



Protein and Proteome Analysis by Mass Spectrometry

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What is the proteome?

The proteome

The Proteome (Keith Williams and Marc Wilkins, 1994)

“The protein equivalent to the genome”

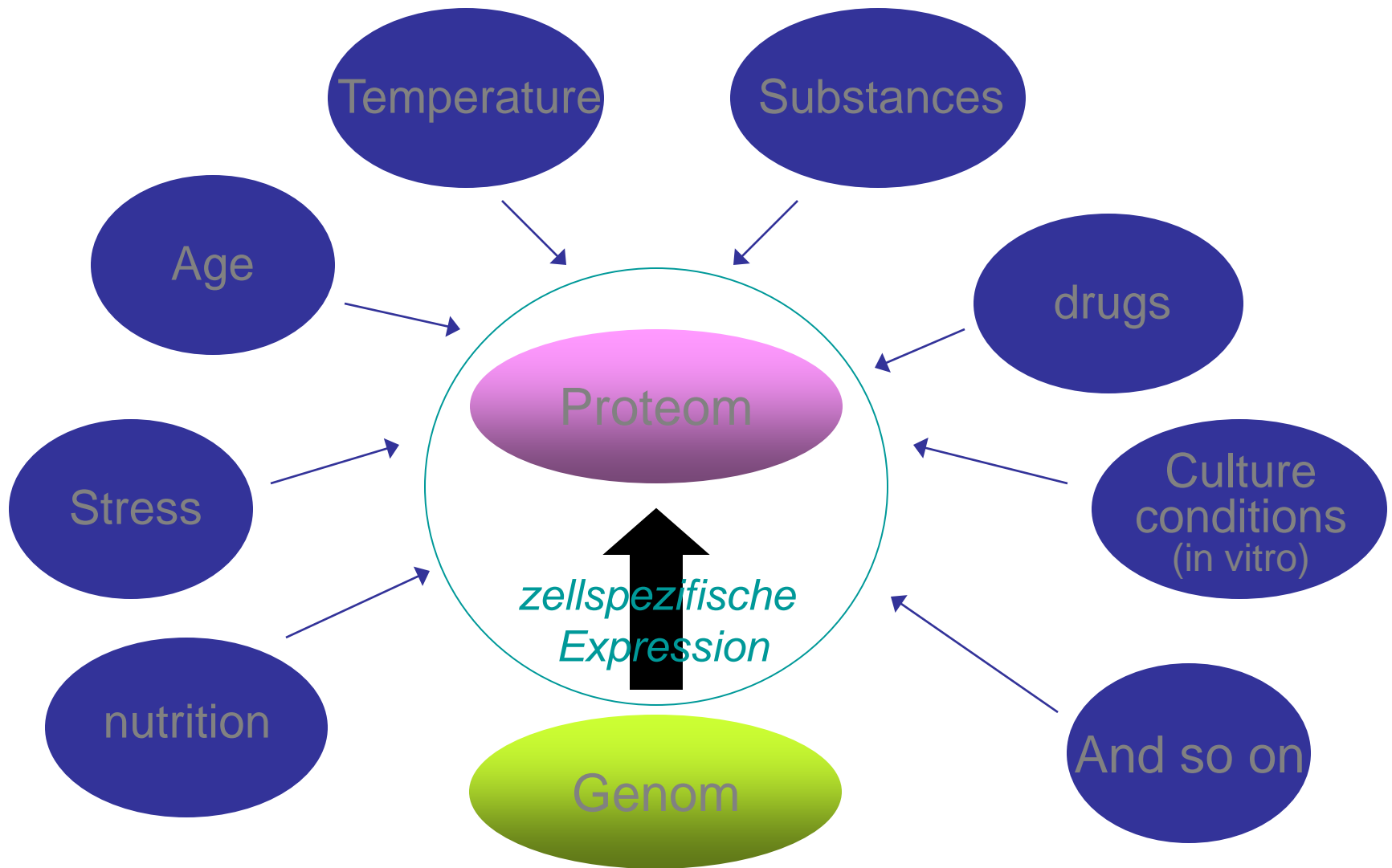
The proteome describes the entirety of proteins of an organism at a **certain point in time and in a certain state.**

*In contrast to the genome, the proteome is not static but **highly dynamic***

*One species,
the same genome,
different proteome*



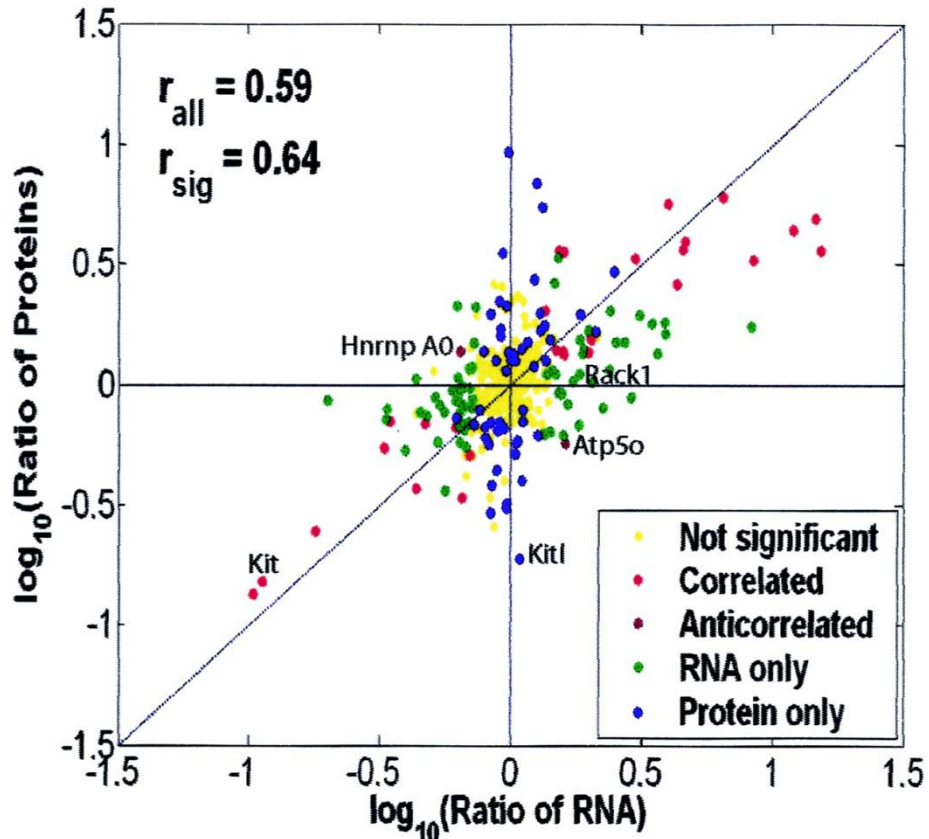
The proteome influenced by the environment



Why do we need proteome analysis?

Correlation with RNA expression

Scatter plot of mRNA versus cognate protein expression ratios (log₁₀) of MPRO:EML.



- Protein expression levels are strongly regulated by post-transcriptional regulation
- RNA expression levels can only capture around 40% of variation in protein expression

➔ Protein expression analysis can give valuable additional information

Tian Q et al. Mol Cell Proteomics 2004;3:960-969

Analysis of sub-proteomes

- Proteomics enables the analysis of sub-proteomes like:
 - Proteomes of organelles (mitochondria, cilia, outer segments of photoreceptors,...)
 - Body fluids
 - Protein complexes
 - ...

- Analysis of post-translational modifications:
 - Phosphorylation
 - Acetylation
 - Sumoylation
 - ...

How can we analyse the proteome and what are the challenges?

Analysing the proteome

➤ *Methods for proteome analysis:*

➤ *Gel based approaches*

- *Labour intensive, hard to standardize*
- *MS necessary for protein identification*

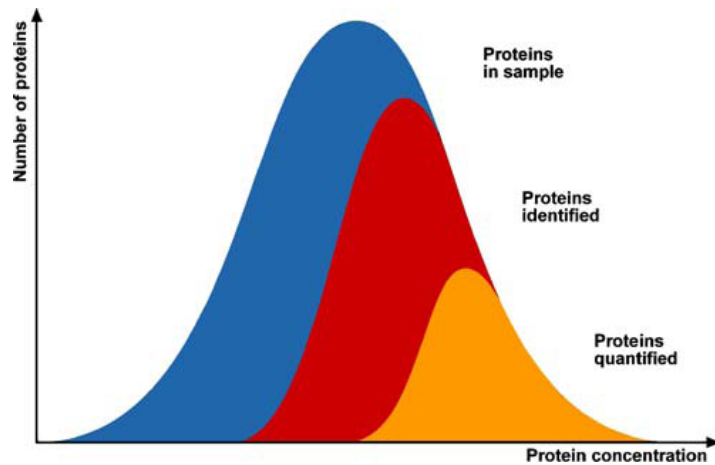
➤ *Gel-free, MS-based approaches*

- *Standardization possible*
- *Automation possible*
- *Great improvement in sensitivity, speed and resolution within the last 10 years*
- *Automated quantification possible*

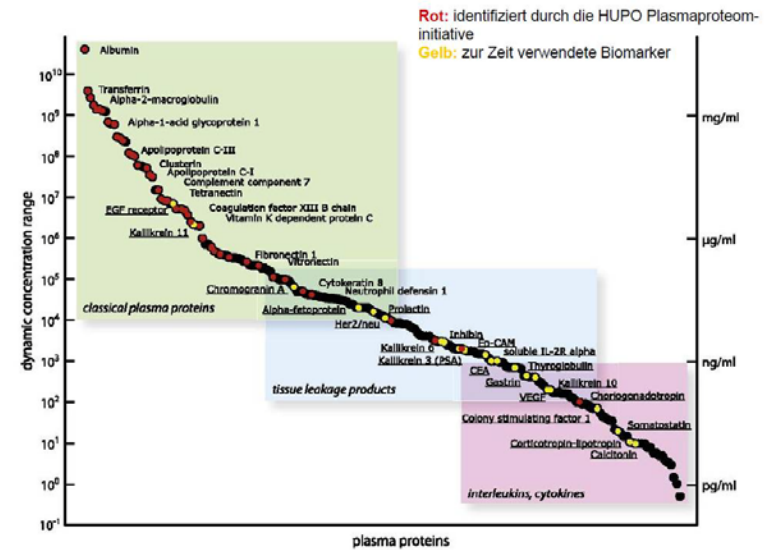
→ *Gel-free, MS-based approaches are highly suitable for proteome analysis*

Major challenges in proteom analysis

- High complexity:
 - ~ 20 000 genes
 - 50 000 - 100 000 proteins
- Modifications lead to even higher complexity
- Huge dynamic range (10 orders of magnitude)



Banschkeff et al., Anal. Bioanal. Chem., 2007

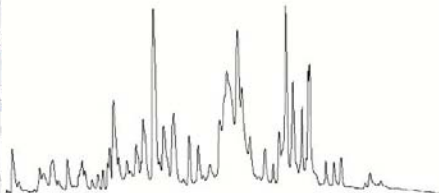
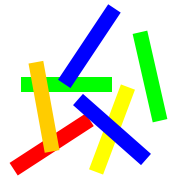


Protein identification by MS: The basics

Bottom-up protein analytics by MS

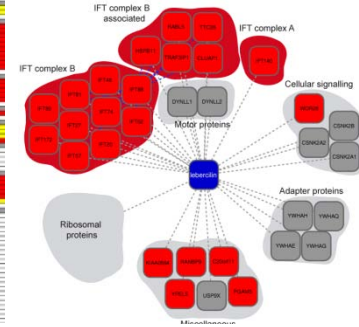


cleavage



Liquid-Chromatography/
Tandem Mass spectrometry

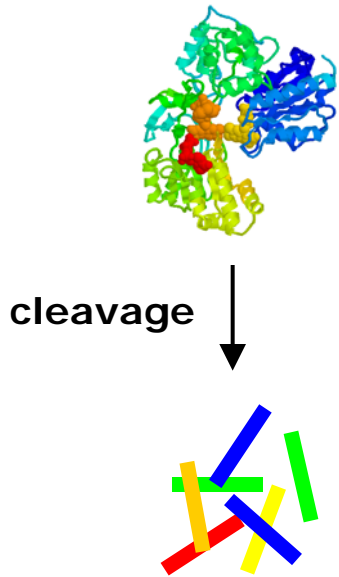
Protein
identification/quantification



Why do we need proteolytic cleavage

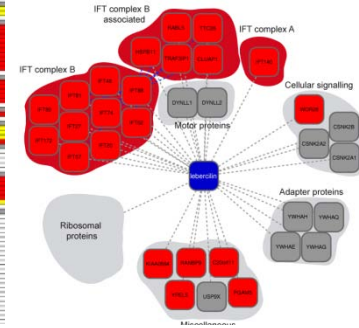
- Molecular weight of proteins varies greatly (10-500 kilo Dalton)
- MS machines are mainly suited for the analysis of molecules of up to some Dalton
- Proteolytic cleavage produces peptide fragments making the high throughput analysis and identification by MS possible
- Major disadvantage: Even higher complexity of the sample

Bottom-up protein analytics by MS

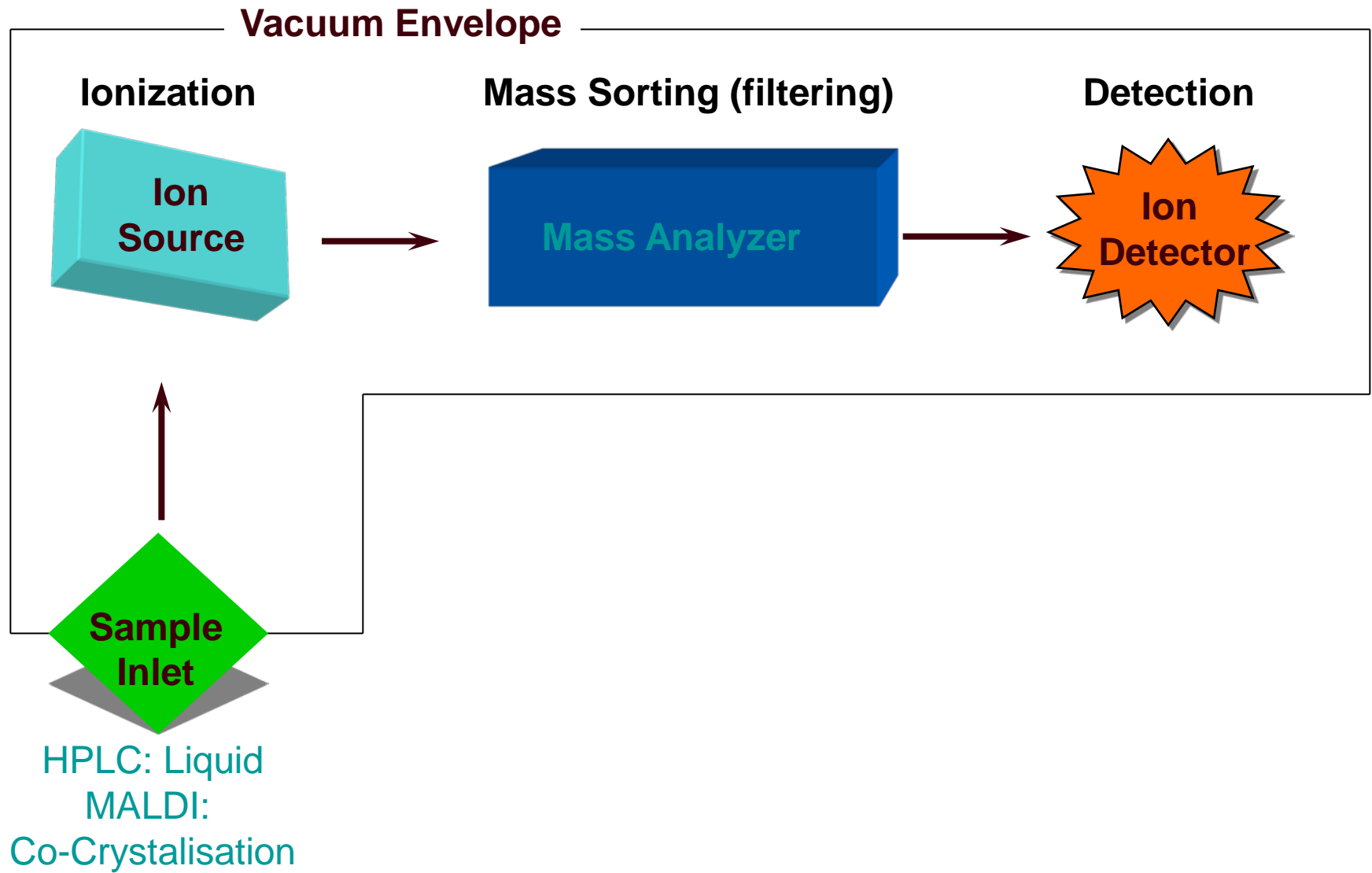


Liquid-Chromatography/
Tandem Mass spectrometry

Protein identification/quantification



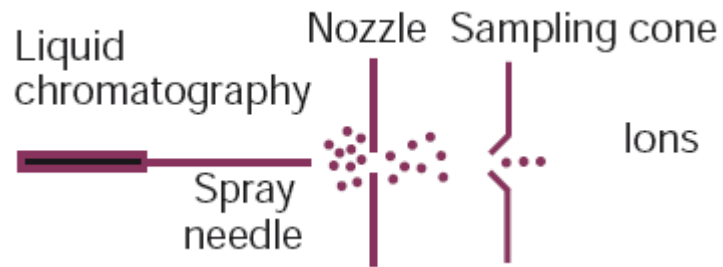
Components of a MS: Ion source



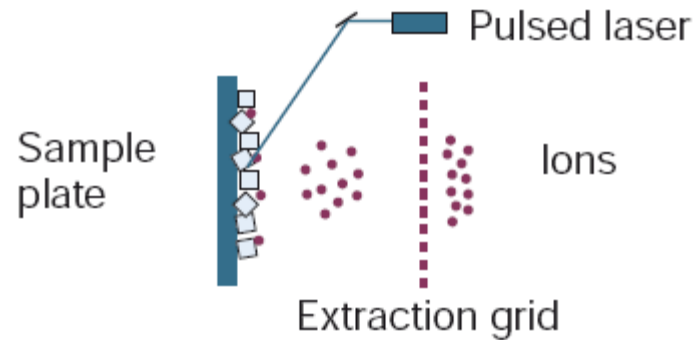
Components of a MS: Ion source



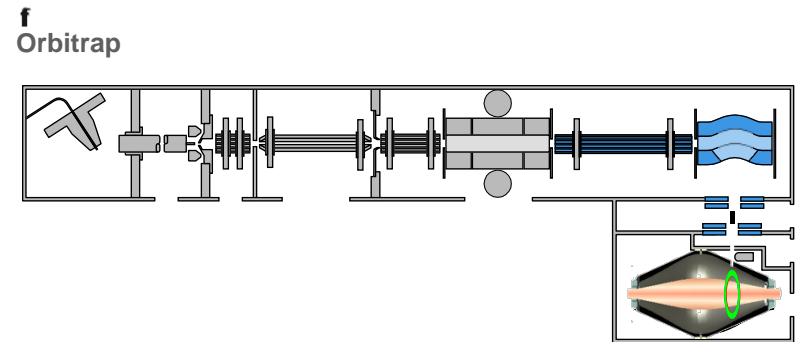
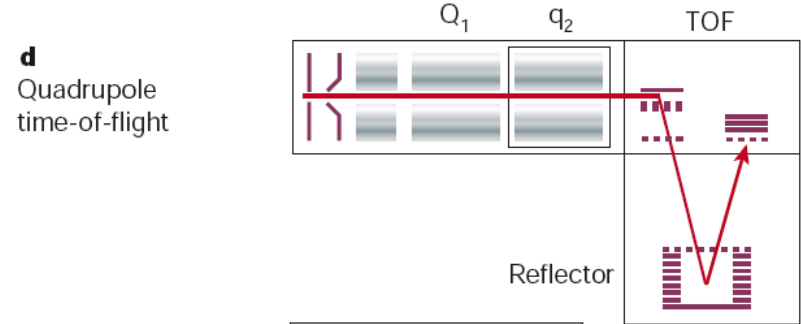
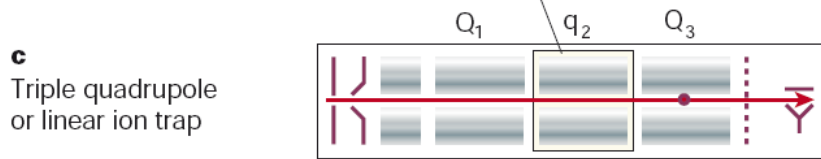
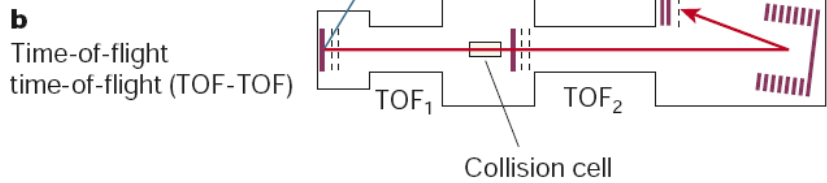
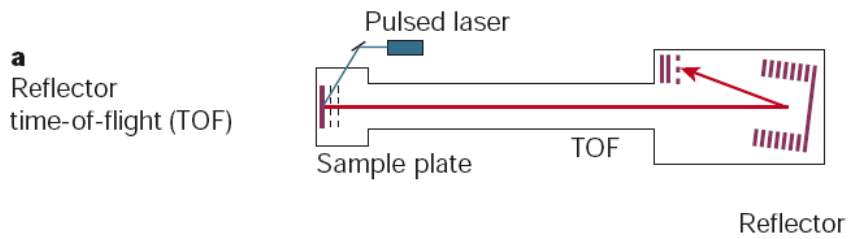
Electro-Spray Ionization
ESI



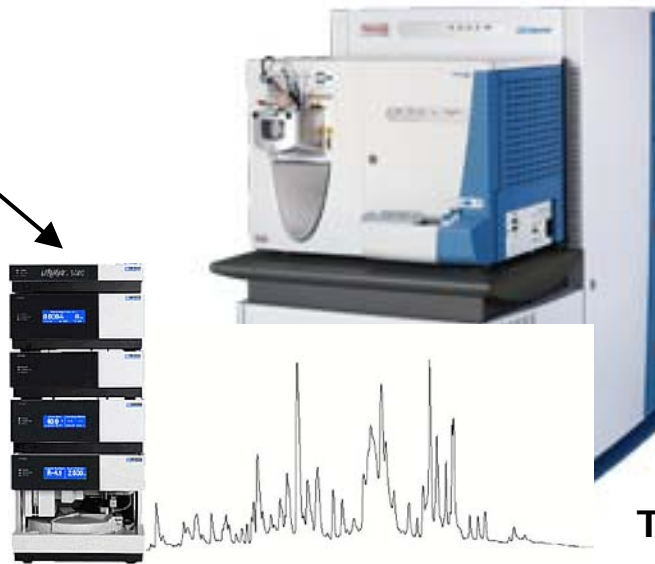
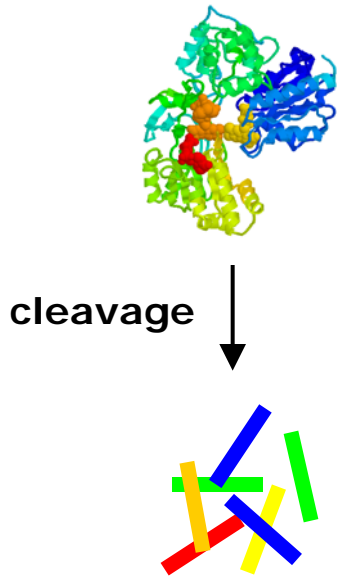
Matrix-Assisted laser desorption/ionization
(MALDI)



Components of a MS: Mass analyser



Bottom-up protein analytics by MS



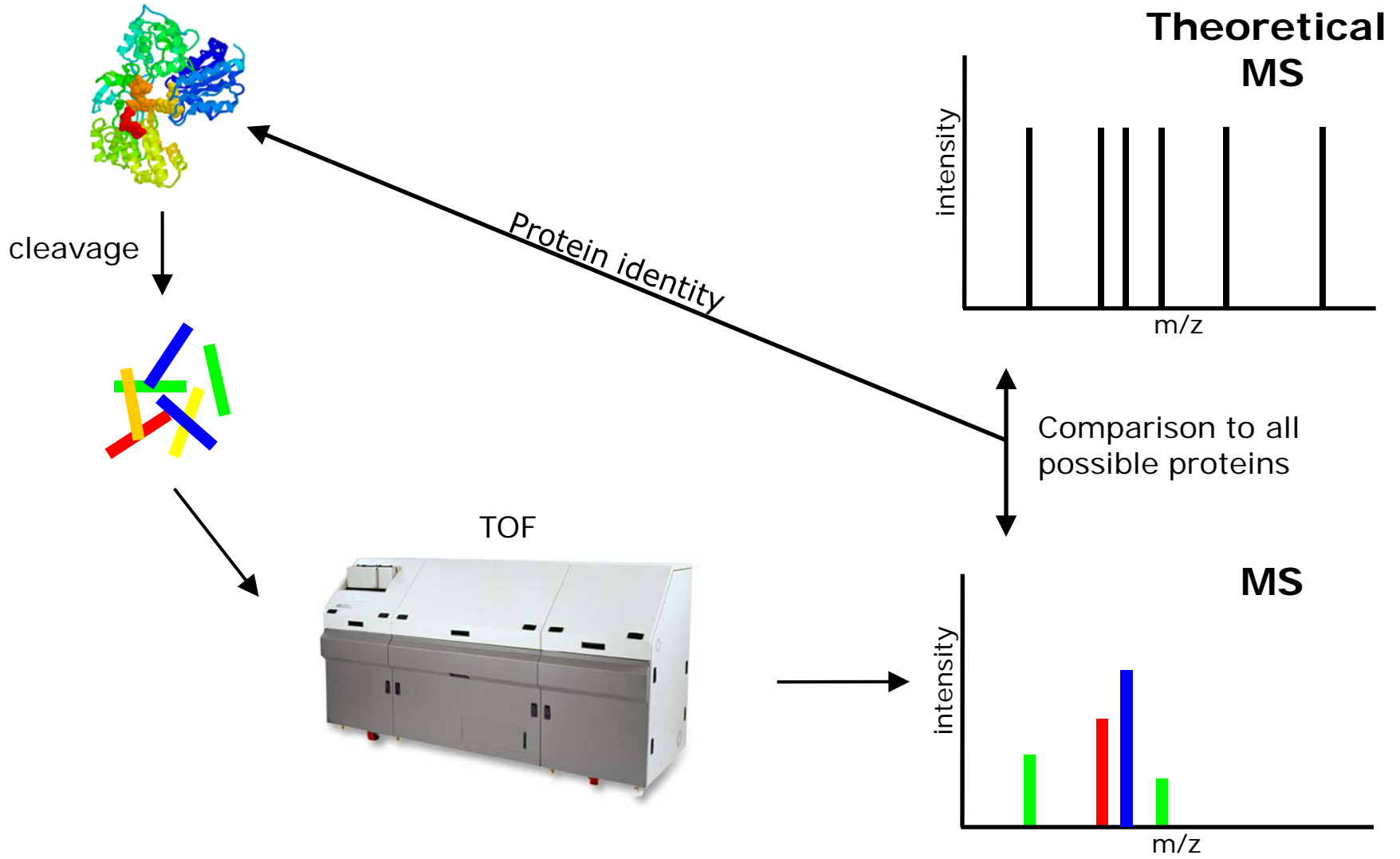
Protein identification/quantification

This section contains a data table and a protein network diagram. The table shows protein identification results with columns for protein name, accession number, and various scores. The network diagram shows a central node labeled "spord2" connected to various protein complexes and pathways, including IFT complex B associated, IFT complex A, Cellular signalling, Motor proteins, Adapter proteins, and Ribosomal proteins.

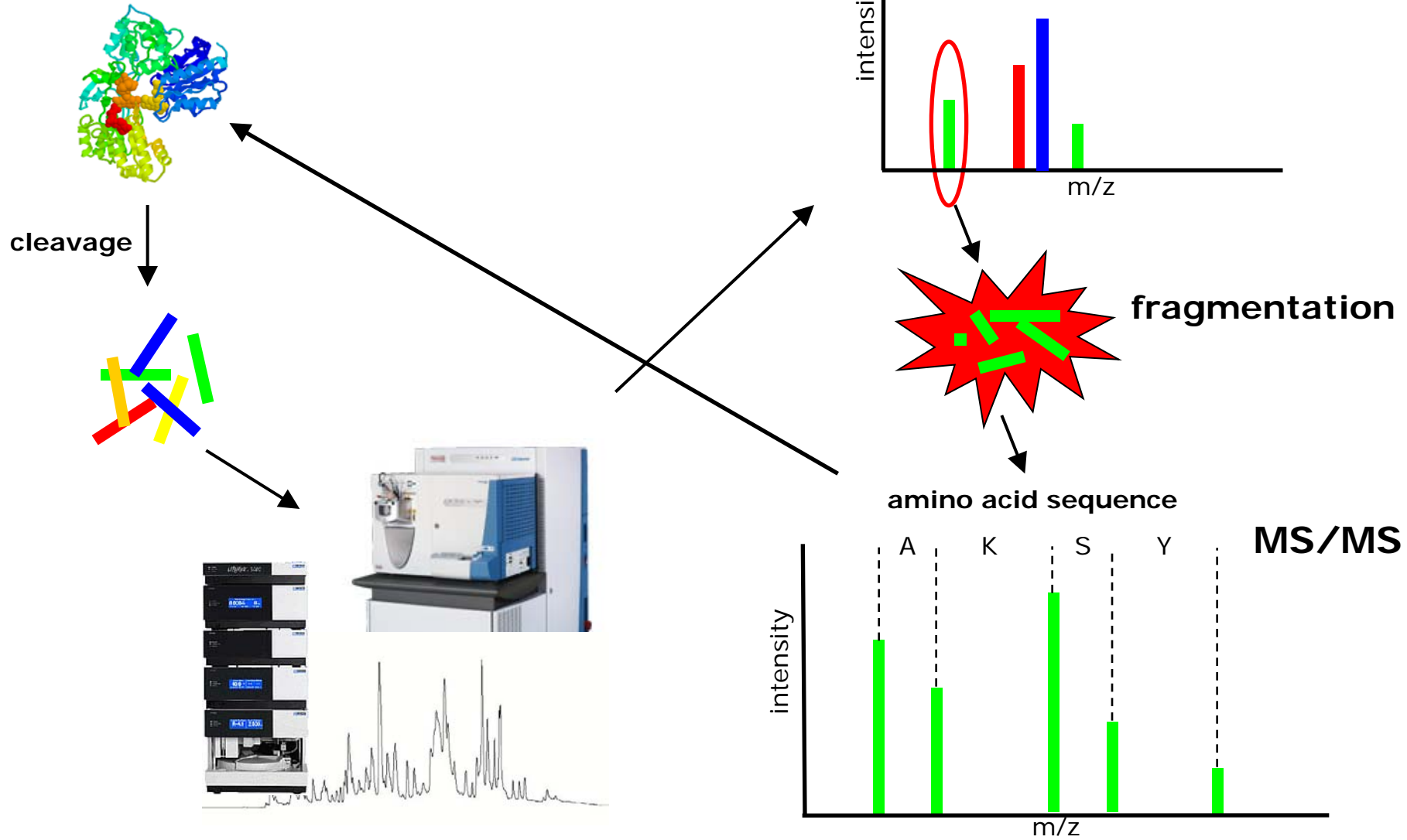
| Protein | Accession | Score | Label |
|------------|--------------|----------|----------|
| Protein 1 | Accession 1 | Score 1 | Label 1 |
| Protein 2 | Accession 2 | Score 2 | Label 2 |
| Protein 3 | Accession 3 | Score 3 | Label 3 |
| Protein 4 | Accession 4 | Score 4 | Label 4 |
| Protein 5 | Accession 5 | Score 5 | Label 5 |
| Protein 6 | Accession 6 | Score 6 | Label 6 |
| Protein 7 | Accession 7 | Score 7 | Label 7 |
| Protein 8 | Accession 8 | Score 8 | Label 8 |
| Protein 9 | Accession 9 | Score 9 | Label 9 |
| Protein 10 | Accession 10 | Score 10 | Label 10 |

Liquid-Chromatography/
Tandem Mass spectrometry

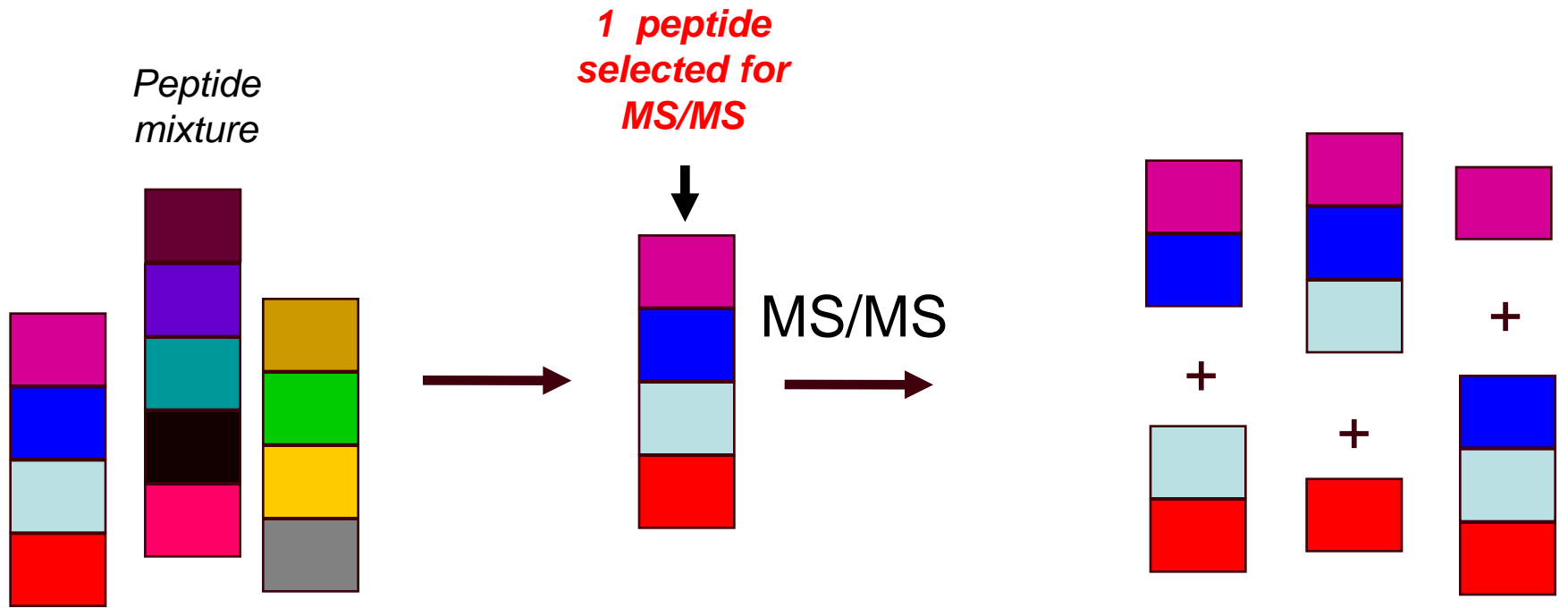
Identification by Peptide Mass Fingerprint



Identification by fragment ions

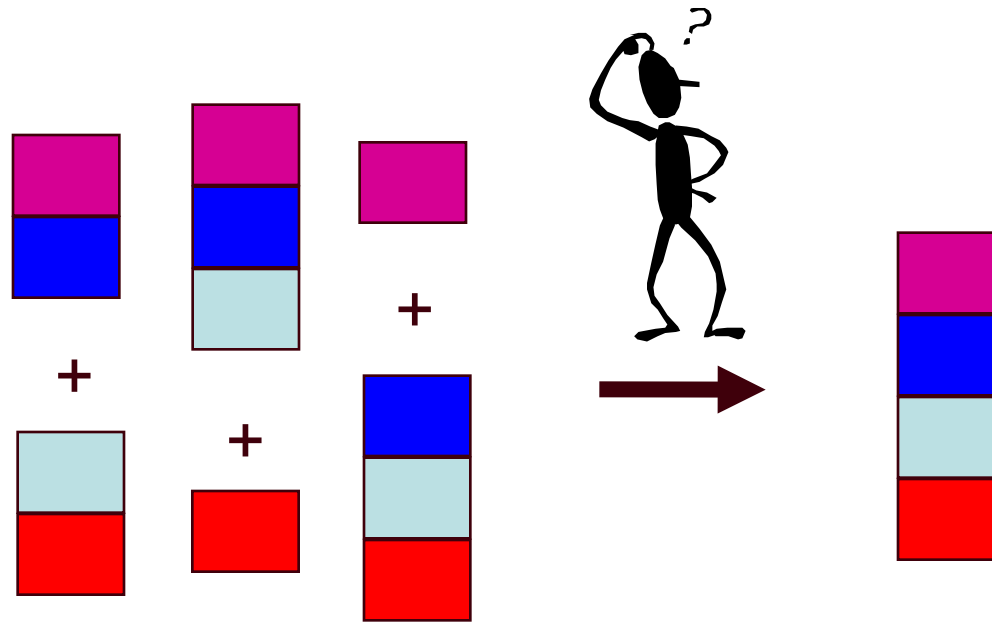


Identification by fragment ions



The masses of all the pieces give an MS/MS spectrum

Identification by fragment ions



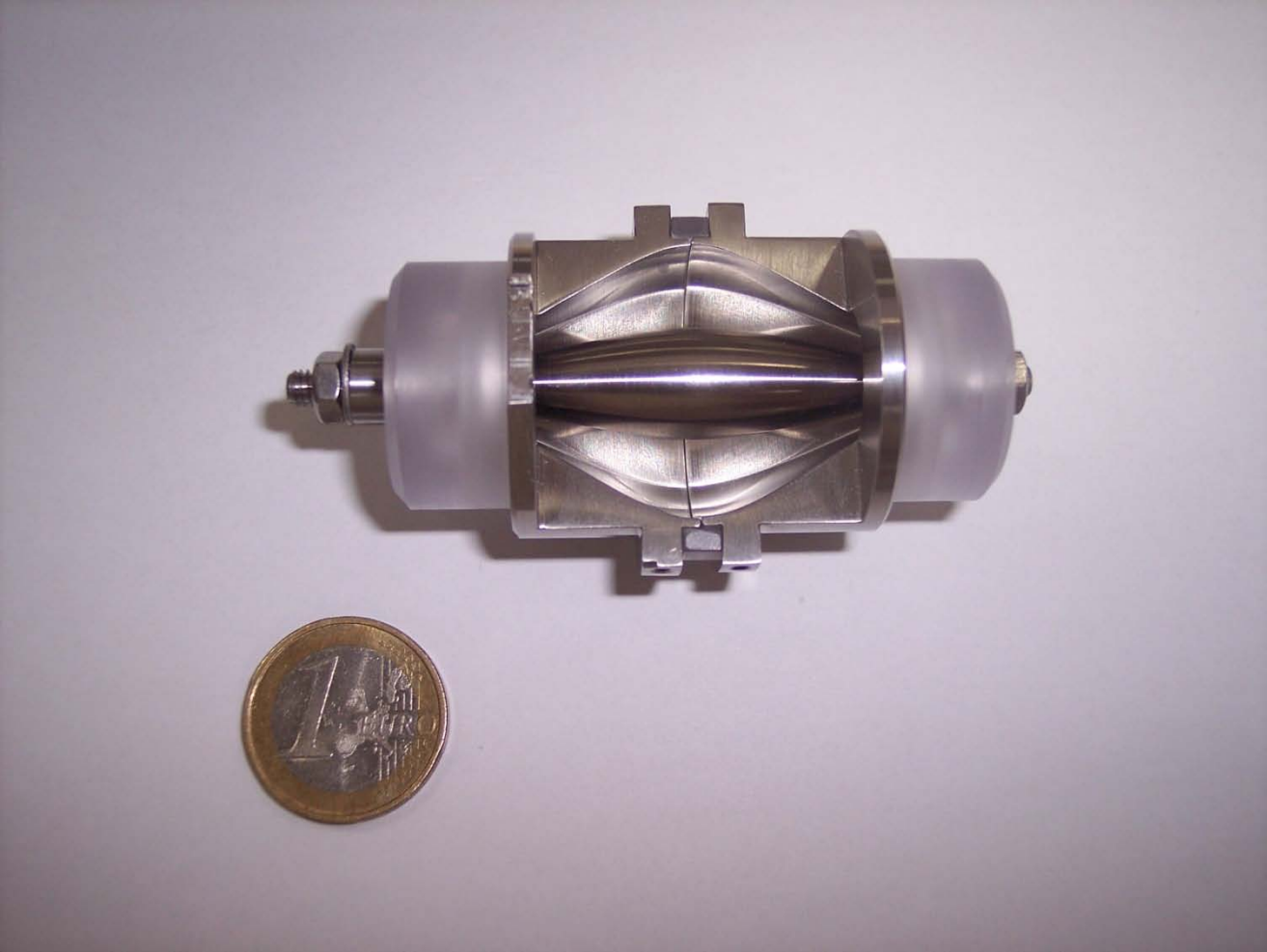
- Use the fragment ion masses as specific pieces of the puzzle to help piece the intact molecule back together
 - Several thousands of peptides/analysis → highly complex job
- Search algorithms make this job

Identification by fragment ions

| B | B Ions | B+2H | B-NH3 | B-H2O | AA | Y Ions | Y+2H | Y-NH3 | Y-H2O | Y |
|----|---------|---------|---------|---------|------|---------|---------|---------|---------|----|
| 1 | 115.1 | | 98.0 | | N | 2,270.1 | 1,135.5 | 2,253.0 | 2,252.1 | 19 |
| 2 | 228.1 | | 211.1 | | I | 2,156.0 | 1,078.5 | 2,139.0 | 2,138.0 | 18 |
| 3 | 357.2 | | 340.2 | 339.2 | E | 2,042.9 | 1,022.0 | 2,025.9 | 2,024.9 | 17 |
| 4 | 470.3 | | 453.2 | 452.3 | L | 1,913.9 | 957.5 | 1,896.9 | 1,895.9 | 16 |
| 5 | 583.3 | | 566.3 | 565.3 | I | 1,800.8 | 900.9 | 1,783.8 | 1,782.8 | 15 |
| 6 | 743.4 | 372.2 | 726.3 | 725.4 | C+57 | 1,687.7 | 844.4 | 1,670.7 | 1,669.7 | 14 |
| 7 | 871.4 | 436.2 | 854.4 | 853.4 | Q | 1,527.7 | 764.4 | 1,510.7 | 1,509.7 | 13 |
| 8 | 1,000.5 | 500.7 | 983.5 | 982.5 | E | 1,399.6 | 700.3 | 1,382.6 | 1,381.6 | 12 |
| 9 | 1,114.5 | 557.8 | 1,097.5 | 1,096.5 | N | 1,270.6 | 635.8 | 1,253.6 | 1,252.6 | 11 |
| 10 | 1,243.6 | 622.3 | 1,226.5 | 1,225.6 | E | 1,156.6 | 578.8 | 1,139.5 | 1,138.5 | 10 |
| 11 | 1,300.6 | 650.8 | 1,283.6 | 1,282.6 | G | 1,027.5 | 514.3 | 1,010.5 | 1,009.5 | 9 |
| 12 | 1,429.6 | 715.3 | 1,412.6 | 1,411.6 | E | 970.5 | 485.8 | 953.5 | 952.5 | 8 |
| 13 | 1,543.7 | 772.3 | 1,526.6 | 1,525.7 | N | 841.5 | 421.2 | 824.4 | 823.4 | 7 |
| 14 | 1,658.7 | 829.9 | 1,641.7 | 1,640.7 | D | 727.4 | 364.2 | 710.4 | 709.4 | 6 |
| 15 | 1,755.7 | 878.4 | 1,738.7 | 1,737.7 | P | 612.4 | | 595.4 | | 5 |
| 16 | 1,854.8 | 927.9 | 1,837.8 | 1,836.8 | V | 515.3 | | 498.3 | | 4 |
| 17 | 1,967.9 | 984.5 | 1,950.9 | 1,949.9 | L | 416.3 | | 399.2 | | 3 |
| 18 | 2,096.0 | 1,048.5 | 2,078.9 | 2,077.9 | Q | 303.2 | | 286.2 | | 2 |
| 19 | 2,270.1 | 1,135.5 | 2,253.0 | 2,252.1 | R | 175.1 | | 158.1 | | 1 |

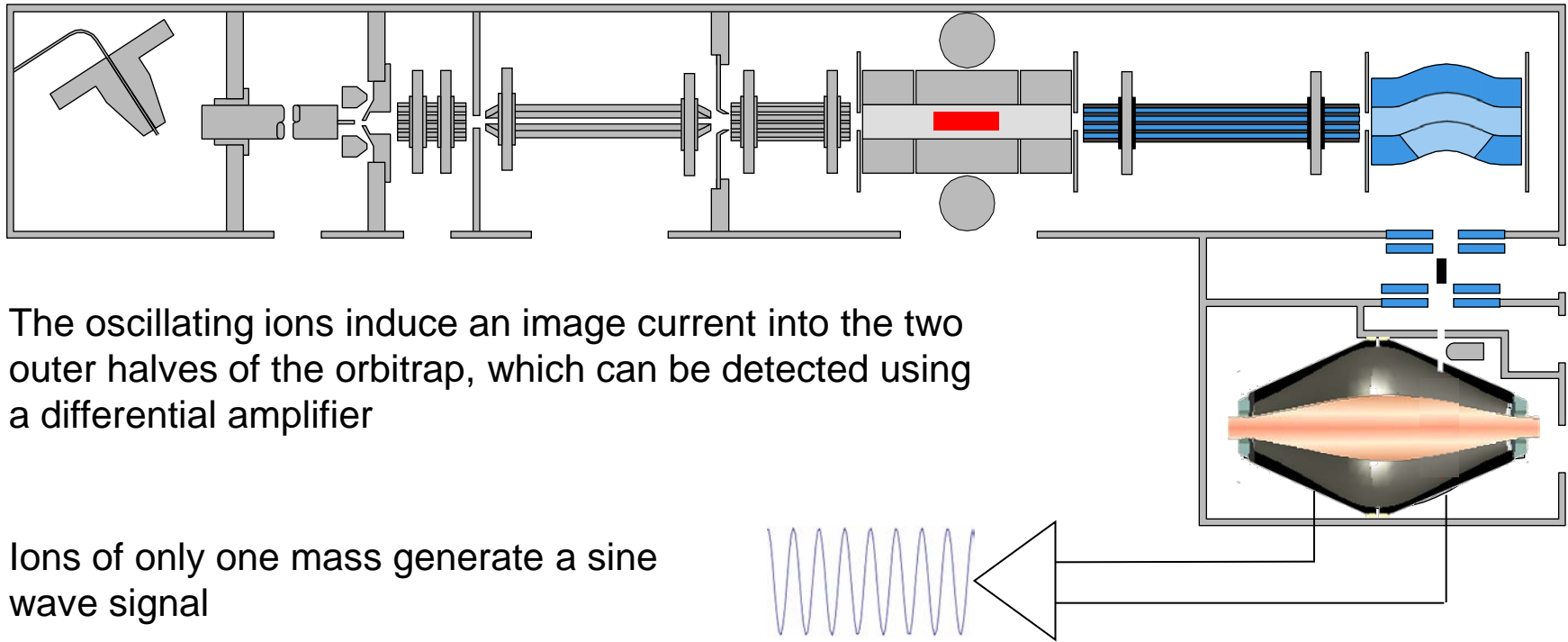
In real life (almost): MSMS on an Orbitrap

The Orbitrap



LC-MSMS on an Orbitrap

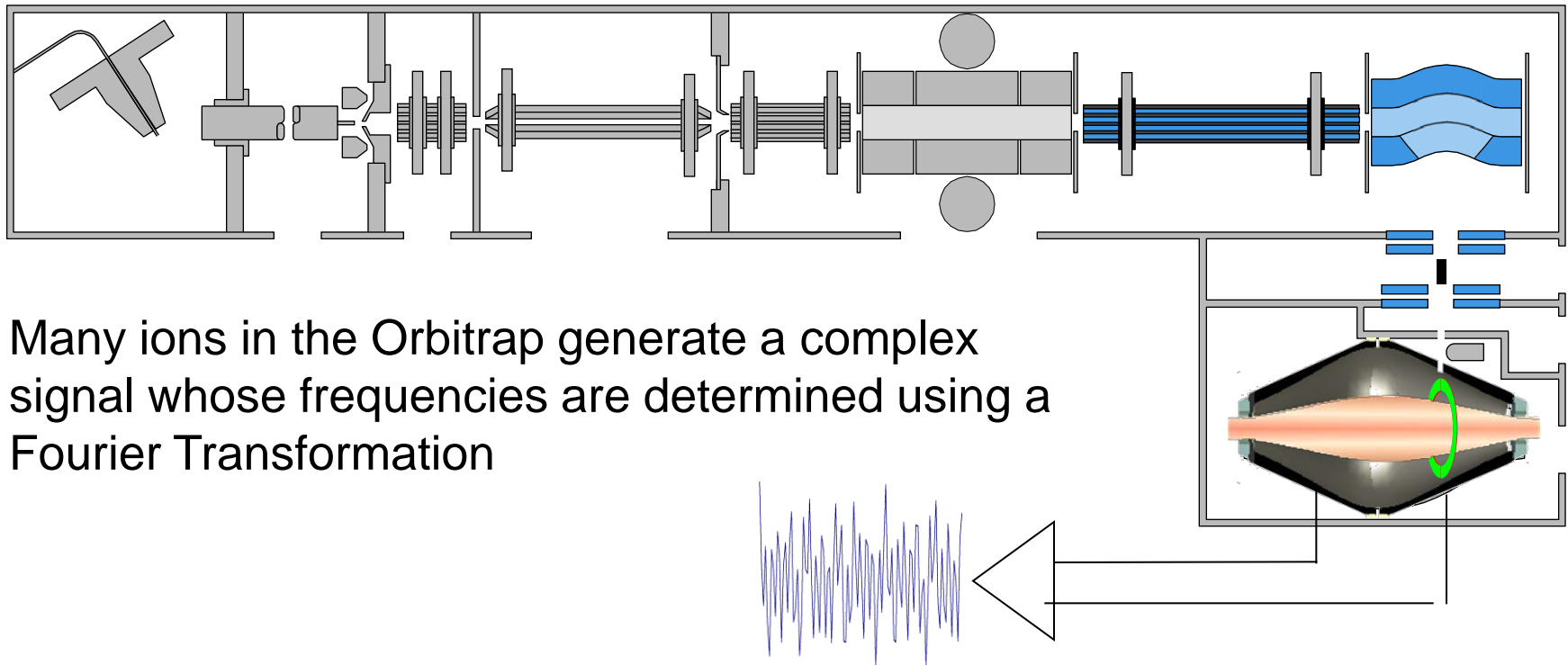
1. Ions are stored in the Linear Trap
2. are axially ejected
3. and trapped in the C-trap
4. they are squeezed into a small cloud and injected into the Orbitrap
5. where they are electrostatically trapped, while rotating around the central electrode and performing axial oscillation



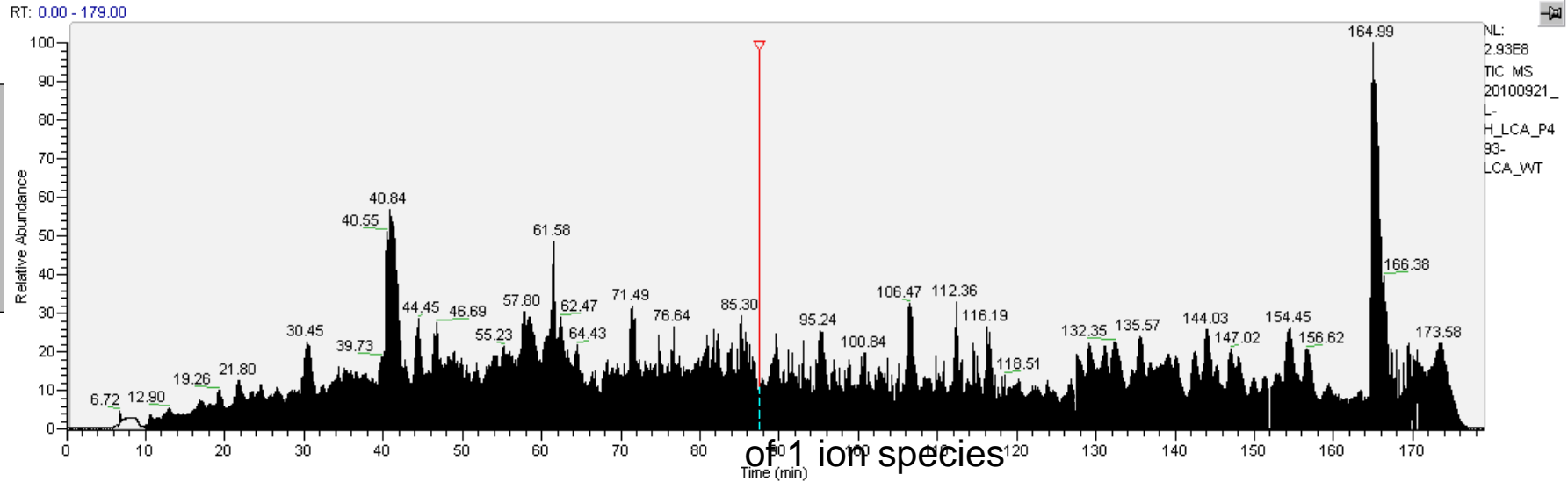
The oscillating ions induce an image current into the two outer halves of the orbitrap, which can be detected using a differential amplifier

Ions of only one mass generate a sine wave signal

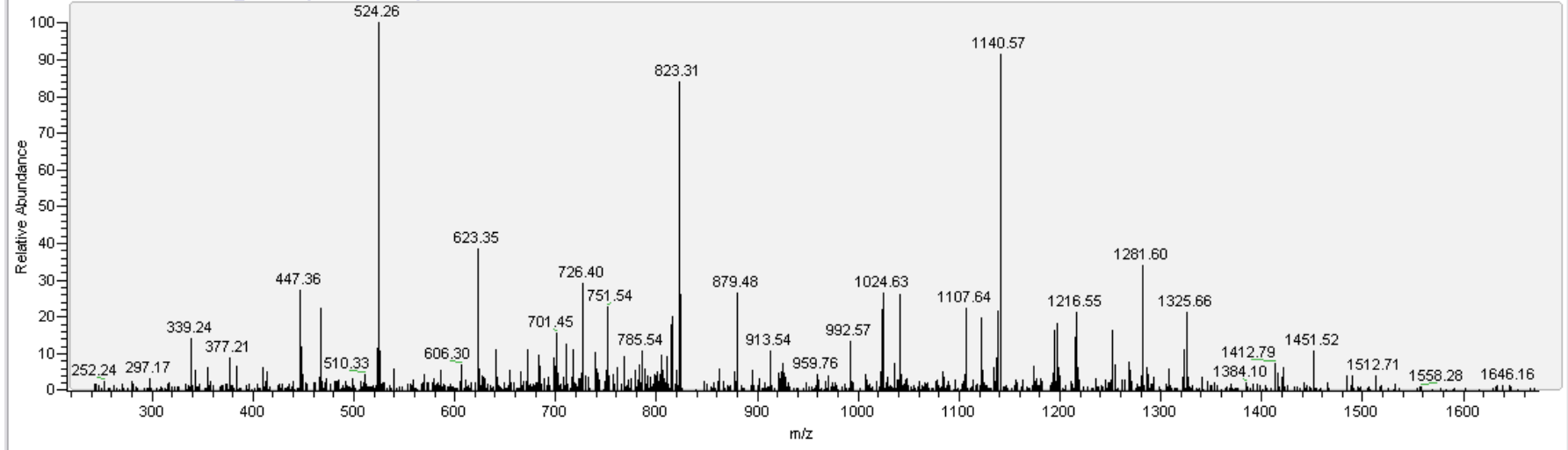
LC-MSMS on an Orbitrap



LC-MSMS on an Orbitrap



20100921_L-H_LCA_P493-LCA_WT #18502 RT: 87.46 AV: 1 NL: 9.72E3
T: ITMS + c NSI d Full ms2 832.46@cid35.00 [215.00-1675.00]



LC-MS/MS on an Orbitrap

Scaffold Viewer - Samples - 20101007_Dorus
File Edit View Experiment Export Quant Window Help

Min Protein: 95.0% Min # Peptides: 2 Min Peptide: 80%

Display Options: Number of Unique Peptides Req Mods: No Filter Search:

Probability Legend:

- over 95%
- 80% to 94%
- 50% to 79%
- 20% to 49%
- 0% to 19%

MS/MS View:
Identified Proteins (132)

BioSample 1

| # | Protein | Molecular Weight | 01_20101005_wash-01.raw (F0...) | 02_20101005_WT.raw (F01541) | 03_20101005_wash-02.raw (F0...) | 04_20101005_wash-02a.raw (F...) | 05_20101005_YEELS.raw (F011...) | 06_20101005_wash-03.raw (F0...) | 07_20101005_wash-03a.raw (F...) | 08_20101005_wash-03b.raw (F...) | 09_20101005_VMEA.raw (F015...) | 10_20101005_wash-04.raw (F0...) | 11_20101005_wash-04a.raw (F...) | 12_20101005_wash-04b.raw (F...) | 13_20101005_NEK1.raw (F015...) | 14_20101005_wash-05.raw (F0...) | 15_20101005_wash-05a.raw (F...) | 16_20101005_wash-05c.raw (F...) | 17_20101005_RMD5A.raw (F00...) | 18_20101005_wash-06.raw (F0...) | 19_20101005_wash-06a.raw (F...) | 20_20101005_wash-06b.raw (F...) | 21_20101005_LC451.raw (F011...) | 22_20101005_wash-07.raw (F0...) | 23_20101005_wash-07a.raw (F...) | 24_20101005_wash-07b.raw (F...) | 25_20101005_CDR2.raw (F015...) | 26_20101005_wash-08.raw (F0...) | 27_20101005_wash-08a.raw (F...) | 28_20101005_wash-08b.raw (F...) | 29_20101005_EEL1.raw (F01569) | 30_20101005_wash-09.raw (F0...) | 31_20101005_wash-10.raw (F0...) | | | |
|----|---------------------------------------|------------------|---------------------------------|-----------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|--------------------------------|---------------------------------|---------------------------------|---------------------------------|--------------------------------|---------------------------------|---------------------------------|---------------------------------|--------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|--------------------------------|---------------------------------|---------------------------------|---------------------------------|-------------------------------|---------------------------------|---------------------------------|---|---|--|
| 1 | Heat shock 70kDa protein 8 isoform... | 71 kDa | | | | 3 | | | | | 25 | | | | | | | | 19 | | | | | 23 | 2 | | | 14 | | | | | | | | |
| 2 | cDNA FLJ54408, highly similar to ... | 64 kDa | | | | 7 | | | | | 21 | 4 | 2 | | 10 | | | | 12 | | | | | | | | | | | | | | | | | |
| 3 | Tubulin beta polypeptide n=1 Tax=... | 48 kDa | | | | | | | | | 17 | 2 | | 2 | 7 | | | | 12 | | | | | | | | | | | | | | | | | |
| 4 | Ran-binding protein 9 n=1 Tax=H... | 78 kDa | | | | | | | | | 13 | | | | | | | | 11 | | | | | | | | | | | | | | | | | |
| 5 | UPI0001D3B08F related cluster n... | 49 kDa | | | | | | | | | 32 | 7 | 5 | 4 | 2 | | | | 10 | | | | | | | | | | | | | | | | | |
| 6 | cDNA FLJ55707, highly similar to ... | 72 kDa | | | | | | | | | 13 | | | | | | | | 10 | | | | | | | | | | | | | | | | | |
| 7 | Propionyl-CoA carboxylase alpha ... | 77 kDa | | | | | | | | | | | | | | | | | 10 | | | | | | | | | | | | | | | | | |
| 8 | cDNA FLJ58802, highly similar to ... | 82 kDa | | | | | | | | | 9 | | | | | | | | 8 | | | | | | | | | | | | | | | | | |
| 9 | T-complex protein 1 subunit beta ... | 57 kDa | | | | | | | | | | | | | | | | | 8 | | | | | | | | | | | | | | | | | |
| 10 | cDNA FLJ37398 fis, clone BRAMY2... | 46 kDa | | | | | | | | | | | | | | | | | 7 | | | | | | | | | | | | | | | | | |
| 11 | Isoform 2 of WD repeat-containin... | 70 kDa | | | | | | | | | | | | | | | | | 7 | | | | | | | | | | | | | | | | | |
| 12 | cDNA FLJ54209 n=1 Tax=Homo s... | 17 kDa | | | | | | | | | | | | | | | | | 7 | | | | | | | | | | | | | | | | | |
| 13 | Protein C20orf11 n=1 Tax=Homo ... | 27 kDa | | | | | | | | | | | | | | | | | 7 | | | | | | | | | | | | | | | | | |
| 14 | ADP/ATP translocase 2 n=1 Tax=... | 33 kDa | | | | | | | | | | | | | | | | | 6 | | | | | | | | | | | | | | | | | |
| 15 | cDNA FLJ55427, highly similar to ... | 113 kDa | | | | | | | | | | | | | | | | | 6 | | | | | | | | | | | | | | | | | |
| 16 | Biotin carboxylase n=4 Tax=Hom... | 266 kDa | | | | | | | | | | | | | | | | | 6 | | | | | | | | | | | | | | | | | |
| 17 | Putative uncharacterized protein ... | 56 kDa | | | | | | | | | | | | | | | | | 6 | | | | | | | | | | | | | | | | | |
| 18 | cDNA FLJ53379, highly similar to ... | 58 kDa | | | | | | | | | | | | | | | | | 6 | | | | | | | | | | | | | | | | | |
| 19 | Propionyl Coenzyme A carboxylas... | 61 kDa | | | | | | | | | | | | | | | | | 5 | | | | | | | | | | | | | | | | | |
| 20 | cDNA FLJ78433, highly similar to ... | 60 kDa | | | | | | | | | | | | | | | | | 5 | | | | | | | | | | | | | | | | | |
| 21 | Heat shock protein HSP 90-beta n... | 83 kDa | 4 | | | | | | | | | | | | 30 | | | | 5 | | | | | | | | | | | | | | | | | |
| 22 | cDNA FLJ75516, highly similar to ... | 68 kDa | | | | | | | | | | | | | | | | | 5 | | | | | | | | | | | | | | | | | |
| 23 | Microtubule-associated protein R... | 30 kDa | 2 | | | 2 | | | | | | | | | | | | | 5 | 3 | 3 | 2 | | | | | | | | | | | | | | |
| 24 | Elongation factor 1-alpha n=1 Ta... | 43 kDa | | | | | | | | | | | | | | | | | 5 | | | | | | | | | | | | | | | | | |
| 25 | T-complex protein 1 subunit delta... | 52 kDa | | | | | | | | | | | | | | | | | 4 | | | | | | | | | | | | | | | | | |
| 26 | Ran-binding protein 10 n=1 Tax=... | 67 kDa | | | | | | | | | | | | | | | | | 4 | | | | | | | | | | | | | | | | | |
| 27 | Ataxin-2-like protein n=1 Tax=H... | 113 kDa | | | | | | | | | | | | | | | | | 3 | | | | | | | | | | | | | | | | | |
| 28 | Putative uncharacterized protein ... | 72 kDa | 5 | 2 | 5 | 5 | | 4 | 5 | 6 | 2 | 5 | 6 | 6 | 2 | 4 | 5 | 6 | 3 | 5 | 5 | 6 | 4 | 6 | 6 | 6 | 6 | 3 | 5 | 6 | | | | 4 | 5 | |
| 29 | Isoform 2 of Protein RMD5 homol... | 11 kDa | | | | | | | | | | | | | | | | | 3 | | | | | | | | | | | | | | | | | |
| 30 | Sarcoplasmic/endoplasmic reticu... | 115 kDa | | | | | | | | | | | | | | | | | 3 | | | | | | | | | | | | | | | | | |
| 31 | cDNA FLJ45675 fis, clone DFNES20... | 25 kDa | | | | | | | | | | | | | | | | | 3 | | | | | | | | | | | | | | | | | |
| 32 | Probable ATP-dependent RNA heli... | 54 kDa | | | | | | | | | | | | | | | | | 3 | | | | | | | | | | | | | | | | | |
| 33 | cDNA, FLJ95068, highly similar to ... | 71 kDa | | | | | | | | | | | | | | | | | 3 | | | | | | | | | | | | | | | | | |
| 34 | Pyruvate carboxylase, mitochond... | 130 kDa | | | | | | | | | | | | | | | | | 3 | | | | | | | | | | | | | | | | | |
| 35 | Protein arginine methyltransfera... | 71 kDa | | | | | | | | | | | | | | | | | 3 | | | | | | | | | | | | | | | | | |
| 36 | cDNA FLJ77281, highly similar to ... | 43 kDa | | | | | | | | | | | | | | | | | 2 | | | | | | | | | | | | | | | | | |
| 37 | cDNA, FLJ96465, highly similar to ... | 40 kDa | | | | | | | | | | | | | | | | | 2 | | | | | | | | | | | | | | | | | |
| 38 | FabQX117 Light Chain Fragment n... | 24 kDa | 2 | | | | 2 | | | | | | | | | | | | 2 | | | | | | | | | | | | | | | | | |

Protein Information:

Lookup Accession Number In:

Preferred Accession Number:

Protein Name:

Sample Information:

Biological Sample:

Sample Category:

Sample Description:

MS/MS Sample:

MS/MS Sample Notes:

132 Proteins
0.2% Prot %FDR
4060 Spectra
2.8% Pept %FDR

Eberhard-Karls-Universität
UKT
Universitätsklinikum Tübingen

Dept.
Protein
Science

FelhmholtzZentrum munchen
German Research Center for Environmental Health

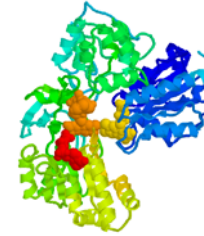
Protein quantification by MS

MS-based quantification: Principle

State A



State B

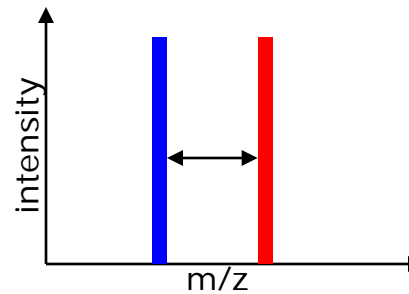


Introduction of a light and HEAVY label before or after cleavage

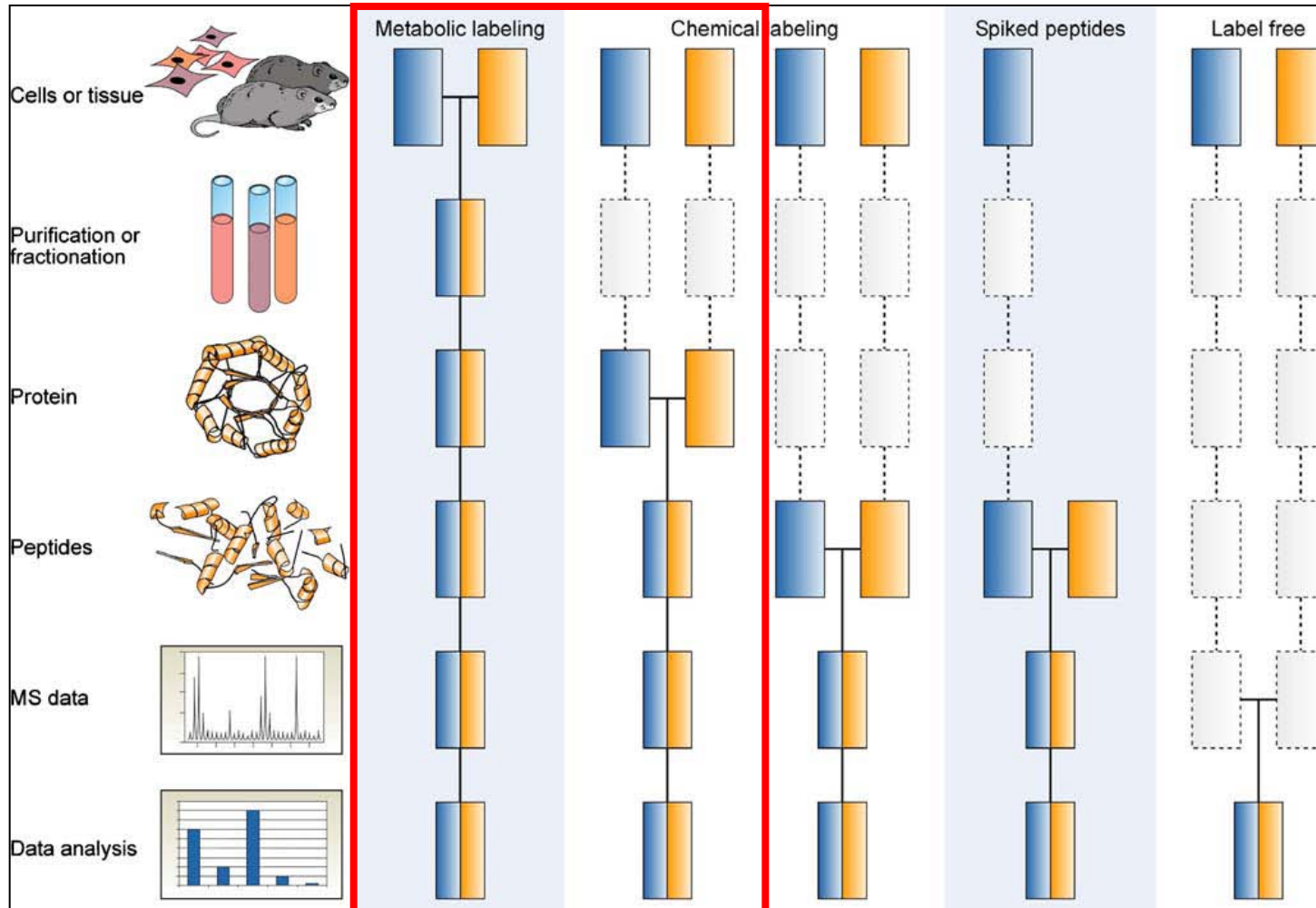


Mix

Relative quantification

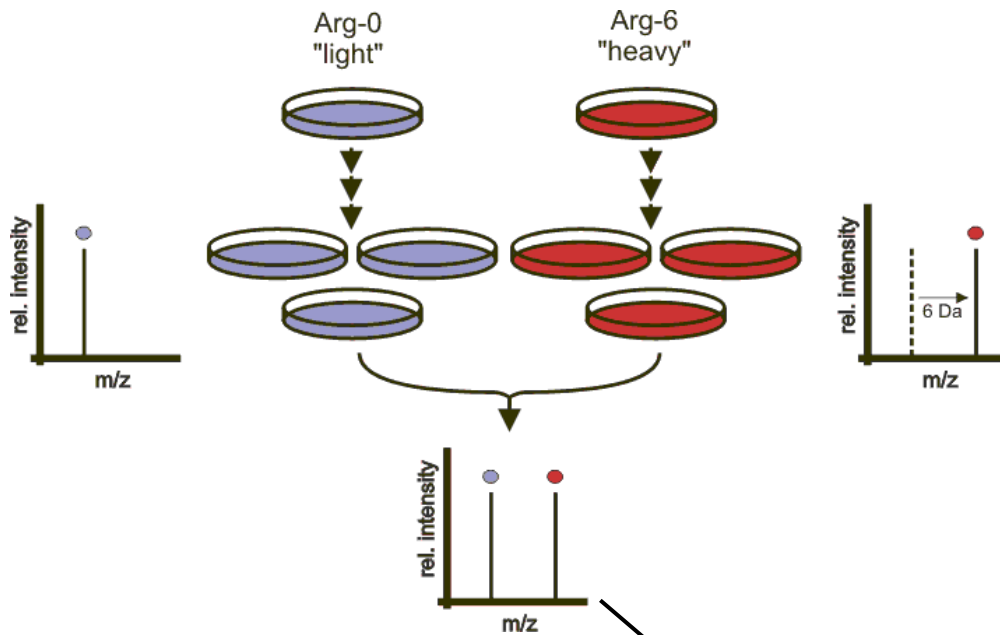


MS-based quantification: an overview

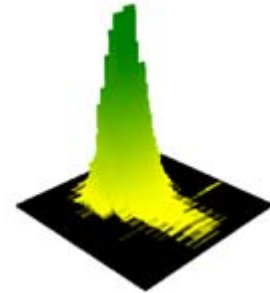


Banscheff et al., Anal. Bioanal. Chem., 2007

MS-based quantification by SILAC

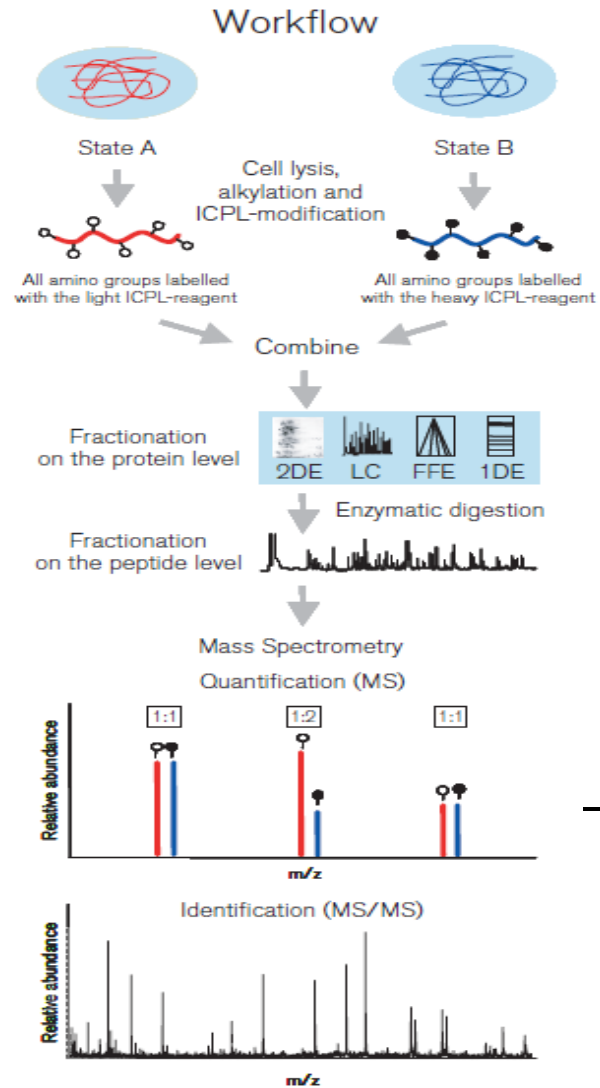


Software based quantification and identification

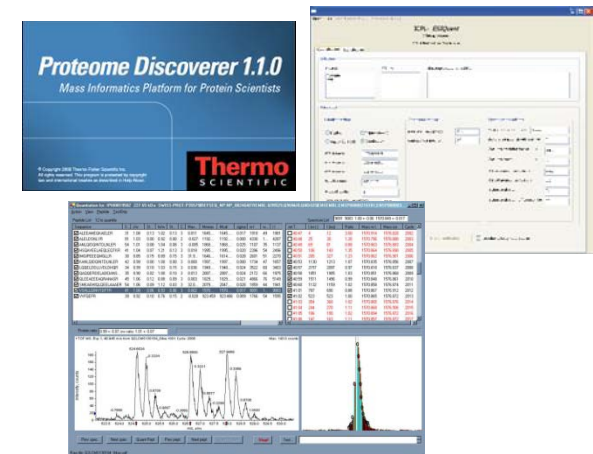


MaxQuant
Cox J, Mann M. Nat Biotechnol. 2008 Dec; 26(12): 1367-72.

MS-based quantification by ICPL



Software based quantification and identification



MSQuant: Mortensen et al.
J Proteome Research, 2010

ICPLQuant: Brunner et al.
Proteomics, 2010

Proteome Discoverer:
ThermoScientific





Andreas Vogt



Norbert Kinkl



Johannes Gloeckner

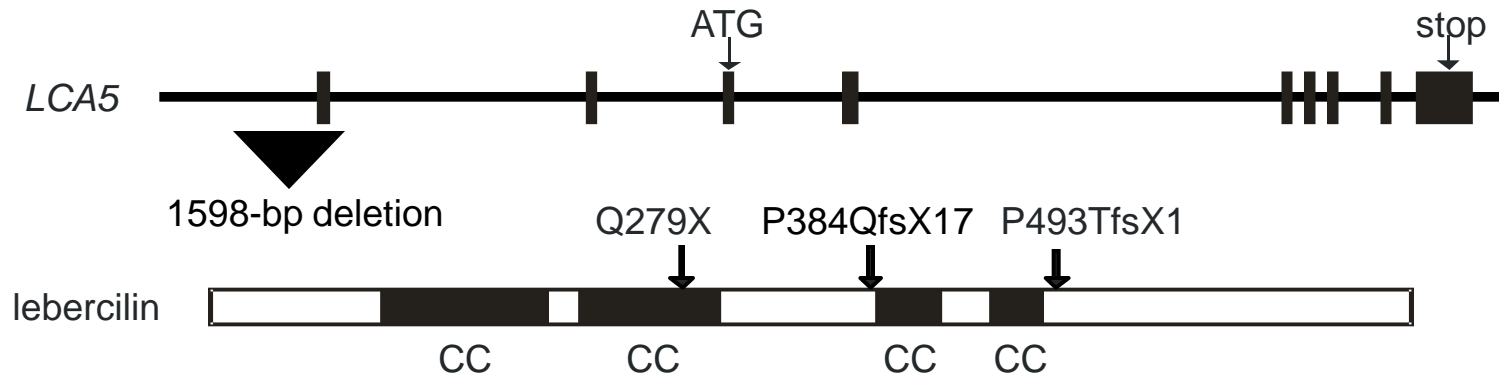
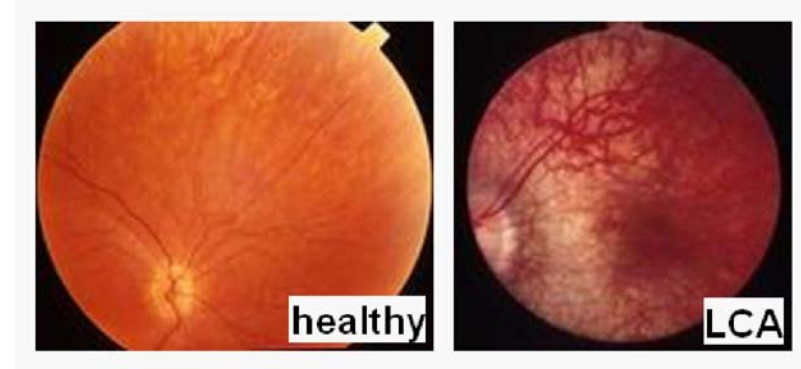


Ronald Roepman

Protein complex analysis of Lebercilin

Mutations in the lebercilin gene cause LCA

- Autosomal recessive
- Severe visual impairment shortly after birth
- Loss of photoreceptors outer segments



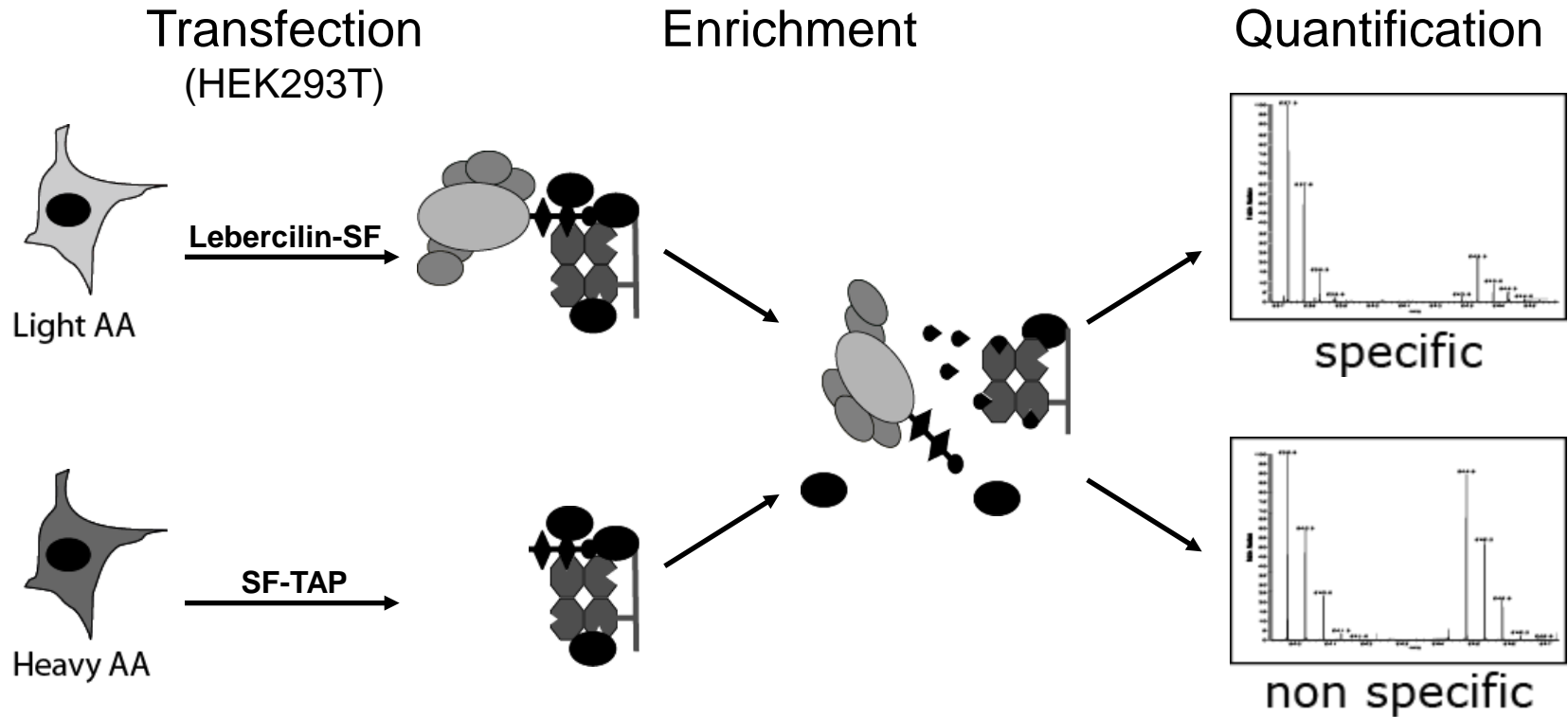
- Ronald Roepman/
Anneke den Hollander → P493TfsX1 and Q297X
- Chris Inglehearn → P384QfsX17
- Irene Maumenee → 1598-bp Promotor Deletion im *LCA5* Promoter

Den Hollander et al. Nat Genet. 2007 Jul; 39(7):889-95.

What does Lebercilin do?

Detection of specific complex components by quantitative protein complex analysis

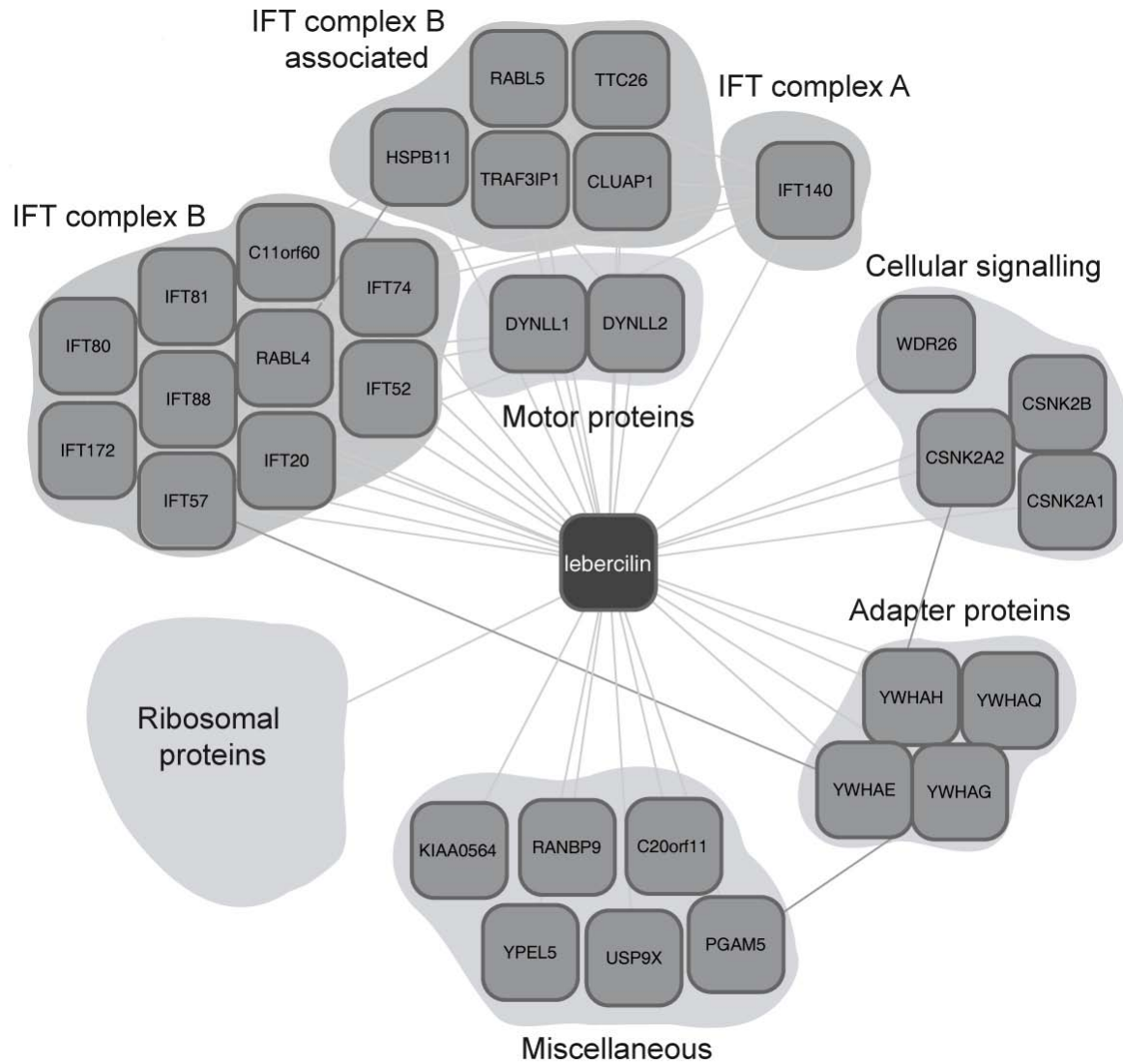
Detection of specific complex components



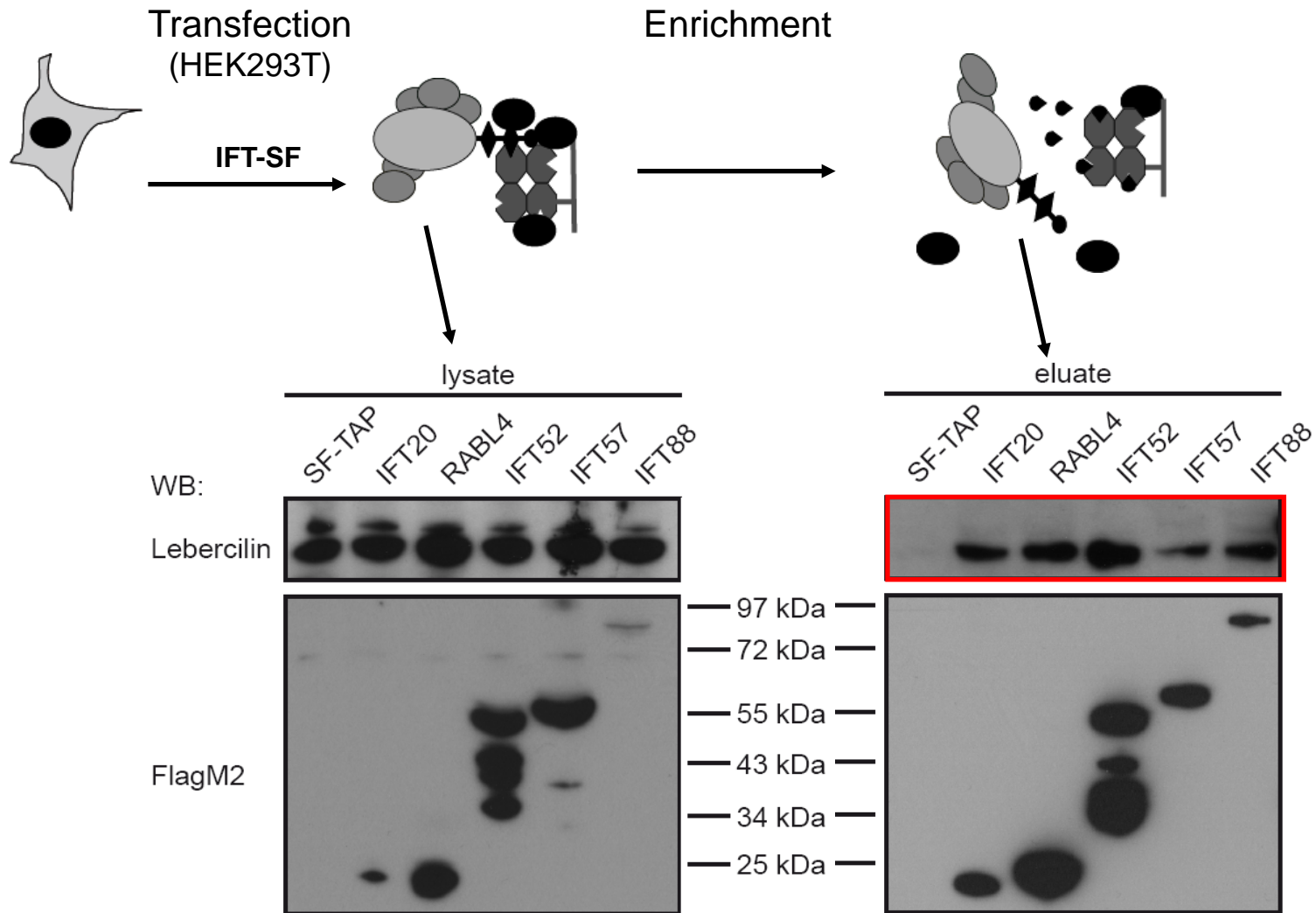
MaxQuant

Cox J, Mann M. Nat Biotechnol. 2008
Dec; 26(12):1367-72.

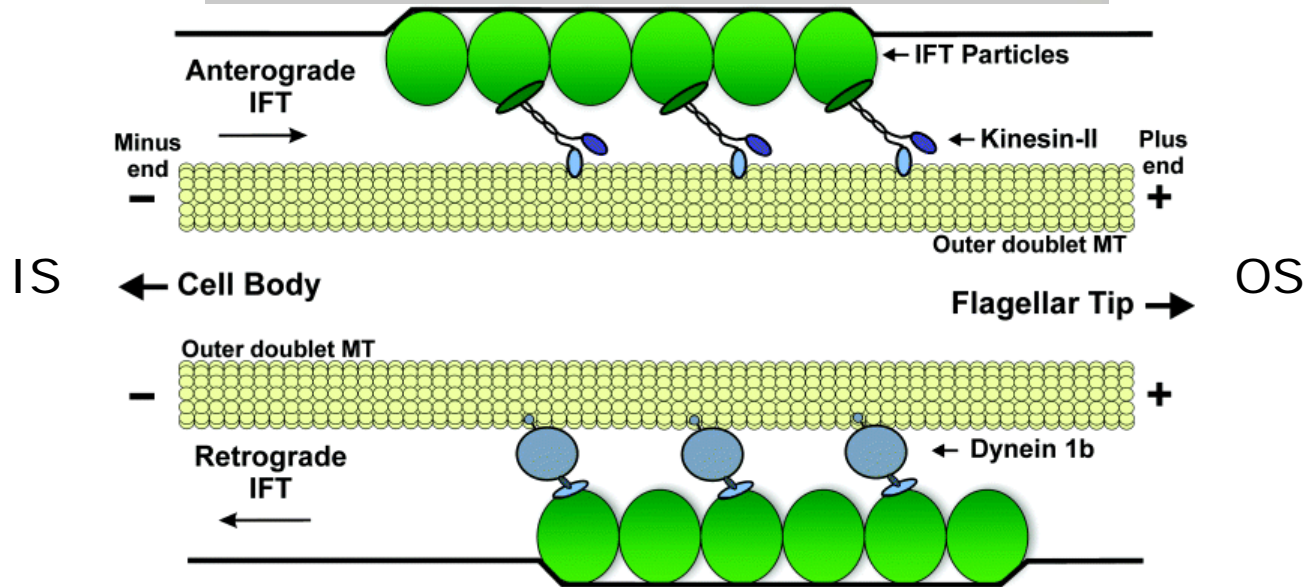
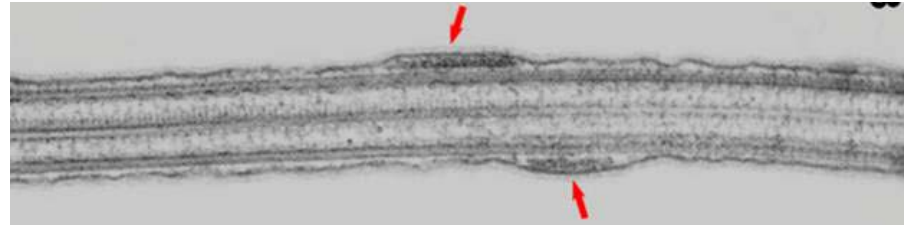
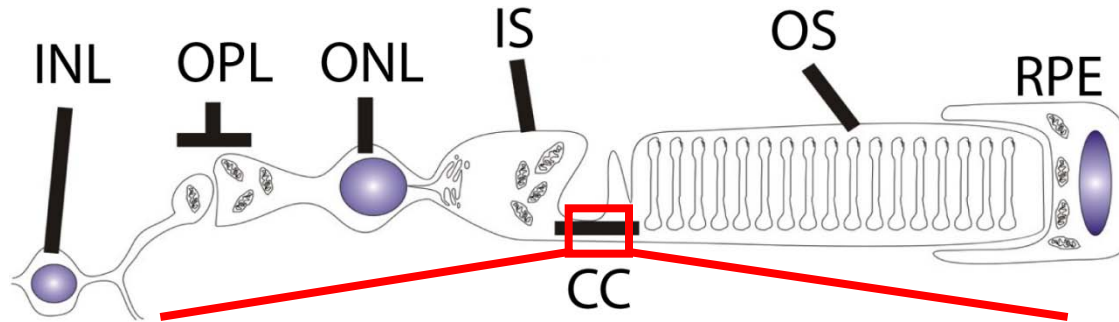
Enrichment of specific interactors



Confirmation of Lebercilin-IFT interaction



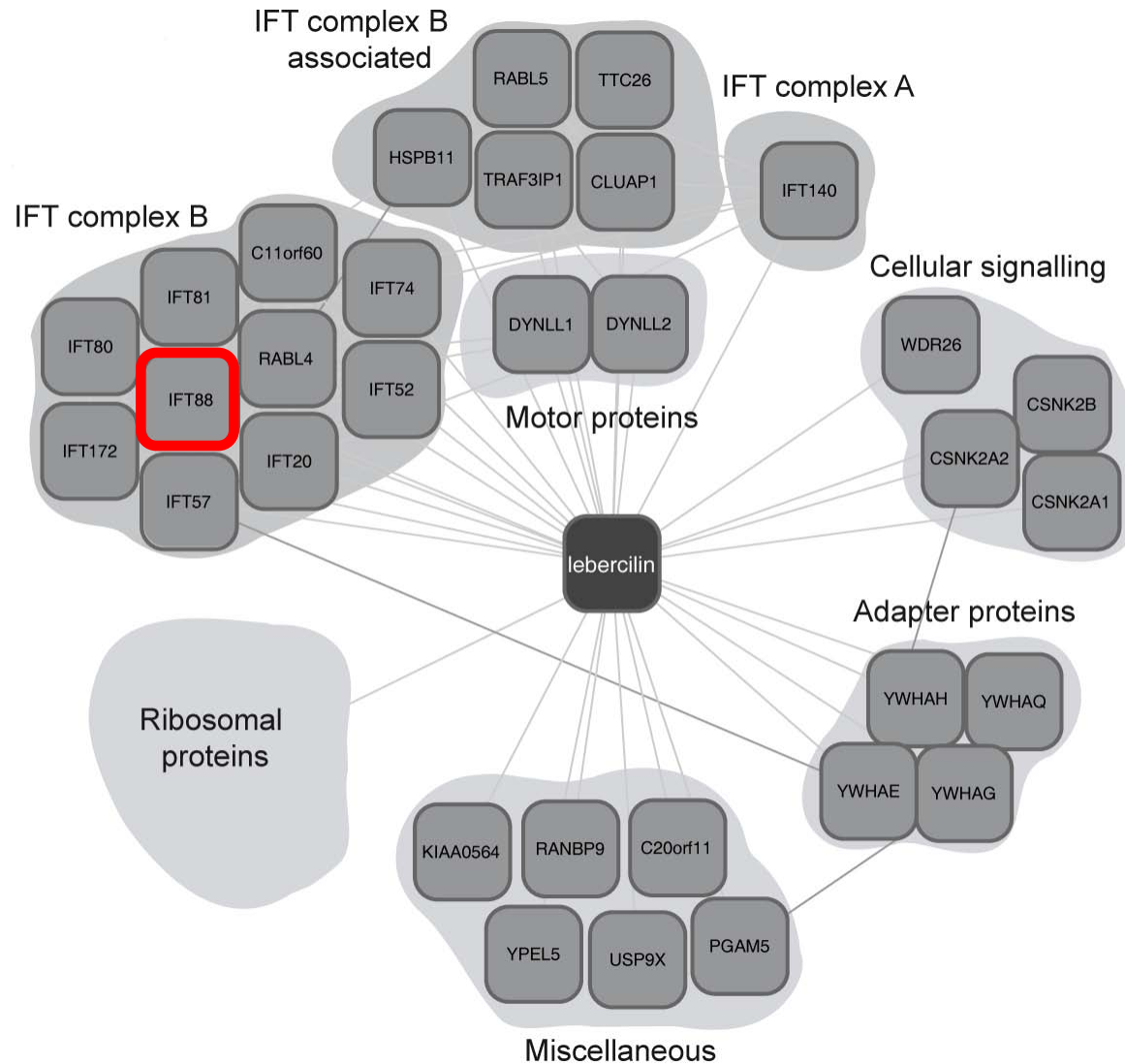
Intraflagellar transport in photoreceptors



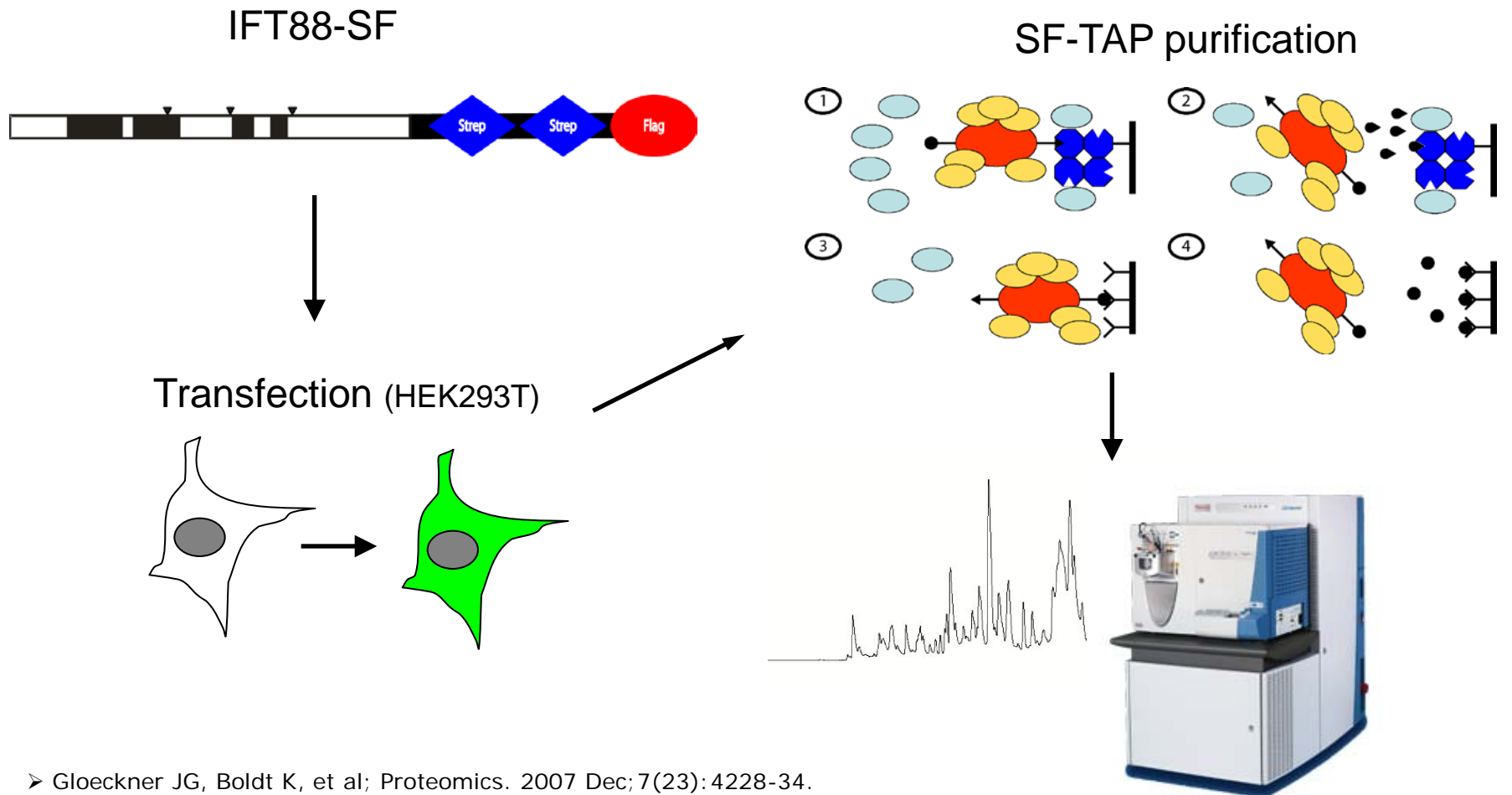
Pedersen et al.,
2006, Curr. Biol.

Cole, 2003,
Traffic

Characterization of the IFT complex B in HEK293T cells

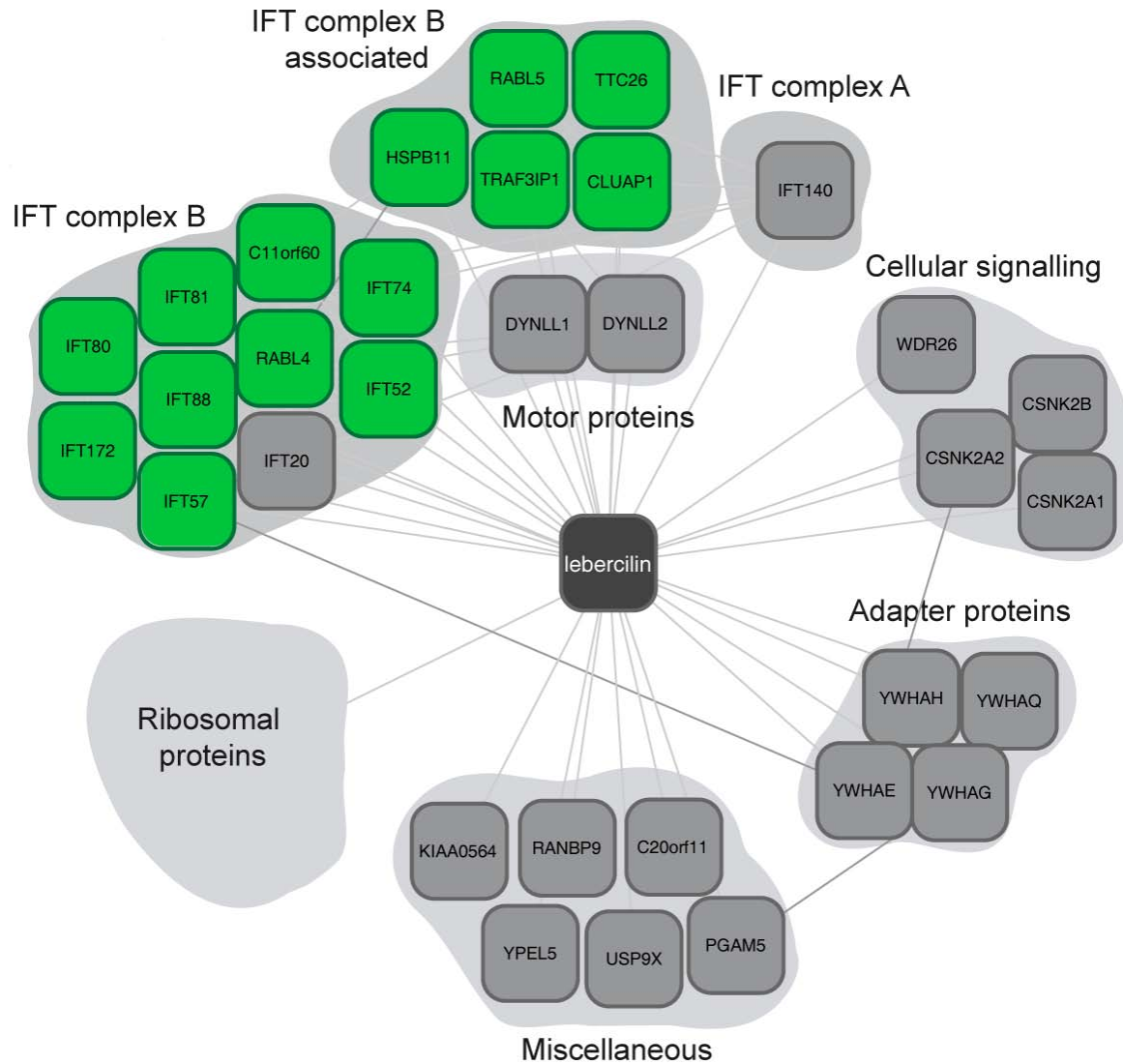


Analysis of the Iebercilin complex by SF-TAP



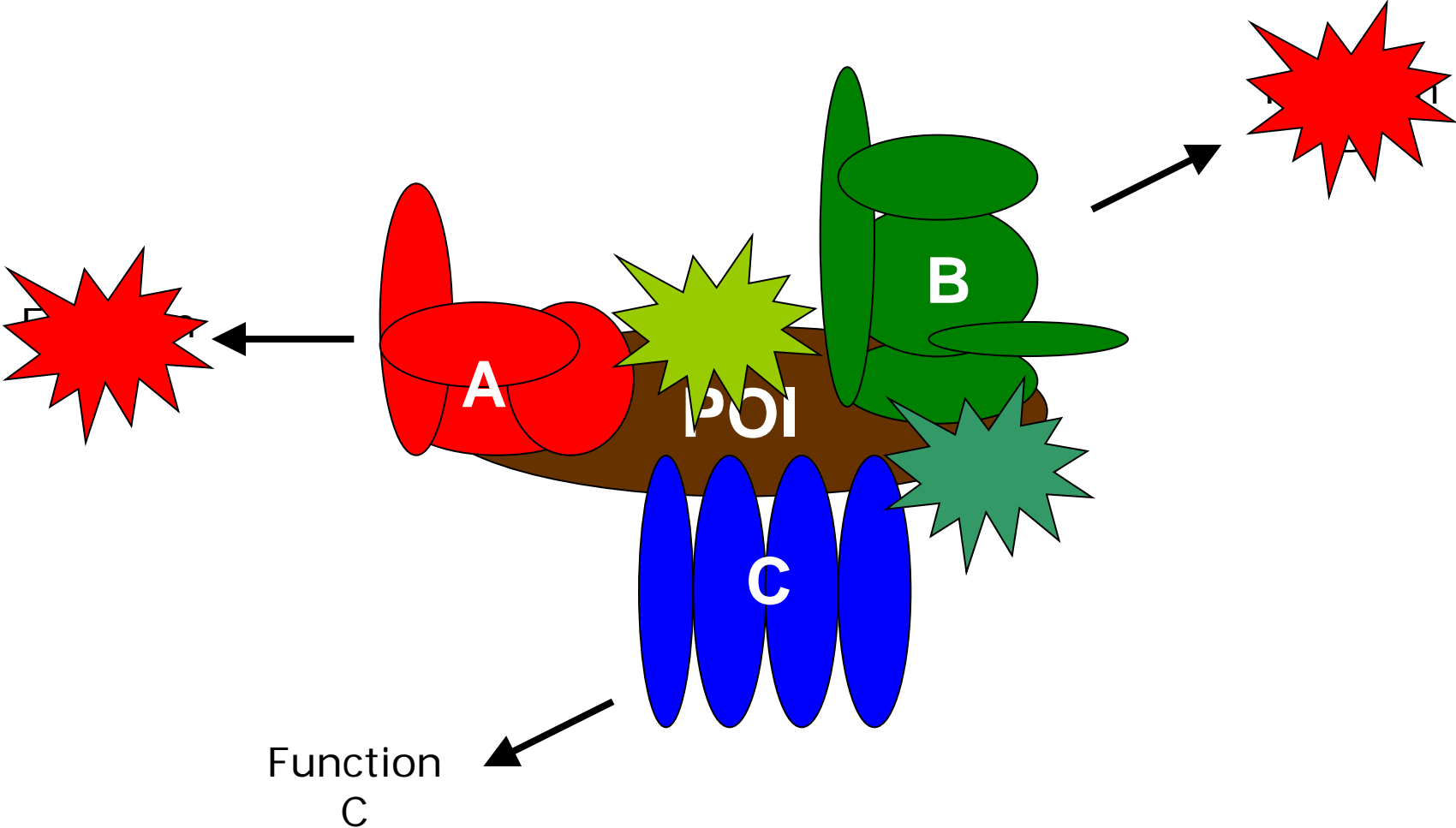
- Gloeckner JG, Boldt K, et al; Proteomics. 2007 Dec; 7(23): 4228-34.
- Den Hollander et.al.; Nat. Genet. 2007 Jul; 39(7): 889-95

The lebercilin-IFT protein complex

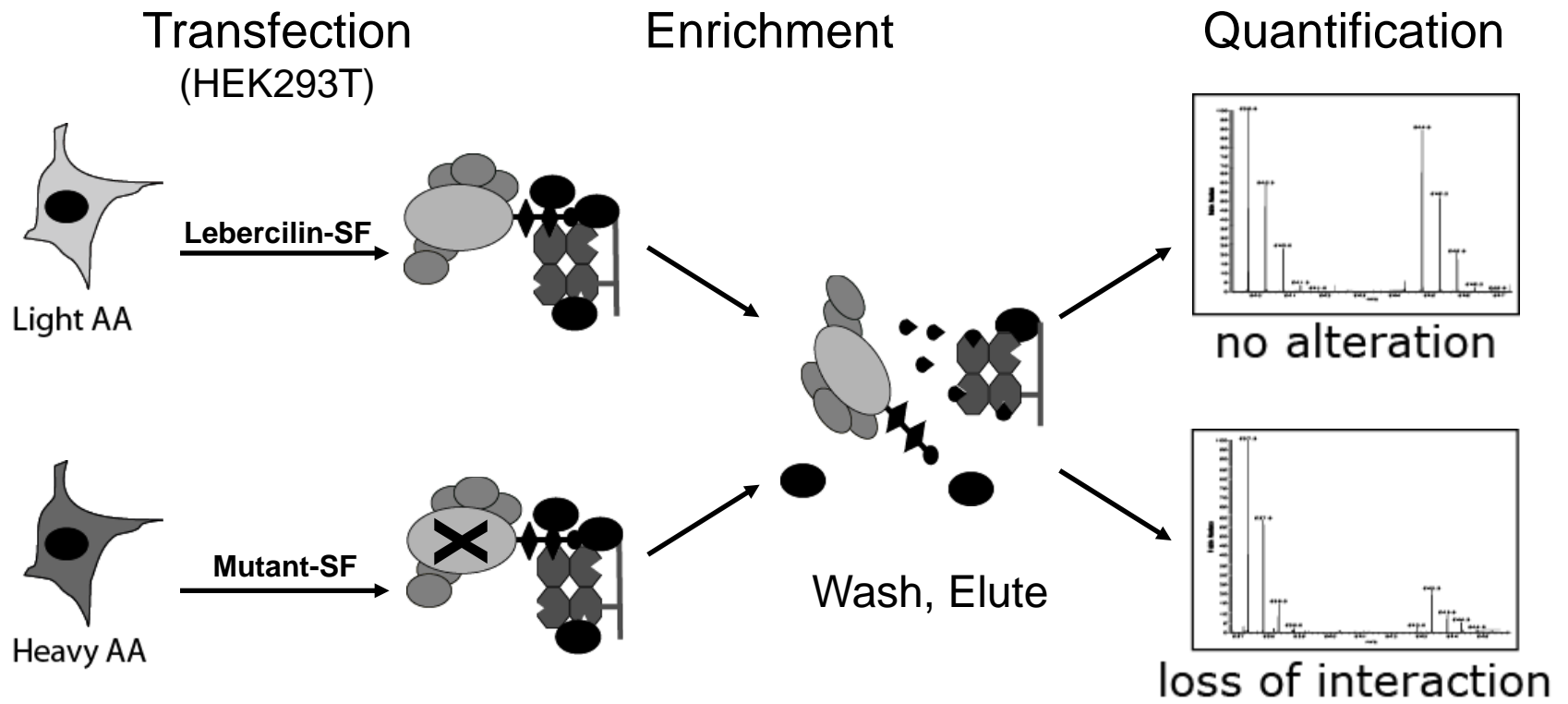


Which effect do mutations have on the complex?

Protein complex alterations due to mutation



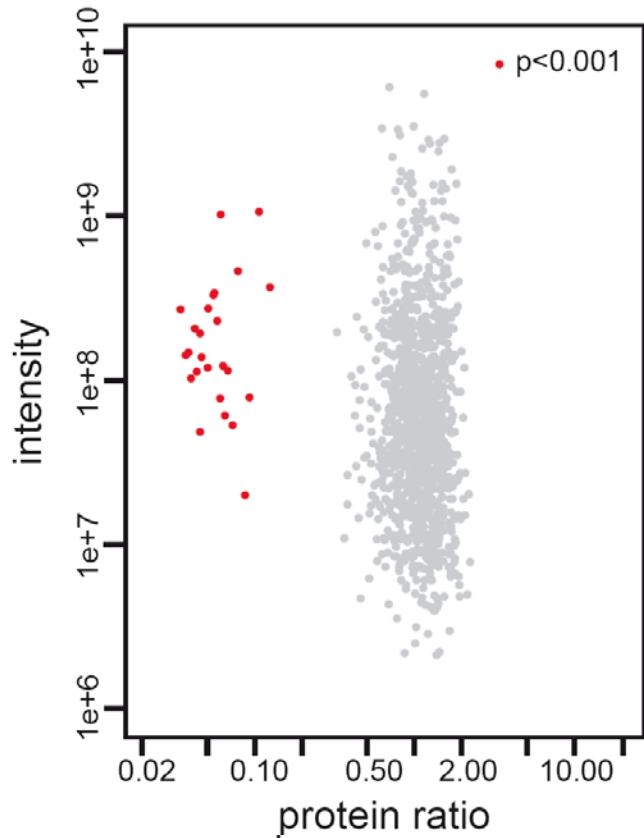
Protein complex comparison



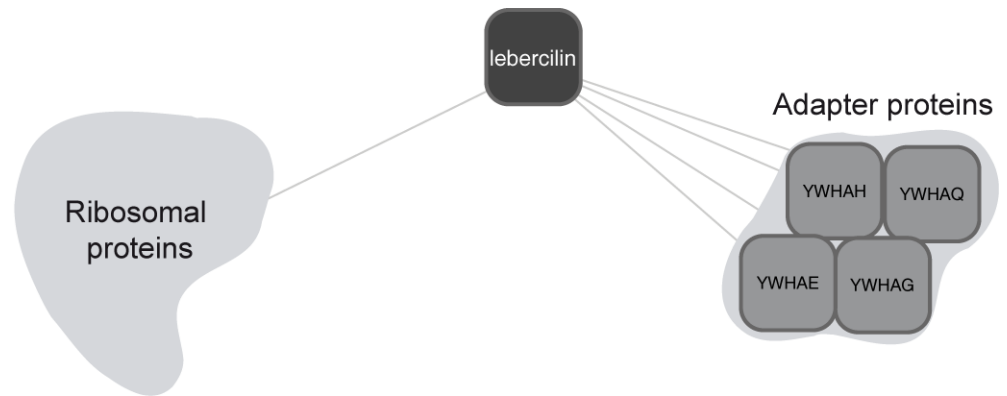
MaxQuant
Cox J, Mann M. Nat Biotechnol. 2008
Dec;26(12):1367-72.

Impact of mutations on the Lebercilin complex

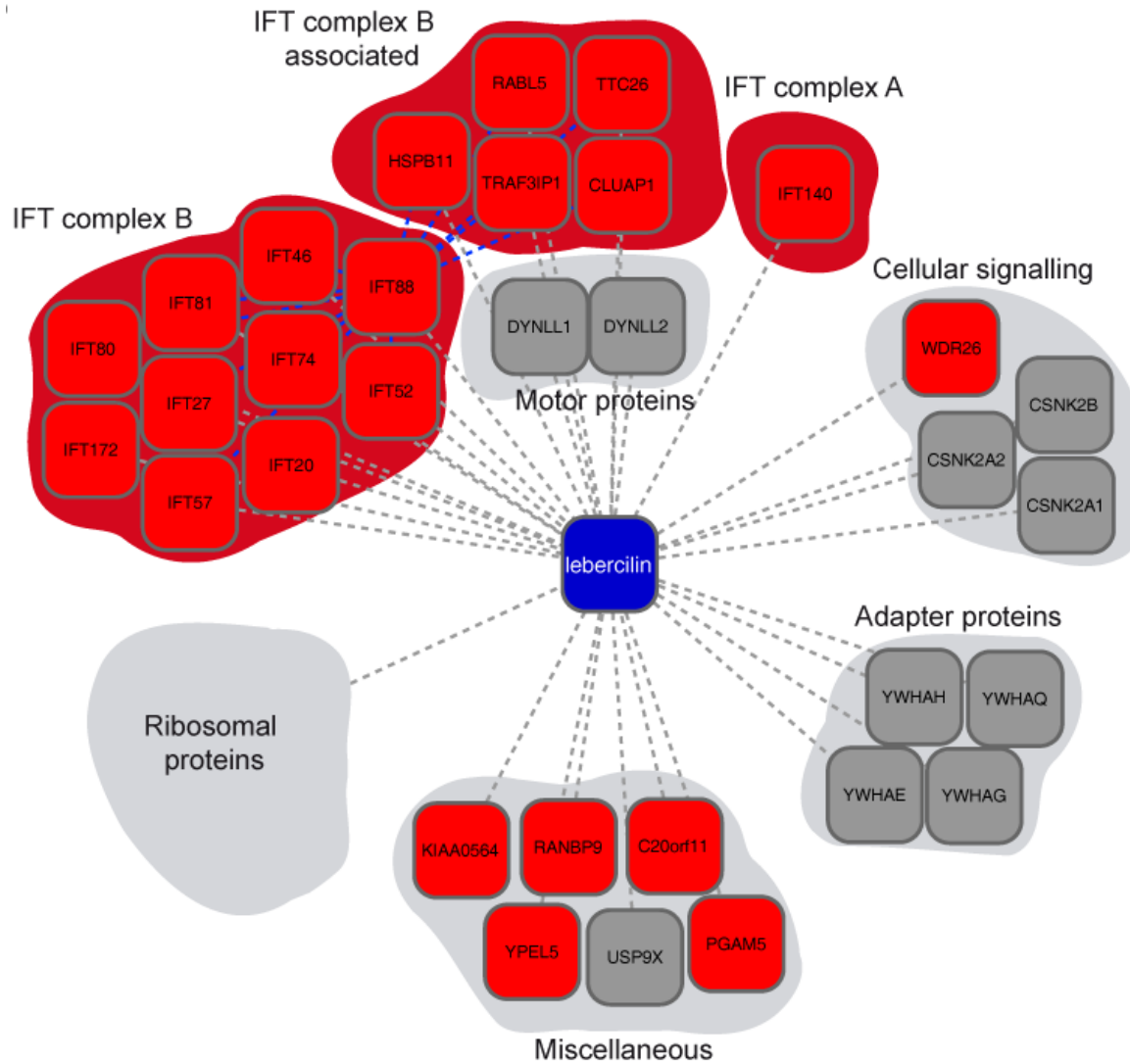
Q1939X 1



Q279X

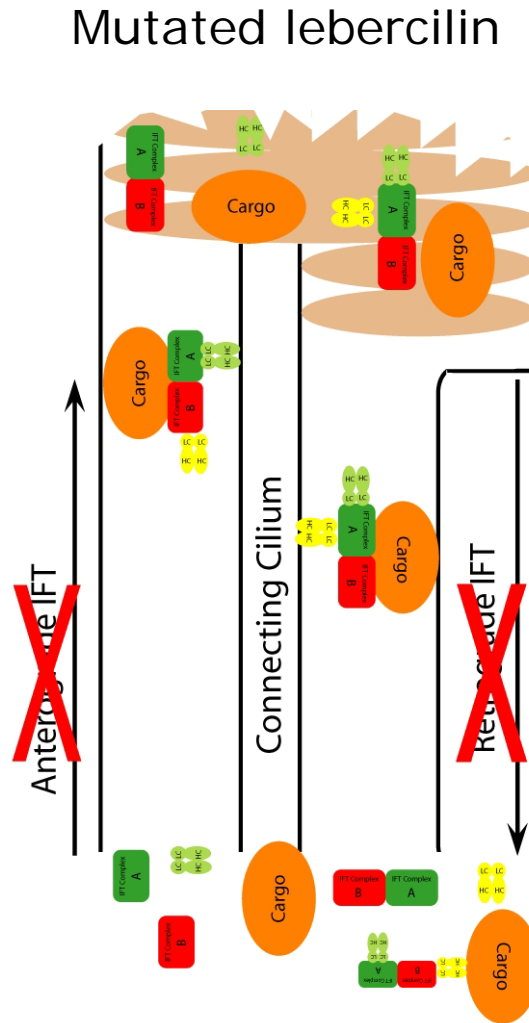
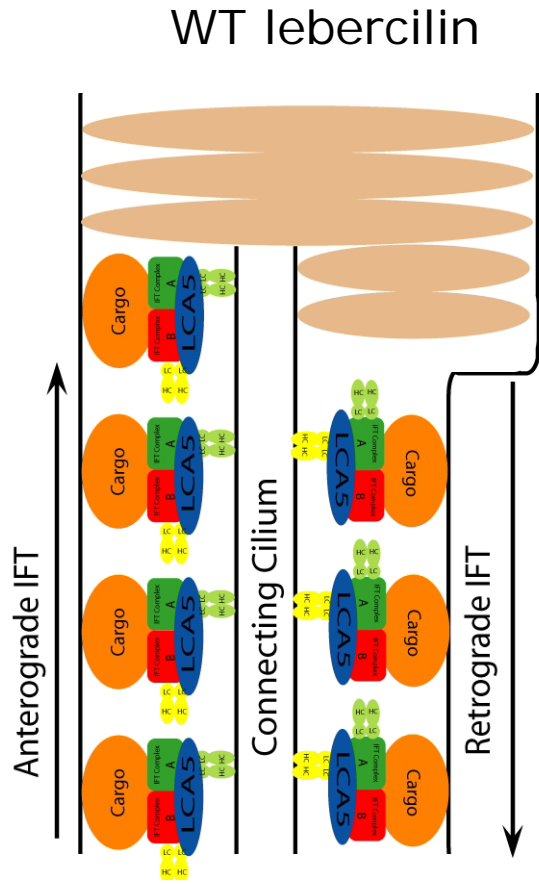


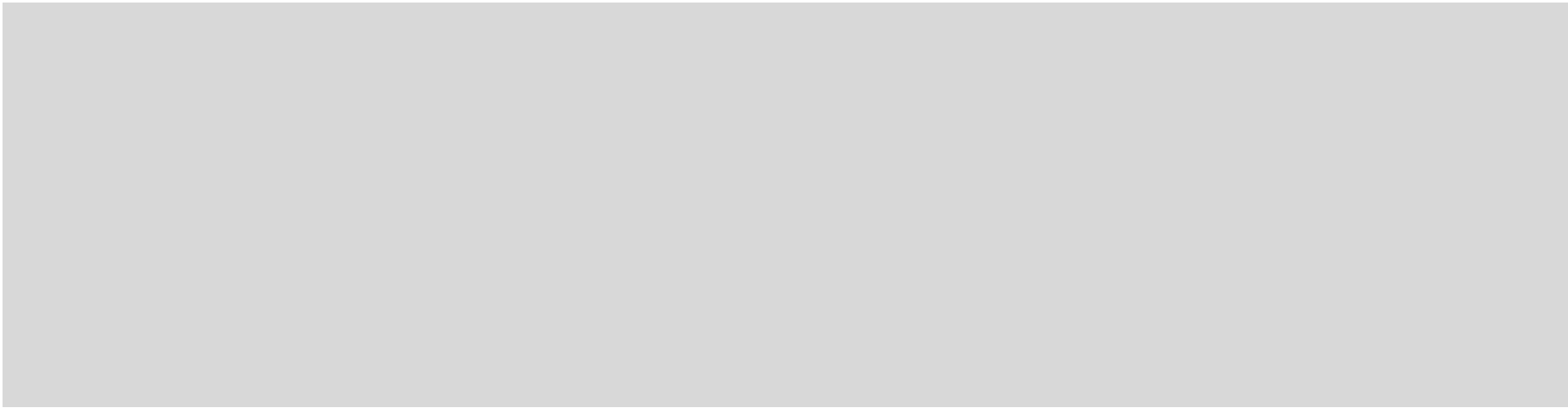
Loss of complex components due to mutation



Boldt et al.
Manuscript in preparation

Loss of Lebercilin's function → Impaired IFT → LCA







Norbert Kinkl



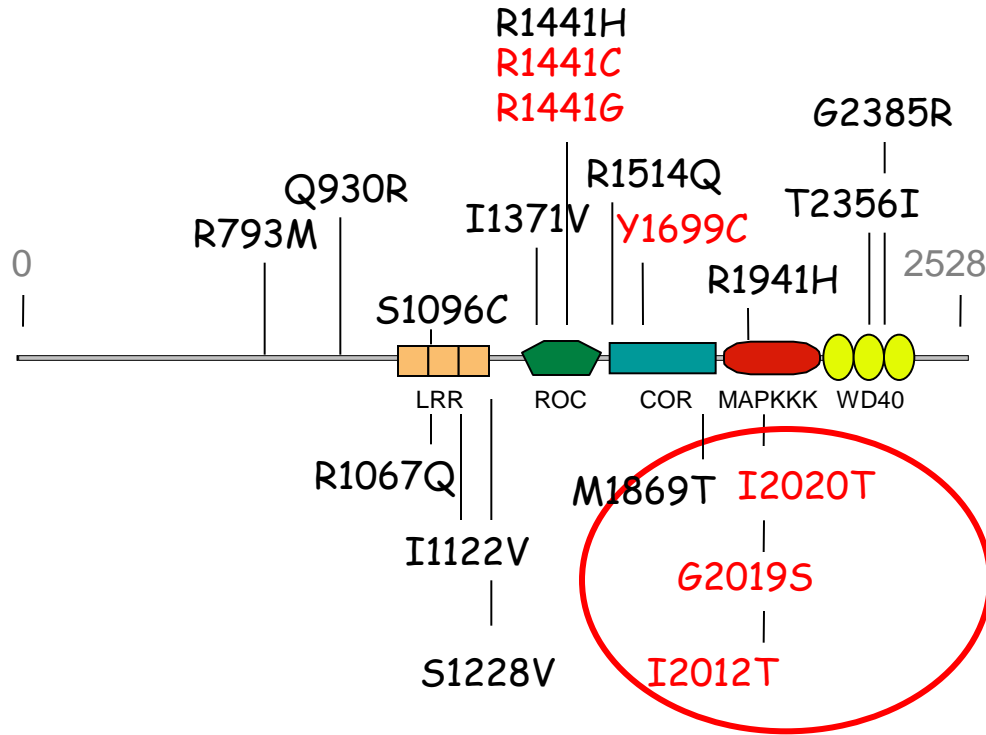
Andrea Meixner



Johannes Gloeckner

LRRK2 auto-phosphorylation and protein complex analysis

LRRK2 mutations associated with Parkinson's disease

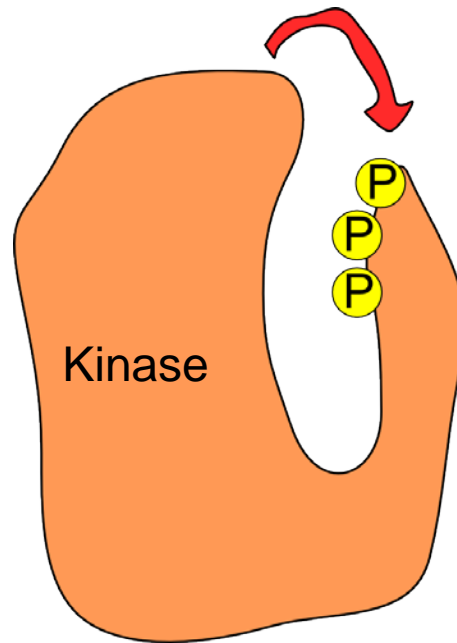


(confirmed segregation, red)

kinase domain

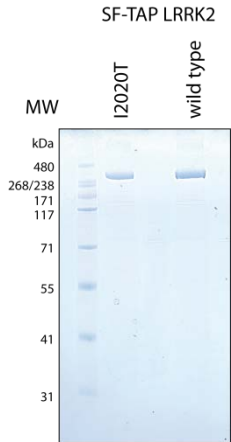
For review see: Taylor et al., 2006

Analysis of LRRK2 autophosphorylation by MS

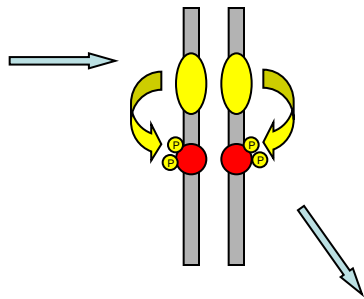


Analytical strategy

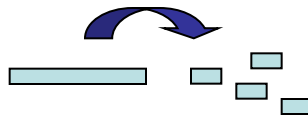
Purification of active LRRK2



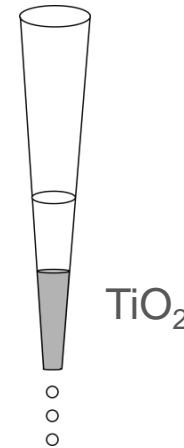
Autokinase assay



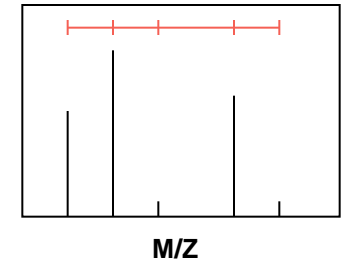
Cleavage



Phosphopeptide enrichment via binding to Titaniumdioxide

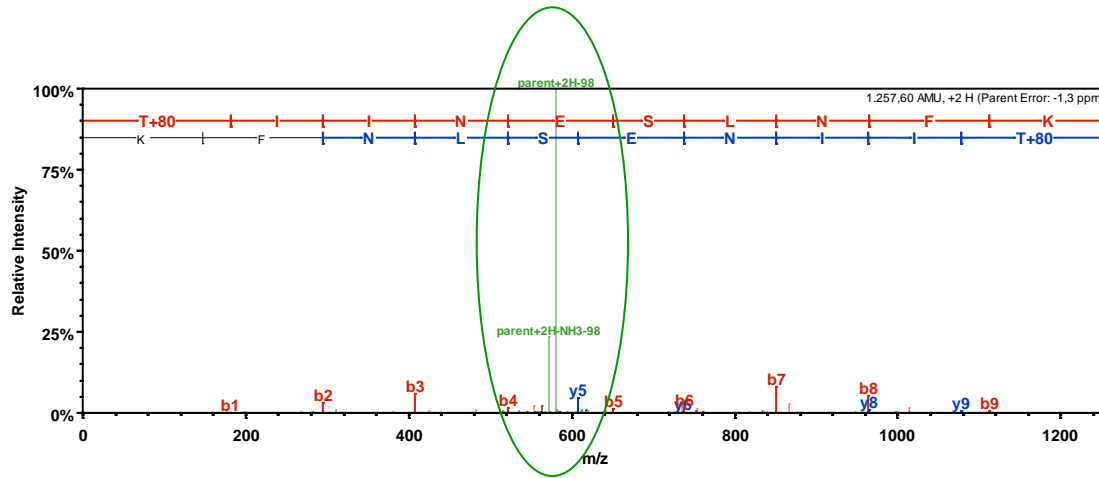


Mass spectrometry



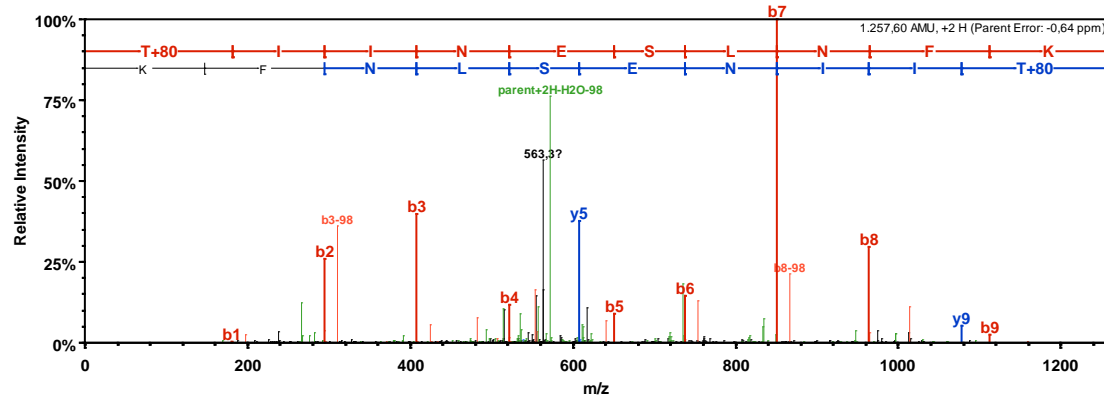
Gloeckner, Boldt et al., J. Proteome Res., 2010

Multi-Stage-Activation for phospho-peptides



MSMS

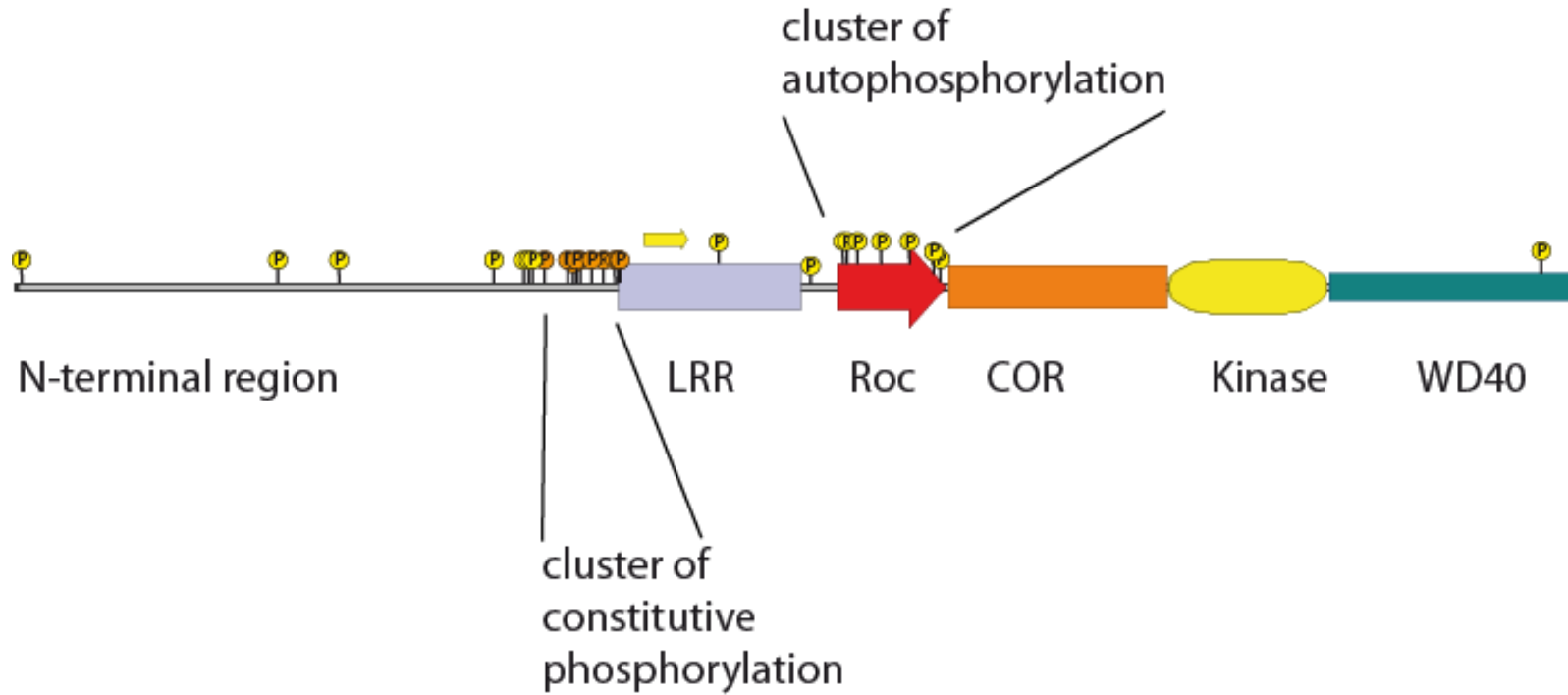
Typical for phosphopeptides:
high neutral loss peak, bad fragmentation
→ Isolate the neutral loss fragment and
perform CID



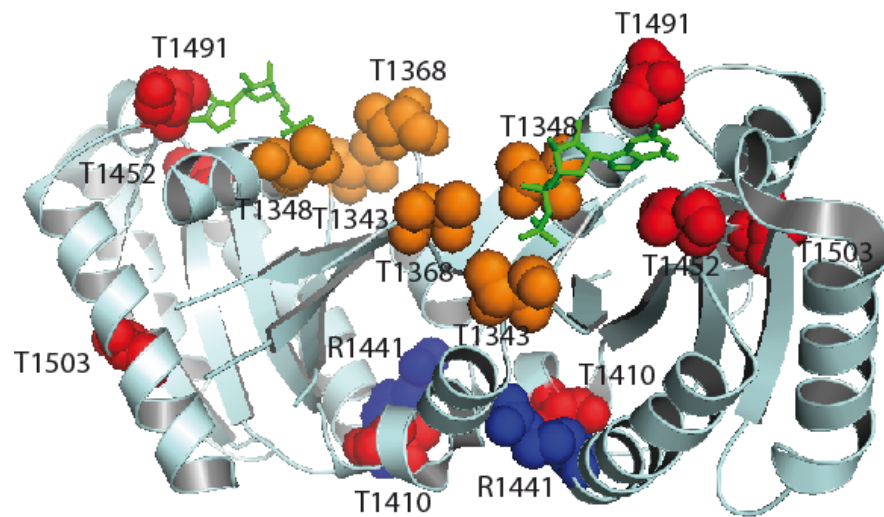
MSA

MSA: better fragment pattern allows mapping of the phosphorylated residue!

Distinct clusters of phosphorylation

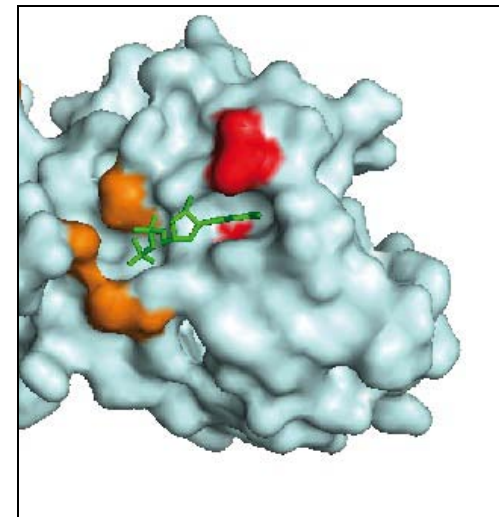


Distinct clusters of phosphorylation at the GTP binding pocket of the Roc domain



- residues mapped unambiguously
- alternative sites (multiphosphorylation possible)
- PD-associated mutation R1441C

GTP binding pocket

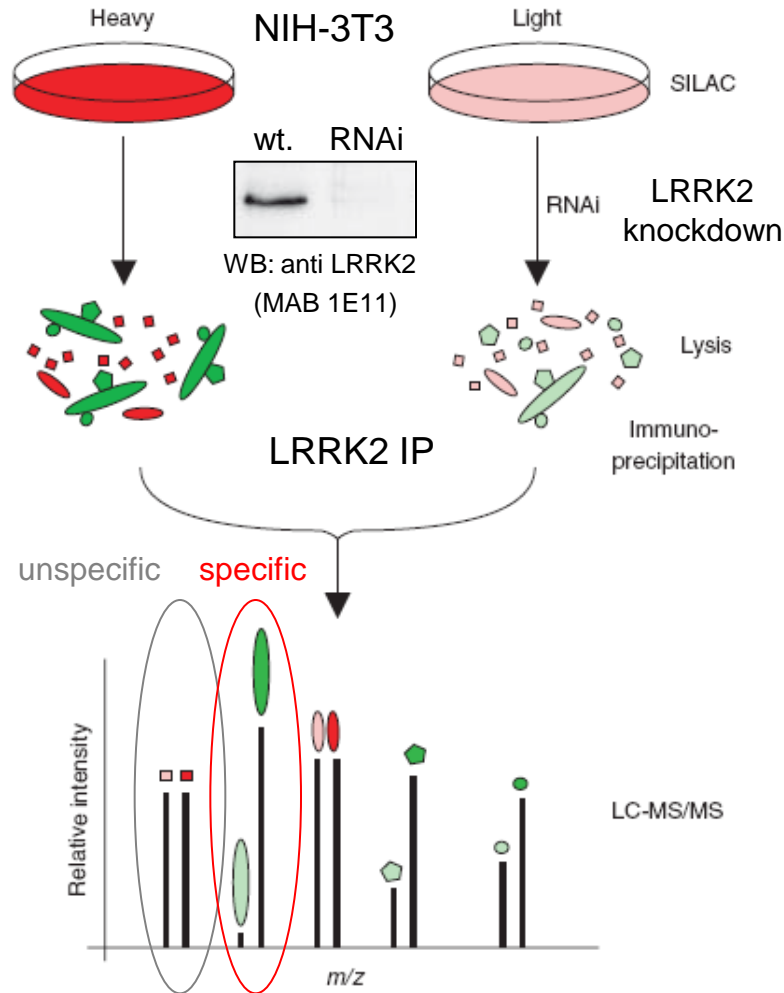


QUICK LRRK2 interaction screen

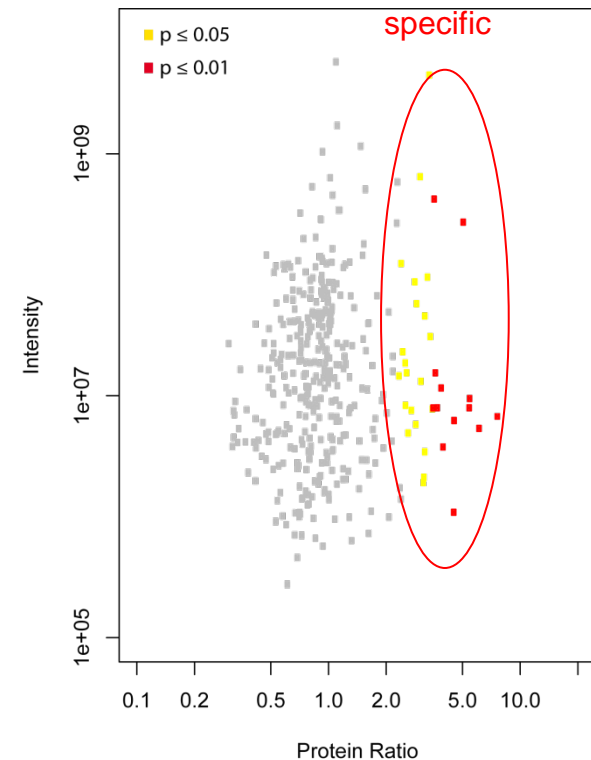
Quantitative Immunoprecipitation combined with knock-down

Quantitative immunoprecipitation combined with knockdown (QUICK)

Selbach, M., and Mann, M. (2006)
Nat Methods 3, 981-983



Ratios „heavy/light“

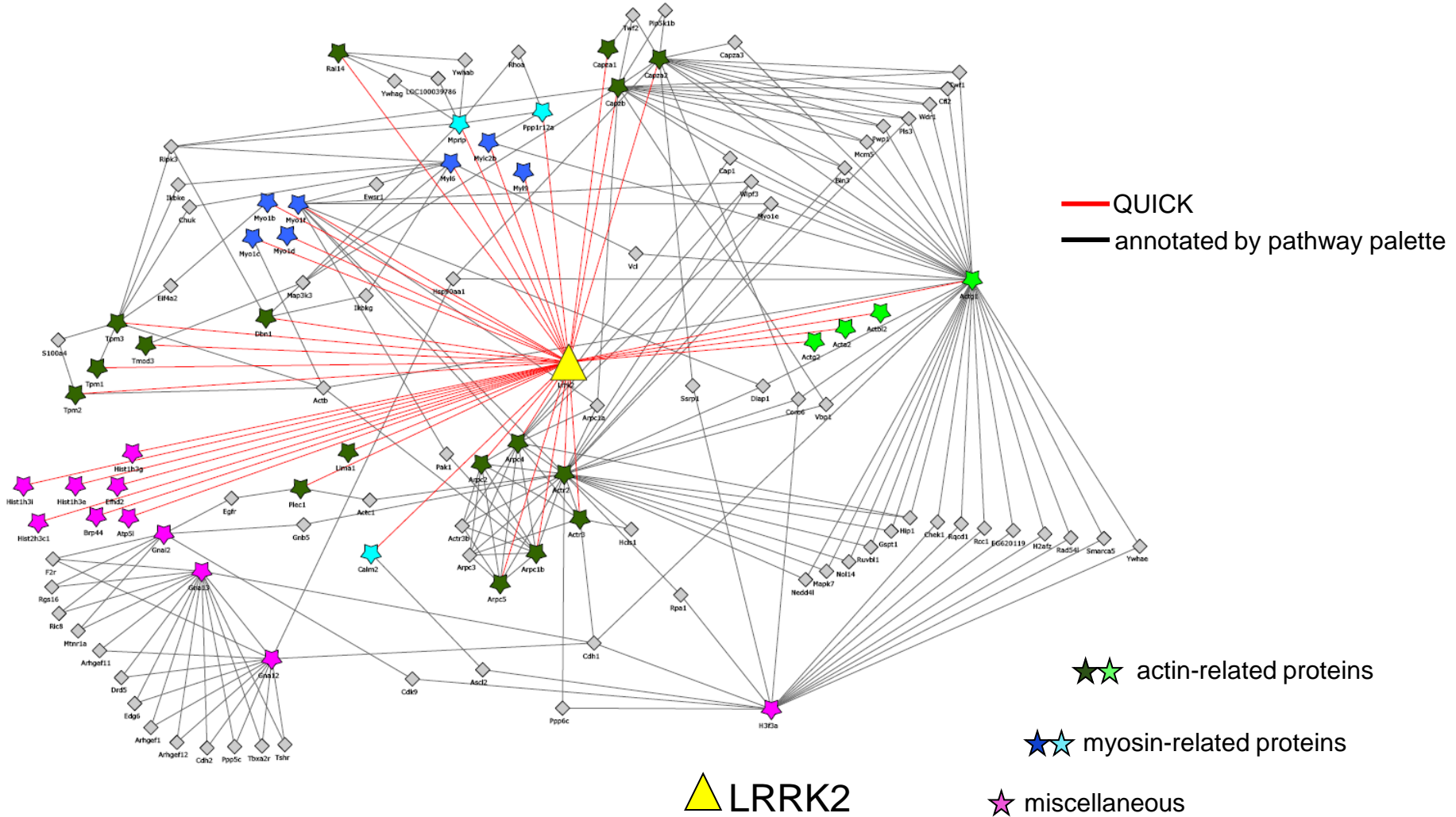


(Meixner, Boldt et al., MCP 2010)

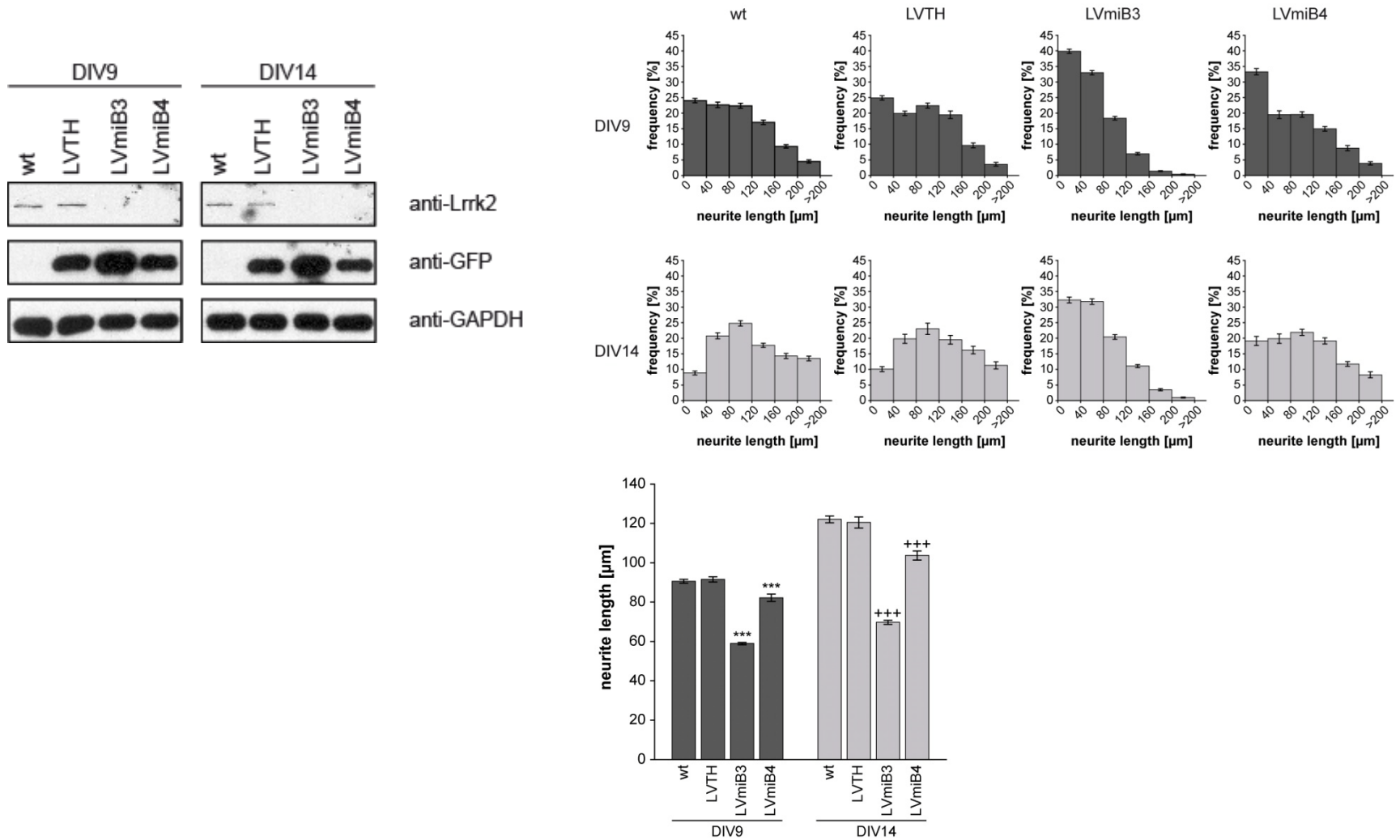
LRRK2 interacts with proteins associated with the actin cytoskeleton

Collaboration with Jarrod A. Mato (Harvard Medical School)

(<http://blaispathways.dfci.harvard.edu/palette.html>)



Knock down of LRRK2 leads to impaired neurite outgrowth in primary VM cultures



Acknowledgements

➤ Tübingen and München

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Sandra Helm

Ludwig Wiesent

➤ Nijmegen

Ronald Roepman

Dorus Mans

Jeroen van Reeuwijk

Stef Letteboer

Anneke den Hollander