl diglyceride
O-GlcNac	palmitoylation	phosphorylation
pyridoxal phosphate	phospho-pantetheine	pyrrolidone-carboxylic acid
sulfation		

Wilkins M.R., Gasteiger E., Gooley A., Herbert B., Molloy M.P., Binz P.A., Ou K., Sanchez J.-C., Bairoch A., Williams K.L., Hochstrasser D.F. *High-throughput Mass Spectrometric Discovery of Protein Post-translational Modifications*. [Journal of Molecular Biology, 289, p. 645-657 \(1999\)](#)

Gasteiger E., Hoogland C., Gattiker A., Duvaud S., Wilkins M.R., Appel R.D., Bairoch A.; *Protein Identification and Analysis Tools on the ExPASy Server*; (In) John M. Walker (ed): [The Proteomics Protocols Handbook. Humana Press \(2005\)](#).

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Site for compilation of PTMs

<https://www.abrf.org/index.cfm/dm.home>

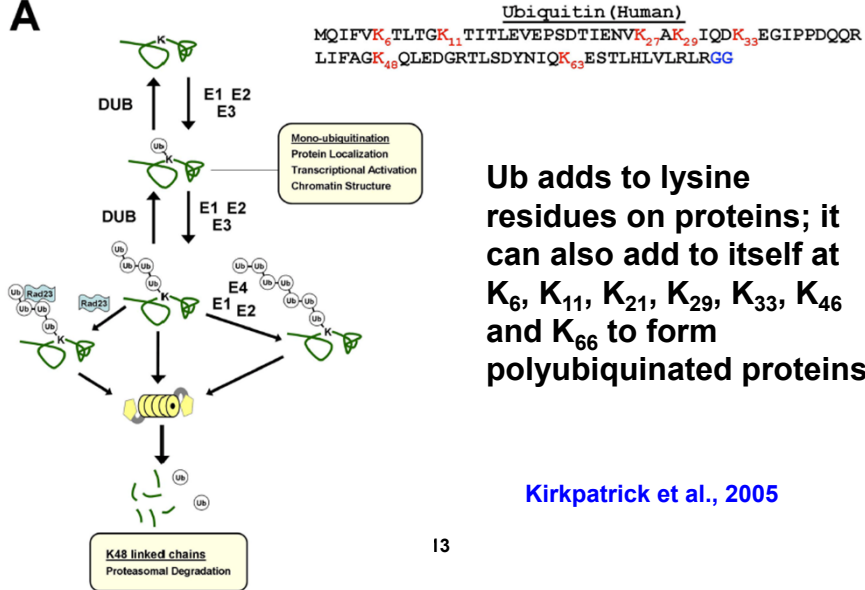
This site was put together by Ken Mitchelhill, Len Packman and friends

Currently ranges from dephosho (-79) to (Hex)3-HexNAc-(dHex)HexNAc (+1,039)

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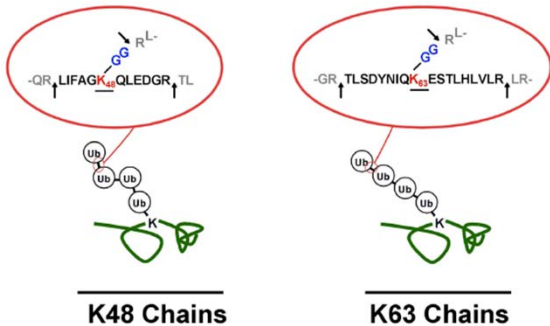
Ubiquitination

A

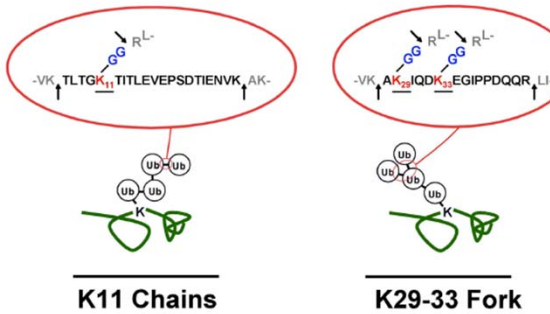


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Sites of attachment of Ub groups

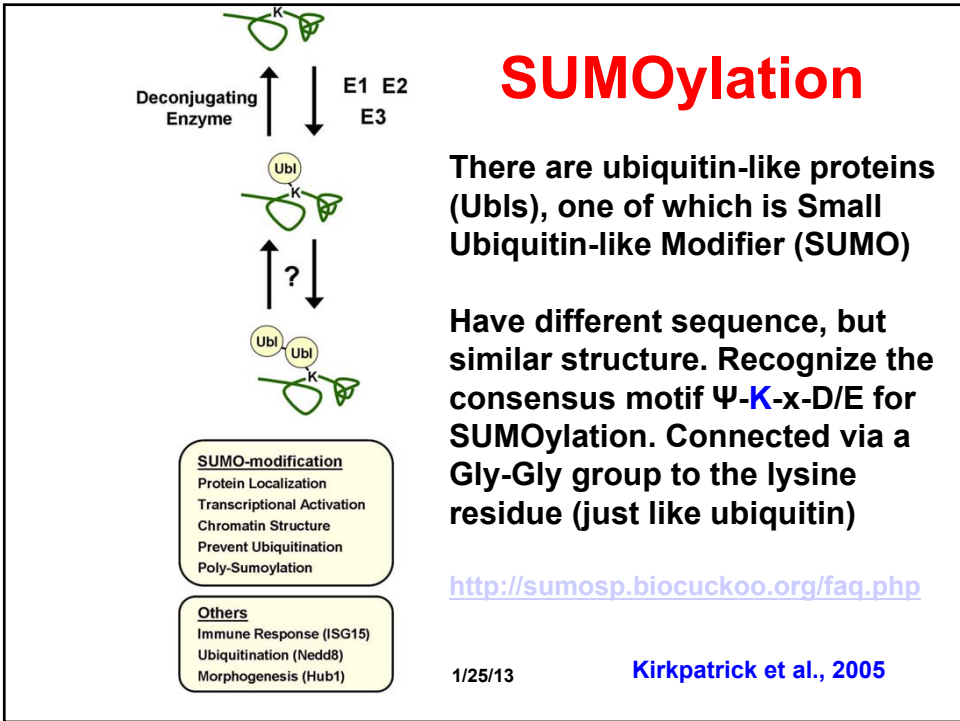
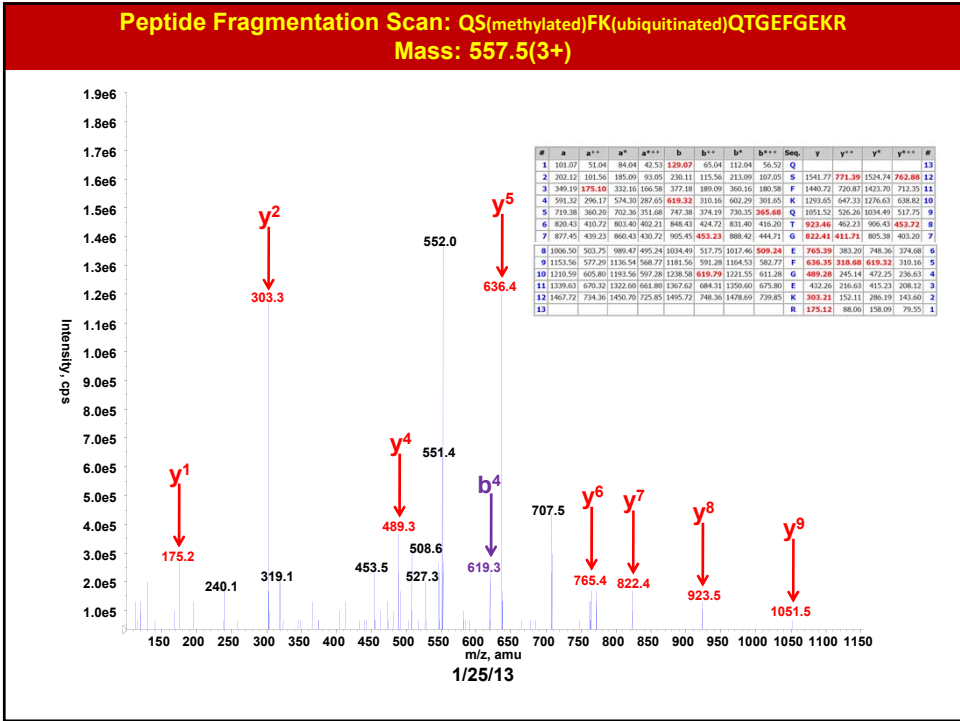


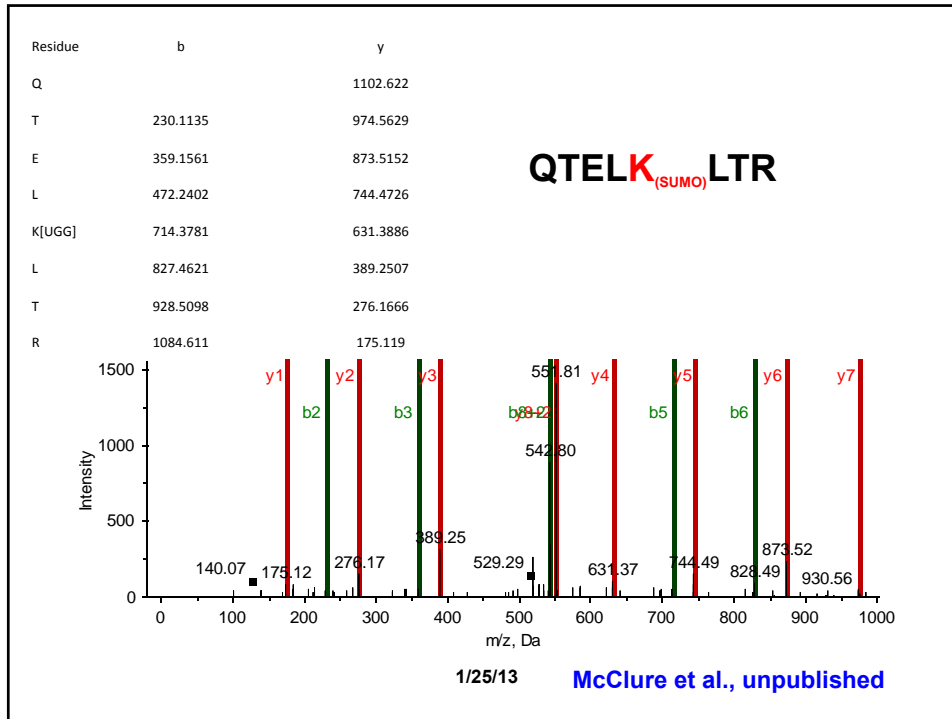
Note that Ub is attached through the side chain of lysine via the two C-terminal glycines of Ubq.



The next residue is Arg – therefore, trypsin hydrolysis will yield a peptide modified by the addition of 114 (*m/z* 261)

Kirkpatrick et al., 2005





Ubiquitin versus SUMO

```

Human Ubiquitin  ...GKQLEDGRTLSDYNIQKESTLHLVLRIRGG
Human NEDD8     ...GKQMNDEKTAADYKILGGSVLHLVLAIRGG
Human ISG15    ...GKPLEDQLPLGEYGLKPLSTVFMNLRIRGG
Human SUMO-1   ...GQRIADNHTPKELGMEEDVIEVYQEQTGG
Human SUMO-2   ...GQPINETDTPAQLEMEDEDTIDVFQQQTGG
Human SUMO-3   ...GQPINETDTPAQLEMEDEDTIDVFQQQTGG
Drosophila SUMO-3 ...GQPINENDTPTSLEMEEGDTIEVYQQQTGG
Arabidopsis SMT3 ...GRRLRAEQTPDELEMEDGDEIDAMLHQTGG
Yeast SMT3     ...GIRIQADQTPEDIDMEDNDIIEAHREQIGG
  
```

So, trypsin will not cut SUMO sites (unlike for ubiquitin) to generate a Gly-Gly modified Lysine

1/25/13 Knuesel et al., 2005

Detecting PTMs

- If we don't have any idea about what the modification(s) is(are), how do we proceed?
- We won't have the modification on MASCOT or Protein Prospector
- No antibodies or convenient affinity phases

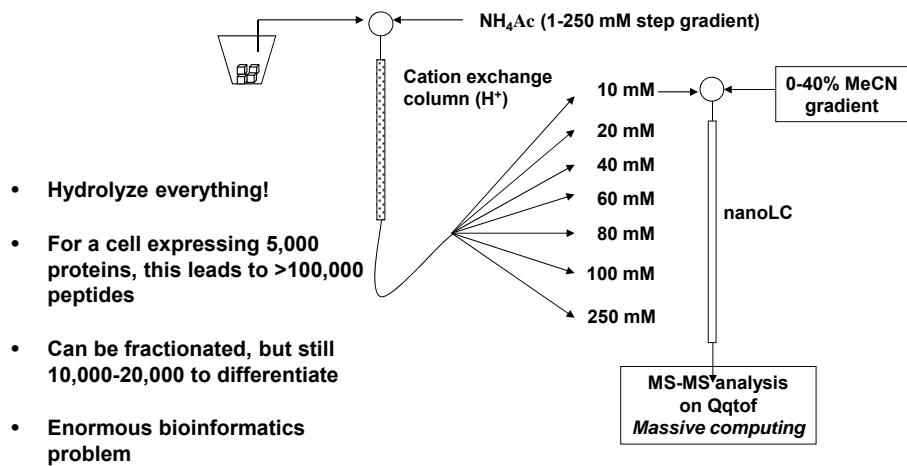
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Can we approach this globally?

- It's asking a lot
 - Too many degrees of freedom
 - Endless modifications
- Should we try?
 - John Yates' group did
 - [MacCoss et al. PNAS 99:7900 \(2002\)](#)

1/25/13

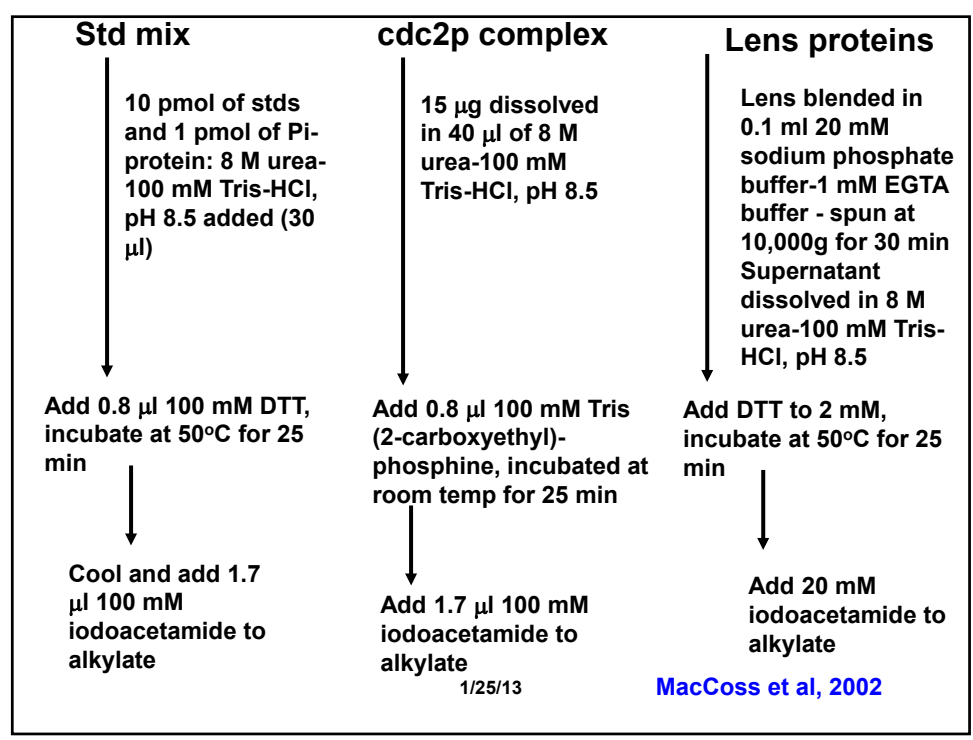
MudPIT - Multi-dimensional Protein Identification Technology



- Hydrolyze everything!
- For a cell expressing 5,000 proteins, this leads to >100,000 peptides
- Can be fractionated, but still 10,000-20,000 to differentiate
- Enormous bioinformatics problem

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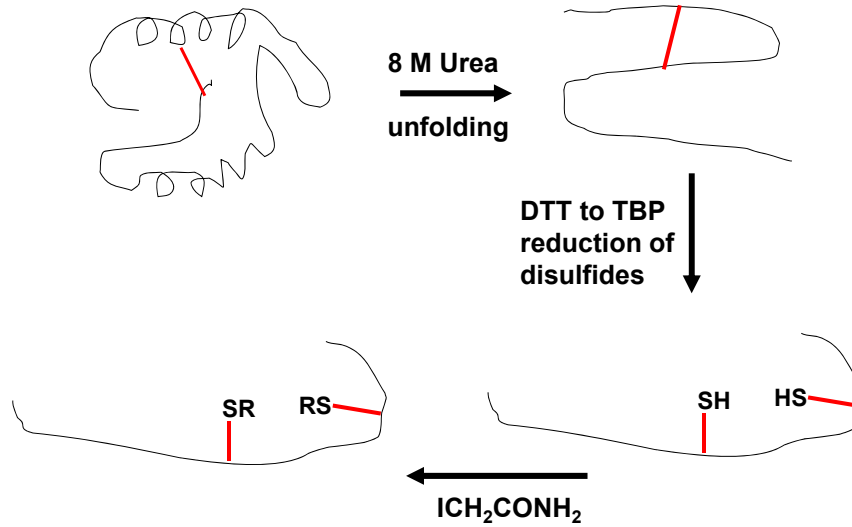
John Yates



1/25/13

MacCoss et al, 2002

Preparing proteins for digestion



1/25/13

Hydrolysis procedures

Reduced, carboxymethylated in 8 M urea

Diluted x 3 with
100 mM Tris-HCl,
pH 8.5

100 mM CaCl_2
added to 1 mM

Roche **trypsin** 1:50,
incubated 12-24 h
at 37°C

quenched with
90% formic acid
to 4%

Diluted x 3 with 4.8
M Urea-100 mM Tris-
HCl, pH 8.5

subtilisin added
1:50, incubated 2-3
hr at 37°C

quenched with
90% formic acid
to 4%

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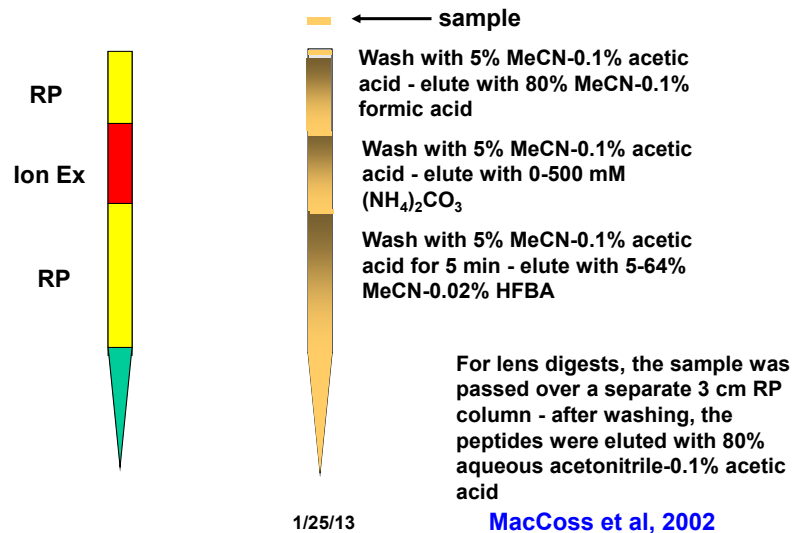
Diluted x 3 with
100 mM Tris-HCl,
pH 8.5

incubated with
elastase 1:50 12 h
at 37°C

quenched with
90% formic acid
to 4%

MacCoss et al, 2002

Elution from a triphasic column



Automated MS-MS analysis

- Limit analysis to 2⁺ or 3⁺-charged peptides
- Delete poor quality spectra
- Identify peptides with 98-Da neutral loss
- Analyze the remaining spectra with SEQUEST (operating on a 31-node beowulf computer cluster) to identify proteins
- Search sequences of identified proteins for PTMs by 80 (STY phosphorylation), 42 (K acetylation), 16 (MWY oxidation) and 14 (K methylation)

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[MacCoss et al, 2002](#)

Results

Protein standard mixture:

- The three digests were combined - 83.7% and 95.4% coverage for glycogen phosphorylase and BSA
- Identified the glycogen phosphorylase phosphorylation site as well as the two known sites (S69/S345) in ovalbumin
- N-acetylation found plus many sites of methionine oxidation (? due to work up or real)
- New sites found - phosphorylation at S237/S241 in ovalbumin and methylation at R652

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MacCoss et al, 2002

Results

Cdc2p complex:

- 200 proteins - 20 showed >40% sequence coverage
- Expected Y15 and T167 phosphorylation sites on Cdc2p found
- New phosphorylation sites found on cyclin partners Cdc13 and Cig1p
- Multiple methylation sites on Cdc2p

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MacCoss et al, 2002

Results

Lens proteins:

- Found 272 proteins - 52 had >40% sequence coverage
- 90% are crystallins
- PTMs accumulate over your lifetime
- Used 18-step MudPIT because of complexity
- 73 different PTMs found on the 11 crystallins
- Found the 13 of 18 PTMs previously described in all species
- Found 60 other new PTMs in phosphorylation, oxidation, acetylation and methylation

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MacCoss et al, 2002

Summary

- The proteome is very complex
- Some proteins are in pro-forms and can be found in many, often small, but active fragments
- In addition, proteins are heavily modified
- Modification can account for marked differences in turnover
- Selective fractionation is needed to demonstrate PTMs that are in low abundance

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Paper to read

- McClure M, DeLucas LJ, Wilson L, Ray M, Rowe S, Wu X, Dai Q, Hong JS, Sorscher EJ, Kappes JC, Barnes S. Palmitoylation and other post-translational modifications of CFTR with relevance to gating and processing. [*Protein Eng Des Sel.* 2012 Jan;25\(1\):7-14.](#)

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