Chapter 8

Phosphoproteomics-Based Characterization of Cancer Cell Signaling Networks

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Additional information is available at the end of the chapter

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1. Introduction

Signal transduction systems regulate complex biological events such as cell proliferation and differentiation via phosphorylation/dephosphorylation kinetic reactions. Therefore, dysregulation of these systems lead to a variety of diseases such as diabetes, abnormal bone metabolism, autoimmune disease and cancer [1-4]. Above all, cancer is well-known to be caused by aberrant regulation of signaling pathways. Although a large number of studies regarding phosphorylation events in cancer cell networks were performed, a global view of these complex systems has not been fully elucidated. Recent technological advances in mass spectrometry-based proteomics have enabled us to identify thousands of proteins in a single project [5-7] and, in combination with relative quantitation techniques such as Stable Isotope Labeling by Amino acids in Cell culture (SILAC), quantitative analysis regarding signaling-related molecules can also be performed [8,9]. Recently, establishment of phosphorylation-directed peptide/protein enrichment technology has led us to capture the comprehensive status of phosphorylated cellular signaling molecules in a time-resolved manner [10-12]. Tyrosine-phosphoproteome analysis conducted by utilizing anti-phosphotyrosine antibodies unveils key regulatory signaling dynamics triggered by tyrosine kinases such as epidermal growth factor receptor (EGFR) in various contexts of cancer cell signaling. Furthermore, chemistry-based phosphopeptide enrichment technologies such as immobilized metal affinity chromatography (IMAC) [13,14] and metal oxide chromatography (MOC) including titanium dioxide (TiO_2) allows us to describe a serine/threonine/tyrosine-phosphorylation dependent global landscape of cellular signaling at the network level [15,16]. In this chapter, we introduce recent technological development regarding quantitative phosphoproteomics and discuss the future direction of cancer research toward exploration of drug targets in complex signaling networks from a system-level point of view.

2. Shotgun proteomics technology

2.1. Mass spectrometry-based proteomics methodology

Recent progress in mass spectrometry-based proteomics technique has greatly contributed to elucidation of the regulatory networks constituted by a small amount of signaling-related molecules [17]. Especially, modern mass spectrometers termed linear ion trap (LTQ) Orbitrap instrument coupled to nano-flow liquid chromatography (nanoLC) enables us to identify and quantify thousands of signaling factors, leading to characterize diverse aspects of biological processes [18,19]. This system is made up of LTQ [20] and Orbitrap [21], which permits reliable peptide identification with high sensitivity, high mass resolution and high mass accuracy. In principle, there are two methodologies (in-gel digestion and in-solution digestion) for mass spectrometric sample preparation (Figure 1). Recently, liquid-fractionation entrapment technology has also been developed to improve comprehensiveness as well as sensitivity.

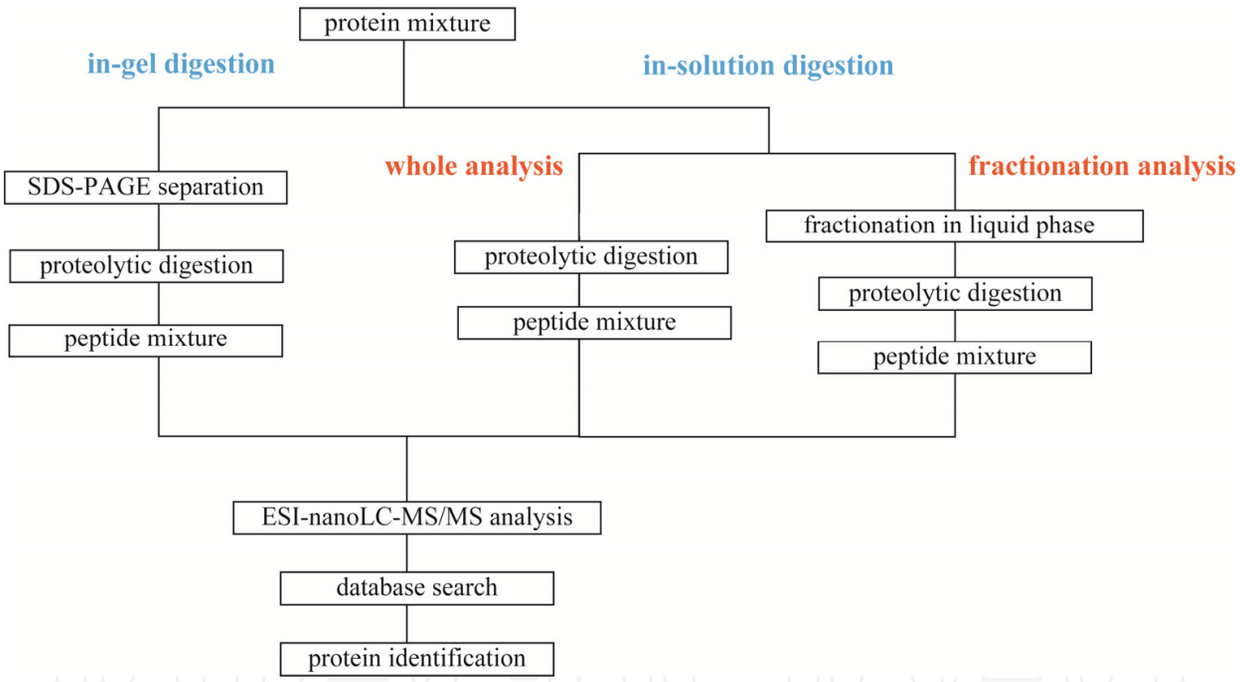


Figure 1. Experimental workflow for advanced mass spectrometry-based proteomics. Two standard methodologies (in-gel digestion and in-solution digestion) are usually applied to sample preparation.

2.2. In-solution fractionation techniques

In order to achieve peptide identification more comprehensively, in-solution fractionation techniques including two dimensional (2D) nanoLC system, Gelfree 8100 Fractionation System (Protein Discovery) [22] and 3100 OFFGEL Fractionator (Agilent) [23] have been developed for further sample separation. 2D nanoLC system consists of on-line strong cation exchange (SCX) and reversed-phase (RP) columns (Figure 2A), whereas off-line fractionation systems such as Gelfree 8100 Fractionation System and 3100 OFFGEL Fractionator separate proteins by molecular weight and isoelectric point, respectively

(Figure 2B, 2C). These systems enable us not only to reduce the complexity of samples but also to minimize the amount of starting materials compared with in-gel digestion.

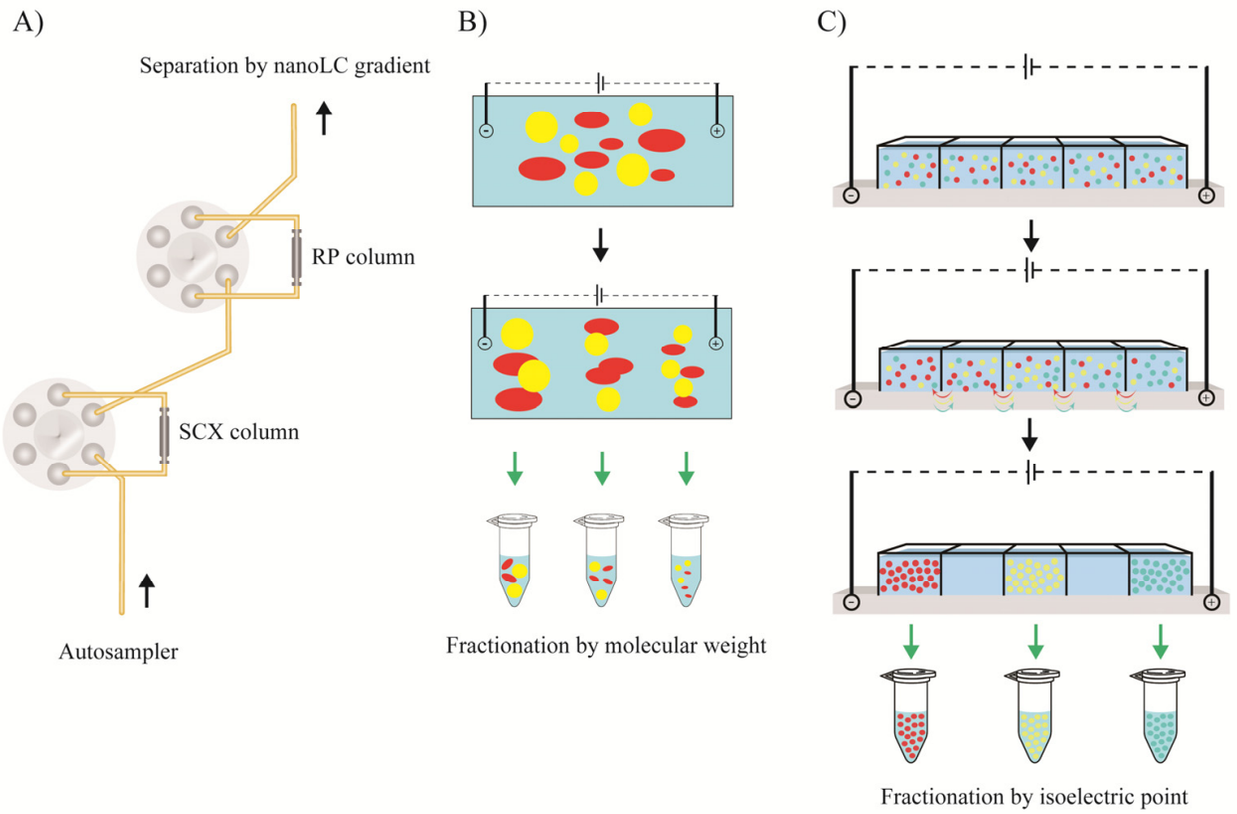


Figure 2. Schematic illustrations for in-solution protein/peptide separation techniques based on fractionation A) using SCX and RP columns (2D nanoLC system), B) by molecular weight (Gelfree 8100 Fractionation System) and C) by isoelectric point (3100 OFFGEL Fractionator).

3. Quantitative proteomics

Quantitative description based on mass spectrometry is not readily available because of the principle that ionization efficiency for mass spectrometric detection depends on the chemical property of each peptide. In recent years, several methods have been intensively developed for absolute and relative quantification [24]. The former methodology enables us to determine the absolute amount of proteins using standard peptides or proteins that are labeled by stable isotopes [25-27]. Meanwhile, the latter can provide information on the relative change in protein/peptide amount. There are two major approaches for relative quantification termed label-free and stable isotope-based methods.

3.1. Label-free methods

The label-free methods that utilize spectral counting or signal intensity for relative quantitation (Figure 3) are simple and economical but less accurate than isotope-based methods [28,29].

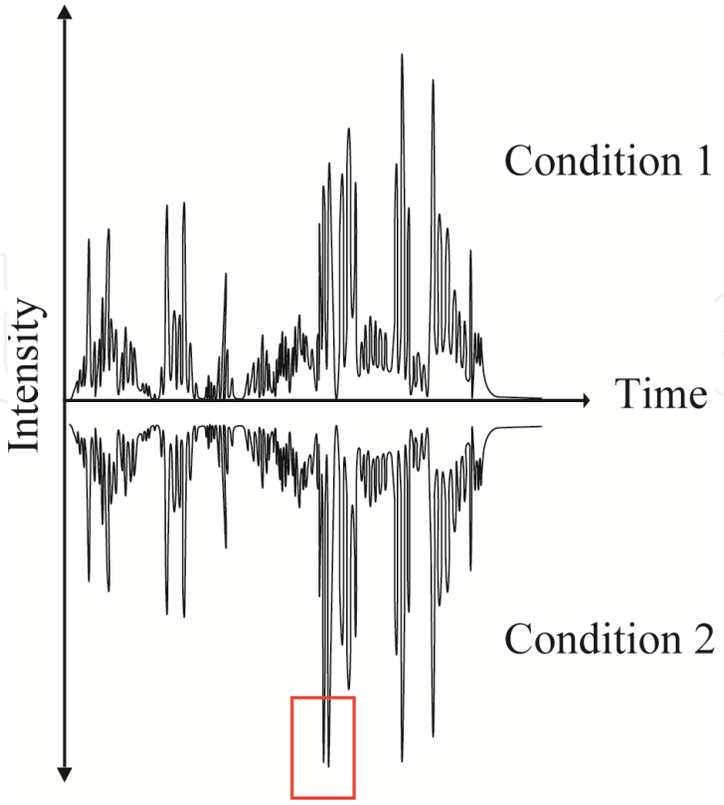


Figure 3. Representative chromatograms acquired under two different conditions. Relative quantitation can be performed by comparing these chromatograms. The red rectangle indicates the peak intensities increased in condition 2 compared with condition 1.

3.2. Stable isotope-based methods

Stable isotope-based methods allow us to distinguish the status of protein/peptide amount of even post translational modifications (PTMs) in a more accurate manner. Stable isotope-labeled reagents were incorporated into specific amino acids by chemical derivatization or metabolic labeling. Isotope-Coded Affinity Tag (ICAT) [30,31], isobaric Tag for Relative and Absolute Quantitation (iTRAQ) [32-34] and Tandem Mass Tag (TMT) [35,36] belong to the former chemical derivatization techniques. As for metabolic labeling strategies, Stable Isotope Labeling by Amino acids in Cell culture (SILAC) technique [37,38] is known as the most useful and accurate for relative quantitation.

3.2.1. ICAT

The chemical structure of the ICAT reagent consists of three regions: a reactive group with cysteine, an isotopically coded linker and a biotin tag (Figure 4). In order to perform a quantitative analysis, the cellular proteomes in two different conditions are labeled with light and heavy ICAT reagents, respectively. After the two samples are combined, they are proteolytically digested and purified with avidin affinity chromatography. The differential analyses are sequentially performed by detecting mass shift using liquid chromatography combined with tandem mass spectrometry (LC-MS/MS).

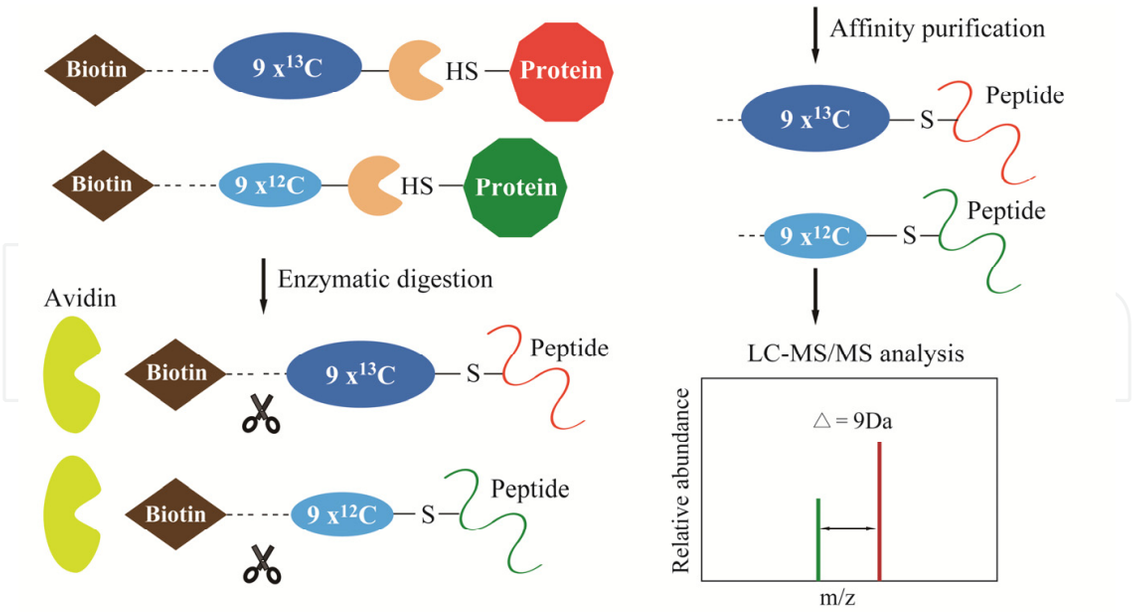


Figure 4. Peptide quantitation using cleavable ICAT. Differentially labeled peptides with ICAT tag at cysteine residues are preferentially enriched and analysed by LC-MS/MS. The ratio of heavy (red peak) to light (green peak) area indicates relative abundance of each peptide.

3.2.2. Isobaric reagents (iTRAQ and TMT)

The isobaric reagents such as iTRAQ and TMT contain an isobaric tag and an amine specific peptide reactive group. This strategy enables us to label all peptides derived from samples. Relative quantification of the mixed sample is performed at the MS/MS fragmentation stage (Figure 5).

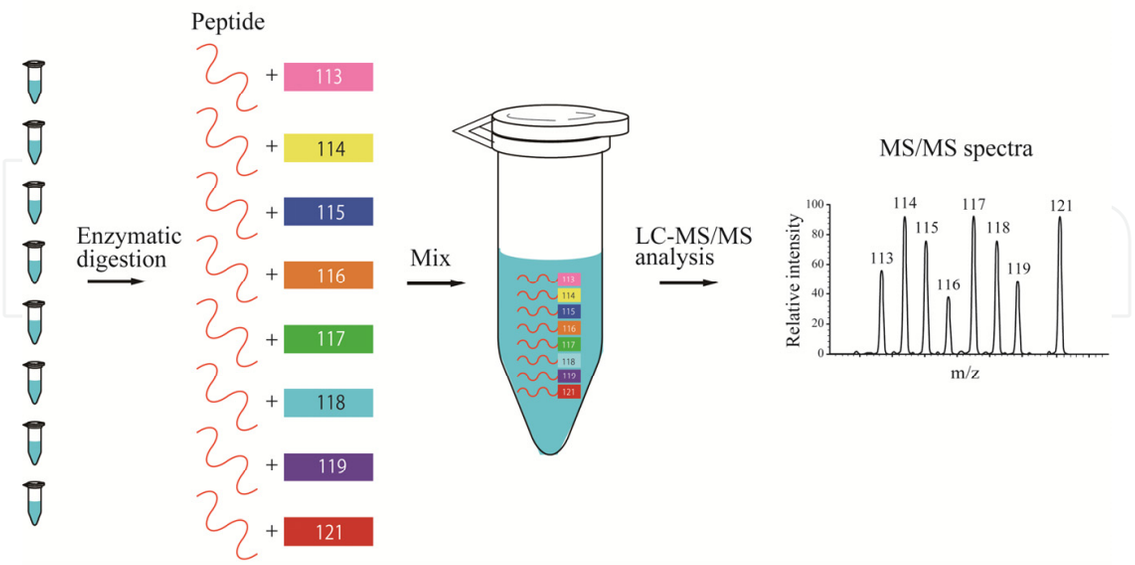


Figure 5. Peptide quantitation using iTRAQ. Peptides labeled by isobaric tags on the N-termini and lysine side chains are mixed and analyzed by LC-MS/MS. After fragmentation, MS/MS spectra of reporter ions are observed in the low mass region. The ratio of these peaks represents a relative amount of each peptide.

3.2.3. SILAC

As for metabolic labeling, Stable Isotope Labeling by Amino acids in Cell culture (SILAC) technique has widely been used to quantify protein abundance or PTM status in different conditions (Figure 6). Two cell populations are grown in different culture media including light or heavy stable isotopes of arginine and/or lysine. The lysates from these cell populations are equally combined, proteolytically digested and analyzed by LC-MS/MS. Regarding each mass pair detected, the ratio of the peak intensities corresponds to the relative peptide abundance.

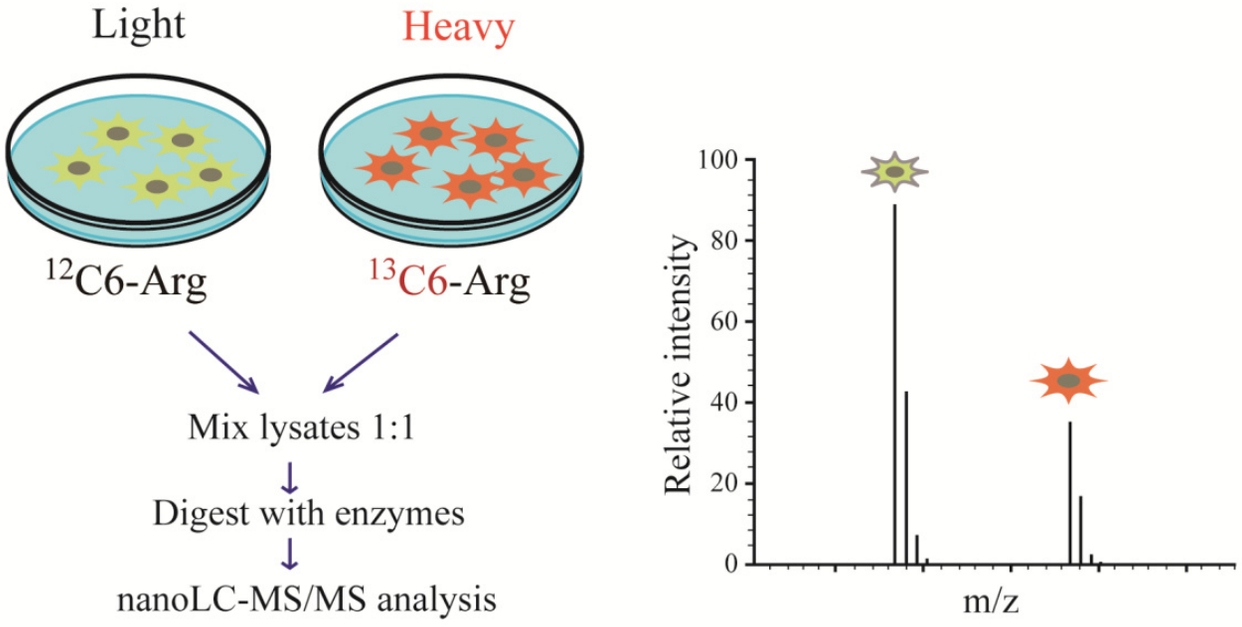


Figure 6. Peptide quantitation using SILAC. Proteins metabolically labeled by differential stable isotopes are combined, proteolytically digested and subjected to nanoLC-MS/MS analysis. The ratio of heavy to light peak area accounts for a relative amount of each peptide.

4. Analytical methodologies for enrichment of phosphorylated molecules

The mechanistic principles for transmitting signals within cellular networks rely greatly on PTMs such as phosphorylation, ubiquitination and acetylation. Although reversible phosphorylation events are well-studied in signal transduction research, a global landscape of phosphorylation-dependent signaling networks remains almost unclear. Here we introduce several phosphoprotein/phosphopeptide enrichment methods for mass spectrometry-based global phosphoproteome analysis.

4.1. Immunoprecipitation using anti-phosphotyrosine antibodies

Anti-phosphotyrosine antibodies are frequently used to enrich tyrosine-phosphorylated proteins (Figure 7A) for analyzing phosphotyrosine-based biological networks using mass spectrometry. These are some previous studies in which this methodology was successfully

applied for phosphotyrosine-related signaling networks in leukemia cells [39] and human HeLa cells [10]. Salomon et al. identified 64 phosphorylation sites on 32 distinct proteins in leukemia cells by treatment with STI571 (Gleevec) [39]. Blagoev et al. showed that 81 signaling related molecules including 31 novel effectors were activated in response to epidermal growth factor (EGF) stimulation in a time-dependent manner [10]. These researches provided the key aspects of cellular regulation in each signaling context.

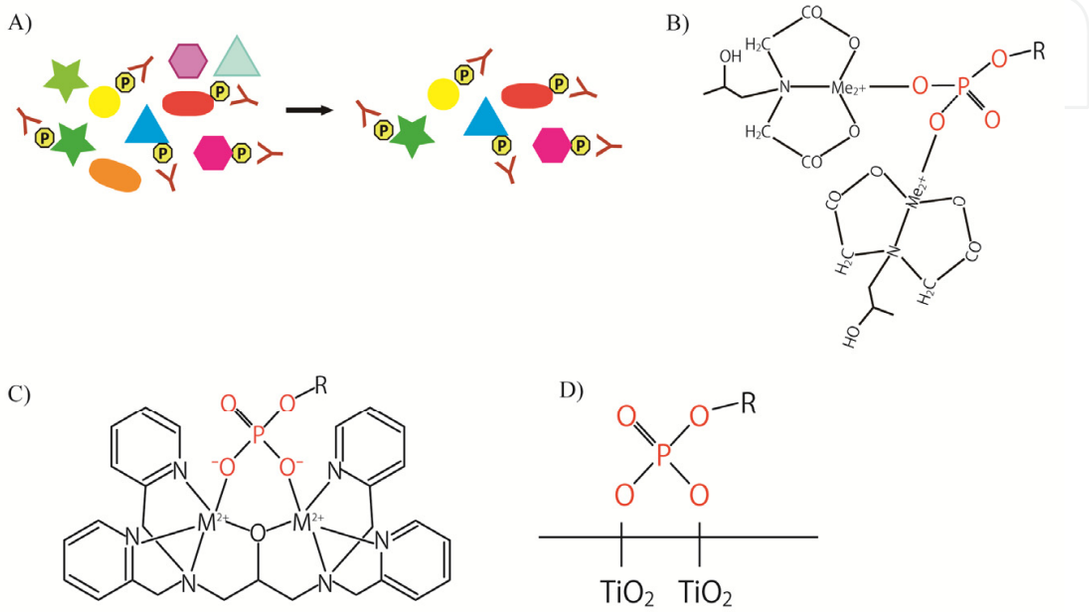


Figure 7. Overview of the affinity status of phosphorylated molecules with A) anti-phosphotyrosine antibody, B) IMAC, C) Phos-Tag and D) TiO_2

4.2. IMAC

Immobilized Metal Affinity Chromatography (IMAC) is based on the notion that phosphate groups can chelate with metal ions such as iron, zinc or gallium (Figure 7B). Stensballe et al. showed that some phosphopeptides could be unambiguously identified using only low-picomole of samples by Fe(III)-IMAC technique [13]. This approach is also known to be suitable for identification of multiply phosphorylated peptides rather than singly modified ones.

4.3. Phos-Tag

Phos-Tag has a vacancy on two metal ions that is accessible for phosphomonoester dianion (Figure 7C). The peptides with phosphorylated serine, threonine and tyrosine residues can be all captured by the chemical structure [40,41].

4.4. TiO_2

Titanium dioxide (TiO_2)-based method is one of the most frequently used technique for phosphopeptide enrichment (Figure 7D) [15,16]. Olsen et al. detected 6,600 phosphorylation

sites on 2,244 proteins in human HeLa cells and showed that 14 % of the identified phosphorylation sites were altered by at least 2-fold in response to EGF stimulation [16]. The unbiased large-scale phosphoproteome data provided more extensive insights regarding phosphorylation-dependent cellular processes.

5. Proteomics-driven computational analysis

In recent years, several functional annotation and network analysis tools have been developed to understand cellular processes from a system-level point of view. Here we introduce two representative computational tools for analyzing large-scale proteome data. Database for Annotation, Visualization and Integrated Discovery (DAVID) [42] (http://david.abcc.ncifcrf.gov/home.jsp), which consists of an integrated biological knowledgebase and some analytical tools, enables extraction of the related information from the functional annotation databases (Figure 8).

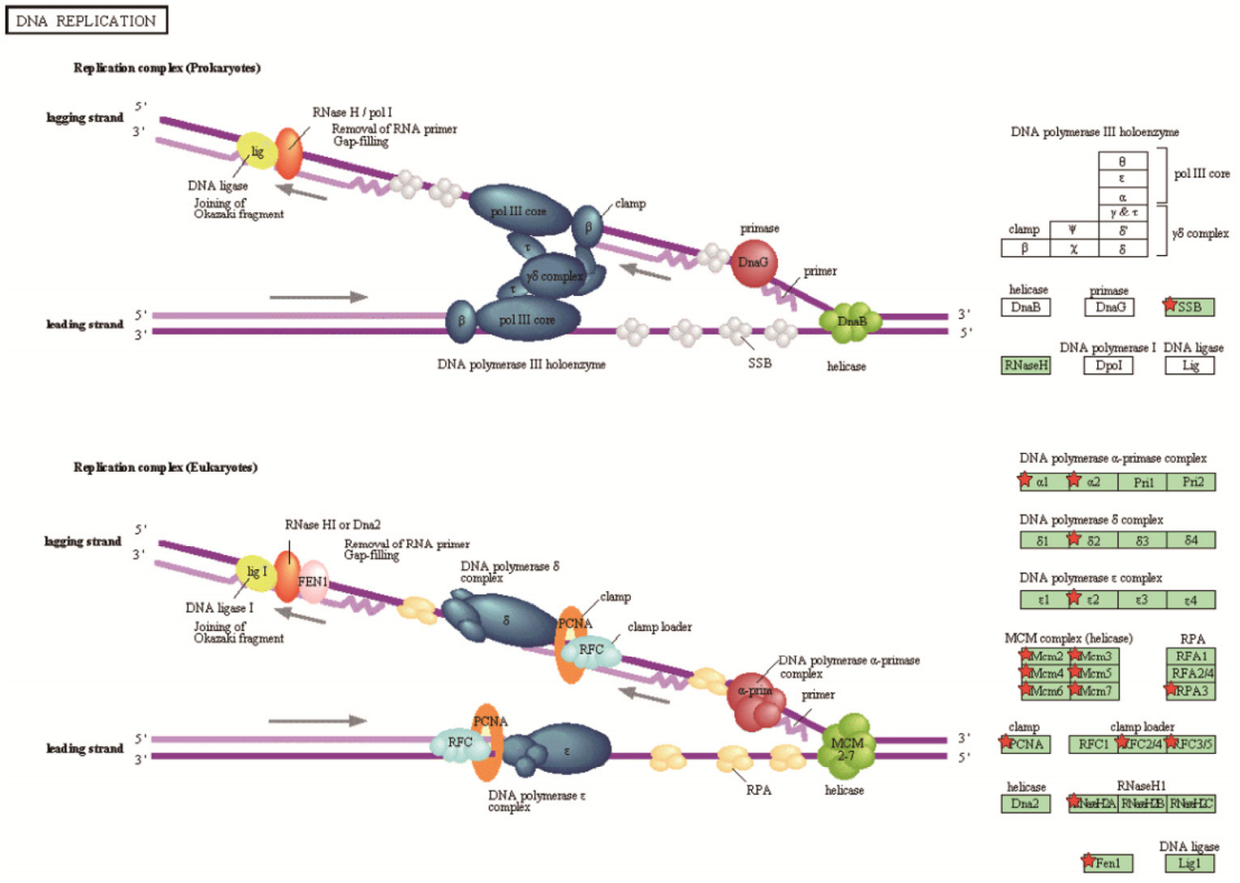
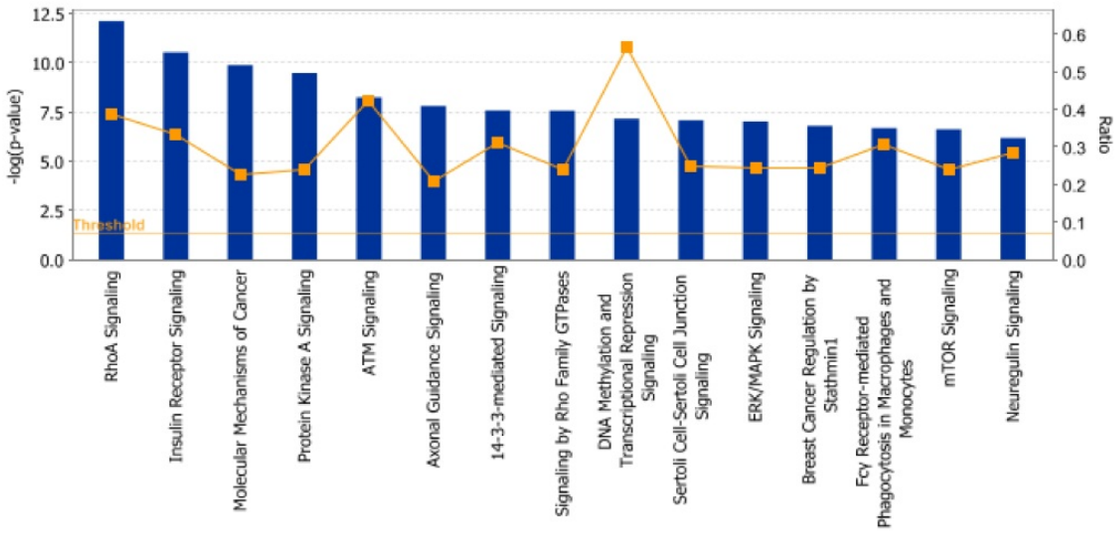


Figure 8. DAVID-based functional description of DNA replication (KEGG pathway). Red symbols indicate the molecules detected by the shotgun proteome analysis of glioblastoma stem cells [43].

Ingenuity Pathways Analysis (IPA) software (http://www.ingenuity.com) (Ingenuity Systems) is used to find networks in relation to experimental proteome data using the Ingenuity Knowledge Base derived from thousands of peer-reviewed journals (Figure 9).

A)



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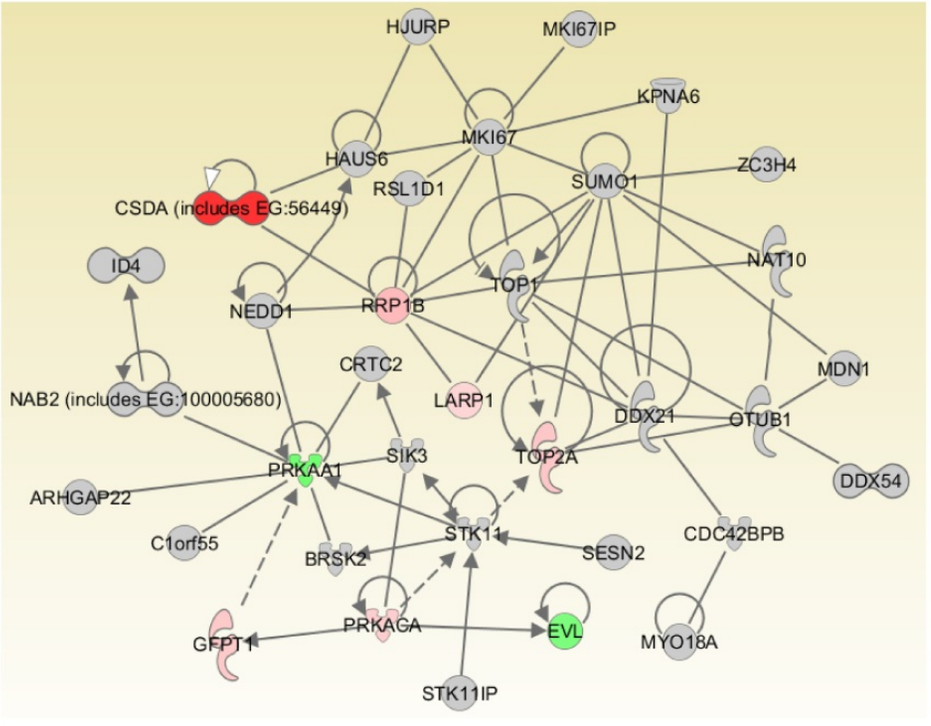


Figure 9. Representative description using IPA software. A) Statistical classification of canonical pathways extracted from experimental data. B) Pathway analysis based on quantitative proteome data.

6. Proteomics-based description of cancer signaling networks

6.1. Phosphoproteome dynamics in cancer cells

Signal transduction systems regulated by tyrosine phosphorylation events are widely known to play a crucial role in fundamental biological processes such as cell proliferation, differentiation and migration. Thus, phosphoproteomics-based approaches have first been applied to reveal the molecular mechanisms governed by tyrosine phosphorylation in response to external growth factors such as EGF [10,11,44,45], fibroblast growth factor (FGF) [46] or heregulin (HRG) [47]. Schulze et al. identified interaction partners of the four members belonging to the ErbB receptor family (EGFR, ErbB2, ErbB3 and ErbB4) using the corresponding synthetic peptides as baits in an unbiased proteomic manner [45]. They revealed that most interaction partners to tyrosine residues were located at the C-terminal end outside the kinase domain of each ErbB family member. Hinsby et al. demonstrated that 28 components were induced by basic fibroblast growth factor (bFGF) stimulation in FGFR-1 expressing cells [46]. The effect of EGF stimulation on human epithelial carcinoma A431 cells was also examined in a time-resolved manner [11] (Figure 10A). Among a total of 136 proteins identified, 56 molecules were quantified by more than 1.5-fold changes upon EGF stimulation. Moreover, the temporal perturbation effects of the Src-family kinase inhibitor, PP2, on the prolonged activation phase were also evaluated regarding various cellular proteins including Src-family kinase substrates. Consequently, the effect of PP2 on the molecules which belong to cell adhesion such as Catenin δ showed significant down-regulation, whereas the impact on the factors related to classical cascades such as EGFR was modest (Figure 10B). IPA analysis was then performed to elucidate the PP2 effects on the EGF-induced A431 cells at the network level (Figure 11). These results clearly showed the differences in tyrosine-phosphorylation levels in the presence or absence of PP2. Thus, these data provide further insight into how such complex biological systems would function in response to external perturbation.

By combining quantitative phosphoproteome and transcriptome data *in silico*, Oyama et al. performed a system-level analysis regarding cellular information networks in wild-type (WT) and tamoxifen-resistant (TamR) human breast adenocarcinoma MCF-7 cells in response to HRG and 17β-estradiol (E2) stimulation [47] (Figure 12). The integrative analysis of phosphoproteome and transcriptome in MCF-7 cells revealed that activation of glycogen-synthase kinase 3β (GSK3β) and mitogen-activated protein kinase (MAPK) 1/3 signaling might be associated with altered activation of CREB and AP-1 transcription factors in TamR MCF-7 cells, which potentially defines drug-resistance properties against tamoxifen (Figure 13).

6.2. Large-scale proteomic characterization of cancer stem/initiating cells

Cancer cells are widely known to be heterogeneous, even though they were derived from a single transformed cell [48]. Some of them show resistance to anti-cancer drugs and radiation therapies [49,50] and recent studies also demonstrated the existence of cancer stem cells (CSCs) in various types of cancer cells including leukemia [51], breast cancer [52], glioma [53,54] and colon cancer [55,56]. Moreover, it has been getting clear that CSCs have

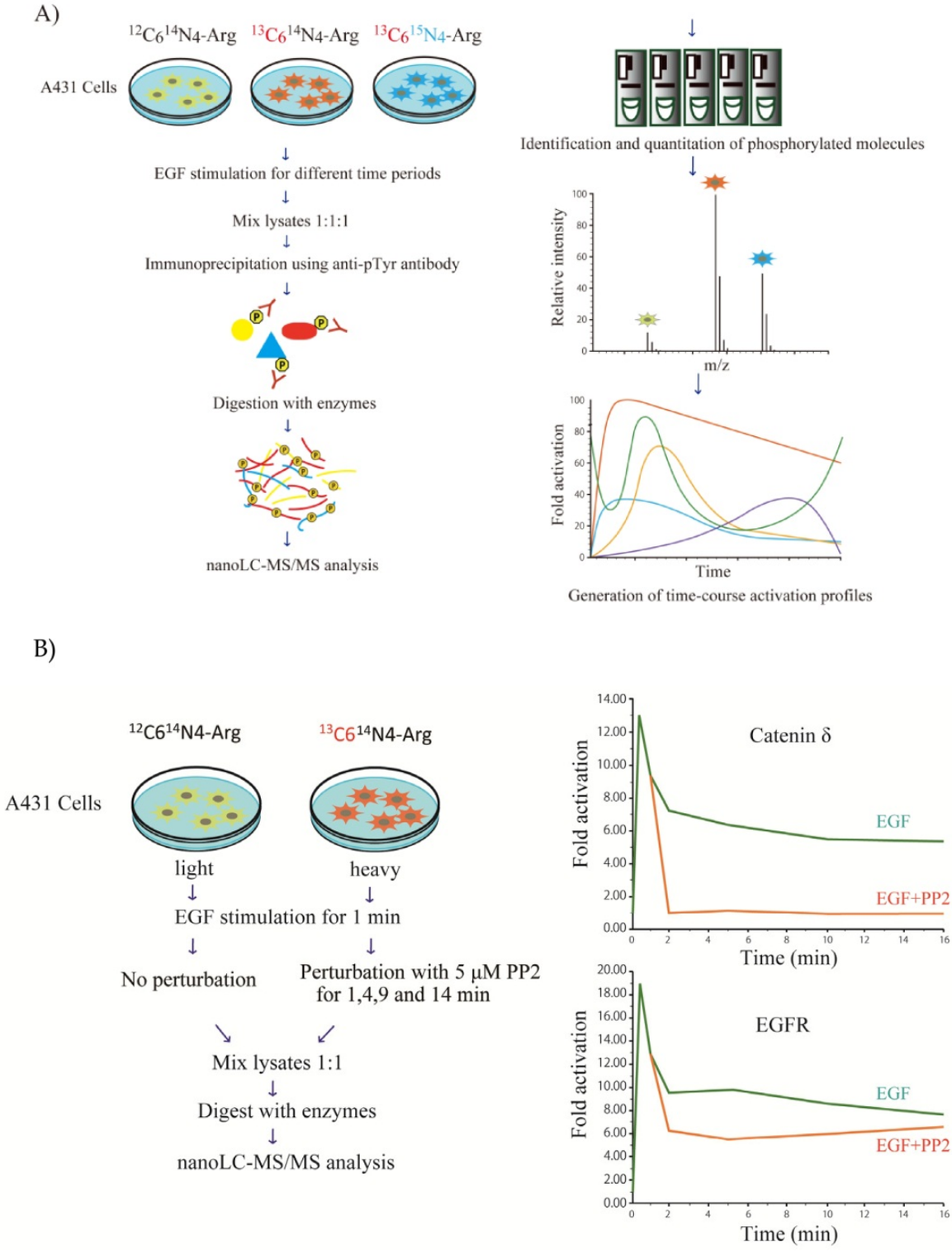
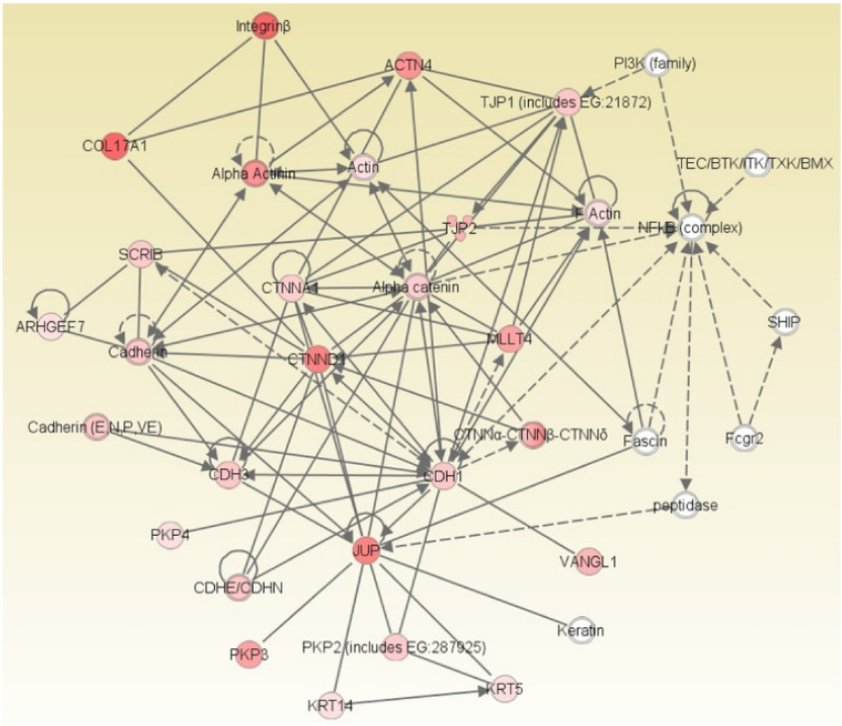


Figure 10. Schematic procedures for identification and SILAC-based quantitation of tyrosine-phosphoproteome in A431 cells [11]. A) The experimental procedure using three different SILAC media to describe tyrosine-phosphoproteome dynamics in response to EGF stimulation. B) Comparative analysis using two distinct SILAC media for evaluation of the perturbation effects by Src-family kinase inhibitor, PP2. Green lines show EGF activation profiles ,whereas red ones indicate temporal perturbation effects by PP2.

A)



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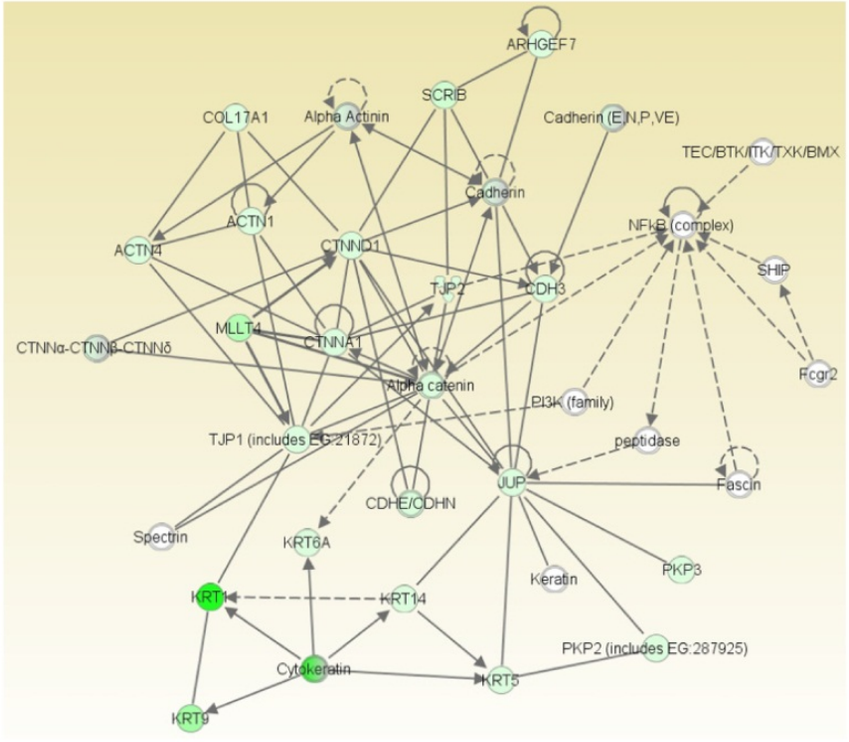

Figure 11. Network analysis of the quantitative phosphoproteome data on A431 cells A) upon EGF stimulation and B) subsequently perturbed by PP2, respectively. Red and green nodes indicate up- and down-regulated signalling molecules, respectively.

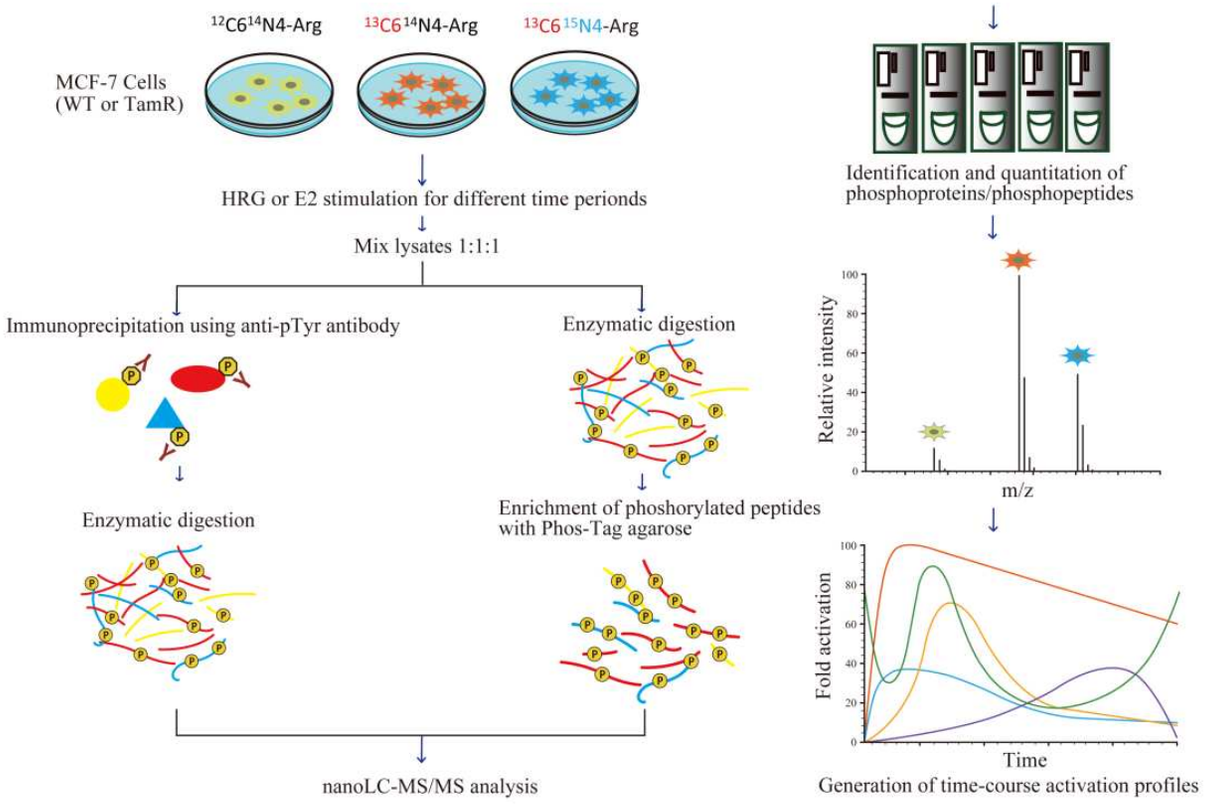

Figure 12. A schematic procedure for identification and quantitation of large-scale phosphoproteome in ligand-stimulated MCF7 cells [47]. The phosphorylated molecules captured by anti-pTyr antibodies or Phos-tag agarose were analysed by nanoLC-MS/MS.

the ability of treatment refractory [57-60] as well as biological properties similar to normal stem cells such as self-renewal and differentiation potency [61]. Recent studies also pointed out the possibility that CSCs were derived from normal stem cells and any non-CSCs might also convert to CSCs [62]. Therefore, comprehensive elucidation of signaling networks in CSCs is considered to be one of the most important steps in cancer research. Thus, we applied mass spectrometry-based shotgun proteomics technology to characterize protein expression profiles [43] and global phosphorylation-dependent signaling networks [63] in glioblastoma stem/initiating cells derived from brain tissues (Figure 14).

In order to gain a comprehensive overview of protein expression in glioblastoma stem/initiating cells, we conducted a shotgun proteome analysis, leading to identification of 2,089 proteins in total [43]. The DAVID-based pathway analysis showed the expressed proteome were enriched in ribosome (Figure 15), spliceosome and proteasome to a high degree. Thus, global protein expression analysis using advanced mass spectrometry offers novel viewpoints for characterization of key factors besides other methodologies such as fluorescence-activated cell sorting (FACS) and gene expression analyses.

The global phosphoproteome analysis of these glioblastoma stem cells also enabled us to determine 6,073 phosphopeptides derived from 2,282 proteins using two fragmentation methodologies of collision induced dissociation and higher energy C-trap dissociation [63]. The IPA analysis of the phosphoproteome data unveiled a variety of canonical pathways

that have been reported to play a crucial role in cancer cells and normal stem cells (Figure 16). Among them, mTOR signaling, which is known to play an important part in stem cell regulation [64,65], was found to be one of the most highly enriched pathways. Very interestingly, the phosphorylation status of EIF4EBP1 and RPS6, which enhance mRNA translation, were up-regulated by EGF stimulation (Figure 17). The analysis also led to identification of various novel phosphorylation sites on the molecules with stem cell-like and glioma properties such as nestin and vimentin [66]. More intriguingly, some novel phosphopeptides derived from undefined regions within the human transcript sequences were also determined from the large-scale phosphoproteome data and the phosphorylation status of the peptide encoded by supervillin-like (LOC645954) was found to be altered upon EGF stimulation (Figure 18).

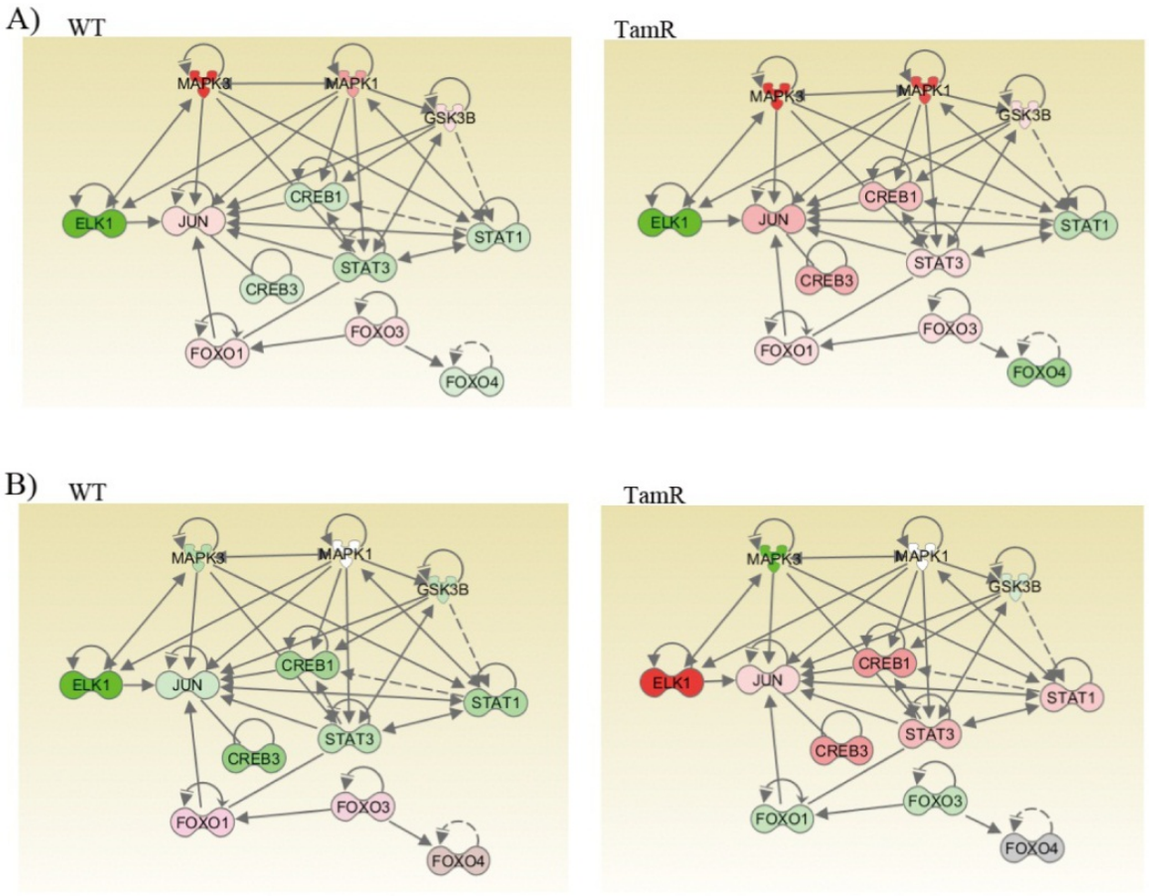

Figure 13. Integrative network analyses of quantitative phosphoproteome and transcriptome data obtained from MCF7 cells A) after HRG stimulation and B) after E2 stimulation. Red and green nodes indicate up- and down-regulated signaling molecules, respectively.

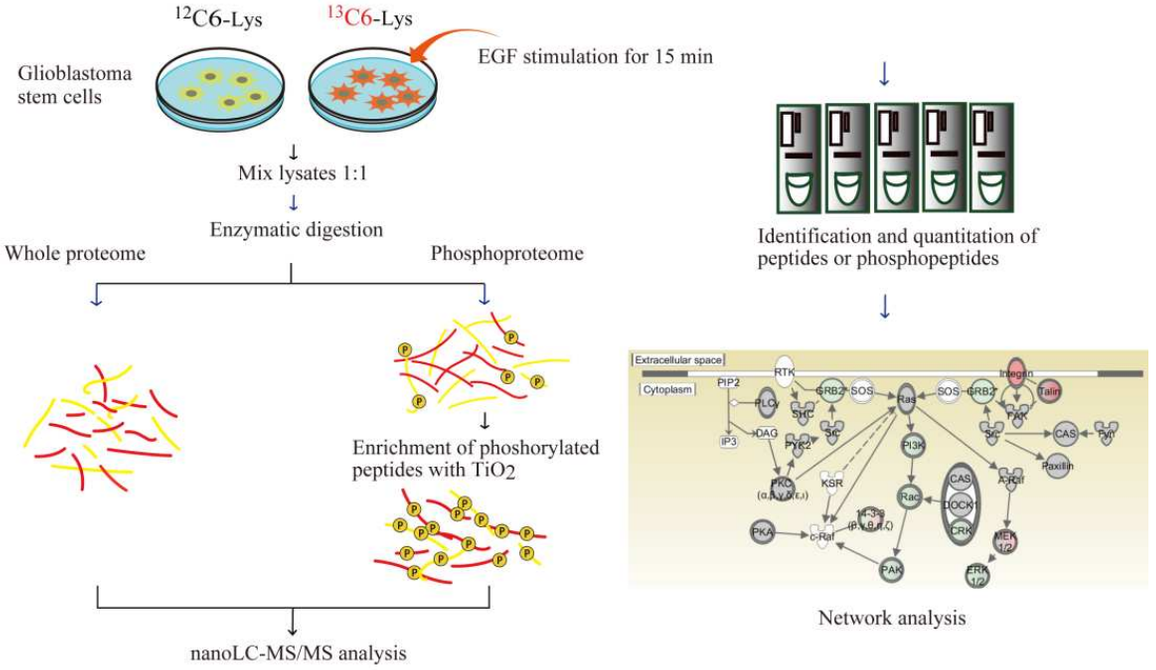

Figure 14. Schematic procedures for identification and quantitation of the expressed proteome and phosphoproteome in glioblastoma stem cells. The whole proteome and phosphoproteome were analysed by nanoLC-MS/MS.

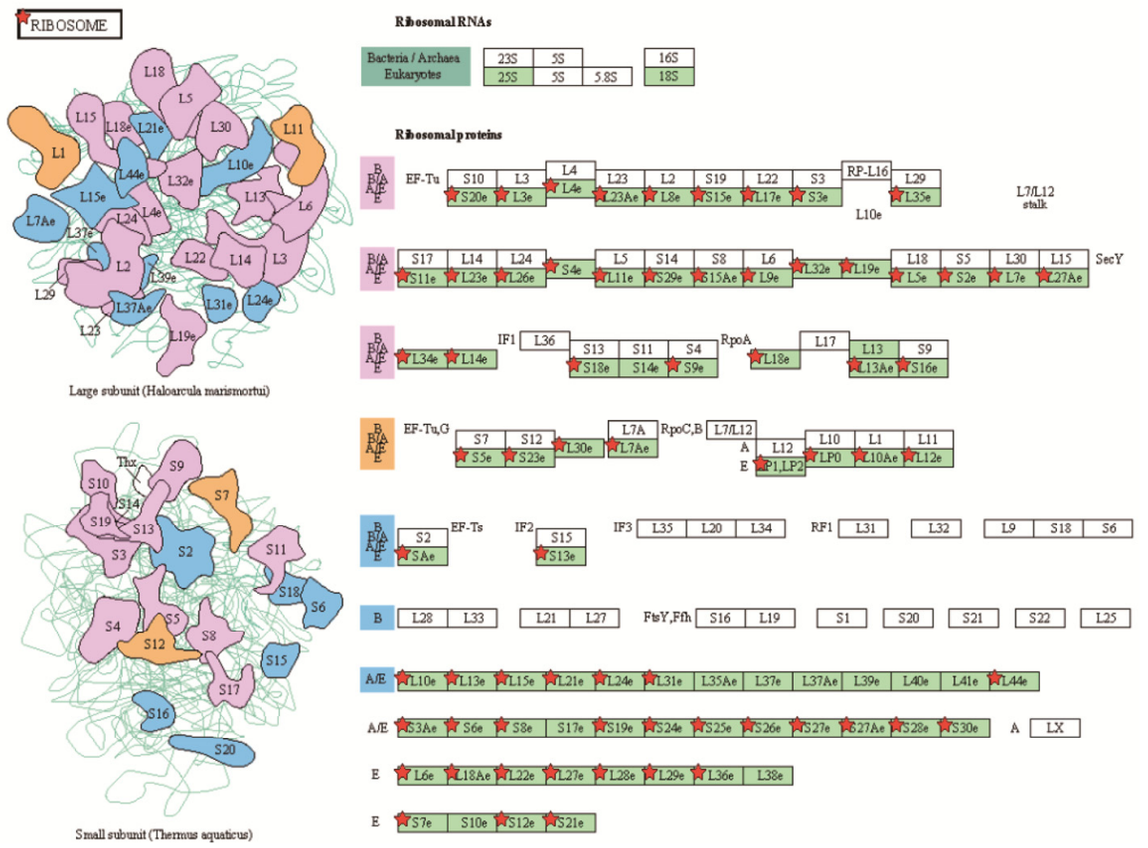

Figure 15. DAVID-based functional description of Ribosome pathway (KEGG pathway). Red symbols indicate the molecules detected in the proteomic analysis of glioblastoma stem cells [43].

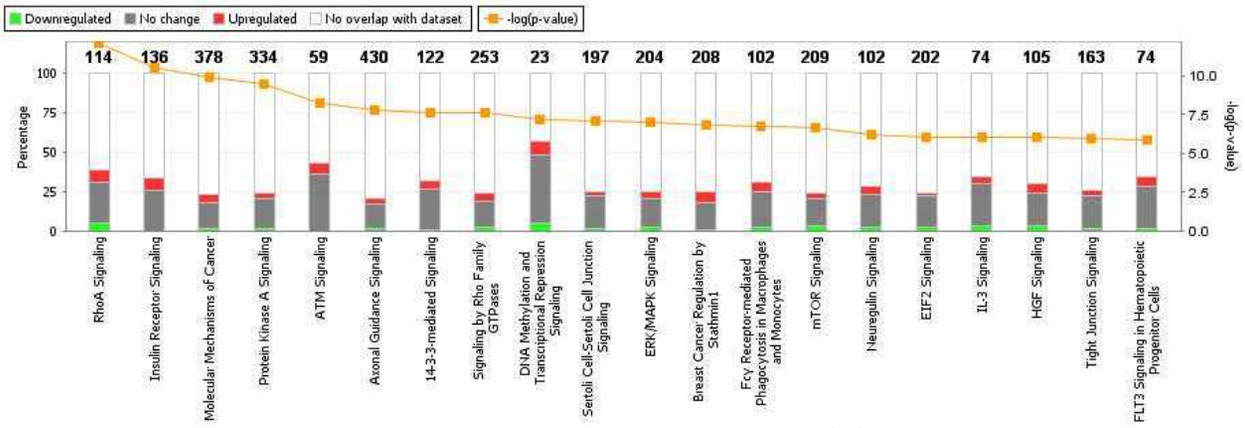

Figure 16. Representative canonical pathways enriched in the phophoproteome of glioblastoma stem cells. Red and green bars indicate up- and down-regulation of phosphorylation levels in response to EGF stimulation, respectively. Orange dots denote –log(p-value) by Fisher's Exact test, indicating the statistical significance of the molecules in each criterion.

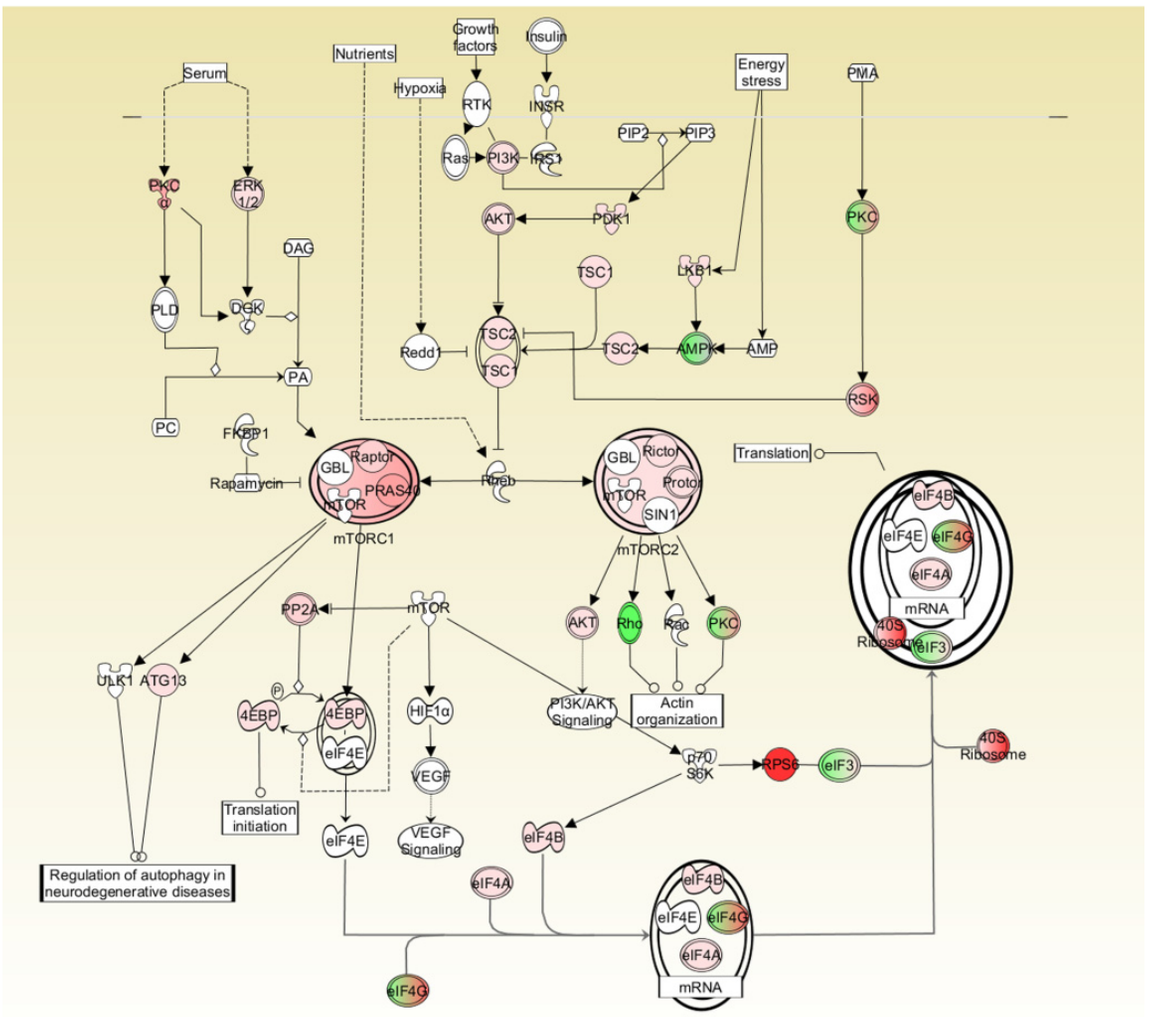

Figure 17. IPA-based network description of mTOR signaling extracted from the large-scale phosphoproteome data on glioblastoma stem cells. Red and green nodes indicate up- and down-regulated signaling effectors in response to EGF stimulation, respectively.

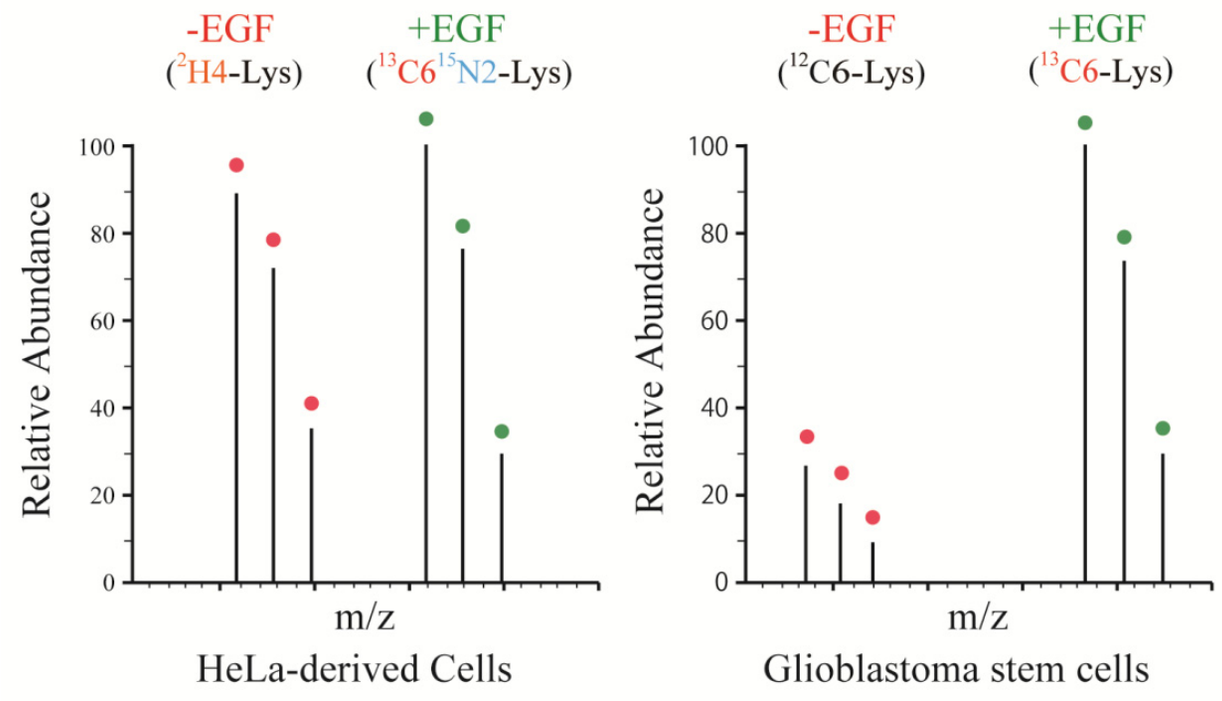

Figure 18. Mass spectra of the novel phosphopeptide encoded by supervillin-like (LOC645954) in HeLa-derived cells and glioblastoma stem cells upon EGF stimulation [63].

7. Conclusion

Advanced mass spectrometry-based proteomics has become a powerful tool for comprehensive understanding of signal transduction networks at the system level. In this chapter, we introduced recent proteomics technologies regarding relative quantitation and enrichment of phosphorylated proteins/peptides for large-scale description of signaling network dynamics. Utilizing these approaches, thousands of phosphorylation sites on diverse signaling-related molecules can now be identified in an unbiased fashion. Quantitative information on the effects of ligand stimulation and inhibitor perturbation also proved beneficial to understand the phosphorylation dynamics at the network level. Furthermore, extensive *in silico* analyses based on comprehensive proteome data enabled us to describe a system-level view of biological networks in a statistical manner. Consequently, mass-spectrometry-based proteomics will pave the way to evaluate molecular hubs in signaling systems and to develop novel targets for treatment of various diseases caused by signaling aberration [67,68].

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