

プロテオーム解析における 質量分析技術と応用研究例

Mass Spectrometry-based Proteomics and its Applications

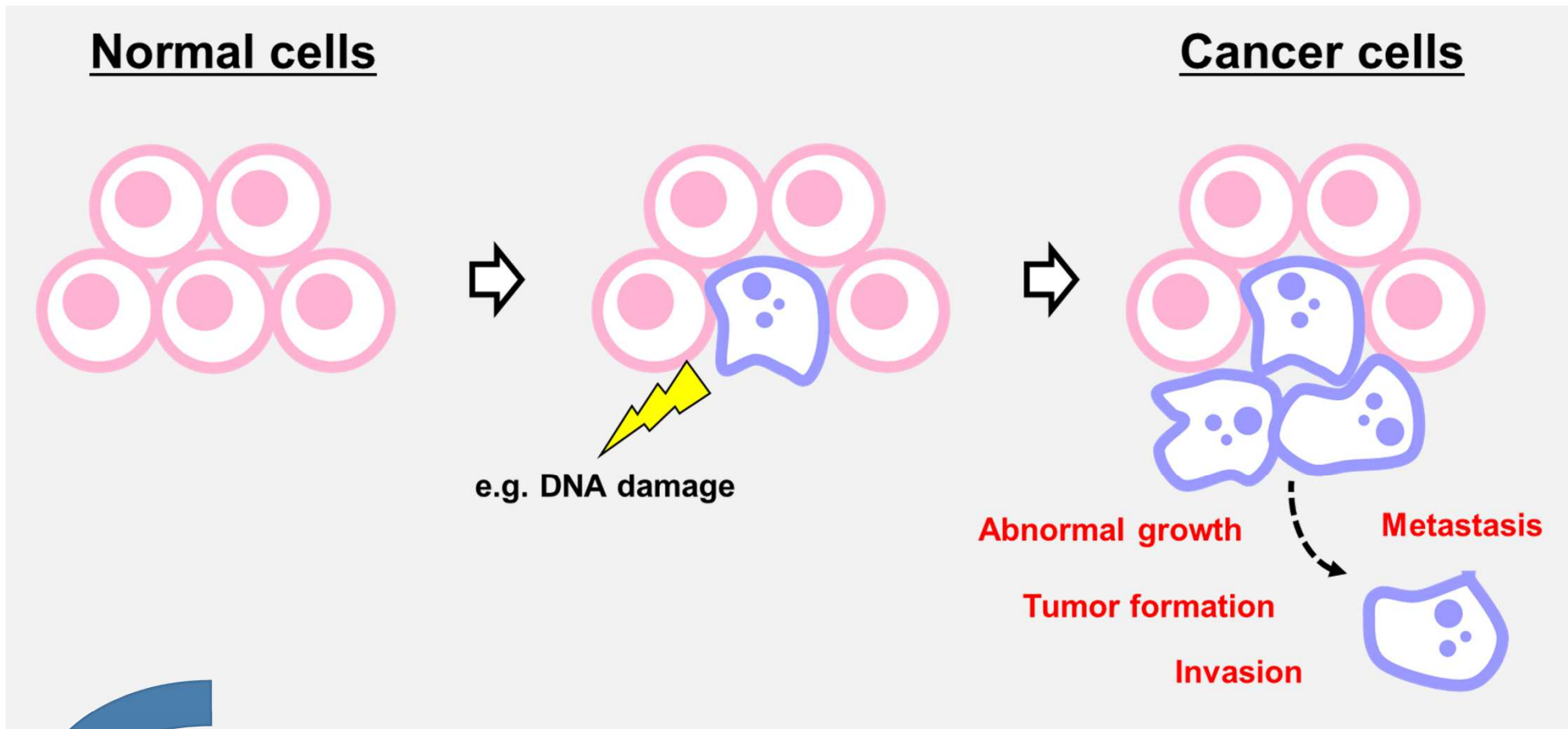
藤田医科大学 研究推進本部
BNCT研究センター
岡西 広樹

Fujita Health University, Research Promotion Headquarter
BNCT Research Center
Hiroki Okanishi

Today's topics

- 1. Mass spectrometry-based proteomics**
- 2. Phosphoproteomics study on cancer cells treated with a molecular targeted drug**
- 3. LC-MS in CoMIT Omics Center (COC)**

Normal cells vs. cancer cells



The expression of proteins which contribute to growth, survival and function is dysregulated in cancer cells.



Analyze expression changes in proteins associated with diseases

How to analyze protein expression

Western blotting, immunocytochemistry Immunohistochemistry, ELISA

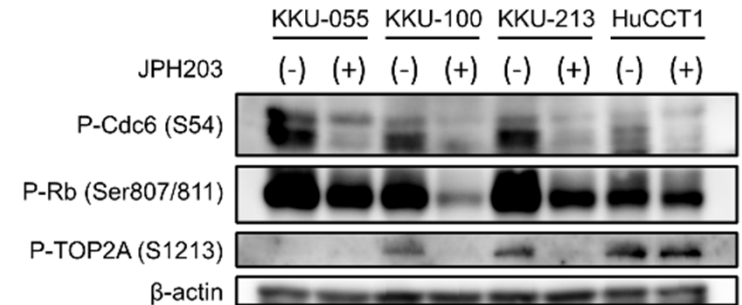
- Detect target proteins using antibodies
- Can quantify expression of targeted proteins and determine their subcellular localization
- Need specific antibodies

Mass spectrometry-based proteomics (Shotgun proteomics)

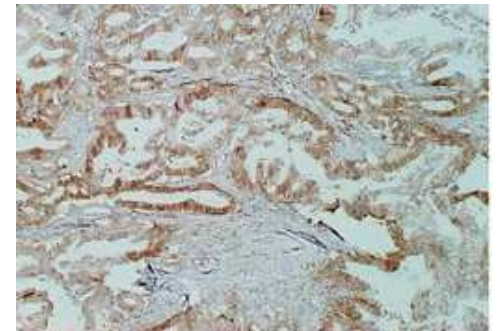
- Identify proteins using mass spectrometer
- Can quantify expression of thousands of proteins
- Need no antibodies (need protein sequence database)

Genome = Gene (遺伝子) + Ome (全体)
Proteome = Protein (タンパク質) + Ome (全体)

Western blotting

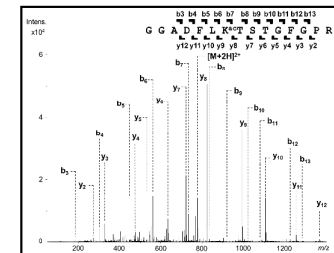


Immunohistochemistry



Kaira et al. (2012)

Mass spectrometry-based proteomics



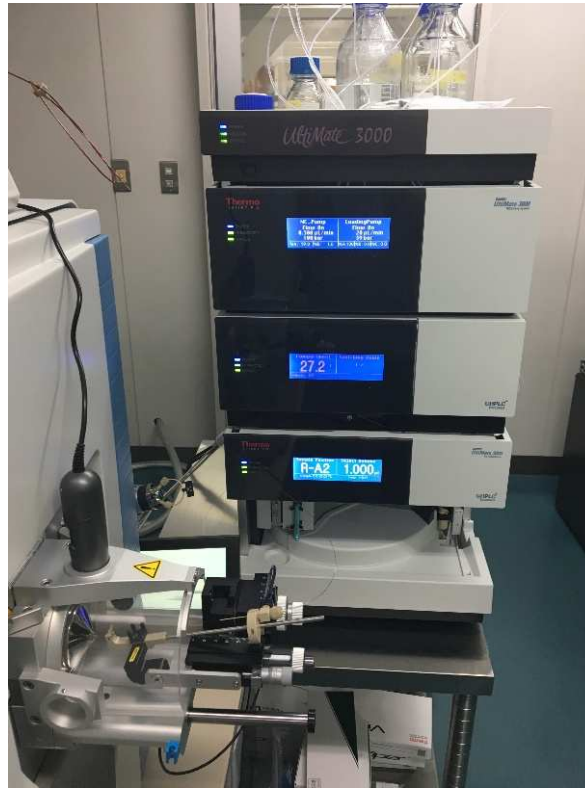
Mass spectrometer

Mass spectrometer combined with nano liquid chromatography system (nLC-MS)

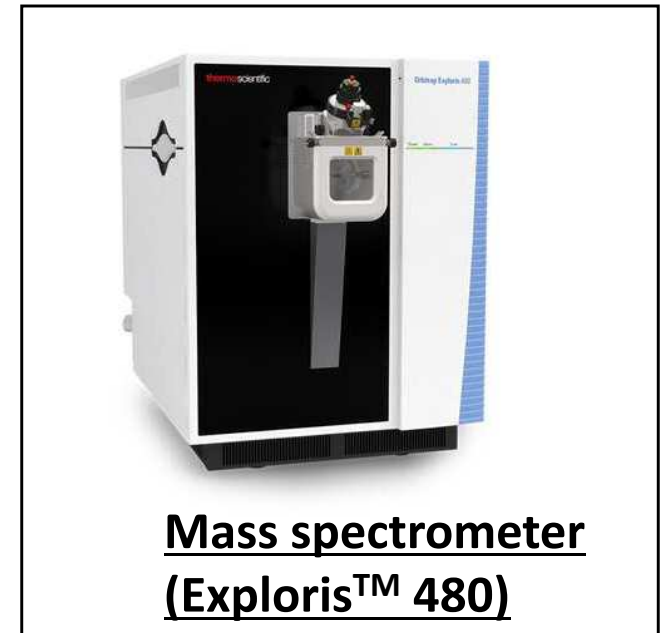
Mass spectrometer
(Q Exactive™ mass spectrometer)



Nano liquid chromatography system
Ultimate™ 3000 HPLC system



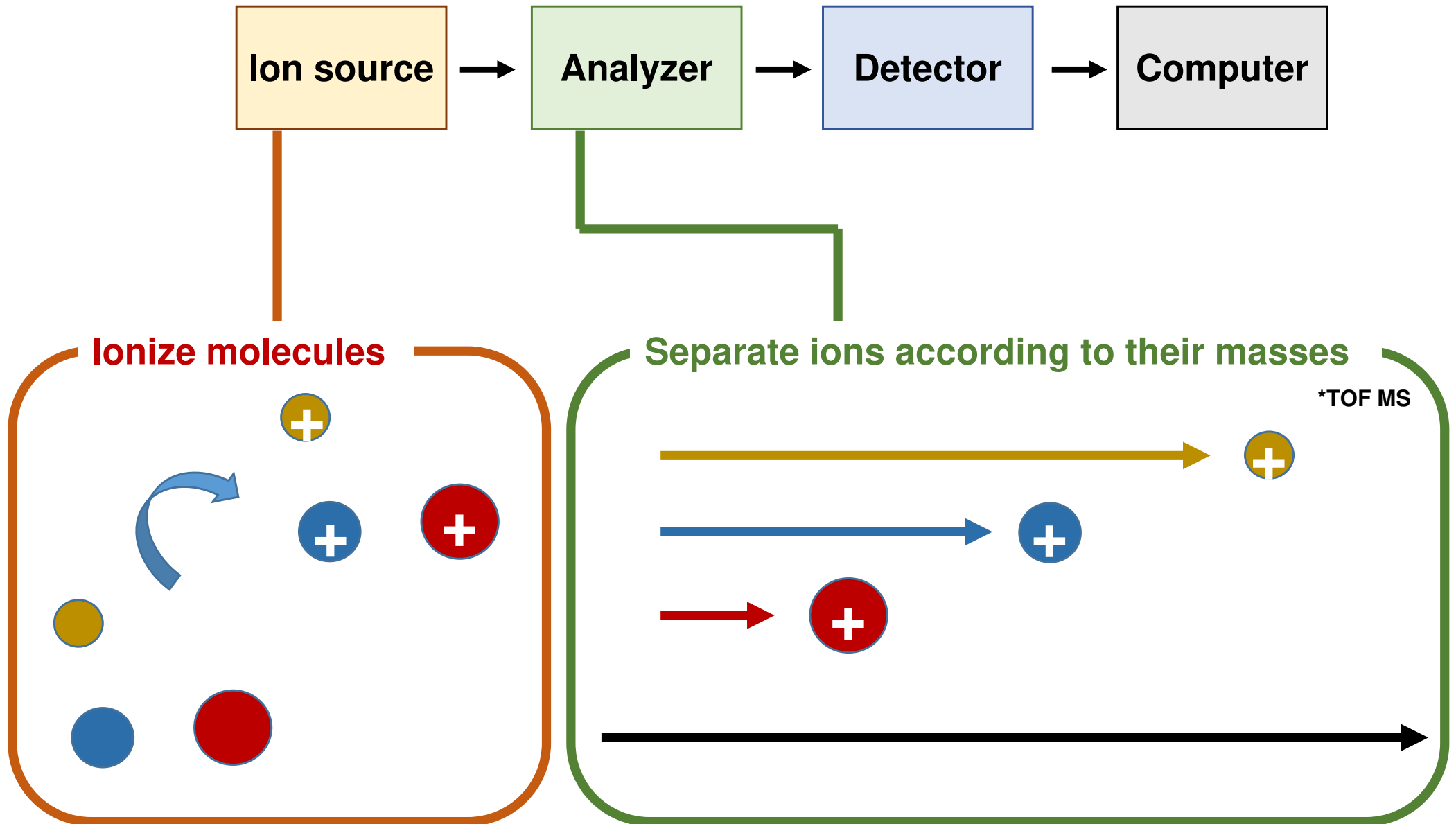
**Mass spectrometer can measure the masses
of molecules
(Resolution: 140,000 [maximum] Q-Exactive)**



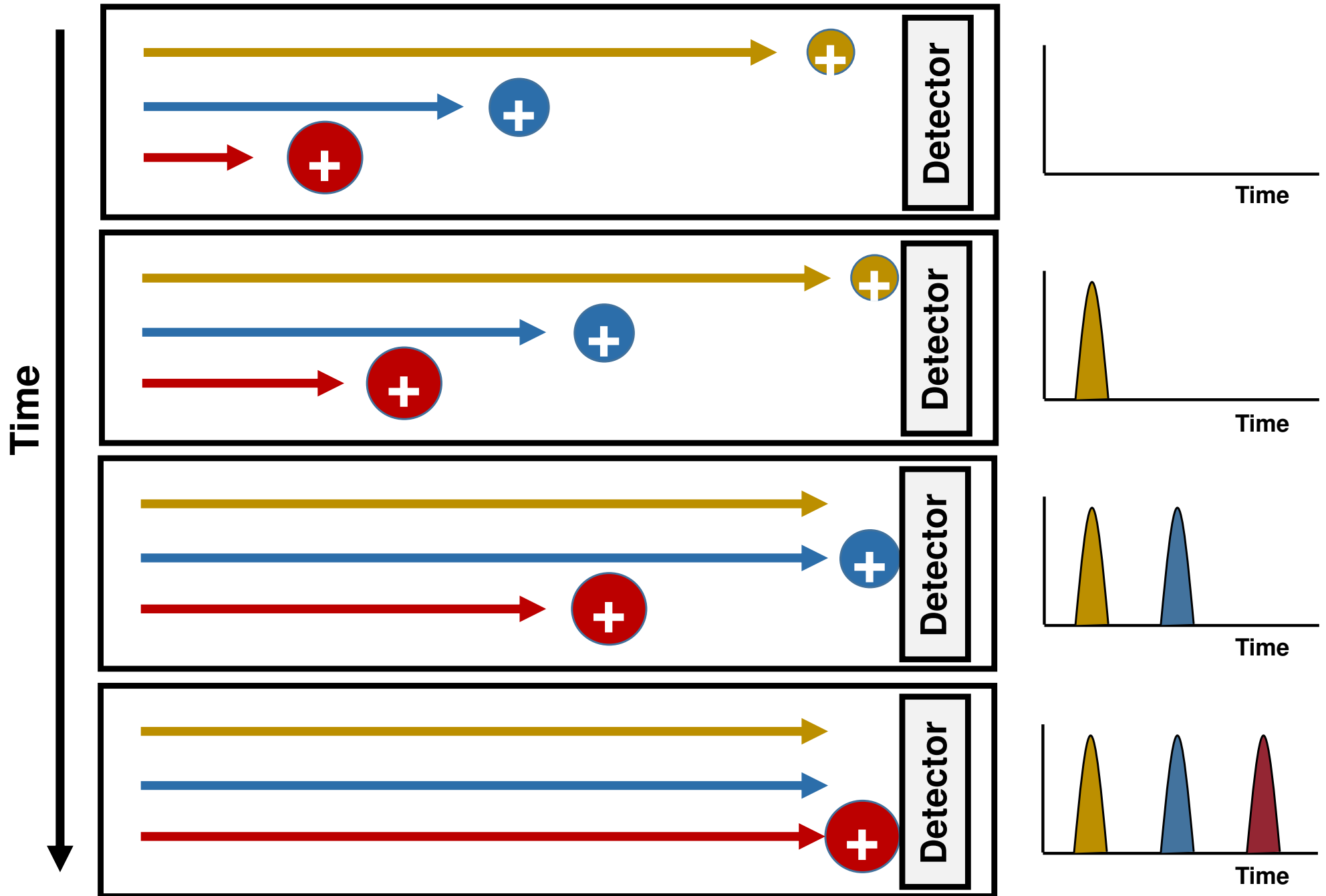
**Mass spectrometer
(Exploris™ 480)**

Mass spectrometer can determine the mass of molecules

Construction of mass spectrometer

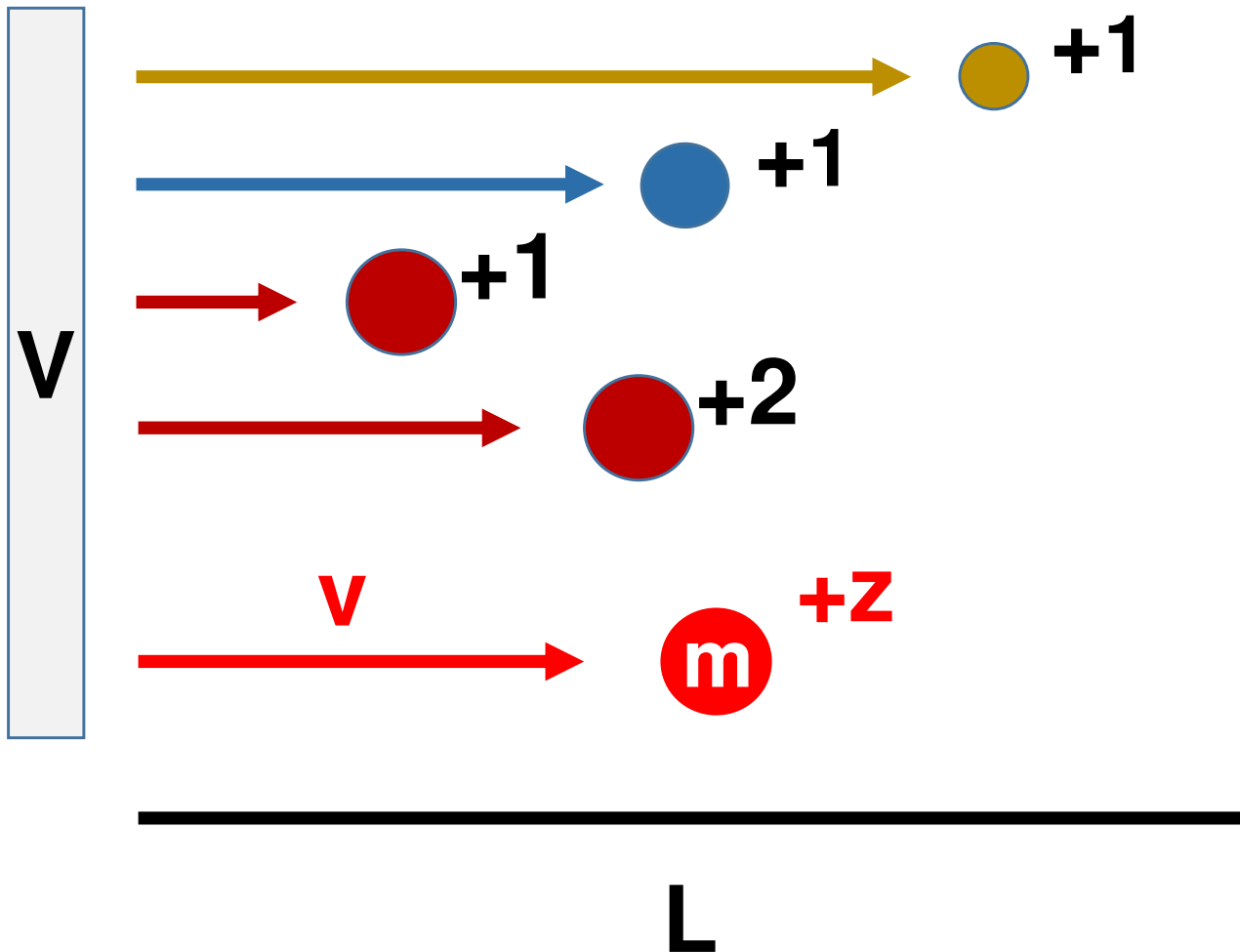


Time-of-flight mass spectrometer



m/z

m/z : mass/charge number



$$zeV = \frac{1}{2}mv^2$$

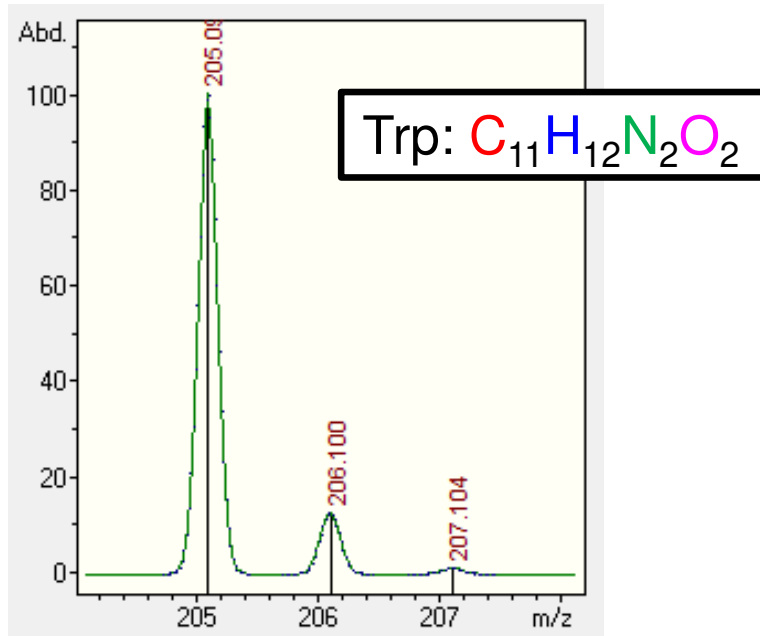
$$L = vt$$

$$zeV = \frac{1}{2}m\left(\frac{L}{t}\right)^2$$

$$t = L \sqrt{m/z \times \frac{1}{2eV}}$$

Average and monoisotopic mass

C, H, O, and N have isotopes
 $[^{13}\text{C}, ^2\text{H (D)}, ^{15}\text{N}, ^{17}\text{O}, ^{18}\text{O}]$



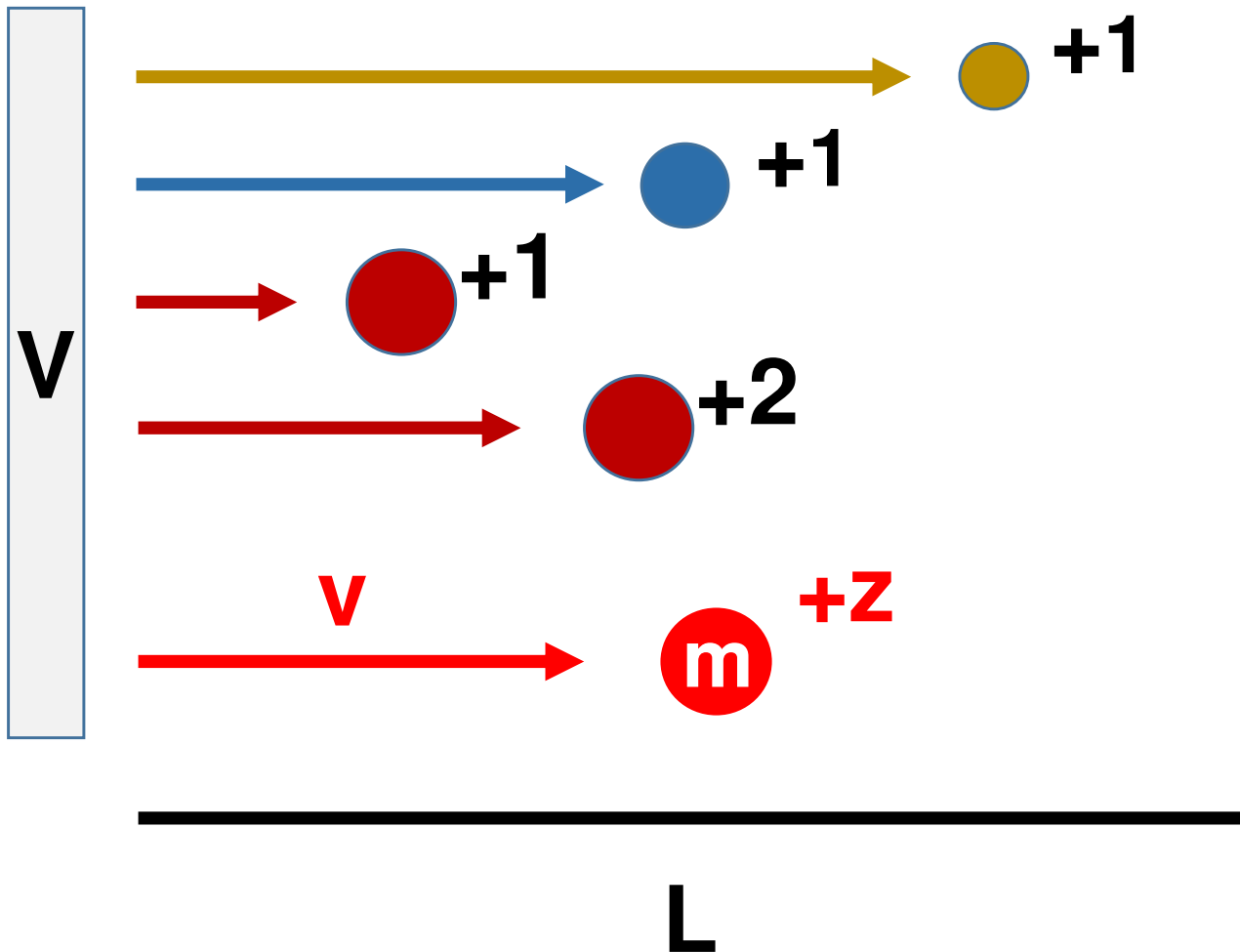
↑
 ↑
 ↑
 Isotopic peaks

↑
 Monoisotopic peak

| 3-letter code | Mass of amino acid residue | |
|---------------|----------------------------|--------------|
| | Average | Monoisotopic |
| Ala | 71.079 | 71.037 |
| Arg | 156.188 | 156.101 |
| Asn | 114.104 | 114.043 |
| Asp | 115.089 | 115.027 |
| Cys | 103.139 | 103.009 |
| Glu | 129.116 | 129.043 |
| Gln | 128.131 | 128.059 |
| Gly | 57.052 | 57.021 |
| His | 137.141 | 137.059 |
| Ile | 113.159 | 113.084 |
| Leu | 113.159 | 113.084 |
| Lys | 128.174 | 128.095 |
| Met | 131.193 | 131.040 |
| Phe | 147.177 | 147.068 |
| Pro | 97.117 | 97.053 |
| Ser | 87.078 | 87.032 |
| Thr | 101.105 | 101.048 |
| Trp | 186.213 | 186.079 |
| Tyr | 163.176 | 163.063 |
| Val | 99.133 | 99.068 |

m/z

m/z : mass/charge number



$$zeV = \frac{1}{2}mv^2$$

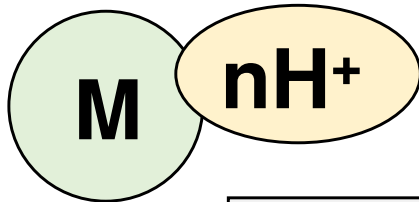
$$L = vt$$

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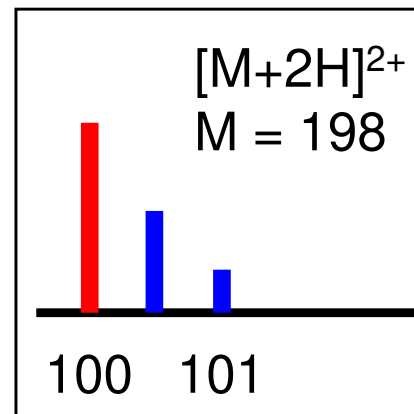
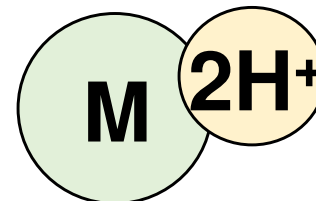
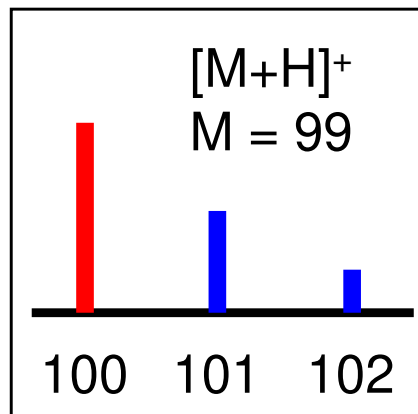
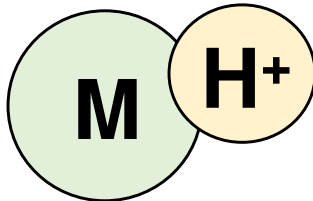
The mass can be calculated by m/z and isotopic peak pattern

Molecules ionized by protonation



$$m = M + n$$
$$z = n$$

$$m/z = \frac{M + n}{n}$$



Monoisotopic peak

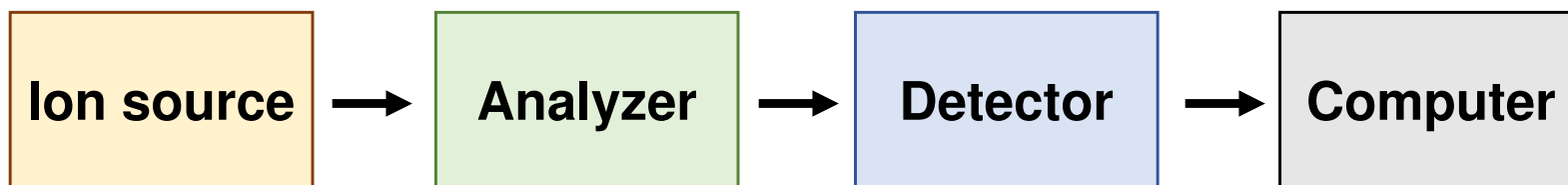
→ m/z

Isotopic peaks

→ z

Type of mass spectrometer

Construction of mass spectrometer



Ion source

MALDI (Matrix Assisted Laser desorption/ionization)

ESI (Electrospray ionization)

Electron ionization

FAB (Fast atom bombardment)

Chemical ionization

Analyzer

TOF (Time-of-flight)

Quadrupole

Ion trap

FT-ICR (Fourier-transform ion cyclotron resonance)

Orbitrap

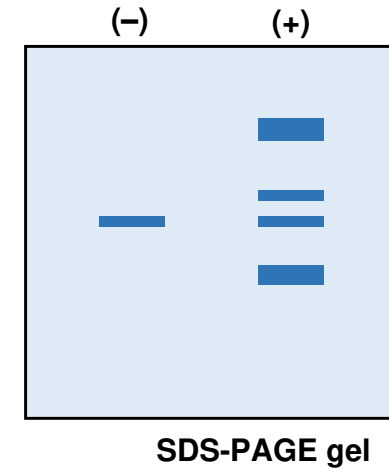
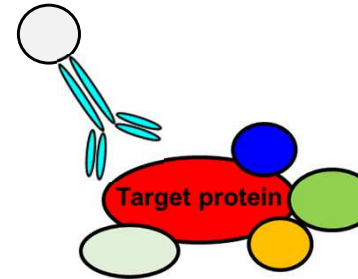
Magnetic sector

Mass spectrometry-based protein analysis

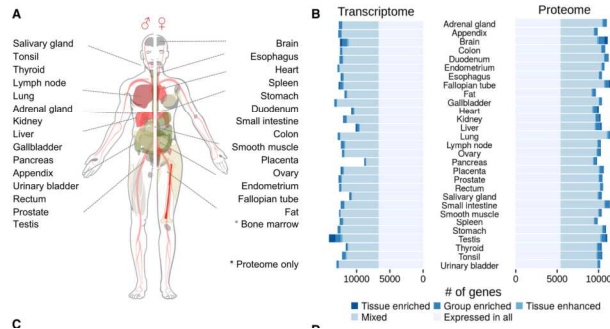
(1) Identification of unknown protein



(3) Identification of proteins interacted with target protein

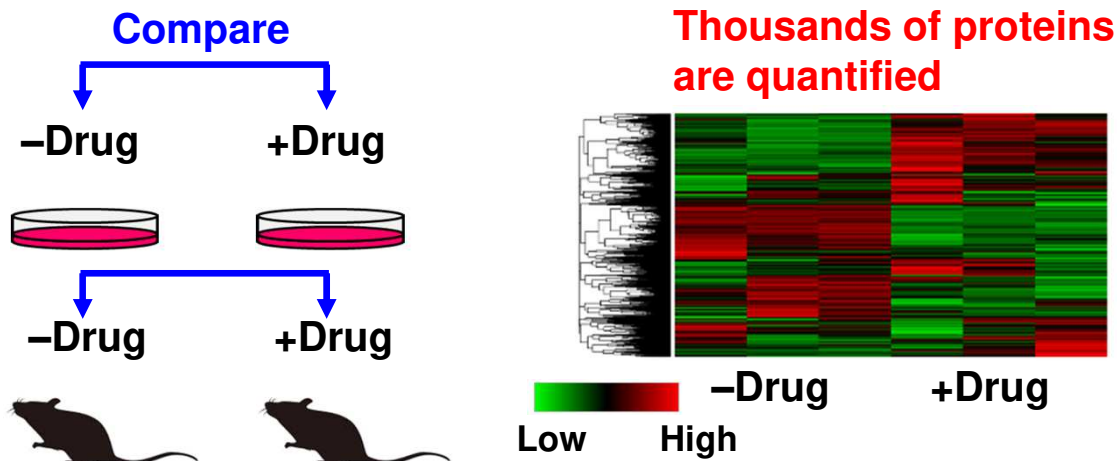


(2) Profiling of proteome in cells or tissues



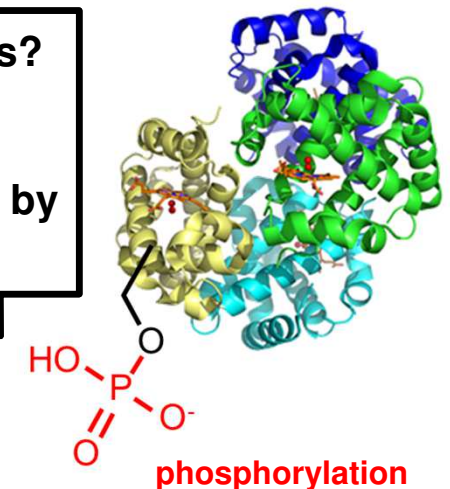
Wang et al. (2019) *Mol. Syst. Biol.*

(4) Comparative quantification of protein expression



(5) Identification of phosphorylation sites (& PTM sites)

What types of PTMs?
Which sites?
When?
Up/down-regulated by drug?



Protein sequence database is available



BLAST Align Retrieve/ID mapping Peptide search SPARQL

UniProtKB - Q01650 (LAT1_HUMAN)

Display [Help video](#) [BLAST](#) [Align](#) [Format](#) [Add to basket](#) [History](#) [Add a publication](#) [Feedback](#)

- Entry
- Publications
- Feature viewer
- Feature table

Protein Large neutral amino acids transporter
Gene SLC7A5
Organism *Homo sapiens (Human)*
Status Reviewed - Annotation score:

- None
- Function
 - Names & Taxonomy
 - Subcell. location
 - Pathol./Biotech
 - PTM / Processing
 - Expression
 - Interaction
 - Structure
 - Family & Domains
 - Sequences (1+)
 - Similar proteins
 - Cross-references
 - Entry information
 - Miscellaneous
- [Top](#)

Function:

The heterodimer with SLC3A2 functions as sodium-independent large neutral amino acids such as phenylalanine, tyrosine, tryptophan, methionine, and leucine (PubMed:9751058, PubMed:10049700, PubMed:11551135, PubMed:11311135, PubMed:11564694, PubMed:1211135, PubMed:30867591). Functions as an amino acid exchanger (PubMed:12225859, PubMed:30867591). May play a role in the transport of leucine across the inner blood-retinal barrier (By similarity). May act as the major transporter of brain leucine (By similarity). May be involved in the transport of thyroid hormones triiodothyronine (T3) and thyroxine (T4) across the inner blood-retinal barrier (PubMed:12225859). When associated with LAPT4B, it is involved in the transport of leucine into lysosomes to promote leucine uptake into these organelles (PubMed:25998567). Involved in the uptake of toxic L-homocysteine complexes (PubMed:12117417). Involved in the transport of L-homocysteine nitrosothiols, via the stereoselective transport of L-homocysteine (PubMed:15769744). [By similarity](#) [1 Publication](#)

Miscellaneous:

The uptake of leucine, tyrosine and tryptophan is inhibited by small zwitterionic amino acids (e.g. methionine, cysteine) and by glutamine and asparagine. The uptake of leucine, tryptophan, tyrosine, and phenylalanine, or 2-methyl-3-isobutylamino-L-alanine is inhibited by the L-system substrate L-cysteine complex and structurally related S-ethyl-L-

Uniprot: <https://www.uniprot.org/>

Display [Help video](#)

Sequence (1+)

- Entry
- Publications
- Feature viewer
- Feature table

Sequence status: Complete.
This entry has 1 described isoform and 1 potential isoform that is computationally mapped. [Show all](#)

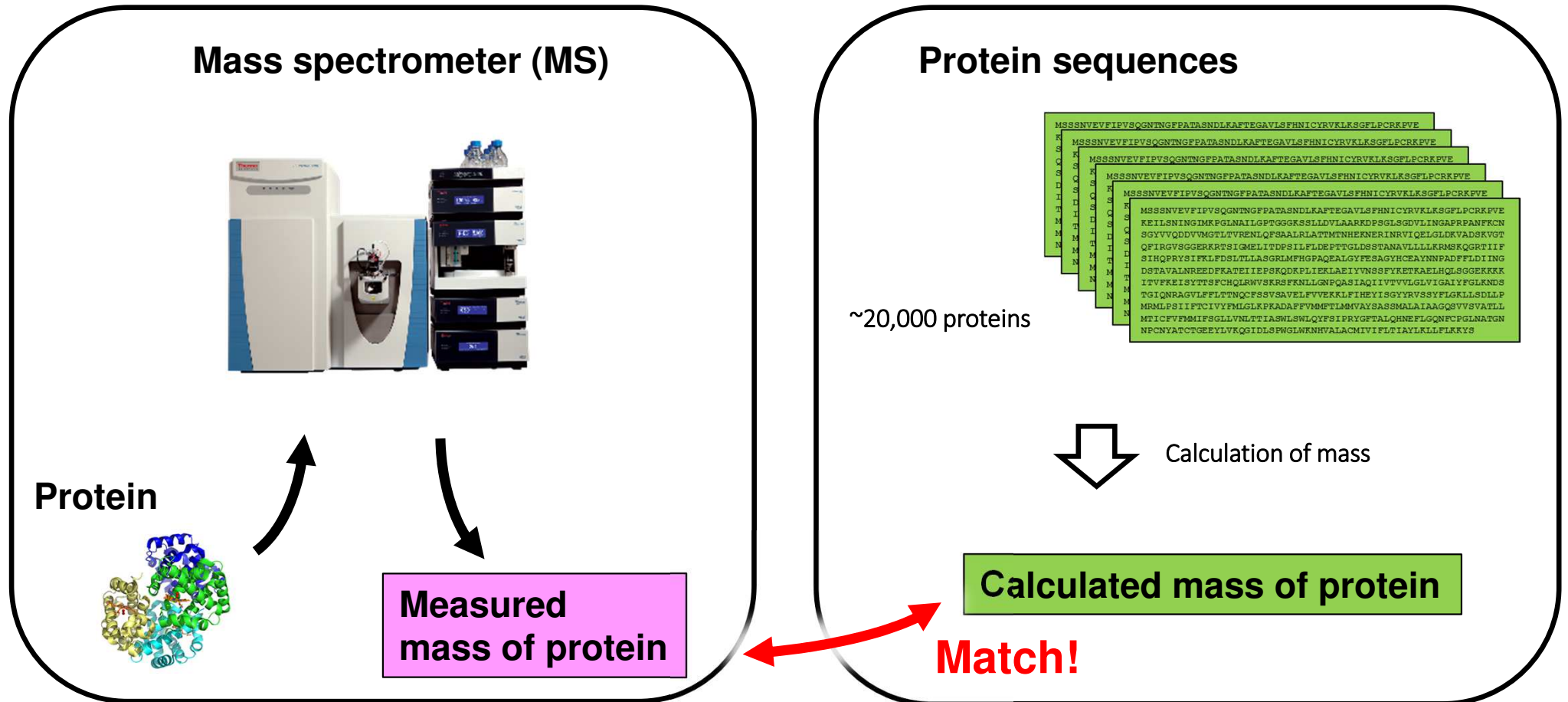
[Align All](#)
Q01650-1 [UniParc] [FASTA](#) [Add to basket](#)

« Hide

| | | | | |
|------------|------------|-------------|------------|------------|
| 10 | 20 | 30 | 40 | 50 |
| MAGAGPKRRA | LAAPAAEKE | EAREKMLAAK | SADGSAPAGE | GEGVTLQRNI |
| 60 | 70 | 80 | 90 | 100 |
| TLINGVAIIV | GTIIGSGIFV | TPTGVLKEAG | SPGLALVVWA | ACGVFSIVGA |
| 110 | 120 | 130 | 140 | 150 |
| LCYAELGTTI | SKSGGDYAYM | LEVYGSLPAP | LKLWIELLII | RPSSQIIVAL |
| 160 | 170 | 180 | 190 | 200 |
| VFATYLLKPL | FPTCFVPEEA | AKLVACLQVL | LLTAVNCYSV | KAATRVQDAF |
| 210 | 220 | 230 | 240 | 250 |
| AAAKLLALAL | IILLGFVQIG | KGDVSNLDPN | FSFEGTKLDV | GNIVLALYSG |
| 260 | 270 | 280 | 290 | 300 |
| LFAYGGWNYL | NFVTEEMINP | YRNLFPLAIII | SLPIVTLVYV | LTNLAYFTTL |
| 310 | 320 | 330 | 340 | 350 |
| STEQMLSSEA | VAVDPGNYHL | GVMSWIIPVF | VGLSCFGSVN | GSLFTSSRLF |
| 360 | 370 | 380 | 390 | 400 |
| FVGSREGHLP | SILSMIHPQL | LIPVPSLVFT | CVMTLLYAFS | KDIFSVINFF |
| 410 | 420 | 430 | 440 | 450 |
| SFFNWLCVAL | AIIGMIWLRH | RKPELERPIK | VNLALPVFFI | LACLFLIAVS |
| 460 | 470 | 480 | 490 | 500 |
| FWKTPVECGI | GFTIILSGLP | VYVFGVWKN | KPKWLLQGI | STIVLCQKLM |
| QVVPQET | | | | |

Length: 507
Mass (Da): 55,010
Last modified: January 24, 2001 - v2
Checksum: 767F3C60B62C0F02
[BLAST](#) [GO](#)

How does mass spectrometry identify protein? (1)

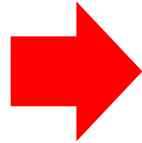


Peptide mass fingerprinting (PMF) & MS/MS

Problem of MS level identification (PMF) regarding peptide sequence

| Peptide sequence | Monoisotopic mass |
|------------------|-------------------|
| DETTIVGGK | 918.465826 |
| DIVGGGETTK | 918.465826 |

These different peptides (different sequence) have same mass



In order to distinguish these two peptides, peptide sequence information is needed.

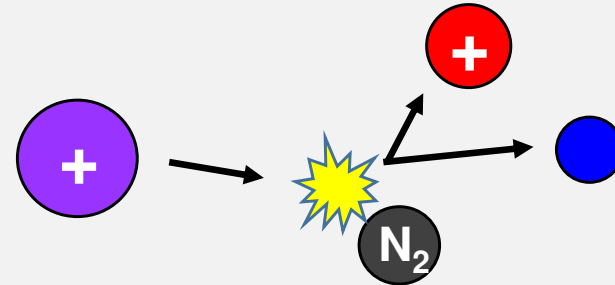
Identification of peptides by Tandem MS (MS/MS)

MS/MS by mass spectrometry

DETTIVGGK

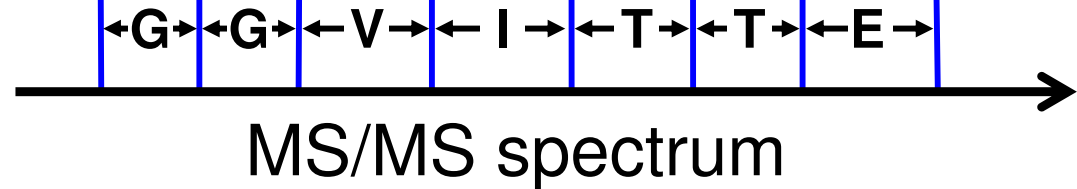
Fragmentation in machine
(CID, ETD, ...)

Collision-induced dissociation (CID)



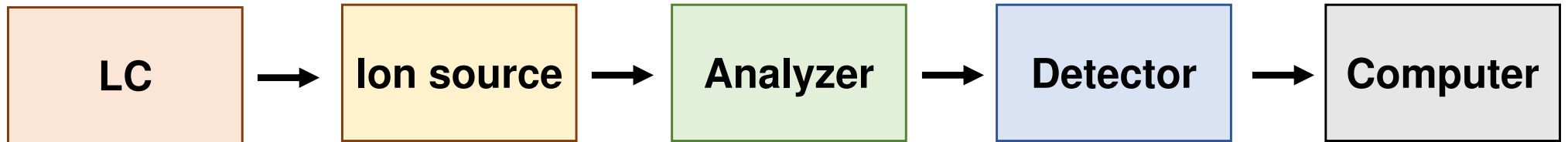
D
DE
DET
DETT
DETTI
DETTIV
DETTIVG
DETTIVGG

ETTIVGGK
TTIVGGK
TIVGGK
IVGGK
VGGK
GGK
GK
K

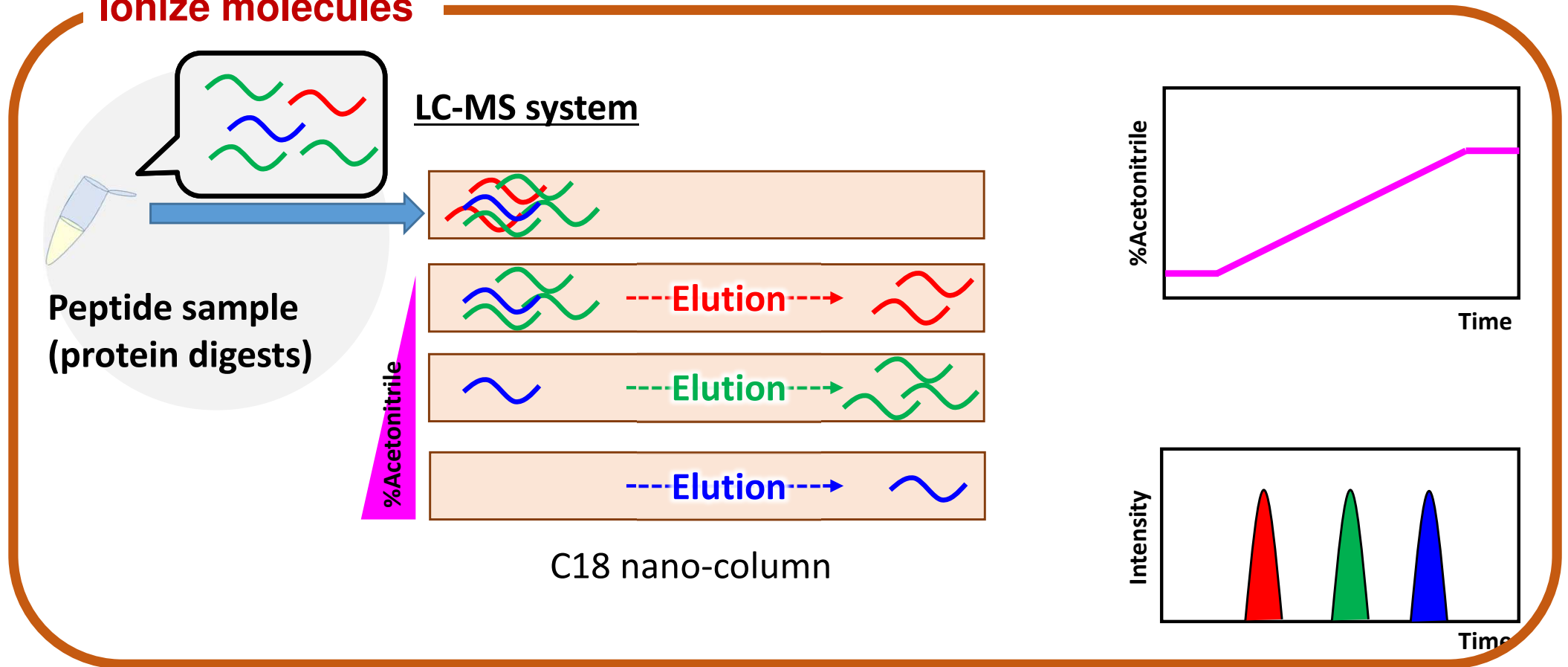


Liquid chromatography-mass spectrometry system (LC-MS system)

Construction of LC-MS



Ionize molecules



Today's topics

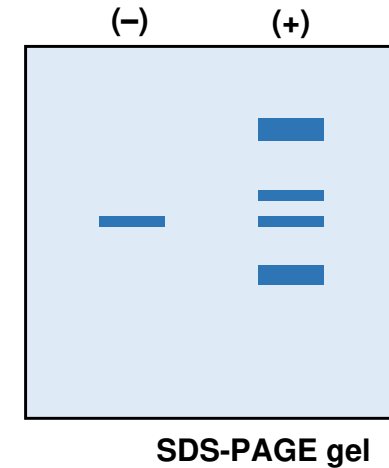
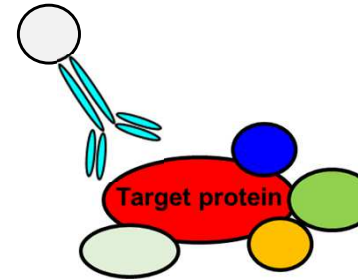
1. **Mass spectrometry-based proteomics**
2. **Phosphoproteomics study on cancer cells treated with a molecular targeted drug**
3. **LC-MS in CoMIT Omics Center (COC)**

Mass spectrometry-based protein analysis

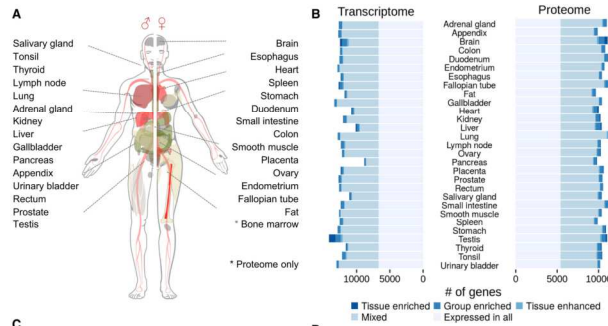
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(3) Identification of proteins interacted with target protein

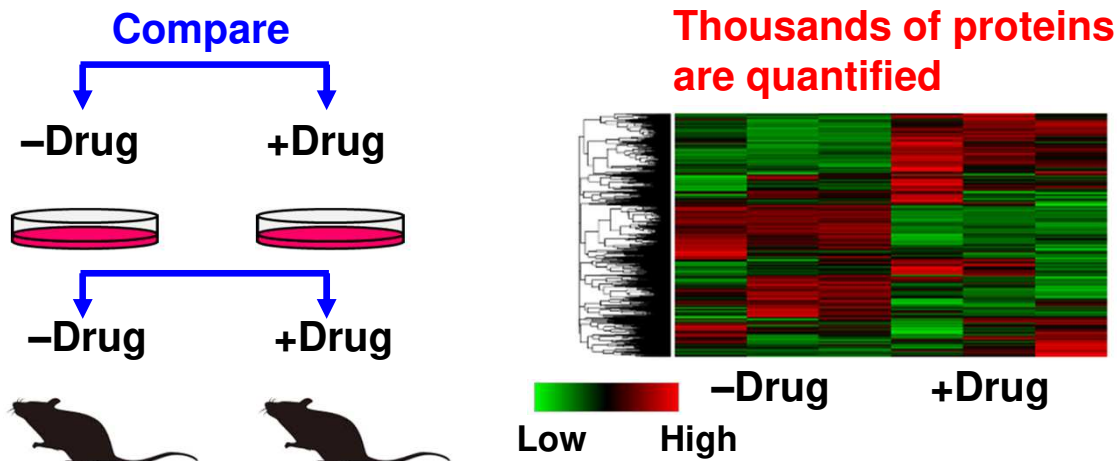


(2) Profiling of proteome in cells or tissues



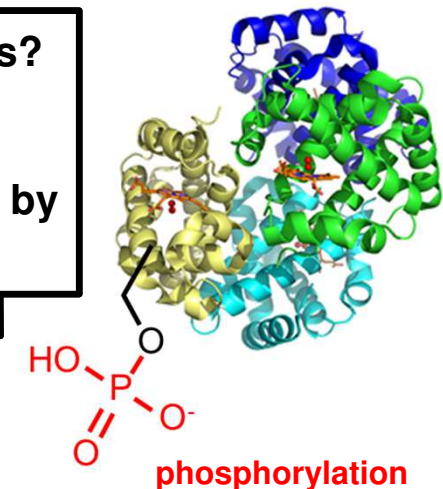
Wang et al. (2019) *Mol. Syst. Biol.*

(4) Comparative quantification of protein expression

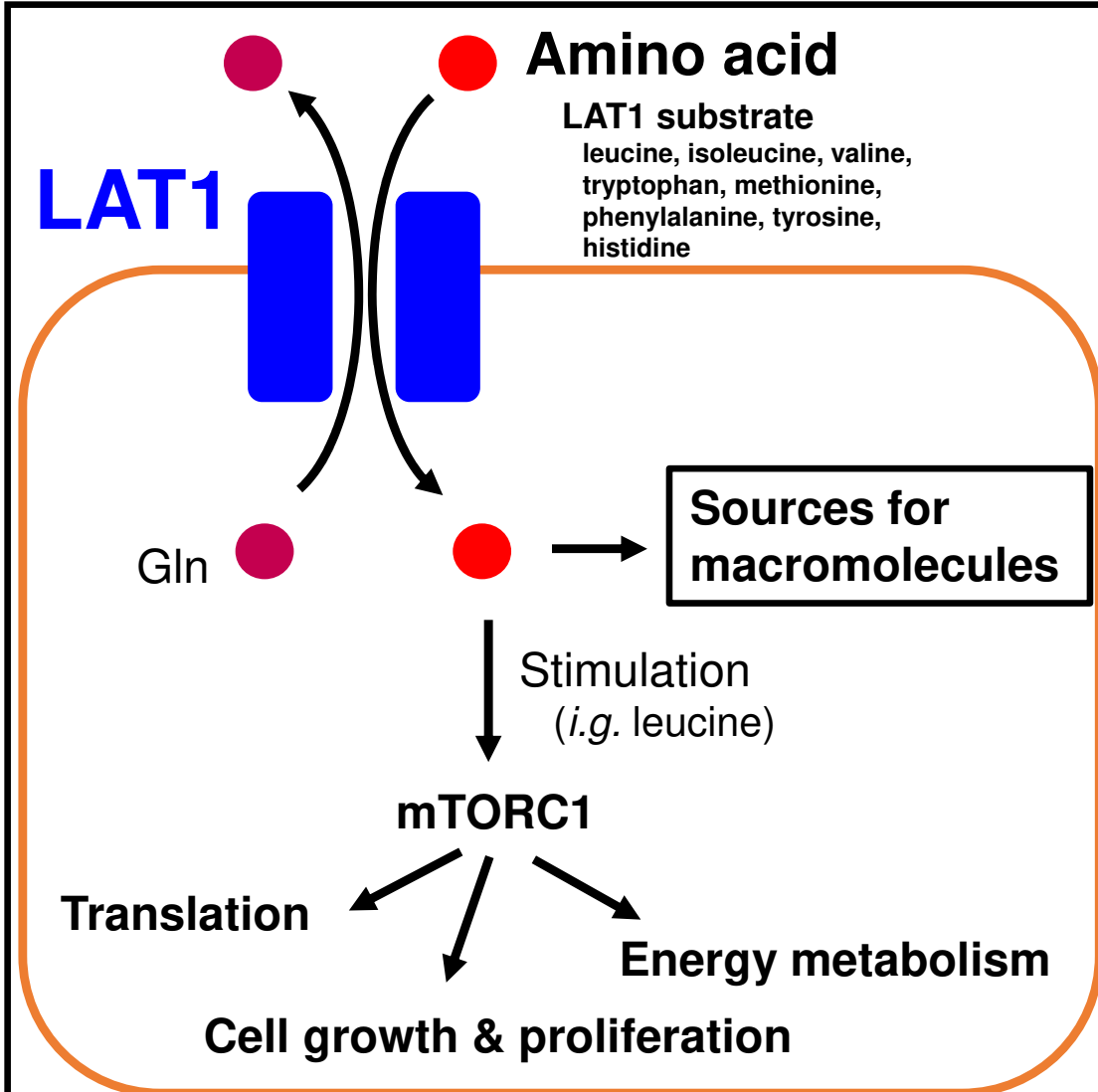


(5) Identification of phosphorylation sites (& PTM sites)

What types of PTMs?
Which sites?
When?
Up/down-regulated by drug?

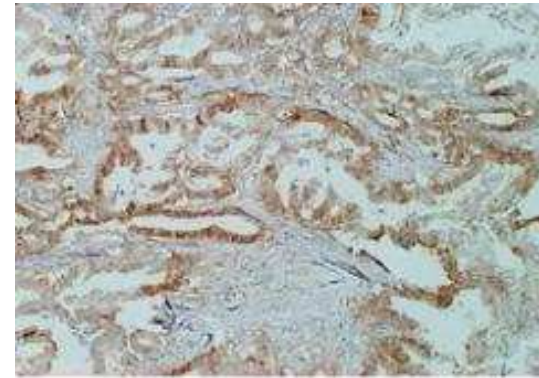


LAT1: Amino acid transporter highly expressed in cancer cells



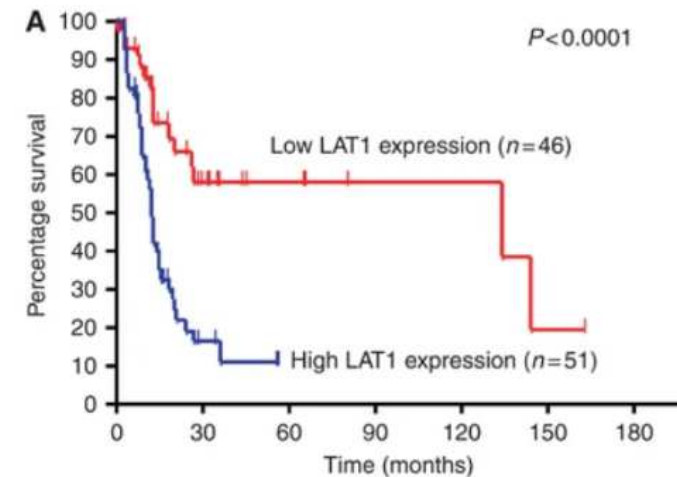
LAT1 substrates function (1) as sources of macromolecules needed for rapid growth, and (2) as signaling molecules

LAT1 is highly expressed in tumor



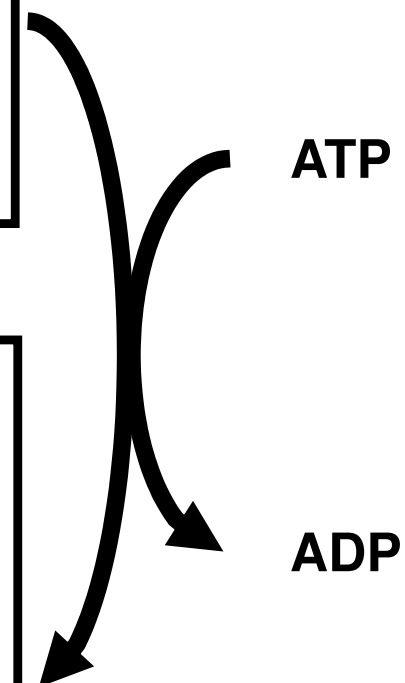
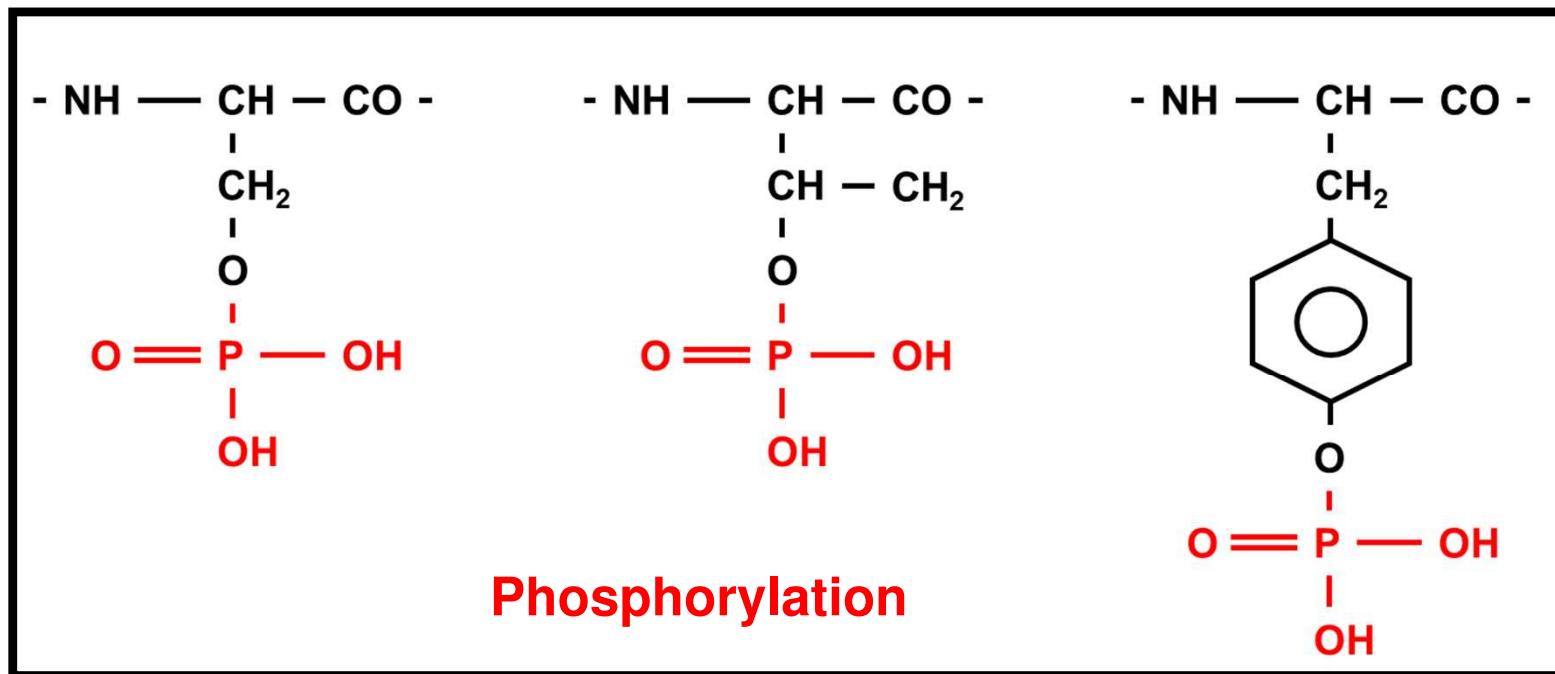
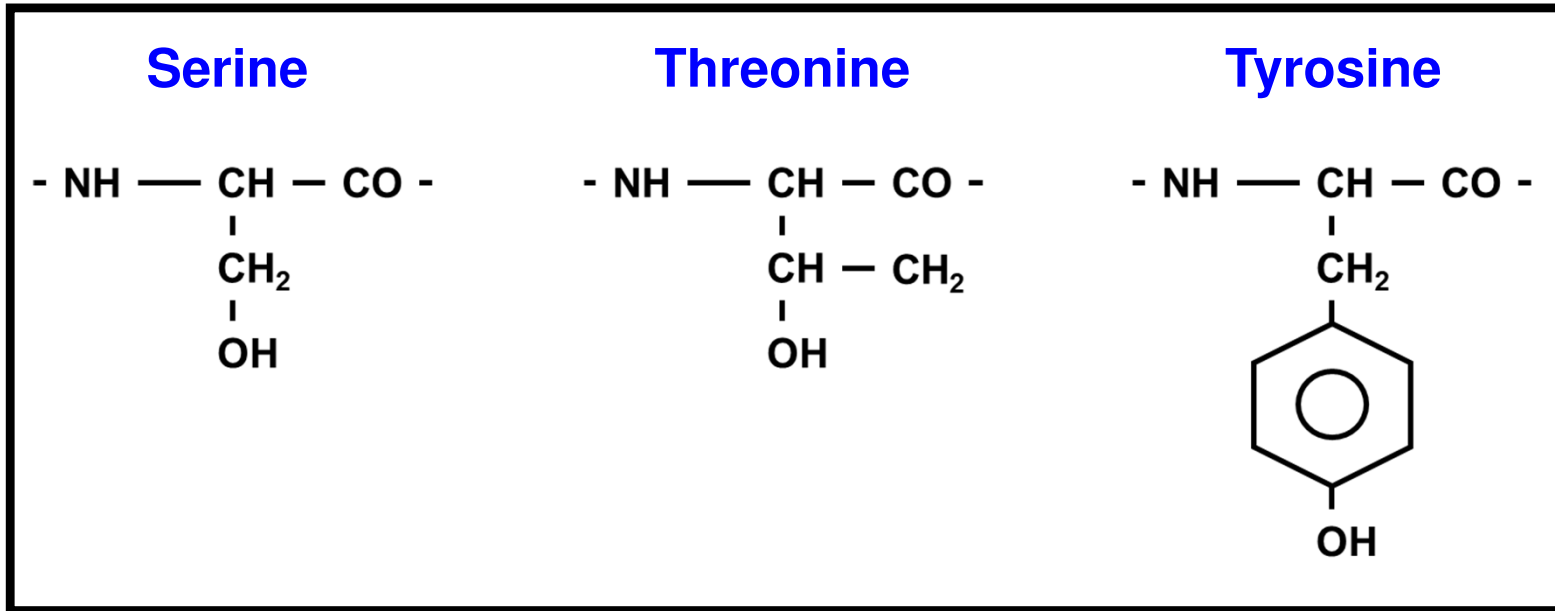
Kaira, K., et al. (2012). *British journal of cancer*, 107, 632–638.

Poor prognosis of patient with highly expressed LAT1



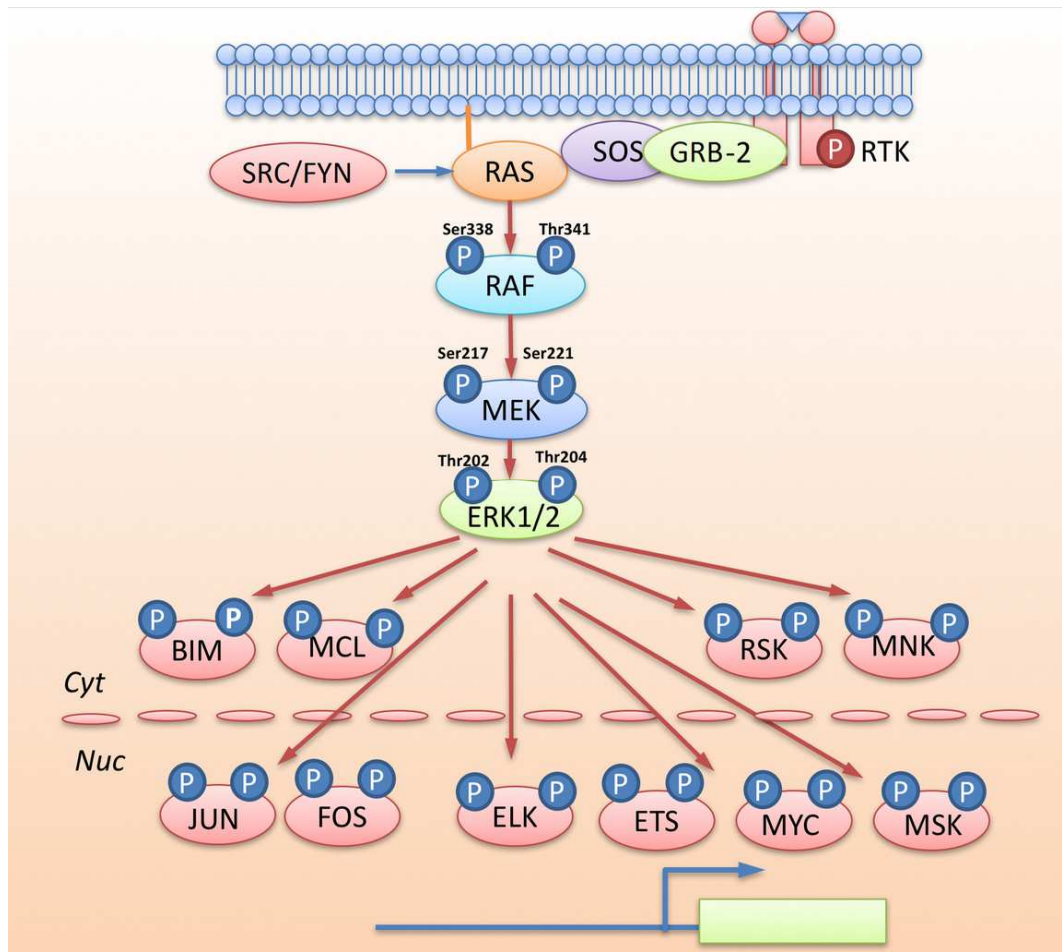
Kaira, K., et al. (2012). *British journal of cancer*, 107, 632–638.

Ser, Thr, Tyr phosphorylation



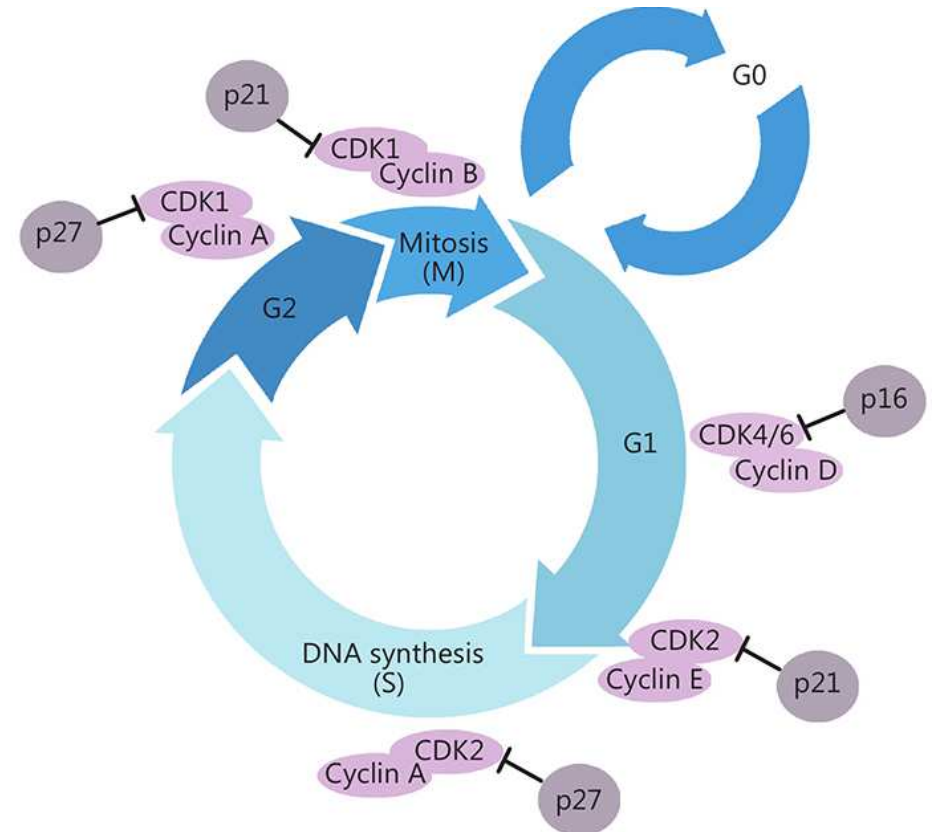
Phosphorylation signaling pathways

MAPK pathway



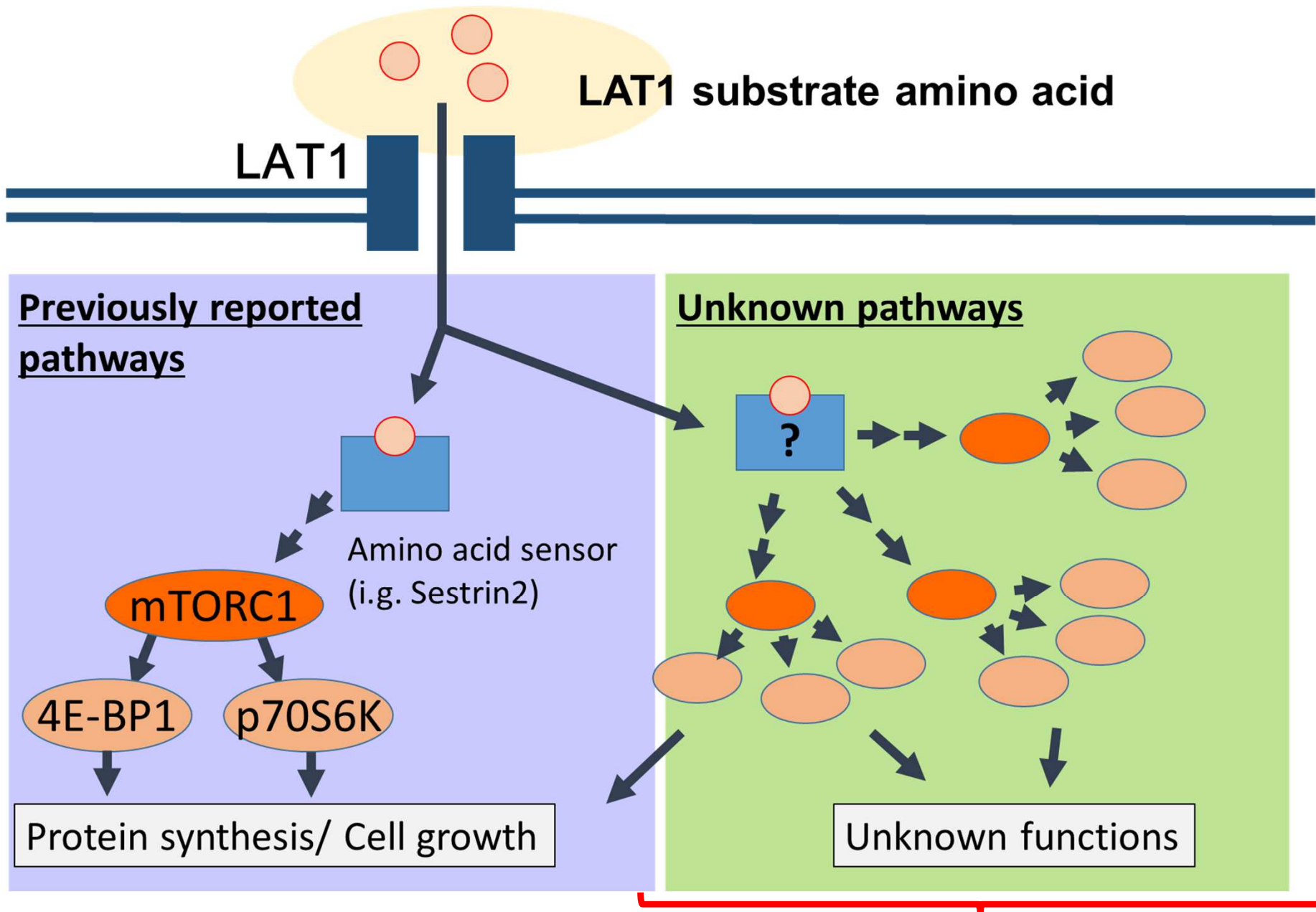
Liu, F. et al. (2018). *Acta pharmaceutica Sinica. B*, 8(4), 552–562.

Cell cycle



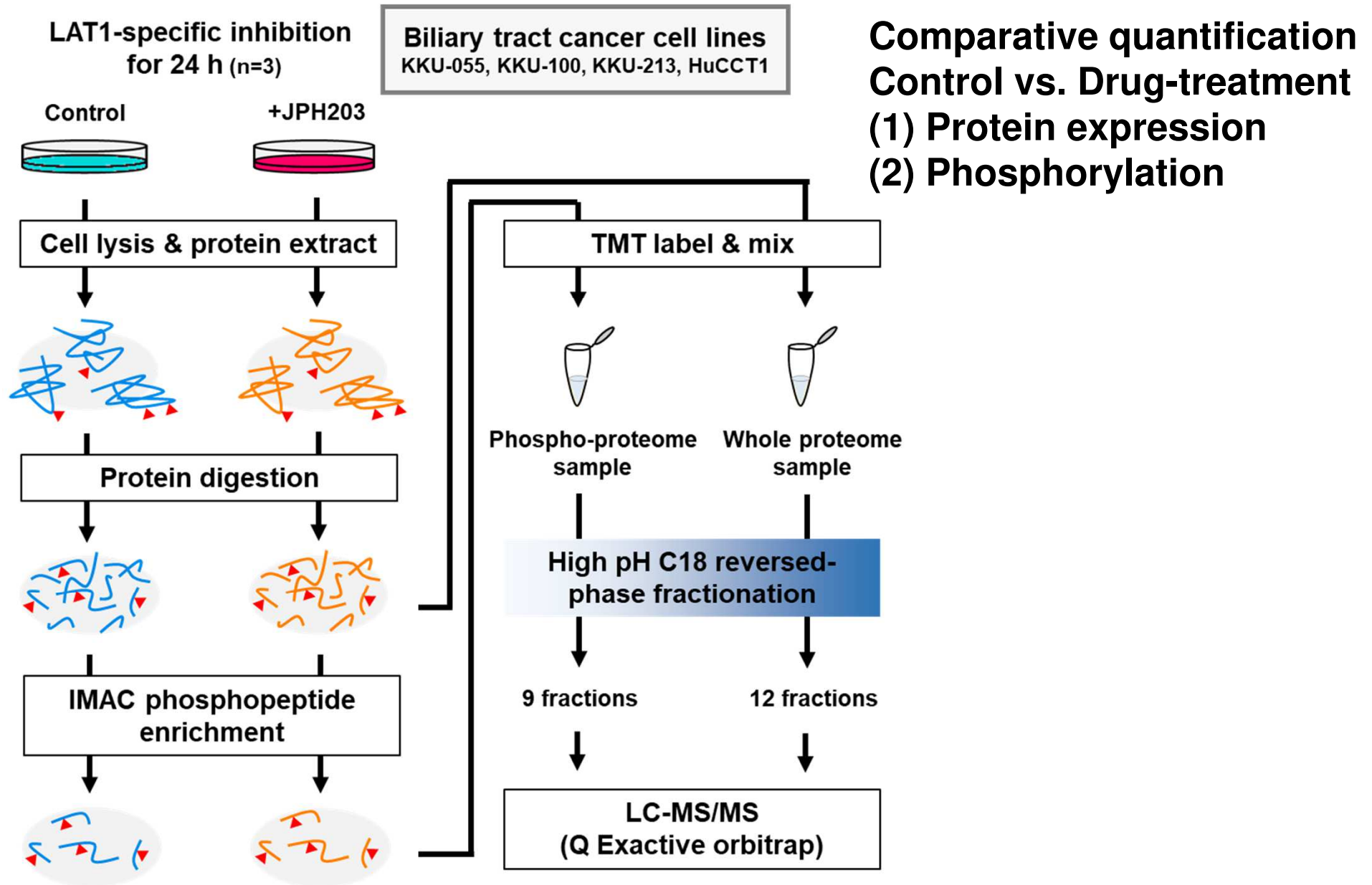
Bai, J., Li, Y., & Zhang, G. (2017). *Cancer biology & medicine*, 14(4), 348–362.

LAT1 and phosphorylation signaling



Proteomics and phosphoproteomics study

Phosphoproteomics workflow



Okanishi, H., *et al.* (2021). Proteomics and phosphoproteomics reveal key regulators associated with cytostatic effect of amino acid transporter LAT1 inhibitor. *Cancer science*, 112, 871–883.

Quantitative proteomics methods

Labeling quantification

Reporter ion-based

iTRAQ (Isobaric Tag for Relative and Absolute Quantitation)

TMT (Tandem Mass Tag)

Precursor ion-based

SILAC (Stable Isotope Labeling)

Dimethyl labeling

^{18}O labeling

Label-free quantification

Precursor ion-based

Chromatogram peak area-based

Data-independent

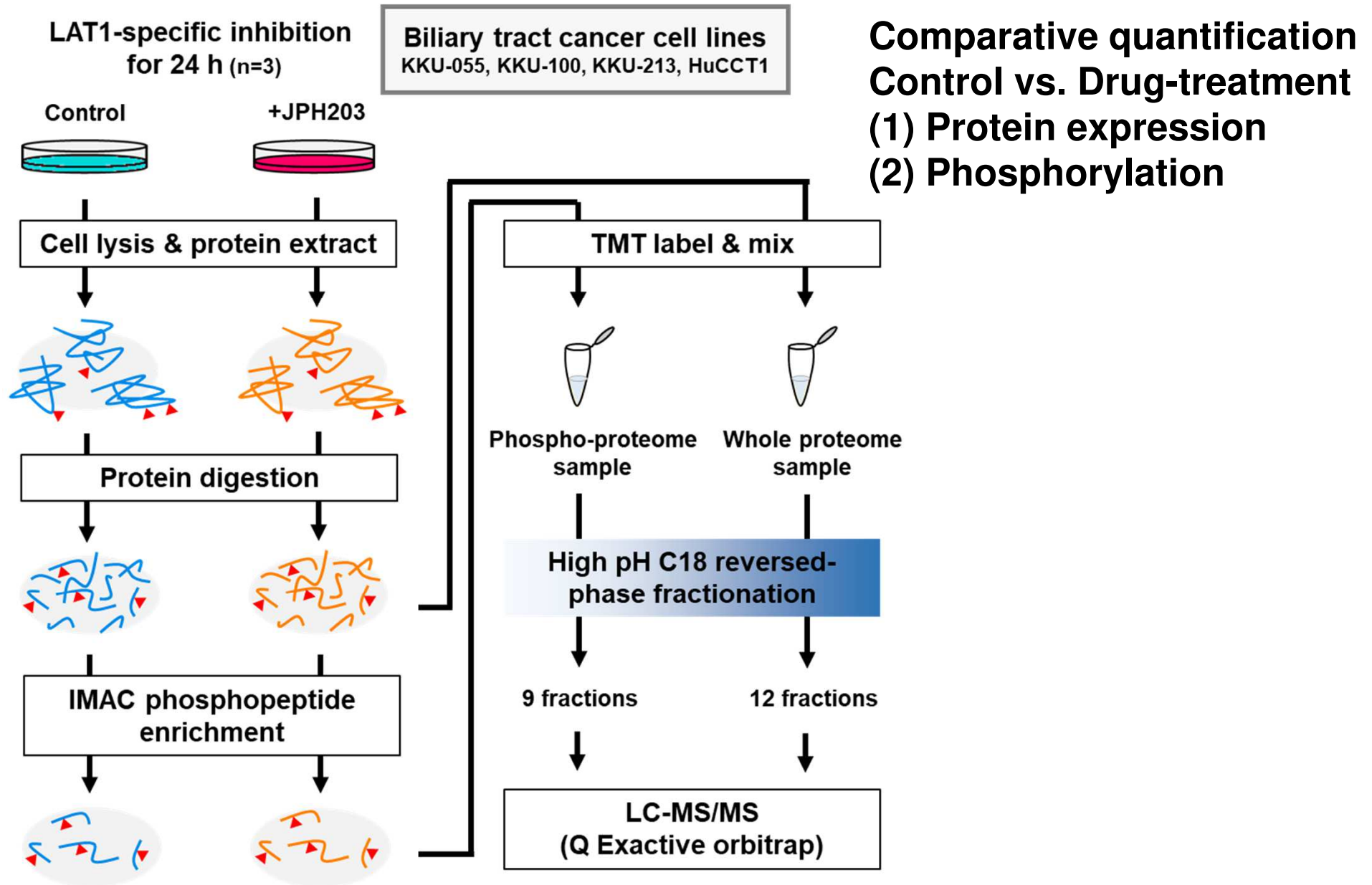
SWATH-MS

Others

Spectral counts

emPAI

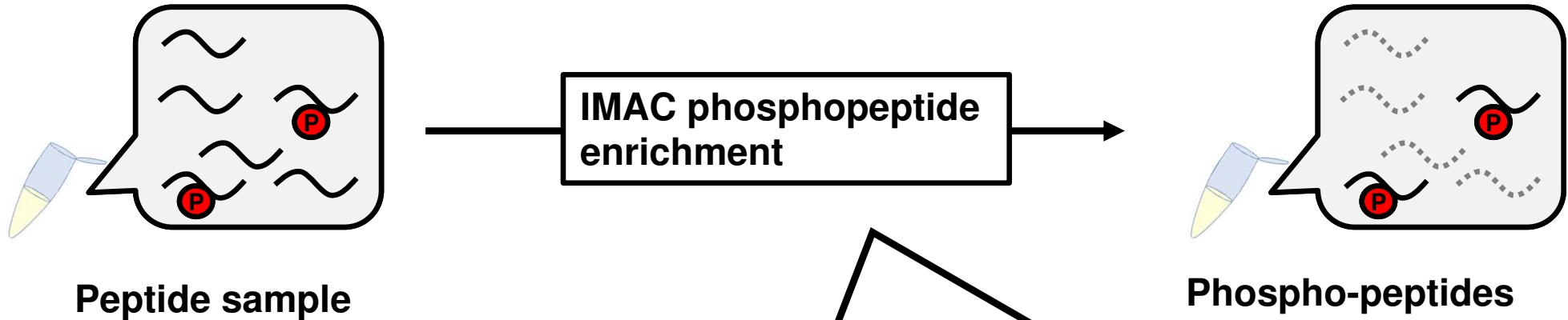
Phosphoproteomics workflow



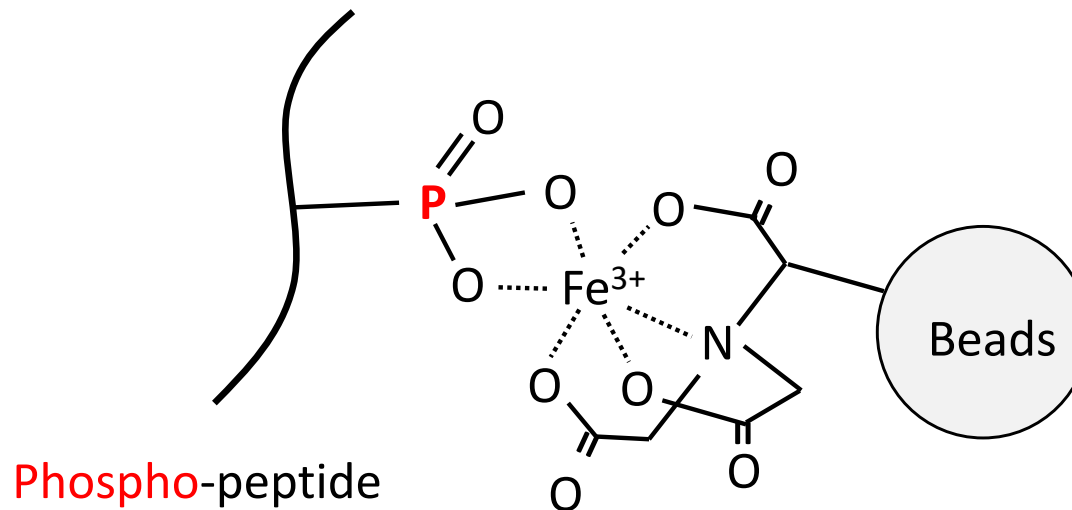
Okanishi, H., *et al.* (2021). Proteomics and phosphoproteomics reveal key regulators associated with cytostatic effect of amino acid transporter LAT1 inhibitor. *Cancer science*, 112, 871–883.

Phosphopeptide enrichment technique: Immobilized metal affinity chromatography (IMAC)

Too many unmodified peptides!



Immobilized metal affinity chromatography (IMAC)



Identification and quantification

Identification summary

Average identified peptides:

54106 peptides (q -value < 0.01) from **6431 proteins**

Average identified phosphopeptides:

21340 phosphopeptides (q -value < 0.01)



Quantification summary

Average quantifiable proteins:

4983 proteins (#unique ≥ 2 with q -value < 0.01)

Average quantifiable phosphorylation sites:

15019 phosphorylation sites (q -value < 0.01, Site probability > 0.75)



Differentially expressed proteins and phosphorylated sites

(p -value < 0.05 and fold change ≥ 1.2)

Proteomics

348 proteins in KKU-055

342 proteins in KKU-100

252 proteins in KKU-213

1315 proteins in HuCCT-1

Phosphoproteomics

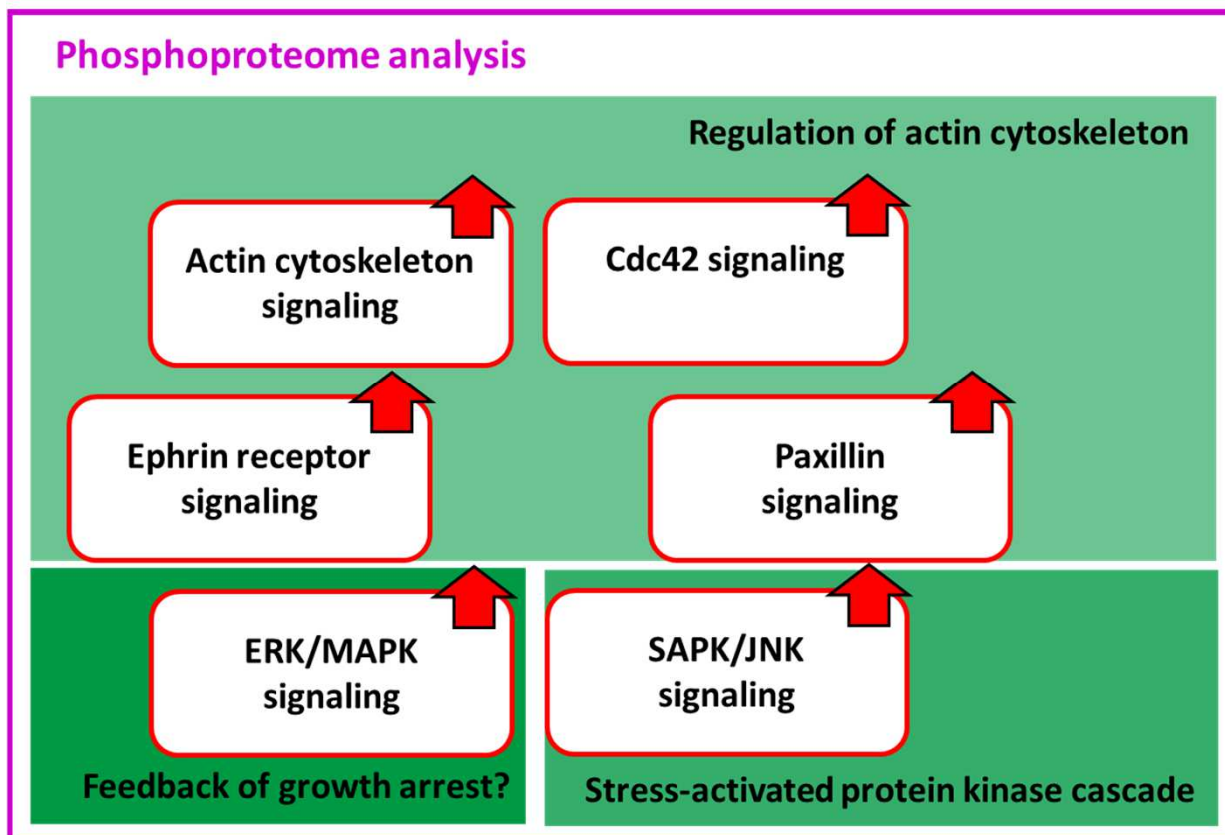
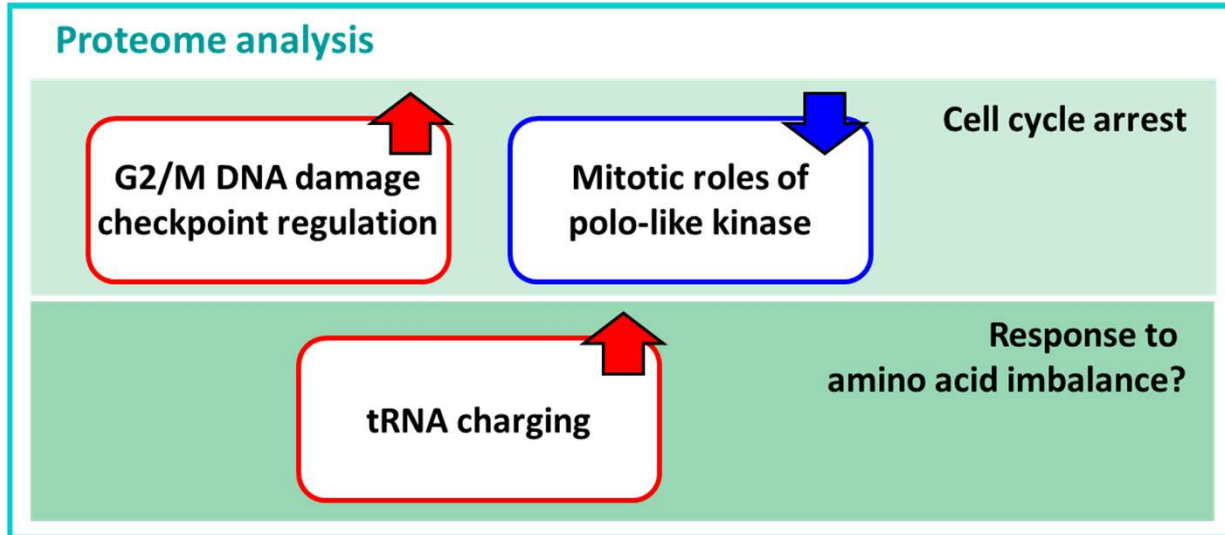
4777 sites in KKU-055

3943 sites in KKU-100

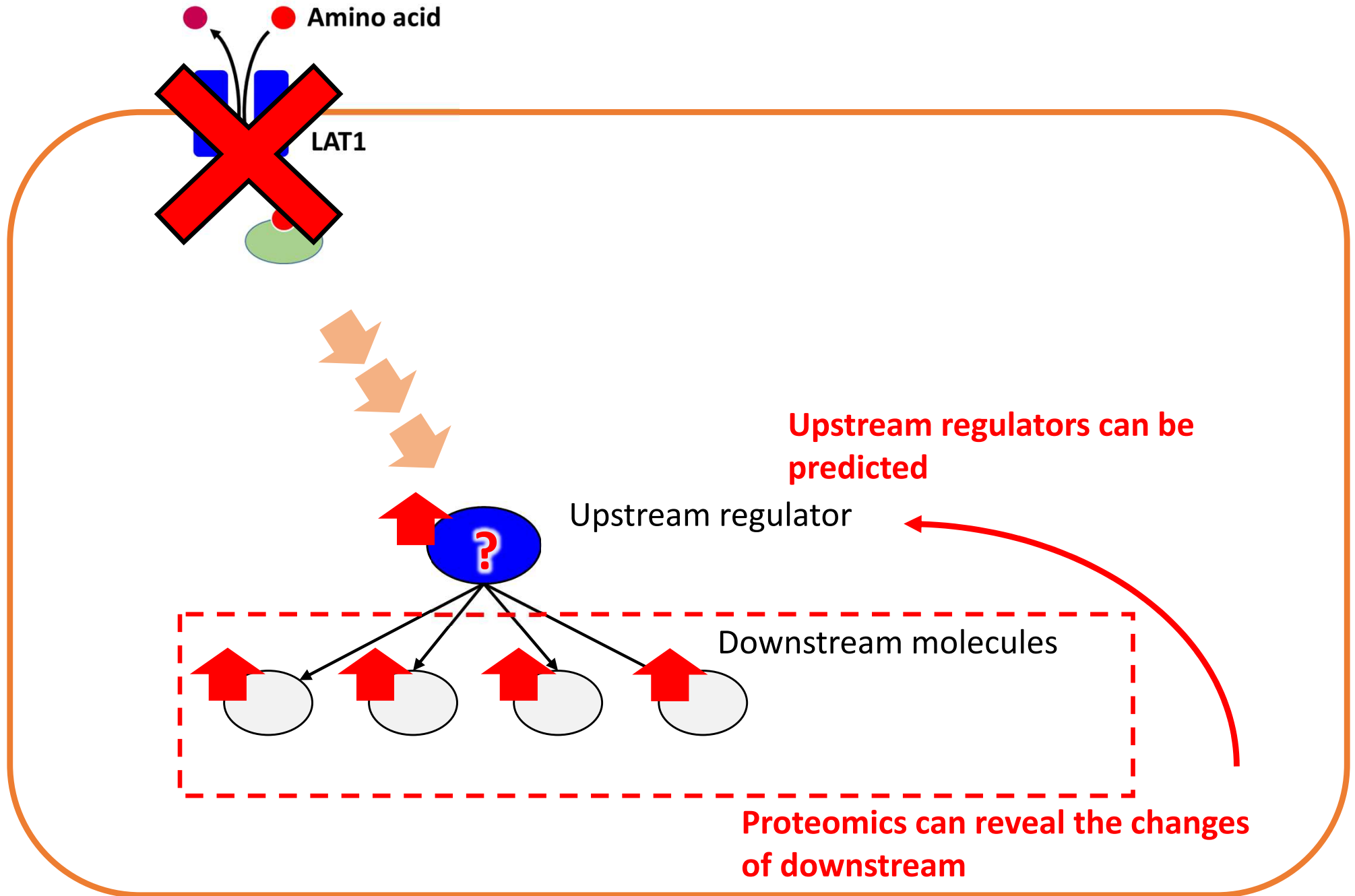
3990 sites in KKU-213

5574 sites in HuCCT1

Commonly activate/inactivated pathways

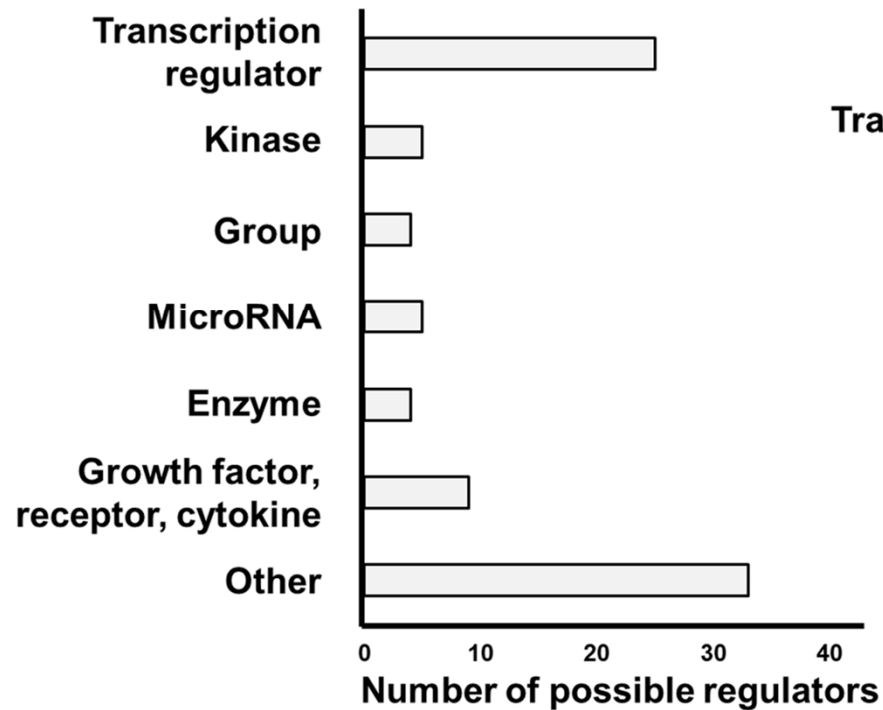


Upstream regulator analysis

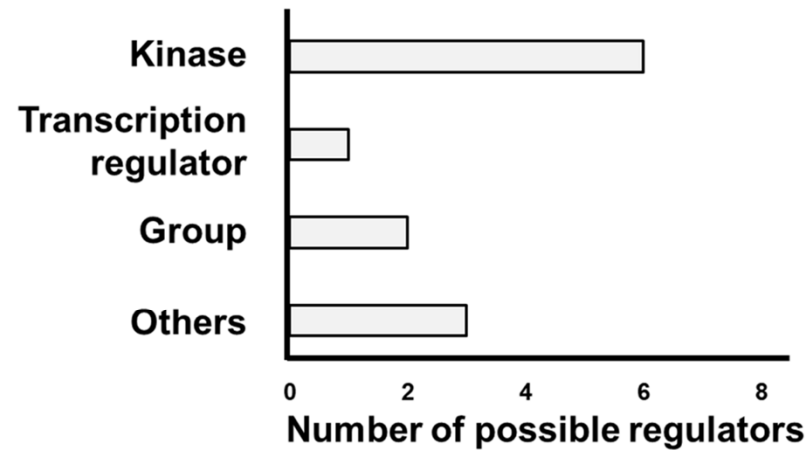


Upstream regulator analysis

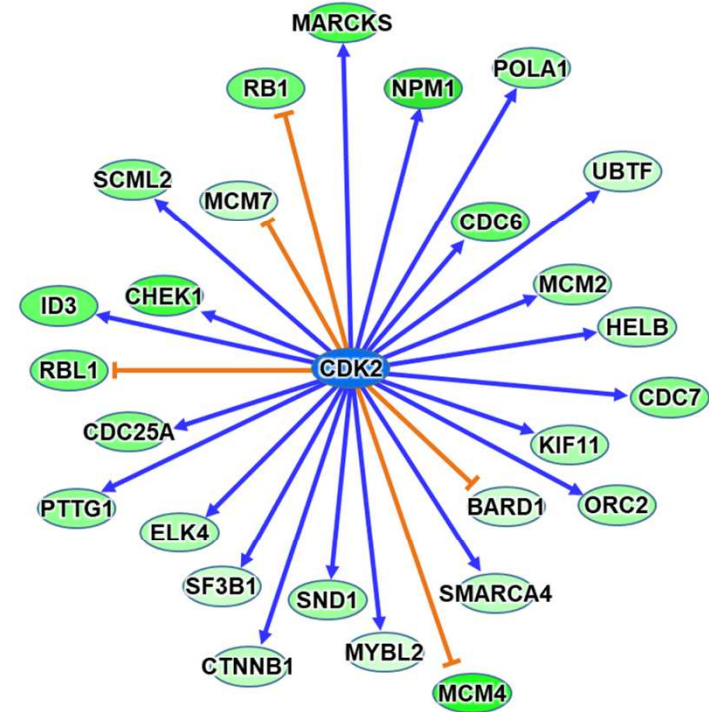
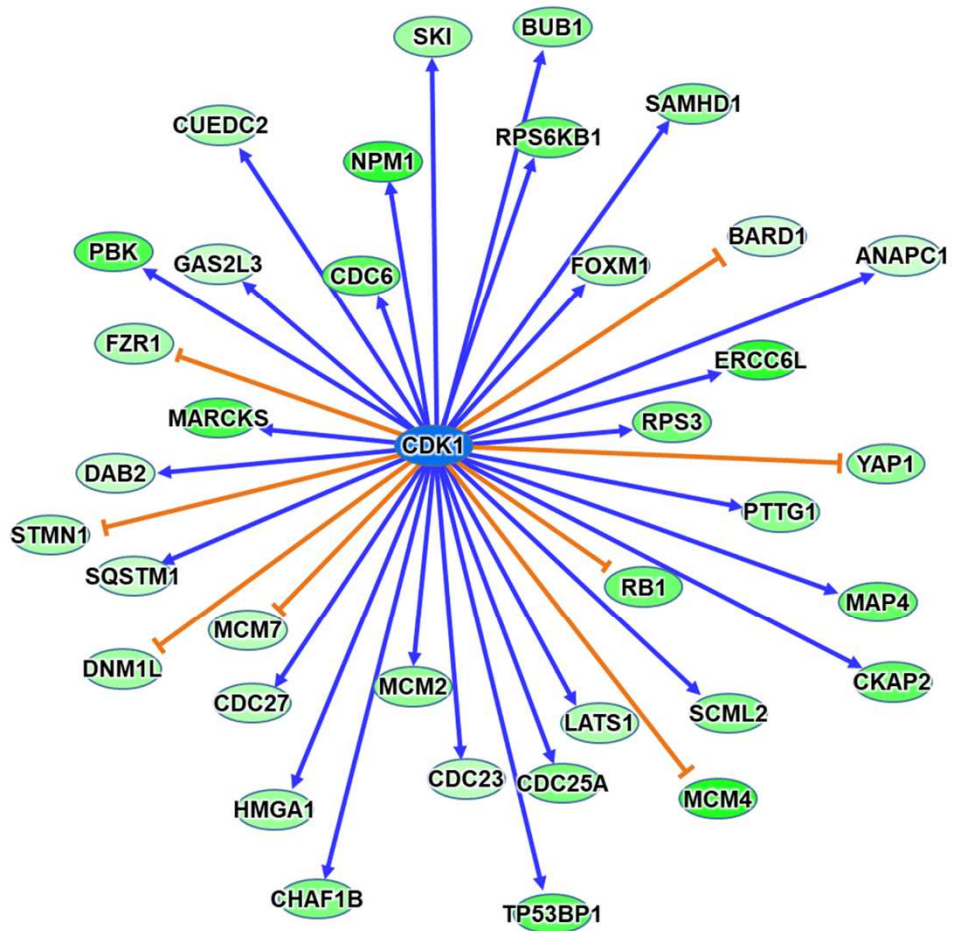
Proteomics



Phospho-proteomics

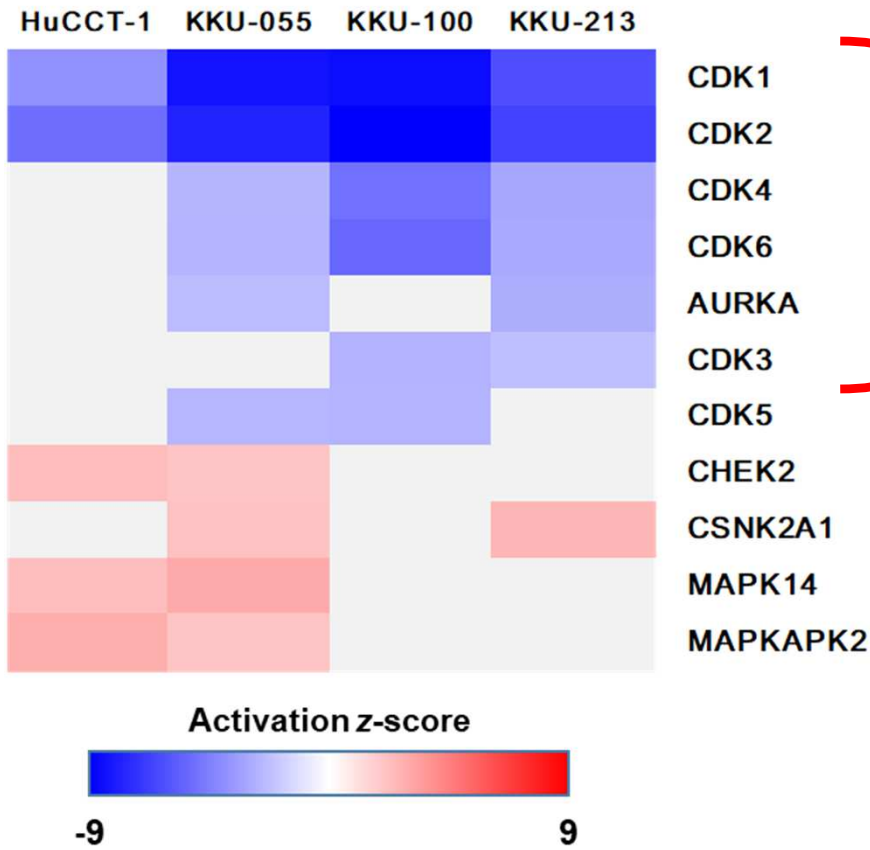


Upstream regulator analysis



**Upstream regulator:
Inactivation of cell cycle proteins (CDK1 and CDK2)**

Kinase substrate enrichment analysis (KSEA)

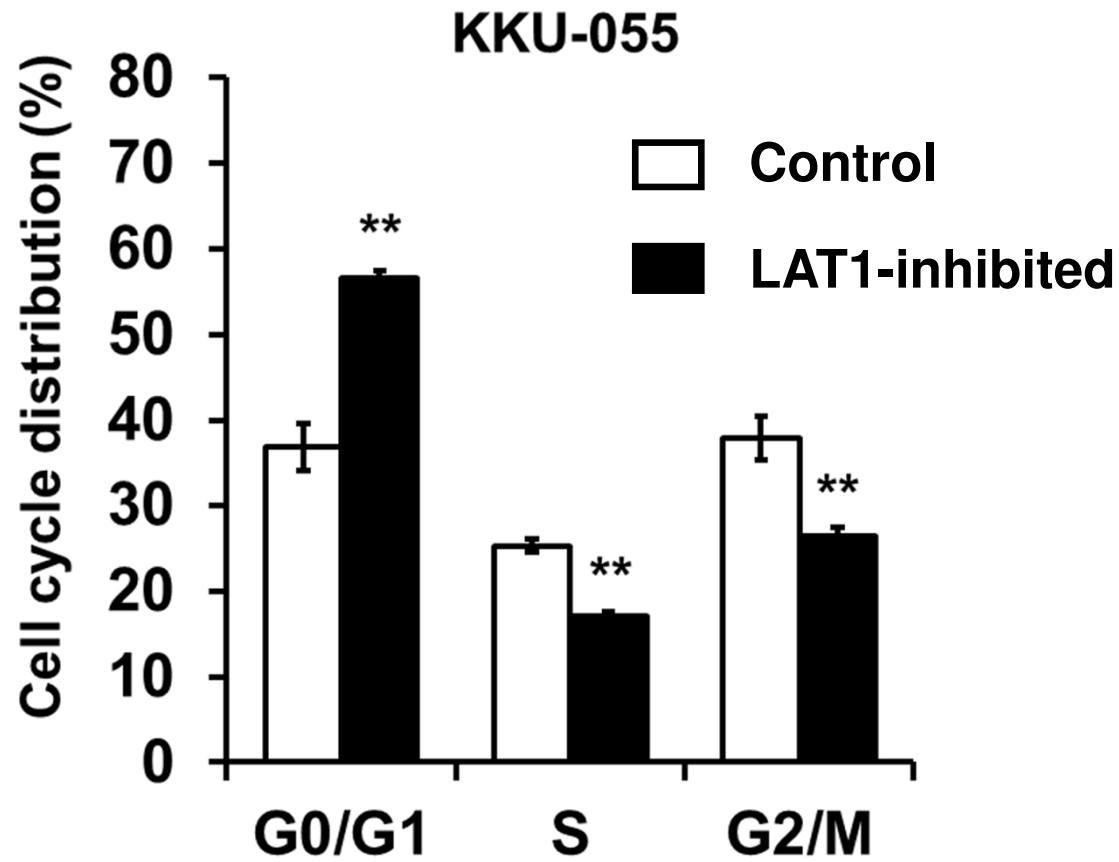


Inactivation of cell cycle-related kinases

CDK1, CDK2, CDK3, CDK4, CDK6
AURKA (aurora kinase A)

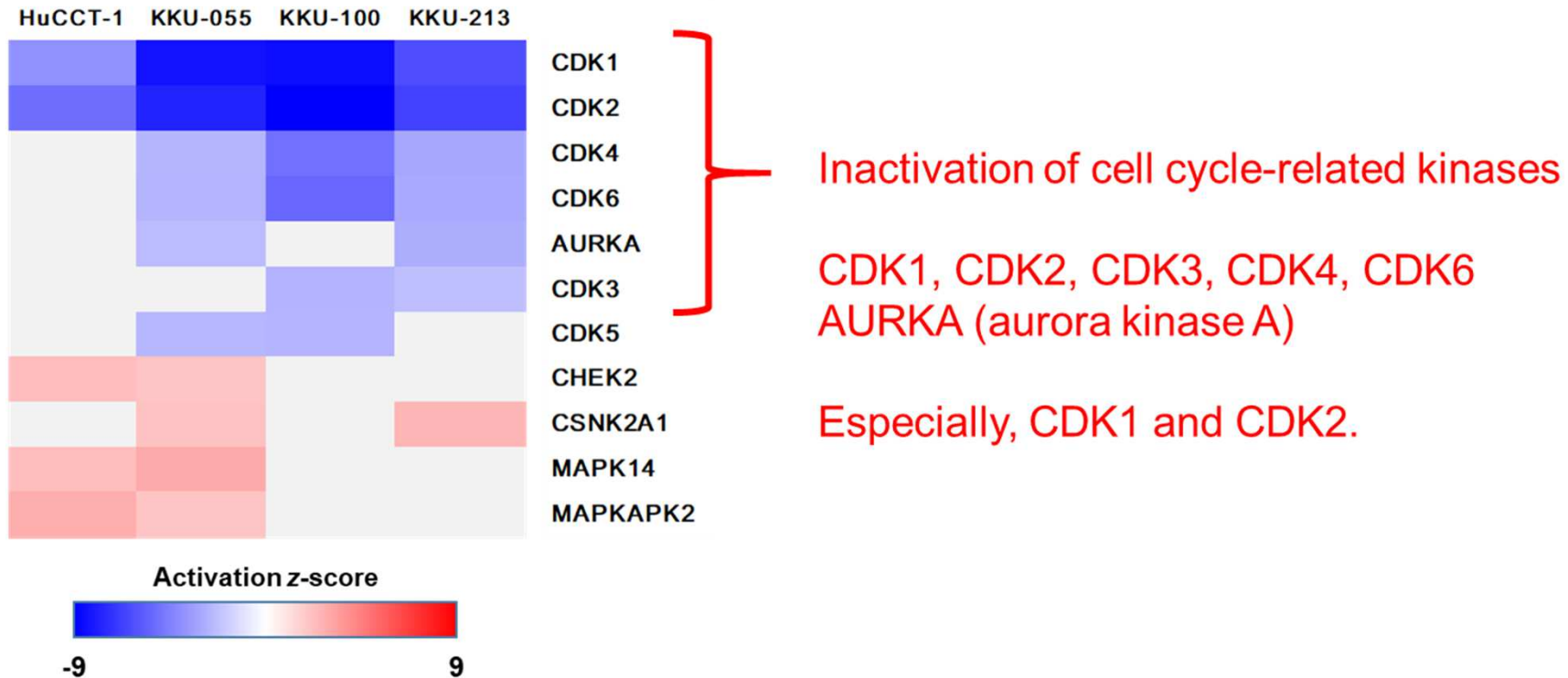
Especially, CDK1 and CDK2.

Cell cycle assay



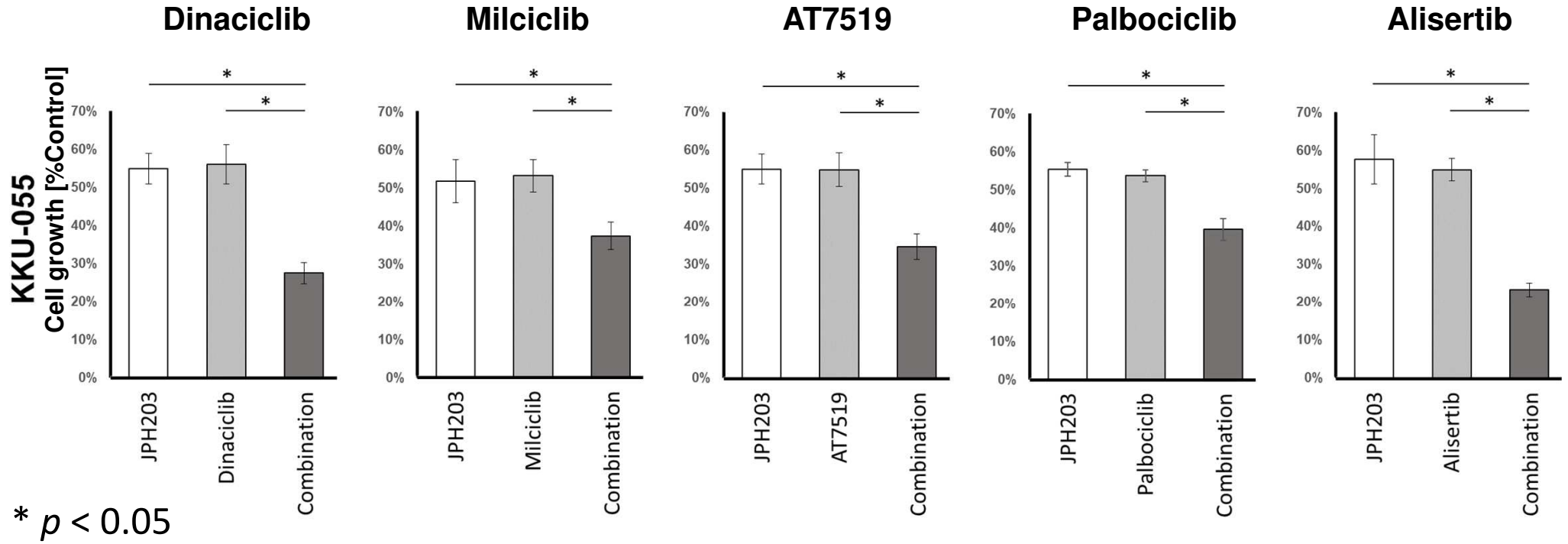
Increase of G0/G1 phase cells by LAT1 inhibition

Combination of LAT1 inhibitor and CDKs inhibitors

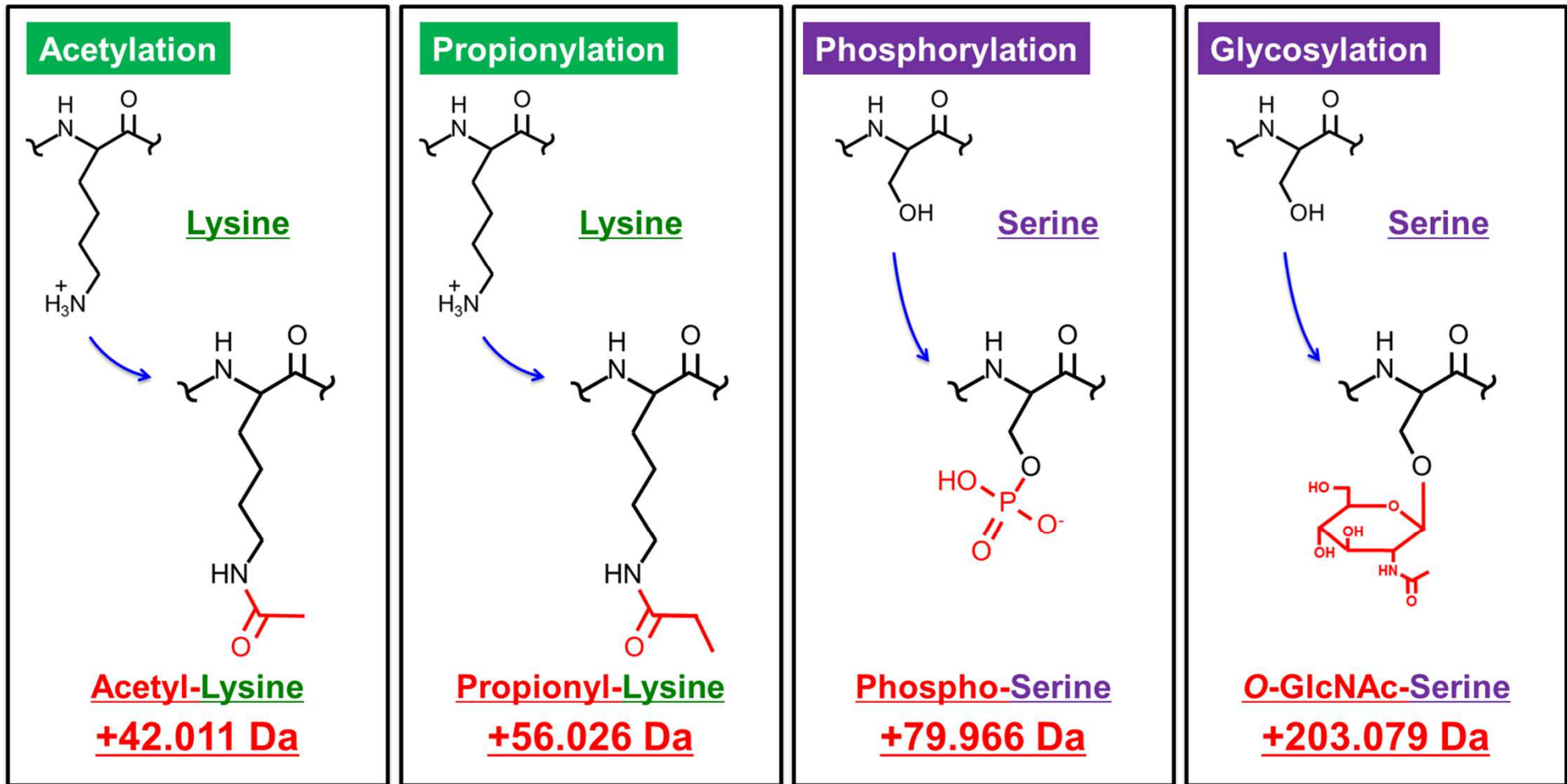


**Inhibitors of these CDKs promote anti-cancer effect of LAT1 inhibitor?
(Dinaciclib, Milciclib, AT7519, Palbociclib, ...)**

Evaluation of drug combinations



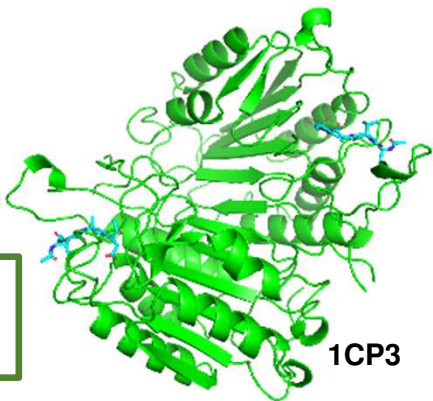
Identification of various PTMs by MS



PTMs are identified by their unique mass shifts from unmodified residues.

Protease specificity profiling & N-terminomics using MS

> 600 proteases in human

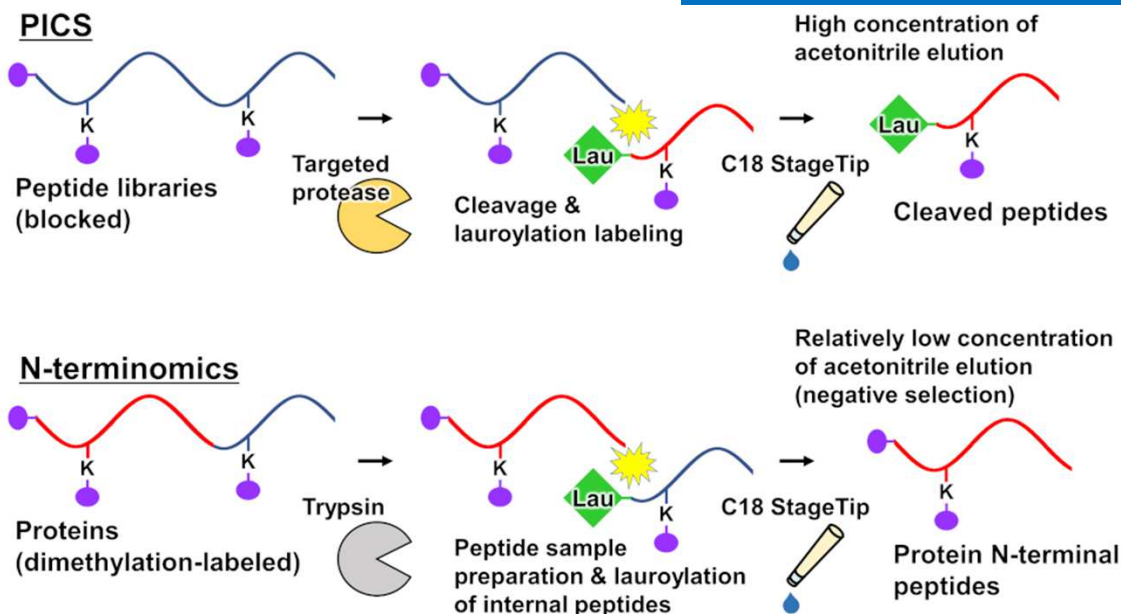


1. Dysregulation of protease activity has been implicated in various pathological conditions, including cancer and inflammation.
2. Proteases play critical roles in infectious diseases and are major components of snake venoms.

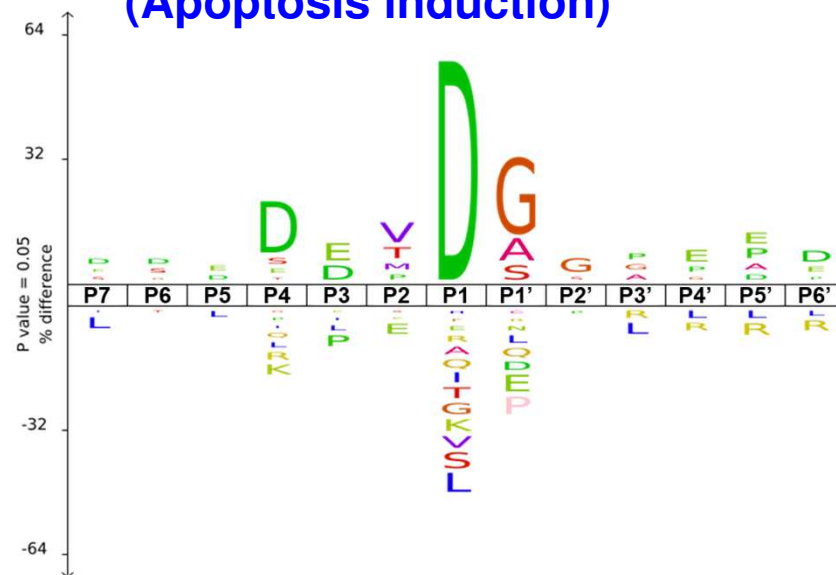
Caspase-3
DEVD↓G

MS-based method:

- Proteomic Identification of Cleavage Sites (PICS)
- N-terminomics



Etoposide treatment (Apoptosis induction)

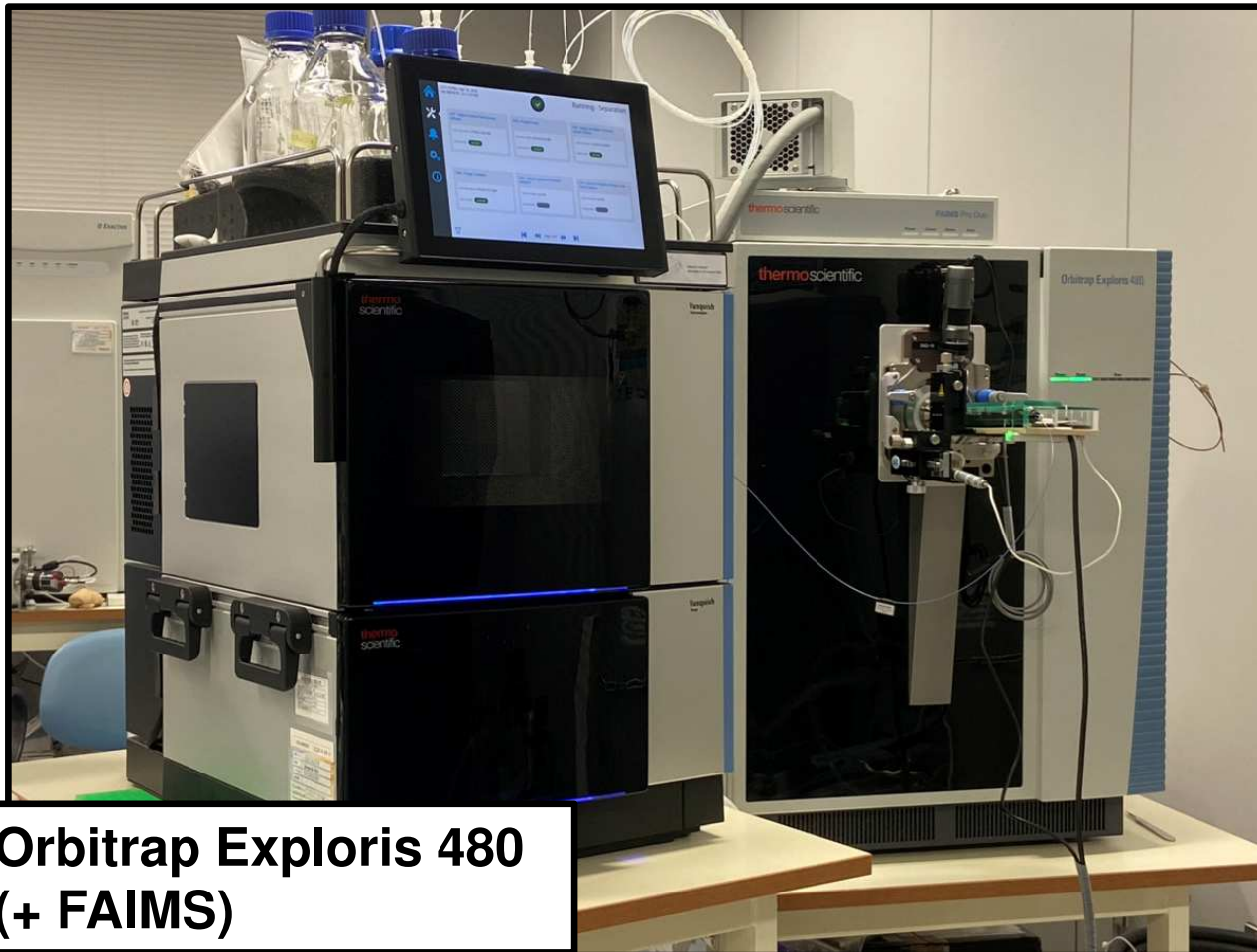


Okanishi, H., *et al.* (2026). A sensitive, specific, and cost-effective lauroylation-assisted workflow for profiling peptide-level protease specificity using proteomic identification of cleavage sites (PICS), with applicability to protein-level N-terminomics *J. Proteome Res.*, doi: 10.1021/acs.jproteome.5c00903. Online ahead of print.

Today's topics

- 1. Mass spectrometry-based proteomics**
- 2. Phosphoproteomics study on cancer cells treated with a molecular targeted drug**
- 3. LC-MS in CoMIT Omics Center (COC)**

Mass spectrometry-based protein analysis

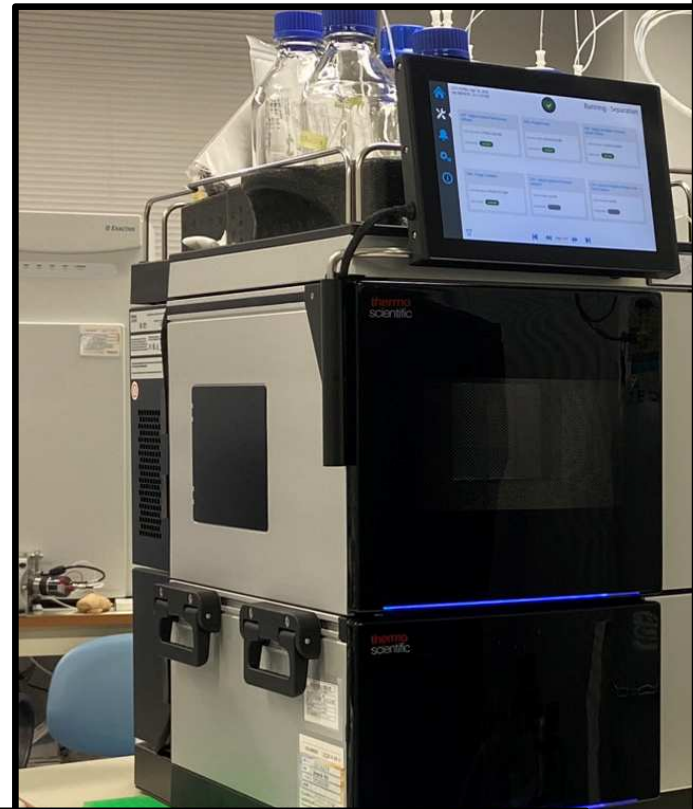


**Orbitrap Exploris 480
(+ FAIMS)**

**Maximum resolution:
48,000
Max scan speed:
>40 Hz
Turbo TMT available
FAIMS Pro available**

Mass spectrometry-based protein analysis

Co-IP sample (comparative quantitative):
 489 identified proteins
 78 candidates response to drug

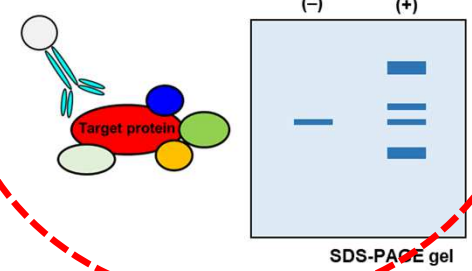
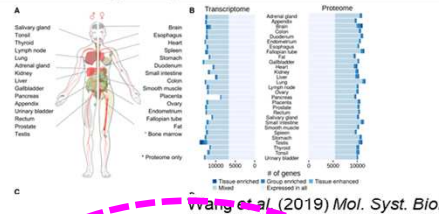


**Orbitrap Exploris 480
 (+ FAIMS)**

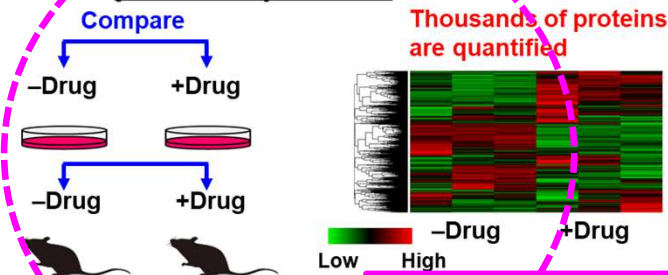
unknown protein

Interaction of proteins
 interacted with target protein

(2) Profiling of proteome in cells or tissues

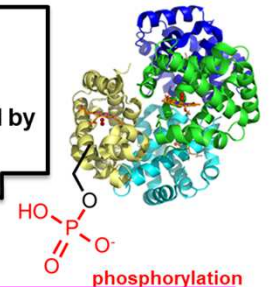


(4) Comparative quantification of protein expression



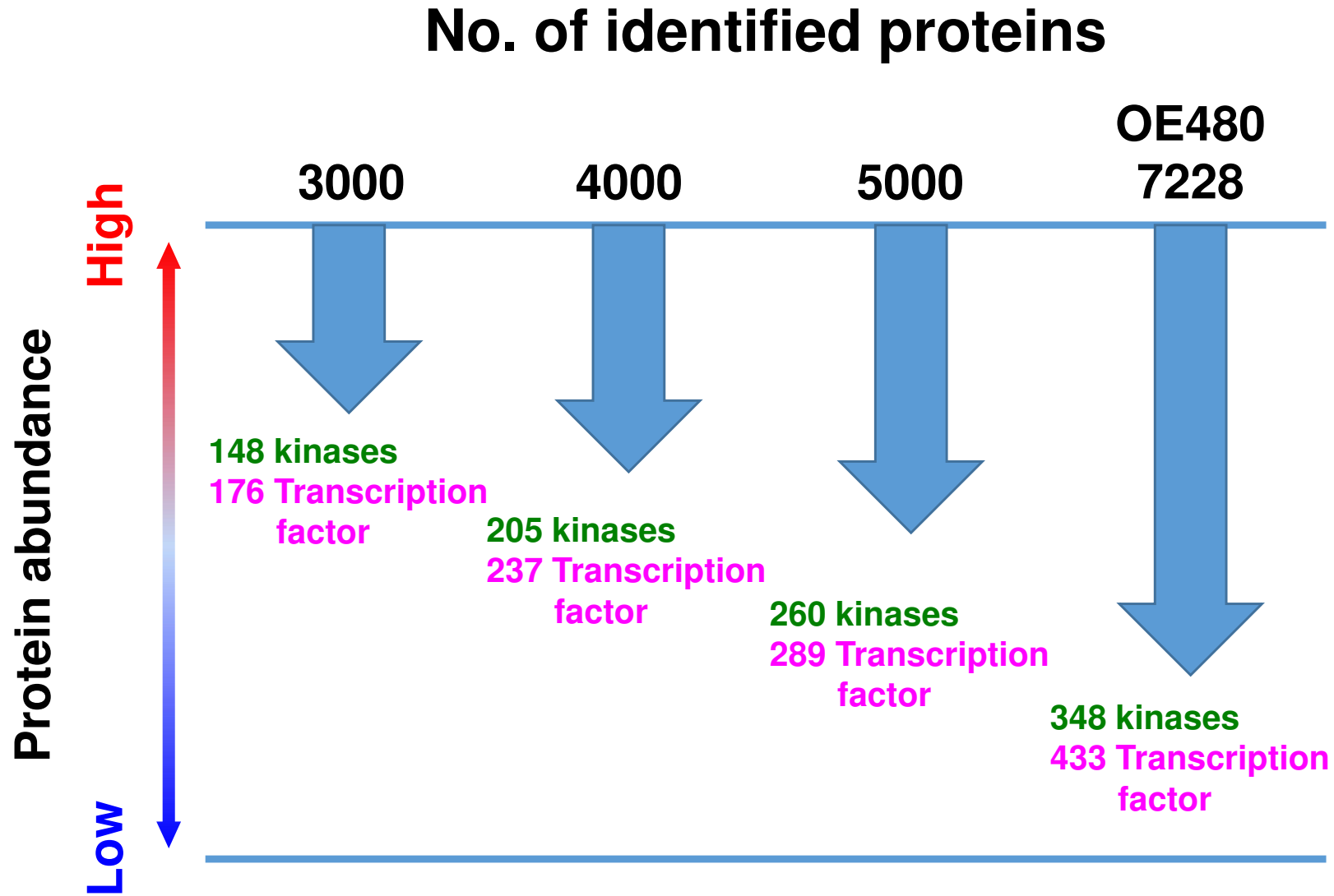
(5) Identification of phosphorylation sites (& PTM sites)

What type PTM?
 Which sites?
 When?
 Up/down-regulated by drug?



Proteome sample (whole cell):
 7228 identified proteins
 (3 h gradient, $n=3$)

Mass spectrometry-based protein analysis

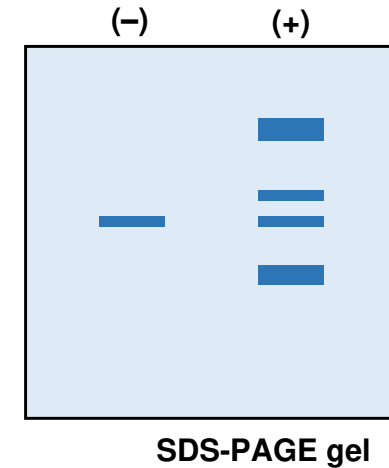
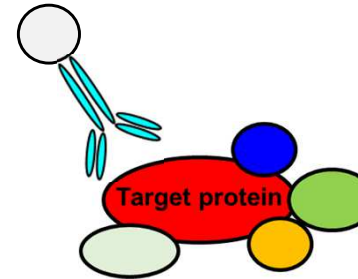


Mass spectrometry-based protein analysis

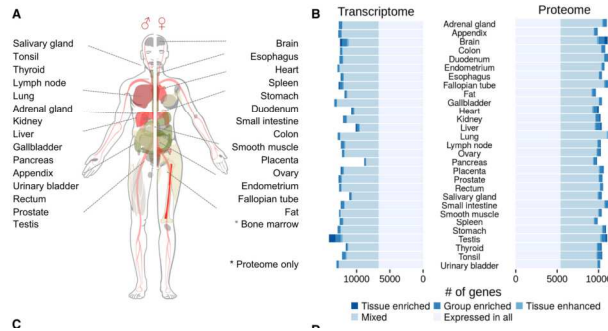
(1) Identification of unknown protein



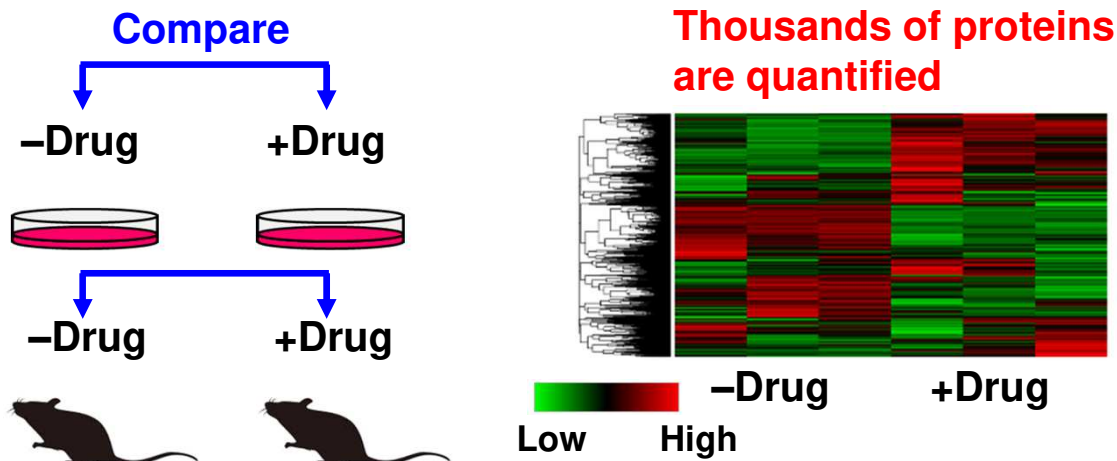
(3) Identification of proteins interacted with target protein



(2) Profiling of proteome in cells or tissues



(4) Comparative quantification of protein expression



(5) Identification of phosphorylation sites (& PTM sites)

What types of PTMs?
Which sites?
When?
Up/down-regulated by drug?

