Biochimica et Biophysica Acta 1828 (2013) 23–34

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Review

Connexin multi-site phosphorylation: Mass spectrometry-based proteomics fills the gap ☆

Vincent C. Chen [a,b,*], Joost W. Gouw [a], Christian C. Naus [b,1,2], Leonard J. Foster [a,1,3]

[a] Department of Biochemistry and Molecular Biology, Centre for High-Throughput Biology, University of British Columbia, 2125 East Mall, Vancouver, British Columbia, Canada V6T 1Z4
[b] Department of Cellular and Physiological Sciences, Life Sciences Institute, University of British Columbia, 2350 Health Sciences Mall, Vancouver, British Columbia, Canada V6T 1Z3

ARTICLE INFO

Article history:
Received 15 December 2011
Received in revised form 19 February 2012
Accepted 28 February 2012
Available online 6 March 2012

Keywords:
Connexin
Cx43
Gap junction
J
Phosphorylation
Proteomic
Multisite phosphorylation

ABSTRACT

Connexins require an integrated network for protein synthesis, assembly, gating, internalization, degradation and feedback control that are necessary to regulate the biosynthesis, and turnover of gap junction channels. At the most fundamental level, the introduction of sequence-altering, modifications introduces changes in protein conformation, activity, charge, stability and localization. Understanding the sites, patterns and magnitude of protein post-translational modification, including phosphorylation, is absolutely critical. Historically, the examination of connexin phosphorylation has been placed within the context that one or small number of sites of modification strictly corresponds to one molecular function. However, the release of high-profile proteomic datasets appears to challenge this dogma by demonstrating connexins undergo multiple levels of multi-site phosphorylation. With the growing prominence of mass spectrometry in biology and medicine, we are now getting a glimpse of the richness of connexin phosphate signals. Having implications to health and disease, this review provides an overview of technologies in the context of targeted and discovery proteomics, and further discusses how these techniques are being applied to "fill the gaps" in understanding of connexin post-translational control. This article is part of a Special Issue entitled: The Communicating junctions, roles and dysfunctions.

© 2012 Elsevier B.V. All rights reserved.

Contents

1. Introduction . . . 23
2. Proteomics . . . 24
3. Mass spectrometry (MS) . . . 24
4. Discovery proteomics: linear trapping quadrupole — orbital trap . . . 26
5. Discovery proteomics: quadrupole quadrupole time-of-flight (QQTOF) . . . 26
6. Database searches and significance in discovery proteomics . . . 27
   6.1. Targeted proteomics: triple-quadrupole (QQQ) MS/MS . . . 27
   6.2. Multi-site phosphorylation . . . 28
7. Understanding kinases and Cx43 sequence multi-site phosphorylation . . . 30
8. Outlook . . . 31
Acknowledgments . . . 32
References . . . 32

1. Introduction

The connexin (Cx) family of proteins are required for a range of biological processes including embryonic development [1,2], homeostasis [3,4], calcium signaling [5], cell migration [2,6] and apoptosis [7]. As a tetraspan-integral membrane protein, resident of the secretory pathway and principal component of gap junctions (GJs), Cx require an integrated network for protein synthesis, transport, assembly, gating, internalization, degradation and feedback control, with each stage likely to be critical in the control of GJ intercellular communication. As

☆ This article is part of a Special Issue entitled: The Communicating junctions, roles and dysfunctions.

* Corresponding author at: Department of Biochemistry and Molecular Biology, Centre for High-Throughput Biology, University of British Columbia, 2125 East Mall, Vancouver, British Columbia, Canada V6T 1Z4. Tel.: +1 604 827 3436.
E-mail addresses: vincent.chen@ubc.ca (V.C. Chen), cnaus@exchange.ubc.ca (C.C. Naus), Foster@chibi.ca (L.J. Foster).
[1] Co-senior authorship.
[2] Tel.: +1 604 827 4383; fax: +1 604 822 2316.
[3] Tel.: +1 604 822 8311; fax: +1 604 822 5227.

0005-2736/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2012.02.028

an especially dynamic component of the proteome, the site-specific phosphorylation/dephosphorylation is thought to occur as Cx subunits are trafficked from one cellular compartment to the other [8–12]. The life-span of most Cxs is incredibly short, and Fig. 1 gives a simplified, but a generally accepted summary related to the "timing" of GJ subunit assembly and turnover. This review starts by discussing how mass spectrometry (MS)-based proteomics may be used to "fill the gaps" in our understanding of GJ/Cx regulation. The specific roles of Cx phosphorylation have been recently discussed [9,10,13], and the purpose of this review is to introduce how proteomics may be applied to better understand the functions, sites, magnitude and sequence of multi-site phosphorylation that encompass the 'Cx code'.

The molecular heterogeneity of a protein's post-translational modification (PTM) is influenced by, and dependent upon, an interplay of enzymes, physiological conditions and the cell-/tissue-type. At the most fundamental level, the introduction of sequence-altering modifications, either dynamic (e.g. phosphorylation, ubiquitylation) or non-reversible (e.g., cleavage, degradation), introduces changes in protein conformation, activity, charge, stability and localization. Furthermore, with more than ten different phosphorylation-specific protein-binding domains [14], the addition of phosphate to one or multiple amino acid residues often modulates docking sites for protein–protein interactions. By SDS-PAGE, Cx43 typically forms multiple bands between ~37 and ~45 kDa, with the fastest migrating species recognized as non-phosphorylated protein. In the early stages of Cx43 assembly the protein is largely non-phosphorylated [11], with extensive modification occurring only after transport to the plasma membrane [15]. Despite the wealth of information related to Cx phosphorylation, our current body of knowledge related to Cx PTMs and their impact on function is often viewed as incomplete, and at times, contradictory [16]. Although monitoring the state of phosphorylation by SDS-PAGE has proven to be useful for Cx43, it should be noted not all phosphoproteins demonstrate detectable electrophoretic shifts, and the identity of individual site(s) and modifying enzyme(s) largely remain unresolved. Driven by the hypothesis that one or a small set of individual sites correspond to one molecular function, the determination of phospho-sites historically required the use of P^{32} radiolabels/2D gels, phospho-specific antibodies, nullifying site-directed conversion of Ser/Thr to Ala or Tyr to Phe, and/or phospho-mimetic mutations to Asp or Glu. The precise role of individual sites of modification alone or in combination within specific subcellular locales has been difficult to pinpoint because of the lack of side chain analogs that allow one to investigate functional contributions of phosphorylation alone. For example, it important to consider that substitution of Ser/Thr/Try to Ala or Phe not only lacks the ability to accept phosphate, but also increases hydrophobicity by displacing the position's ability to accept hydrogen bonds with water. With varying degrees of success [17–21], discussions regarding Glu/Asp phospho-mimetics largely center on obvious differences in the number of oxygen atoms/hydrogen bonds, geometry, numbers of negative charge at neutral pH, size and the lack of a natural pTyr isostere [22]. While individual sites of phosphorylation may be sufficient to induce an on/off response, compounding these challenges PTMs have also recently been found to work in a cooperative manner [23,24]. Best exemplified by gene regulation and the histone code, core histones are reversibly modulated by synergistic phosphorylation, acetylation, methylation and ubiquitylation [25,26]. With similar hypotheses having also been put forward for p53 [27] and tubulin [28], it is now clear multi-site PTMs represent an important mechanism for cell signal transduction. For Cx43 sequence harboring 66 Ser/Thr/Tyr residues, with the majority of confirmed sites residing in the cytoplasmic carboxyl-terminal tail (CT), it is important to recognize that a single Cx43 subunit may encompass upwards of 10^92 (66!) possible phosphate-based signaling combinations. It is clear the consequences of multi-site phosphorylation with respect to Cx trafficking/life-cycle and GJ gating have yet to be fully appreciated.

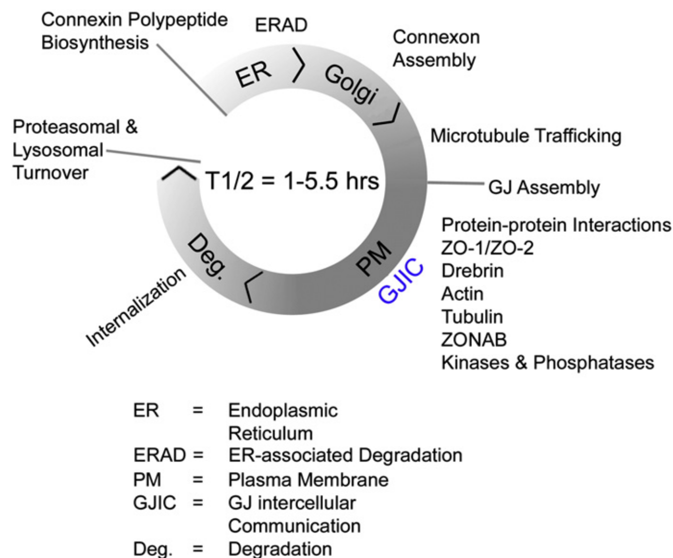


Fig. 1. Overview of the GJ life-cycle, depicting the relative order and timing of Cx subunits. Newly synthesized Cxs undergo synthesis within the ER undergo quality control within the ER-associated degradation pathway (ERAD). Subunits passing this stage undergo vesicular transport to the Golgi where they are oligomerized into heximeric "connexons". From here, connexon enriched vesicles are delivered to the plasma membrane where they dock at sites of cell-to-cell contact to establish or maintain sites of GJ intercellular communication (GJIC). While at GJs, Cxs-bind to an array of proteins, including cytoskeletal proteins (ZO-1, ZO-2, MUPP1, actin, drebrin, tubulin), transcription factors (ZONAB) and a variety of signaling kinases and phosphatases that are thought scaffold channels and potentiate GJ communication levels. A mechanism of GJ degradation involves internalization as double membrane structures called annular GJs that interface with the lysosome and/or proteasome. Most Cxs examined thus far demonstrate a half-life on the order of 1–5.5 h. Such rates of production and turnover, alongside complex patterns of assembly and trafficking, are consistent with a highly integrated system.

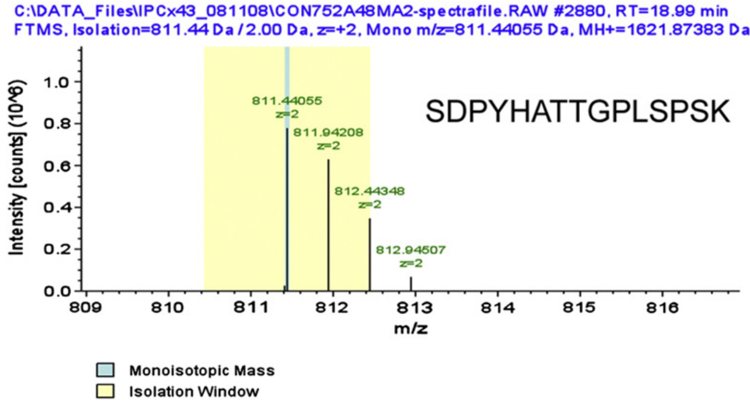
2. Proteomics

In its most classical definition, a proteome is defined as the set of proteins expressed by a genome [29,30]. While omic-based approaches to address specific research questions are often described as "fishing experiments", these strategies have their merits as they provide an unbiased view of biology that could otherwise never have been predicted. Proteomics technology is improving rapidly and now endeavors to systematically examine protein expression, structure, function, protein–protein interaction and dynamics. The proteome is highly dynamic, with a broad range of individual protein concentrations ranging from several million to only a few copies/cell [31]. Although sub-stoichiometric phosphorylation may be sufficient to induce a robust biological signal [32,33], the detection of discrete changes in protein phosphorylation amongst the bulk proteome are not trivial. In general, to identify a protein by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS), the instrument should be able to (i) determine the peptide's mass, (ii) fragment the peptide and analyze fragment mass, and in some instances, (iii) quantify expression or level of biochemical enrichment (Fig. 2). The lack of time for stages i and ii due to the complexity of digests makes sample preparation an important feature of most proteomic endeavors. An ongoing theme in the field is to therefore increase analytical sensitivity, by increasing instrumental speed and/or fractionation of the proteome by chemical, biochemical and genetic methods [34–36].

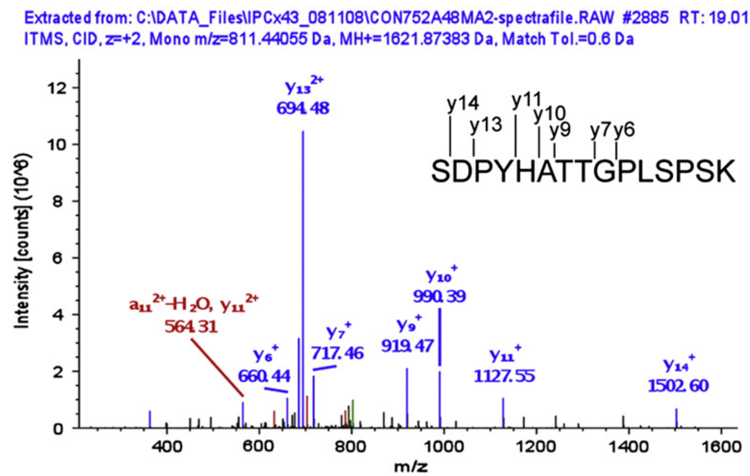
3. Mass spectrometry (MS)

The first step in proteomic analysis involves reducing proteins to peptides with an endoproteinase, such as trypsin. Cleaving carboxy-

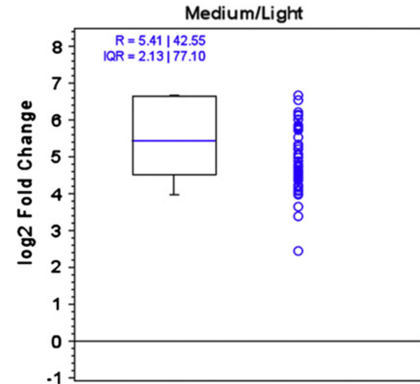
A) Stage 1: MS, Peptide *m/z*



B) Stage 2: MS/MS, Peptide Fragmentation, *m/z*



C) Stage 3: Quantitation



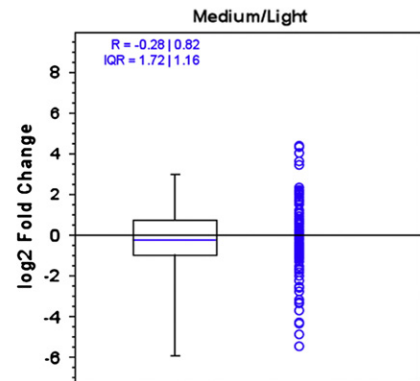


Fig. 2. Stages of proteomic experiment. A) MS of Cx43 peptide SDPYHATTGPLSPSK (A.A. 244–258) that was isotope-encoded by heavy dimethylation to provide a measure biochemical enrichment relative to irrelevant IgG immunoprecipitation (IP) negative control. Monoisotopic mass 1621.8738 (m/z 811.4405, blue) and 2 Da isolation window used for peptide selection for MS/MS (yellow). B) MS/MS CID fragmentation of SDPYHATTGPLSPSK (Mascot peptide score 68) identify peaks primarily assigned to the y-ion (C-terminal) series that corresponds to breakage of the peptide (C−N) bond. Experimentally measured [M + H +] = 1621.8738, theoretical mass [M + H] = 1621.8678. C) Summary of quantified Cx43 peptides using a box plot of Cx43 peptides from experimental and negative control IPs. Here, chromatograms for each isotope-encoded peptides pair are reconstructed to derive integrated peak areas. The peptide ratio chart shows the spread (log2 fold change) of quantified peptides of Cx43 (IP) and enrichment relative to this control. Added in equal amounts, trypsin peptides demonstrate the expected log2 ~ 0 (ratio ~1). MS and MS/MS measurements obtained on an LTQ-OrbitrapXL (ThermoFisher, Germany). Spectra and quantification charts derived using Proteome Discoverer 1.3 (ThermoFisher, Germany). Protein IDs derived with Mascot 2.3 (MatrixScience, USA).

terminal of Arg and Lys residues, trypsin produces peptides of favorable size and charge that further augments efficiency of HPLC and measurement by MS. Most modern-day MS systems are comprised of 4 major components: 1) HPLC, 2) the ion source, 3) the mass analyzer and 4) the detector. At the heart of this system is the ion source, where peptides acquire charge and are transferred into the gas phase. Until the mid-1980s, the underlying problem in biological MS was how to get peptides, proteins and other large biomolecules into the gas phase without extensive decomposition. Recognized by a Nobel Prize (2002), breakthroughs by John Fenn (electrospray ionization, ESI) and Kochi Tanaka (light-desorption ionization) have provided significant contributions that have enabled the unbiased, high-throughput detection of peptides and proteins. Commonly used embodiments of these sources now include nano-electrospray (nESI) [37], and matrix-assisted laser desorption-ionization (MALDI) [38] developed by Franz Hillenkamp and Michael Karas.

The principal outcome of most proteomics experiments is the identification of proteins and the use of high-accuracy, high-precision mass analyzers is therefore necessary to increase the confidence of results. Within the mass spectrometer, gas phase ions are separated and characterized based on their behavior within electric or magnetic fields. It is important to note that MS does not measure the mass, but instead measures mass-to-charge ratio (*m/z*) of peptides, and their diagnostic fragments by tandem-MS, a.k.a. MS/MS. Information garnered from MS and MS/MS measurements is used to identify proteins and isolate important sites of PTM. Primary sequence information can be obtained by MS/MS were peptides are dissociated by introducing intermolecular collisions with a neutral gas (e.g. He, Ar, or N2) and analyzing the resulting fragments in a process called collision-induced dissociation (CID). Peptide breakage during a CID experiment generally occurs through the lowest energy pathway to produce a series of fragment ions containing either the C-terminal (designated x-, y-, or z-ion), or N-terminal ions (a-, b-, or c-ion). For the case of CID, this mode of fragmentation usually results in the cleavage of the peptide (C−N) bond to generate b-/y-ions ion fragment series. To designate the position of the fragment within the polyamino acid sequence, each series of ions is numerically designated — with position 1 identifying the absolute C-/N-terminal amino acid within the original sequence. The described nomenclature for peptide fragmentation is summarized in Fig. 3.

Several different types of MS/MS-enabled instruments are now available, and such instruments now include tandem-in-time (such

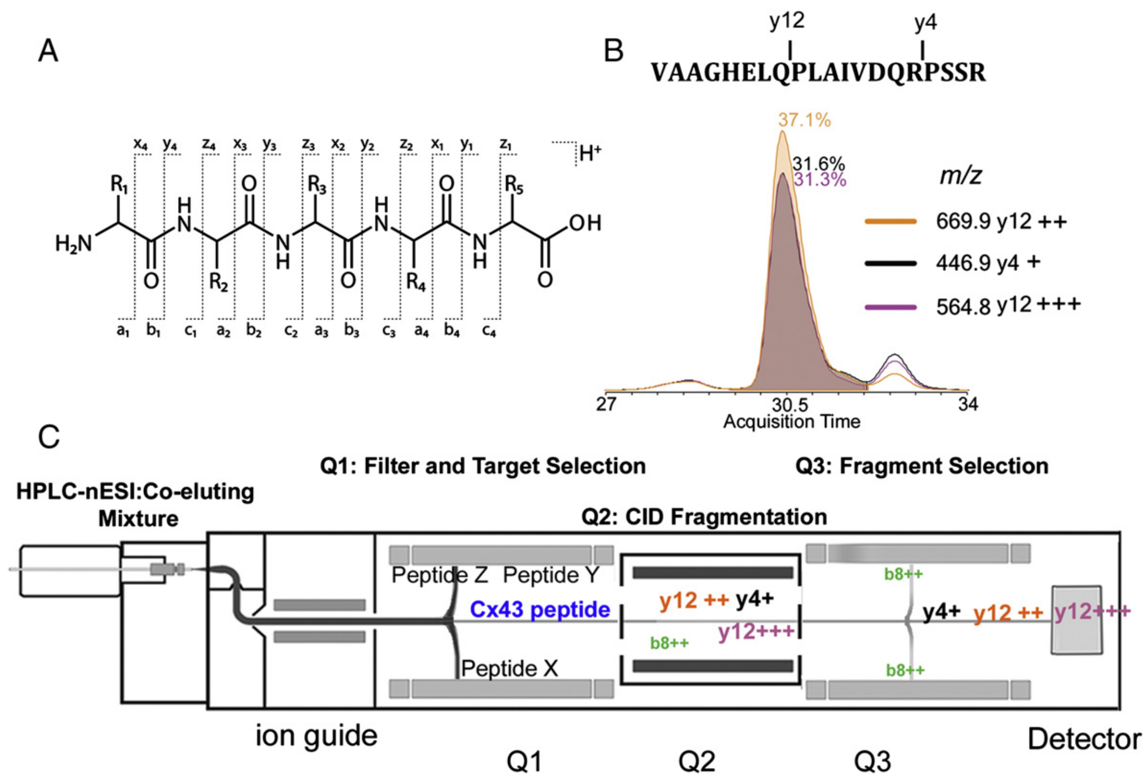


Fig. 3. A) Nomenclature for peptide fragmentation. Positional cleavage along the peptide backbone results in C- (x-, y-, z-) or N-terminal (a-, b-, c-) ions. CID experiments typically follow b-/y-ions, while ECD and ETD-type fragmentation typically produce c- and z-type ions. B) MRM detection of Cx43 peptide VAAGHELQPLAIVDQRPSSR (A.A. 347–366) from lysates of C6 glioma cells by CID-MS/MS. C) Overview of a MRM-QQQ experiment. During an MRM run, Q1 selects peptides based on *m/z* for transfer to the Q2 CID collision cell. Product ions, called transitions, are then specifically selected as they pass through a Q3 isolation window. Each set of transitions at a given LC-elution time uniquely identifies a target protein.

as ion traps) and tandem-in-space spectrometers (e.g. triple quadrupoles). In theory, MS and MS/MS measurements can be used to examine any PTM leading to a difference in mass. For example, the addition of a phosphate to Ser/Thr/Tyr residue is expected to yield a mass increase of 79.9 Da. For enrichment, chromatographic approaches for phosphopeptide analysis generally take advantage of their negative charge (e.g. strong cation (SCX)/anion exchange) [39,40], or affinity for either metal oxides (e.g. TiO_2) [41], or metal cations (immobilized metal affinity chromatography, IMAC) [42]. For quantitative experiments, isotope-encoded reference peptides (e.g. Aqua [43]), chemical modifications (dimethylation [44]) or metabolic labeling (SILAC [45]) all allow accurate evaluation of protein and PTM levels between and within samples. To a high-level of analytical precision, the incorporation of these internal standards permits the high-throughput examination of protein dynamics related to a time course and/or a drug treatment. While there are numerous types of mass analyzer configurations that are capable of doing MS-based experiments, this review will primarily focus on triple-quadrupole (QQQ), quadrupole–quadrupole-time-of-flight (QQTOF) and orbital/ion traps instruments.

4. Discovery proteomics: linear trapping quadrupole — orbital trap

LTQ-Orbitrap is an example of a hybrid instrument employing an orbital trapping device [46] used in parallel with a linear trapping quadrupole (LTQ). In the orbital trap, ions orbit around a central electrode and oscillate harmonically at a frequency characteristic of their *m/z*. The movements of these ions induce a mirroring current on the outer surface of the device. Monitored frequencies of these currents undergo Fourier transformation to generate high-resolution MS spectra of intact peptides at accurate mass. Simultaneous with these measurements, the LTQ component of the mass spectrometer is responsible for the fast generation of sequence-dependant data by repeatedly selecting, fragmenting, and analyzing peptides by CID where fragment ions are sequentially ejected, strike a detector, and recorded. With the aim of enhancing the detection of phosphorylated peptides, complementary modes of MS/MS analysis have now been developed [47]. In contrast to CID, electron-transfer (ETD) and electron-capture dissociation (ECD) proceeds through a mechanism of electron transfer to induce bond rearrangement and spontaneous cleavage of the peptide backbone. This mode of MS/MS preferentially generates c- and z-type fragment ions (Fig. 3). Permitting direct sequencing by MS/MS, an important advantage of ETD/ECD is the connectivity of labile phosphates can now be maintained [47,48].

5. Discovery proteomics: quadrupole quadrupole time-of-flight (QQTOF)

A quadrupole (Q) mass analyzer effectively functions as an *m/z* filter. Depending on their *m/z*, ions flowing into a quadrupole either have a stable trajectory to reach the detector, or have unstable paths and effectively lost. Alternatively, when operated in 'RF-only' mode, these devices may serve as ion guides that permit all ions to pass that are particularly useful as "collision cells" for CID. In a typical quadrupole quadrupole time-of-flight (QQTOF) instrument, Q1 serves as mass selector, Q2 collision cell, with the TOF component used to measure the *m/z* intact peptide and resulting MS/MS fragments [49]. The TOF analyzer itself is unique in the fact it analyzes ions based on their travel within a field free drift tube of known length. Drift in this tube permits ion 'flight times' to be measured and *m/z* to be calculated after impact with the detector. As all ions receive the same amount of kinetic energy, ions of smaller *m/z* travel faster to reach the detector first. Another benefit of the QQTOF configuration is the ability to examine the full complement of MS/MS fragments within a single TOF scan. Relevant to further discussions, as the mechanisms

for mass selection (Q1) and (Q2) fragmentation are largely identical, discovery on QQTOF make them ideally suited for follow up examination by targeted proteomics using triple quadrupole (a.k.a. "triple quad" or QQQ) instrumentation.

6. Database searches and significance in discovery proteomics

Another important and evolving aspect of discovery proteomics is the processing and interpreting the enormous amounts of MS and MS/MS data. The identity of a protein can be unveiled by several different methods for identification, however it should be mentioned the increasing abundance and quality of genomic data sets has greatly simplified these procedures. Historically, protein identities could be revealed by accurately measured masses of intact peptides that were matched against a theoretical list of in silico digested protein. This method, known as peptide mass fingerprinting or PMF [50], was highly successful for rapidly identifying moderately pure proteins originating from a 2D-PAGE spot or a narrow SDS-PAGE band [51]. A caveat to the PMF approach however is that peptides masses together contribute to the reliability of a protein identification and the examination of protein mixtures, or even LC separated peptide materials often confounds identification due to the detection of background proteins. Furthermore, as no direct sequence information can be directly derived from MS-only/PMF data (e.g. peptide HPYTGK will have the same m/z as GTYPHK), these methods cannot be used to confidently assign peptide sequences, or differentiate a peptide carrying a PTM from unrelated peptide of similar mass. Due to the limitations of MS-only information, instrumentation capable of MS/MS sequencing in combination with the accurate mass measurements have largely replaced single-stage MS in proteomics.

The modern-day standard for protein/PTM identification is now based on measuring intact peptide and the resulting fragment ions generated by MS/MS. It is important to note that the presentation of a list of proteins by the search engine is preceded by two important procedures: 1) assigning peptide sequences to proteins, and 2) the separation of correct from incorrect peptide identifications. During a typical MS/MS experiment, fragmentation information (i.e. intensities and *m*/*z* values of all the peaks) from each MS/MS spectrum is submitted to a database search engine, which in turn tries to assign a peptide sequence to a spectrum. Many such MS/MS database search engines exist [52]. Two popular ones, Mascot [53] and Sequest [54], identify peptides using probability-based and autocorrelation algorithms, respectively. In general, these two programs score a peptide fragment ion spectrum based upon how well they match against a theoretical fragment spectra constructed from candidate peptide sequences within a database. This group of candidate peptides depends on user-defined criteria such as mass tolerances, proteolytic enzymes and possible modifications. Each of these candidates is given a (search engine dependant) score and ranked accordingly. However, since the aim of most experiments is to identify protein, the identification of peptide sequences only represents an intermediate step. Difficulties of assembling sequences into protein ID arise from the fact that proteins are digested with a protease. This loss of connectivity complicates computational analysis and biological interpretation, especially when a peptide is ambiguously found in multiple proteins [55]. Under ideal situations, the identification of a discriminating peptide can be used for verification [51]. However, in the absence of such a diagnostic sequence, alternate enzyme(s), targeted proteomic methods (as discussed in subsequent sections), or application of alternate methods to increase sequence coverage may be beneficial.

Incorrect peptide identifications can be the result of a substantial overlap between the experimental MS/MS ion spectrum and a theoretical fragment spectrum from an unrelated precursor that results in a chance random match or so-called "false-positive". Since there is a substantial overlap between correct and incorrect identifications, it is necessary to find a balance between sensitivity (increasing true-positives) and specificity (minimizing false-positives). The latter can be examined by searching against a "decoy" database or where protein sequences are reversed [56], shuffled [57] or by using random sequences [58]. The number of identifications from the target-decoy search strategy is used to calculate a false discovery rate (FDR), which is defined as the percentage of accepted identifications that are incorrect, and to describe the statistical significance of the identifications [59,60]. These procedures are necessary to obtain an independent measure of dataset reliability. Alongside target-decoy searches, 'in silico' proteome should also include sequences for human (e.g. keratins) and other commonly observed experimental contaminants (e.g. from bovine serum/culture media, trypsin).

It is important to note that protein identification algorithms, such as MASCOT and Sequest, make the first and foremost assumption that the correct sequences for identification are amongst those being searched. Failure to include all possible sources of protein, either by searching against an irrelevant or searching against an artificially small database, will result in the search engine assigning the highest scoring incorrect sequence as the most reliable answer [61,62]. A recent study by Locke et al. reported the PTM of Cx26 to identify more than 15 sites of PTM (acetylation, hydroxylation, carboxyglutamation, methylation and phosphorylation), at sites of deafness-causing Cx26 mutations [63]. Here, materials originating from human Hela cells, were searched against a database of "connexin only" sequences or a database containing rodent sequences (parameters: 20–40 kDa, pI 0–14, mass tolerance 50 ppm) [63]. Even at the reported confidence search interval (95%), such a search would very likely generate an a high number of false-positive identifications for of the following reasons: 1) MS data was searched against an artificially small database containing only Cx sequences; 2) The majority of PTMs assigned by this study were solely based on accurate mass (the accepted method for PTM site assignment is MS/MS); and 3) Failure to consider human sequences, alongside common contaminants (Cx26 materials were originally obtained from Hela cells). For protein identification, it is very important to stress, that database search programs have all possible sequences to be available for comparison to MS-generated data. Restricting the search space to one, or an artificially small handful of proteins, will result in all spectra being matched back to the one/limited number sequences. In fairness to this study, the re-evaluation of raw data files using an appropriate database (in silico proteome, reverse sequences and contaminants) will be necessary to determine the absolute reliability of PTMs/Cx26 peptides reported. To avoid potential pitfalls, the proteomic community strongly advocates against the use of similar 'Cx only' databases or the use of single-stage MS data to assign sites of PTM control.

6.1. Targeted proteomics: triple-quadrupole (QQQ) MS/MS

Due to complexity of the proteome and limitations of discovery-based MS data acquisitions, a major drawback to discovery-based proteomics is the lack of consistency between sample runs. However, recent applications in proteomics have resulted in the delivery of analytical strategies that permit a set of proteins to be consistently measured across multiple samples [64]. When a specific target is known, a method called multiple-reaction monitoring (MRM, also called selected-reaction monitoring or SRM), Fig. 3B and C, has emerged as a highly sensitive and reproducible technique, even across different labs and manufacturer platforms [65]. These types of detections have the benefit of multiplexing, molecular resolution, absolute quantitation, low-attomole sensitivity and dynamic range upwards of five orders of magnitude [66]. During an MRM analysis, Q1 is instructed to repeatedly sweep through a list of *m*/*z* corresponding to peptide(s) of interest. As only one specific peptide *m*/*z* will be allowed to enter the (Q2) CID collision cell, with only selected fragments ions (called transitions) being recorded, the MRM approach permits a subset of proteins to be reproducibly recorded. As multiple levels of molecular

filtering are now utilized (HPLC-retention time, selected peptide *m/z*, and selected peptide fragment *m/z*), these detections are both highly selective and sensitive. Furthermore, as each set of transitions at a specific HPLC elution time uniquely identifies a peptide, the area under an ion chromatogram curve provides a direct quantitative measure. Recently, public repositories and tools including PeptideAtlas (www.peptideatlas.org) [67], Global Proteome Machine (GPM, www.gpm.org), Protein identification Database (www.ebi.ac.uk/pride/) [68,69] and the Sequence-specific Retention Time Calculator (http://hs2.proteome.ca/SSRCalc/SSRCalcX.html) [70] have been developed with the aim of annotating protein expression [71] and deciphering protein-based signals by MRM [72]. Demonstrating the potential of this technology to identify (QQTOF) and track (QQQ) phosphorylation dynamics with phosphotyrosine-IPs and IMAC enrichment, Wolf-Yadlin et al. followed the temporal profiles of 222 tyrosine phosphorylation sites across seven time points after EGF treatment [73]. Developing a simplified method to quantifying the absolute stoichiometry of protein phosphorylation by MRM, Domanski et al. developed a novel approach based upon the addition of single isotope-encoded non-phosphorylated synthetic peptide and enzymatic dephosphorylation. Relative increases in non-phosphorylated peptide with alkaline phosphatase treatment provided an absolute measure of protein residing in the phosphorylated from [74]. However as phosphorylated species are collapsed to it's unmodified native form, it should be noted that contribution from individual multi-phosphorylated sites cannot be distinguished using this method.

As only a certain set of representative peptides will be detectable within a complex mixture by MRM, selection of peptides and their most abundant fragment ions will be absolutely critical [64]. To expedite the use of MRMs in Cx expression profiling and similar phosphorylation stoichiometry studies, our lab has developed a list of Cx43 peptide transitions (Table 1). From immunoprecipitation of Cx43 (<1% FDR), we have compiled consistently and confidently identified peptides by LC-MS/MS. These peptides were chemically synthesized and analyzed by QQQ-MS/MS to determine the most abundant/sensitive fragments for MRM transition selection. More specifically, we have employed solid phase chemical synthesis (Intavis MultiPep, Germany) to manufacture Cx peptides (Table 2) for MS/MS examination on a CHiP-QQQ (Aglient 6400 series, 1200 series HPLC, USA). For example, peptide VAAGHELQPLAIVDQRPSSR (Fig. 3) demonstrates the uses of these transitions in the detection of Cx43 from cell lysates from C6 glioma cells. Here, HPLC retention time and abundance of transitions (percent area y12, y4 and y10) from whole cell lysates match the expected values from synthesized standards.

6.2. Multi-site phosphorylation

Cx43 has been shown to be a substrate for MAPK, PKC, CaMK and c-/v-SRC [74–78]. As the process of PTM generates a potentially distinct functional entity, a principal question related to this work is how does phosphorylation by kinases and phosphatases regulate Cxs? Amongst others, work by Laird, Musil, Nagy, Lau, Kardami and Lampe laboratories have established the importance of phosphorylation for Cx subcellular trafficking, GJ gating, function and turnover. To enrich for plasma membrane proteins, Wisniewski et al. developed methods to isolate ion channels and transporters and identified more than 12,000 phosphorylation sites on 4579 brain proteins, including numerous sites within Cx43, Cx32, Cx47 and Cx29 [79]. With the aim of better understanding the role of phosphorylation in different organs, using a combination of SCX and metal-affinity enrichment, Huttlin et al. examined phosphorylation patterns in mouse heart, brain, fat, kidney, pancreas, testes, spleen, lung and liver [34]. This study found upwards of 36,000 phosphorylation sites

Table 1
Cx43 MRM Transitions.

Sequence (A.A.), species	SSRCal: hydrophobicity [108]	Precursor (*m/z*)	Fragment (*m/z*)	Precursor (SILAC: K4/R6)	Fragment (SILAC: K4/R6)	Ion
YGIEEHGK	9.42	466.9++	769.4	468.9++	773.4	y7
(137–144)			599.0		603.0	y5
Ms, Rat, Hu			712.4		716.4	y6
		311.6+++	599.2	312.9+++	603.2	y5
			385.3		387.3	y7++
			341.3		345.3	y3
SDPYHATTGPLSPSK	19.06	520.2+++	678.4	521.5+++	680.4	y13++
(244–258)			452.6		453.9	y13+++
Ms, Rat			418.2		422.2	y4
		779.6++	678.3	781.6++	680.3	y13++
			958.4		962.4	y10
			887.3		891.3	y9
SFPISHVR	22.15	471.9++	708.4	474.9++	714.4	y6
(249–256)			498.3		504.3	y4
Ms, Rat, Hu			354.8		357.8	y6++
		315+++	611.4	317+++	617.4	y5
			498.3		504.3	y4
			354.8		357.8	y6++
GDWSALGK (2–9*)	20.56	417.4++	661.4	419.4++	665.4	y6
Ms, Rat, Hu			475.3		479.3	y5
			331.2		333.2	y6++
VAAGHELQPLAIVDQRPSSR	31.1	537.1++++	669.9	538.6++++	672.9	y12++
(348–366)			564.8		567.8	y10++
Ms, Rat, Hu			446.9		448.9	y12+++
		715.7+++	669.9	717.7+++	672.9	y12++
			365.7		368.7	y6++
			855.0		858.0	y15++
TYIISILFK	44.58	549.5++	833.6	551.5++	837.6	y7
(154–162)			720.4		724.4	y6
Ms, Rat, Hu			607.4		611.4	y5

+ = H^+ charge state (+1, 2, 3 or 4), SILAC: K = +4.025107, R = +6.020129, * n-terminal M removal. SSRCal is a hydrophobicity index used to order and accurately predict HPLC elution time of peptides in targeted proteomic studies. Developed by Oleg Krokhin (University of Manitoba), the higher the value, the later the peptide will elute by reverse-phase chromatography [108]. Precursor values are m/z of intact peptides use by Q1. Fragment m/z present the top 3 highest abundance/most sensitive ions generated by Q2, for monitoring by Q3. Ion fragment type and charge state (+, ++, +++) are provided.

Table 2
Cx43 site and multi-site phosphorylation.

Region	Site	Target-based methods	Site/multi-site Cx43 phosphorylation
NT	Ser5		GDW**pS(5)**ALGK*
CT	Ser244	[16]	
CT	Tyr247	[109]	SDP**pY(247)**HATTGPLSPSK*
CT	Ser/Thr250		SDPYHAT**pT(250)**GPLSPSKDCG**pS(262)**PK•
CT	Thr251		SDPYHAT**pS(251)**GALSPAK*
			SDPYHAT**pS(251)**GAL**pS(255)**PAK°
CT	Ser255	[13,76]	SDPYHATSGAL**pS(255)**PAK•
			SDPYHAT**pS(251)**GAL**pS(255)**PAK°
			SDPYHATTGPL**pS(255)**PSKDCG**pS(262)**PK•
CT	Ser257	[16]	SDPYHATTGPLSP**pS(257)**KDCGSPK•
CT	Ser262	[101,110]	SDPYHAT**pT(250)**GPLSPSKDCG**pS(262)**PK•
			SDPYHATTGPL**pS(255)**PSKDCG**pS(262)**PK•
CT	Tyr265	[111]	**pY(265)**AYFNGCSSPTAPLSPM**pS(282)**PPGYK•
CT	Ser273		YAYFNGCS**pS(273)**PTAPLSPMSPPGYK•
			YAYFNGCS**pS(273)**P**pT(275)**APLSPMSPPGYK•
CT	Thr275		YAYFNGCS**pS(273)**P**pT(275)**APLSPMSPPGYK•
CT	Ser279	[76]	
CT	Ser282	[76]	**pY(265)**AYFNGCSSPTAPLSPM**pS(282)**PPGYK•
CT	Thr290		LV**pT(290)**GDRNNSSCR•
CT	Ser296	[112]	LVTGDRNN**pS(296)**SCR*
			LVTGDRNN**pS(296)pS(297)**CR•
CT	Ser297	[112]	LVTGDRNNS**pS(297)**CR•
CT	Ser306	[112]	QA**pS(306)**EQNWANYSAEQNR*
			QA**pS(306)**EQNWANY**pS(314)**AEQNR•
CT	Tyr313		QASEQNWAN**pY(313)**SAEQNR⊗
CT	S314	[16]	QASEQNWANY**pS(314)**AEQNR*
CT	S325	[113]	MGQAG**pS(325)**TISNSHAQPFDFPDDSQNAK*
			MGQAG**pS(325)pT(326)**ISNSHAQPFDFPDDSQNAK•
			MGQAG**pS(325)**TI**pS(328)**NSHAQPFDFPDDSQNAK•
			MGQAG**pS(325)**TISN**pS(330)**HAQPFDFPDDSQNAK•
			MGQAGSTI**pS(325)**NSHAQPFDFPDD**pS(341)**QNAKK•
CT	S326		MGQAG**pS(325)pT(326)**ISNSHAQPFDFPDDSQNAK•
			MGQAGS**pT(326)**I**pS(228)**NSHAQPFDFPDDSQNAK•
			MGQAGS**pT(326)**ISN**pS(330)**HAQPFDFPDDSQNAK•
			MGQAGSp**T(326)**Ip**S(328)**NSHAQPFDFPDD**pS(341)**QNAKK•
CT	S328	[113]	MGQAGSTI**pS(328)**NSHAQPFDFPDDSQNAK*
			MGQAG**pS(325)**TI**pS(328)**NSHAQPFDFPDDSQNAK•
			MGQAGS**pT(326)**I**pS(328)**NSHAQPFDFPDD**pS(341)**QNAKK•
CT	S330	[113]	MGQAGSTISN**pS(330)**HAQPFDFPDDSQNAKK*
			MGQAG**pS(325)**TISN**pS(330)**HAQPFDFPDDSQNAK•
			MGQAGS**pT(326)**ISN**pS(330)**HAQPFDFPDDSQNAK•
CT	S341		MGQAGSTI**pS(325)**NSHAQPFDFPDD**pS(341)**QNAKK•
			MGQAGS**pT(326)**I**pS(328)**NSHAQPFDFPDD**pS(341)**QNAKK•
CT	S364	[114]	VAAGHELQPLAIVDQRP**pS(364)**SR*
			VAAGHELQPLAIVDQRP**pS(364)pS(365)**RASSR•
			KVAAGHELQPLAIVDQRP**pS(364)pS(365)**RA**pS(368)**SR•
CT	S365	[115]	VAAGHELQPLAIVDQRPS**pS(365)**R*
			VAAGHELQPLAIVDQRPS**pS(365)**RA**pS(368)**SR*
			VAAGHELQPLAIVDQRPS**pS(365)**RAS**pS(369)**R*
CT	S368	[116]	VAAGHELQPLAIVDQRPSpSRA**pS(368)**SR*
			VAAGHELQPLAIVDQRPS**pS(365)**RA**pS(368)**SR*
CT	S369	[115]	VAAGHELQPLAIVDQRPSpSRAS**pS(369)**R*
			VAAGHELQPLAIVDQRPS**pS(365)**RAS**pS(369)**R*
CT	S372	[117]	
CT	S373	[115]	

*[79], •[34], °[118], ⊗[119]. Summary of Cx43 site and multi-site phosphorylation, using respective hypothesis-driven and phosphoproteomic strategies.

on 12,039 proteins. Suggesting the modification plays a significant role in complementing a tissues' physiology, these authors also found some broadly expressed proteins, such as Cx43, were variably modified [34]. However, as a targeted technique to track and reliably quantify sites of modification across multiple tissues has not yet been applied, it should be cautioned that the lack of a phosphorylated species within a tissue may not necessarily be reflective of the physiological state, but a technical constraint linked to sample complexity. Regardless of these perceived limitations, using phosphopeptide datasets generated by the proteomic community, we have compiled a current set of known individual and multi-site Cx43 phosphopeptide sequences.

A large-scale phosphoproteomic analysis of mouse tissues demonstrated kinase activation loops were targets of phosphorylation, indicative of cross-talk between kinases and phosphates [27]. In keeping with this view, a notable study by Breitkreutz et al. additionally observed promiscuity between kinases and phosphatases within more than 1800 interactions [59]. Here, kinases and phosphatase did not appear to interact in a traditional stepwise cascade, but rather a myriad of interactions more similar to a "neural network" capable of computing functions to drive cellular responses. Understanding how Cxs phosphorylation, subcellular localization and protein–protein interactions respond to such highly networked signals will be a particular challenge. The analysis of Cx-protein interactions thus far [80] shows that Cxs are not only associated with kinases, but also with a variety of other proteins presumably through cytoplasmic CT domains. Among these interaction partners are adherens/tight junctional complex such as beta-catenin [81], v-SRC

[82], ZO-1 [83], cytoskeletal proteins such as cortactin [84], MUPP1 [85], drebrin [86] and tubulin [87], as well as caveolin [88], CCN3 [89] and the transcription factor ZONAB [85,90]. To examine structural influences, we next classify Cx43 sites according to predicted secondary structure within regions of protein disorder [91]. Here, based on the Cx43 sequence, the DisPro server (http://scratch.proteomics.ics.uci.edu/) was used to calculate disordered secondary structure and propensity for solvent accessibility. As found in previous studies for other 'hub' proteins [92], we find an overwhelming body of phosphorylation sites within disordered and/or loosely ordered sequences (Fig. 4). These segments include *Region 1* (*disordered*): Ser244, Tyr247, Thr250, Thr251, Ser255, Ser257, Ser262, and Tyr265 *Region 2* (*loosely ordered*): Thr290, Ser296, Ser297, S306, Y313 and Ser314; *Region 3* (*disordered*): Ser325, Thr326, S328 and S330; *Region 4* (*disordered*): Ser364, Ser365, Ser368, Ser369, Ser372 and Ser373, which may serve as 'hotspots' for intracellular signals. Protein–protein interactions, phosphorylation and protein disorder prediction, along with our characterization that intrinsically disordered regions of Cx43 are targets of phosphorylation, further supports the notion that the Cx43-CT may be a highly flexible sensor capable of monitoring the overall health of the cell as proposed by the Kardami and Spray labs [93]. In particular, convergence of these signaling processes onto Cx43 may help to explain why the CT alone appears to influence a seemingly divergent but growing list of cellular functions including growth control and cell proliferation [94], migration [95,96], spreading and adhesion in B-lymphocytes [97] and neural development [2].

7. Understanding kinases and Cx43 sequence multi-site phosphorylation

Phosphorylation and dephosphorylation are catalyzed by more than 500 kinases and 100 phosphatases that are in turn regulated by phosphorylation [34]. With an unprecedented level of detail, richness of phosphate-based signals is beginning to emerge. However, it has also become apparent that the identification of these events is quickly out-pacing our ability to assess their function, and the matching specific sites of phosphorylation with acting kinases still remains a significant challenge. Cx43 contains numerous consensus kinases sites, among them protein kinase A (PKA), protein kinase C (PKC), protein tyrosine kinases, mitogen activated protein kinases (MAP kinases), glycogen synthase kinase (GSK), and casein kinase (CK).

To generate insights into multi-site phosphorylation patterns, the NetPhos informatics server was used to examine consensus sites (Fig. 5). Heat map analysis revealed the majority of phosphorylation sites appear to be recognized by multiple kinases. Hierarchical clustering revealed multiple kinases such as CaM-II, GSK and cdc2 having high probability across multiple sites, while others such as PKB show low-to-negligible phosphorylation potential. Broad patterns for CaMk-II, GSK and cdc2 consensus sites suggest these kinases could be potentially used to induce massive changes in phosphorylation state. Indeed, the necessity of a 'priming' phosphorylation upstream of consensus GSK sites [98] suggests Cx43 itself might serve as a signaling hub and substrate mediating signal cross-talk between GSK, other kinases and phosphatases. In line with the convergence of signaling diversity, some kinases appear to have overlapping consensus residues, while others appear to engage Cx43 through distinctive patterns of phosphorylation. For example, while PKC (T290, S297, T325, T326, S364, S368, S372 and S373) and PKA (S244, S296, S306 and S373) largely differ in phosphorylation threshold, cdk5 and p38MAPK demonstrate similarities in consensus sequences (S255, S262, S273, S279 and S282). Using similar methods for consensus prediction, MAPK was confirmed to phosphorylate Cx43 at S255, S279, and S282 [76], with pS279/282 later shown to be important in decreasing GJ channel open probability [99]. EGF treatment and down-stream simulation of MAPK has been found to correlate with phosphorylation, ubiquitylation and internalization of Cx43 GJs from the plasma membrane [100]. During mitosis, cdk2 has been found to moves to the cortical surface of the plasma membrane where it phosphorylates Cx43 at S255 and S262, and appeared to correlate with GJ internalization [101,102]. Through similar patterns of multi-site phosphorylation, is it possible that signal transduction through either MAPK or cdks, are capable of engaging GJ internalization machinery? It is obvious much work will be necessary to define the mechanisms of multi-site phosphorylation.

An important element of Cx studies is to determine their composition, sites and order of PTMs. In the heart, ischemia is often accompanied by loss of electrical coupling and arrhythmias that coincide with Cx43 dephosphorylation at intercalated disks [103]. The cycling of kinase-phosphatase during ischemia has been proposed to play an important role in GJ remodeling [104,105] and the introduction of phosphatase inhibitors has been found to change their electrophoretic mobility [106]. Using a highly specific antibody for Cx43 phosphorylation at S325, S328, and/or S330, Lampe et al. reported that phosphorylation at these sites became drastically reduced during ischemia [107]. The functional consequence of multi-phosphorylation remains unknown and the specific order by which Cxs are phosphorylated and dephosphorylated largely remains a mystery. Using proteomic datasets, the presence or lack of phosphate at individual sites alone or within a multiphosphorylated peptide provides insight into the order of sequence-dependent dephosphorylation and phosphorylation. As an example of how global proteomic datasets can be

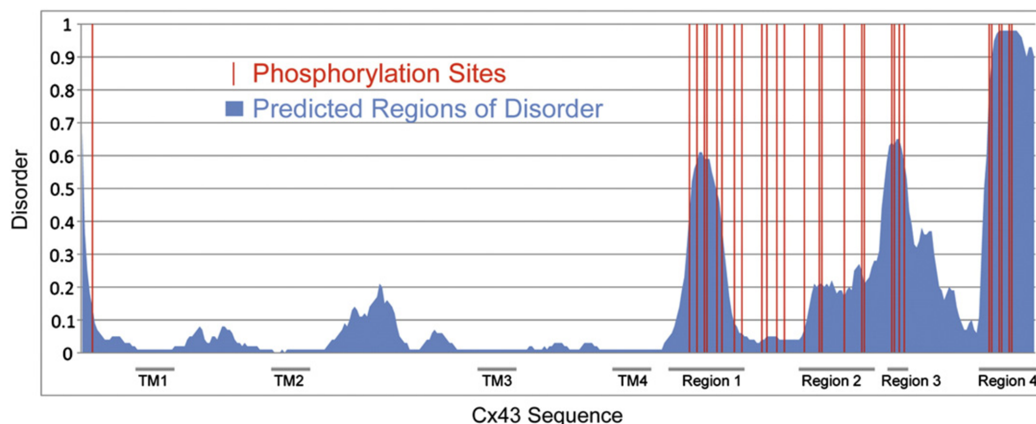


Fig. 4. Protein disorder and Cx43 phosphorylation sites. A proportion of phosphorylated residues (see Table 2) of Cx43 reside in disordered/loosely ordered sequences. Thresholds >0.45 were defined as disorder while regions 0.45 > × > 0.15 were considered loosely ordered. Protein disorder predictions were obtained using DisPro [91] of the SCRATCH protein bioinformatics suite (http://www.ics.uci.edu/~baldig/scratch/).

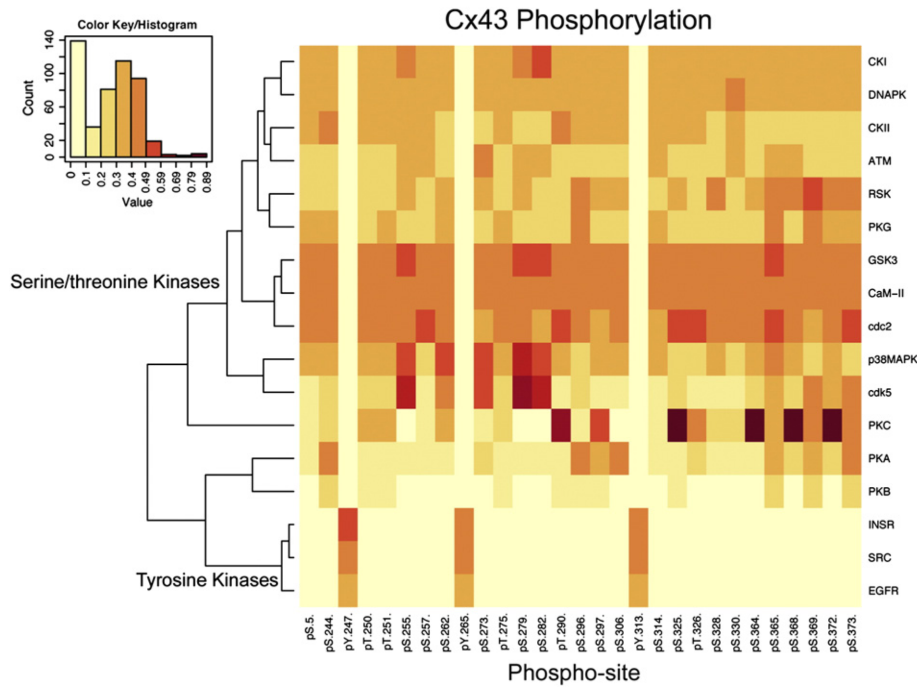


Fig. 5. Heat map distribution of Cx43 phosphorylation sites demonstrates the diversity of kinase signals. Sites of Cx43 phosphorylation were derived from Table 2, and values for preference for individual kinases were derived from NetPhosK [120].

used to provide additional insights, the detection of pS325 and pS328 alone, and with multi-phosphorylated peptides (Table 2), implies these residues are phosphorylated early (and/or dephosphorylated late) relative to S326, S330 and S341 (Fig. 6). In this scenario, experiments working to disrupt Cx43 phosphorylation order, by substituting phosphorylatable residues in mice or cultured cells, may yield important insights into the physiological importance of multi-site phosphorylation. These studies not only confirm multi-site phosphorylation at S325, S328, and S330 [107], but additionally provide direct molecular evidence for the co-existence of pS325 with pS326, and pS325 with pS341.

8. Outlook

As a highly dynamic component of the proteome, Cxs form structures that traffic from one compartment of the endomembrane system to another. An important element of understanding Cx regulation is to determine their structural composition, protein–protein interactions, PTMs and to comprehend how signals work together as an integrated system. For Cx43, the early stages of assembly demonstrate the protein is largely non-phosphorylated, with extensive modification occurring only after transport to the plasma membrane, however the specific sites, order and magnitude of multi-site phosphorylation are poorly resolved. The mechanisms of Cx phosphorylation has been historically been viewed in the literature as one site of modification represents one binary molecular function. The implications of results discussed in this review are multiple. The recent release of large-scale phosphoproteomic datasets currently challenges this dogma by demonstrating Cxs undergoes multiple levels of multi-site phosphorylation. We propose this may manifest in a combinatorial phosphorylation or a 'Cx code' that may be used to help support the functions of cells and tissues. It is also evident, that distinct signal transduction pathways also appear to consolidate onto Cx43 through several phosphorylation-based signaling mechanisms, and a pattern of multi-site phosphorylation has just begun to emerge (Table 2, Fig. 6). This review also found the preferential phosphorylation within structurally disordered regions that for the first time reveals the architecture of Cx43 is intimately linked to multi-site PTMs. Using a variety of bioinformatic tools and peer-reviewed large-scale phosphoproteomic datasets, we demonstrate the principal GJ subunit, Cx43, likely serves as a highly integrated hub capable of consolidating a variety of phosphate-based signaling cascades. A fundamental question that remains unanswered is what are the sites,

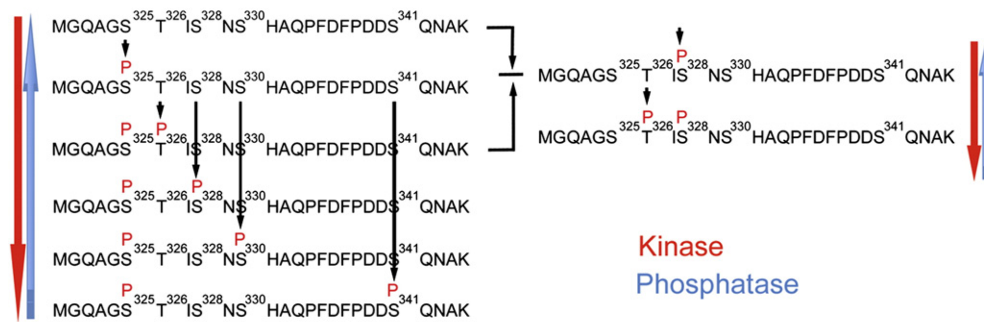


Fig. 6. Determination of relative order of kinases/phosphatases using phosphoproteomic datasets. By transitive relationships, the presence or lack phosphorylation at individual sites can be used to map relative order of Cx43 PTM for kinases (phosphorylation addition, red arrow) and phosphatases (blue arrow). At individual congruent sites, black arrows indicate the order of kinase activity at specific sites, with the opposite direction implying phosphate removal by phosphatases. In this model, phosphorylation at S325 and S328 serves as an early event towards multi-site PTM.

sequence and order, and how these complex events come together to influence Cx subunits and physiology.

Within the context of a Cx life-cycle, a particular challenge of studying phosphorylation arises from the difficulties of isolating specific stages of GJ/Cx maturation. The regulation of Cx subunits appears to be incredibly complex, and we anticipate that proteomic technologies and chemical biology will have an increasing role in the interrogation of protein-based signals. Therefore, when considering interactions and PTMs, it will be important to examine these events within the context of trafficking subunits and ideally within the context of an integrated network — possibly using GJ intercellular communication as an output for system-level models. In the case of functionally distinct, location-specific modifications and interactions, one would expect initiation of these events at fairly precise locations within the cell. It is anticipated that the subcellular fractionation and the systematic examination using discovery proteomics, targeted technologies (such as MRM) and quantitative proteomics together will provide a valuable framework towards fully understanding Cxs. Although an unprecedented view of Cx multi-site phosphorylation is emerging, it appears we have only scratched the surface towards understanding the Cx code.

Acknowledgments

The authors would like to thank anonymous reviewers for critical insights. This work was supported by grants from the Canadian Institutes for Health Research (CCN, LJF), Heart and Stroke Foundation of BC and Yukon (CCN & LJF), and the Canadian Stroke Network (CCN). LJF and CCN are the Canada Research Chairs in Quantitative Proteomics and Gap Junctions and Disease, respectively. VCC holds a Bluma Tischler Postdoctoral Fellowship and was supported by a PDF award from the Heart and Stroke Foundation of Canada/BC and Yukon.

References

[1] C. Cina, J.F. Bechberger, M.A. Ozog, C.C. Naus, Expression of connexins in embryonic mouse neocortical development, J. Comp. Neurol. 504 (2007) 298–313.
[2] C. Cina, K. Maass, M. Theis, K. Willecke, J.F. Bechberger, C.C. Naus, Involvement of the cytoplasmic C-terminal domain of connexin43 in neuronal migration, J. Neurosci. 29 (2009) 2009–2021.
[3] N. Kamasawa, A. Sik, M. Morita, T. Yasumura, K.G. Davidson, J.I. Nagy, J.E. Rash, Connexin-47 and connexin-32 in gap junctions of oligodendrocyte somata, myelin sheaths, paranodal loops and Schmidt–Lanterman incisures: implications for ionic homeostasis and potassium siphoning, Neuroscience 136 (2005) 65–86.
[4] T.W. White, Unique and redundant connexin contributions to lens development, Science 295 (2002) 319–320.
[5] M.L. Cotrina, J.H. Lin, A. Alves-Rodrigues, S. Liu, J. Li, H. Azmi-Ghadimi, J. Kang, C.C. Naus, M. Nedergaard, Connexins regulate calcium signaling by controlling ATP release, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 15735–15740.
[6] L.A. Elias, D.D. Wang, A.R. Kriegstein, Gap junction adhesion is necessary for radial migration in the neocortex, Nature 448 (2007) 901–907.
[7] M.R. Wilson, T.W. Close, J.E. Trosko, Cell population dynamics (apoptosis, mitosis, and cell–cell communication) during disruption of homeostasis, Exp. Cell Res. 254 (2000) 257–268.
[8] J.K. VanSlyke, L.S. Musil, Analysis of connexin intracellular transport and assembly, Methods 20 (2000) 156–164.
[9] P.D. Lampe, A.F. Lau, The effects of connexin phosphorylation on gap junctional communication, Int. J. Biochem. Cell Biol. 36 (2004) 1171–1186.
[10] D.W. Laird, Connexin phosphorylation as a regulatory event linked to gap junction internalization and degradation, Biochim. Biophys. Acta 1711 (2005) 172–182.
[11] D.W. Laird, M. Castillo, L. Kasprzak, Gap junction turnover, intracellular trafficking, and phosphorylation of connexin43 in brefeldin A-treated rat mammary tumor cells, J. Cell Biol. 131 (1995) 1193–1203.
[12] J.I. Nagy, W.E. Li, C. Roy, B.W. Doble, J.S. Gilchrist, E. Kardami, E.L. Hertzberg, Selective monoclonal antibody recognition and cellular localization of an unphosphorylated form of connexin43, Exp. Cell Res. 236 (1997) 127–136.
[13] J.L. Solan, P.D. Lampe, Connexin phosphorylation as a regulatory event linked to gap junction channel assembly, Biochim. Biophys. Acta 1711 (2005) 154–163.
[14] M.B. Yaffe, A.E. Elia, Phosphoserine/threonine-binding domains, Curr. Opin. Cell Biol. 13 (2001) 131–138.
[15] L.S. Musil, D.A. Goodenough, Biochemical analysis of connexin43 intracellular transport, phosphorylation, and assembly into gap junctional plaques, J. Cell Biol. 115 (1991) 1357–1374.
[16] R.Y. Huang, J.G. Laing, E.M. Kanter, V.M. Berthoud, M. Bao, H.W. Rohrs, R.R. Townsend, K.A. Yamada, Identification of CaMKII phosphorylation sites in Connexin43 by high-resolution mass spectrometry, J. Proteome Res. 10 (2011) 1098–1109.
[17] J.L. Solan, L. Marquez-Rosado, P.L. Sorgen, P.J. Thornton, P.R. Gafken, P.D. Lampe, Phosphorylation at S365 is a gatekeeper event that changes the structure of Cx43 and prevents down-regulation by PKC, J. Cell Biol. 179 (2007) 1301–1309.
[18] M.M. Hao, A.M. Lowy, M. Kapoor, A. Deffie, G. Liu, G. Lozano, Mutation of phosphoserine 389 affects p53 function in vivo, J. Biol. Chem. 271 (1996) 29380–29385.
[19] W. Huang, R.L. Erikson, Constitutive activation of Mek1 by mutation of serine phosphorylation sites, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 8960–8963.
[20] W. Zheng, Z. Zhang, S. Ganguly, J.L. Weller, D.C. Klein, P.A. Cole, Cellular stabilization of the melatonin rhythm enzyme induced by nonhydrolyzable phosphonate incorporation, Nat. Struct. Biol. 10 (2003) 1054–1057.
[21] A.H. Zisch, C. Pazzagli, A.L. Freeman, M. Schneller, M. Hadman, J.W. Smith, E. Ruoslahti, E.B. Pasquale, Replacing two conserved tyrosines of the EphB2 receptor with glutamic acid prevents binding of SH2 domains without abrogating kinase activity and biological responses, Oncogene 19 (2000) 177–187.
[22] L.M. Szewczuk, M.K. Tarrant, P.A. Cole, Protein phosphorylation by semisynthesis: from paper to practice, Methods Enzymol. 462 (2009) 1–24.
[23] O.N. Jensen, Interpreting the protein language using proteomics, Nat. Rev. Mol. Cell Biol. 7 (2006) 391–403.
[24] X.J. Yang, Multisite protein modification and intramolecular signaling, Oncogene 24 (2005) 1653–1662.
[25] M.S. Cosgrove, J.D. Boeke, C. Wolberger, Regulated nucleosome mobility and the histone code, Nat. Struct. Mol. Biol. 11 (2004) 1037–1043.
[26] W. Fischle, Y. Wang, C.D. Allis, Binary switches and modification cassettes in histone biology and beyond, Nature 425 (2003) 475–479.
[27] E. Appella, C.W. Anderson, Signaling to p53: breaking the posttranslational modification code, Pathol. Biol. (Paris) 48 (2000) 227–245.
[28] S. Westermann, K. Weber, Post-translational modifications regulate microtubule function, Nat. Rev. Mol. Cell Biol. 4 (2003) 938–947.
[29] M.R. Wilkins, C. Pasquali, R.D. Appel, K. Ou, O. Golaz, J.C. Sanchez, J.X. Yan, A.A. Gooley, G. Hughes, I. Humphery-Smith, K.L. Williams, D.F. Hochstrasser, From proteins to proteomes: large scale protein identification by two-dimensional electrophoresis and amino acid analysis, Biotechnology (N. Y) 14 (1996) 61–65.
[30] C.L. de Hoog, M. Mann, Proteomics, Annu. Rev. Genomics Hum. Genet. 5 (2004) 267–293.
[31] L. Cai, N. Friedman, X.S. Xie, Stochastic protein expression in individual cells at the single molecule level, Nature 440 (2006) 358–362.
[32] J.D. Graves, E.G. Krebs, Protein phosphorylation and signal transduction, Pharmacol. Ther. 82 (1999) 111–121.
[33] S. Barik, Protein phosphorylation and signal transduction, Subcell. Biochem. 26 (1996) 115–164.
[34] E.L. Huttlin, M.P. Jedrychowski, J.E. Elias, T. Goswami, R. Rad, S.A. Beausoleil, J. Villen, W. Haas, M.E. Sowa, S.P. Gygi, A tissue-specific atlas of mouse protein phosphorylation and expression, Cell 143 (2010) 1174–1189.
[35] J.V. Olsen, B. Blagoev, F. Gnad, B. Macek, C. Kumar, P. Mortensen, M. Mann, Global, in vivo, and site-specific phosphorylation dynamics in signaling networks, Cell 127 (2006) 635–648.
[36] L.J. Foster, C.L. de Hoog, Y. Zhang, X. Xie, V.K. Mootha, M. Mann, A mammalian organelle map by protein correlation profiling, Cell 125 (2006) 187–199.
[37] M. Wilm, M. Mann, Analytical properties of the nanoelectrospray ion source, Anal. Chem. 68 (1996) 1–8.
[38] M. Karas, F. Hillenkamp, Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons, Anal. Chem. 60 (1988) 2299–2301.
[39] B.A. Ballif, J. Villen, S.A. Beausoleil, D. Schwartz, S.P. Gygi, Phosphoproteomic analysis of the developing mouse brain, Mol. Cell. Proteomics 3 (2004) 1093–1101.
[40] G. Han, M. Ye, H. Zhou, X. Jiang, S. Feng, R. Tian, D. Wan, H. Zou, J. Gu, Large-scale phosphoproteome analysis of human liver tissue by enrichment and fractionation of phosphopeptides with strong anion exchange chromatography, Proteomics 8 (2008) 1346–1361.
[41] L.D. Rogers, Y. Fang, L.J. Foster, An integrated global strategy for cell lysis, fractionation, enrichment and mass spectrometric analysis of phosphorylated peptides, Mol. Biosyst. 6 (2010) 822–829.
[42] M. Kokubu, Y. Ishihama, T. Sato, T. Nagasu, Y. Oda, Specificity of immobilized metal affinity-based IMAC/C18 tip enrichment of phosphopeptides for protein phosphorylation analysis, Anal. Chem. 77 (2005) 5144–5154.
[43] S.A. Gerber, J. Rush, O. Stemman, M.W. Kirschner, S.P. Gygi, Absolute quantification of proteins and phosphoproteins from cell lysates by tandem MS, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 6940–6945.
[44] J.L. Hsu, S.Y. Huang, N.H. Chow, S.H. Chen, Stable-isotope dimethyl labeling for quantitative proteomics, Anal. Chem. 75 (2003) 6843–6852.
[45] S.E. Ong, B. Blagoev, I. Kratchmarova, D.B. Kristensen, H. Steen, A. Pandey, M. Mann, Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics, Mol. Cell. Proteomics 1 (2002) 376–386.
[46] A. Makarov, E. Denisov, A. Kholomeev, W. Balschun, O. Lange, K. Strupat, S. Horning, Performance evaluation of a hybrid linear ion trap/orbitrap mass spectrometer, Anal. Chem. 78 (2006) 2113–2120.
[47] J.E. Syka, J.J. Coon, M.J. Schroeder, J. Shabanowitz, D.F. Hunt, Peptide and protein sequence analysis by electron transfer dissociation mass spectrometry, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 9528–9533.

[48] A. Stensballe, O.N. Jensen, J.V. Olsen, K.F. Haselmann, R.A. Zubarev, Electron capture dissociation of singly and multiply phosphorylated peptides, Rapid Commun. Mass Spectrom. 14 (2000) 1793–1800.
[49] W. Ens, K.G. Standing, Hybrid quadrupole/time-of-flight mass spectrometers for analysis of biomolecules, Methods Enzymol. 402 (2005) 49–78.
[50] W.J. Henzel, C. Watanabe, J.T. Stults, Protein identification: the origins of peptide mass fingerprinting, J. Am. Soc. Mass Spectrom. 14 (2003) 931–942.
[51] V.C. Chen, X. Li, H. Perreault, J.I. Nagy, Interaction of zonula occludens-1 (ZO-1) with alpha-actinin-4: application of functional proteomics for identification of PDZ domain-associated proteins, J. Proteome Res. 5 (2006) 2123–2134.
[52] A.I. Nesvizhskii, O. Vitek, R. Aebersold, Analysis and validation of proteomic data generated by tandem mass spectrometry, Nat. Methods 4 (2007) 787–797.
[53] D.N. Perkins, D.J. Pappin, D.M. Creasy, J.S. Cottrell, Probability-based protein identification by searching sequence databases using mass spectrometry data, Electrophoresis 20 (1999) 3551–3567.
[54] J.R. Yates III, J.K. Eng, A.L. McCormack, D. Schieltz, Method to correlate tandem mass spectra of modified peptides to amino acid sequences in the protein database, Anal. Chem. 67 (1995) 1426–1436.
[55] A.I. Nesvizhskii, R. Aebersold, Interpretation of shotgun proteomic data: the protein inference problem, Mol. Cell. Proteomics 4 (2005) 1419–1440.
[56] R.E. Moore, M.K. Young, T.D. Lee, Qscore: an algorithm for evaluating SEQUEST database search results, J. Am. Soc. Mass Spectrom. 13 (2002) 378–386.
[57] A.A. Klammer, M.J. MacCoss, Effects of modified digestion schemes on the identification of proteins from complex mixtures, J. Proteome Res. 5 (2006) 695–700.
[58] J. Colinge, A. Masselot, M. Giron, T. Dessingy, J. Magnin, OLAV: towards high-throughput tandem mass spectrometry data identification, Proteomics 3 (2003) 1454–1463.
[59] D.B. Weatherly, J.A. Atwood III, T.A. Minning, C. Cavola, R.L. Tarleton, R. Orlando, A Heuristic method for assigning a false-discovery rate for protein identifications from Mascot database search results, Mol. Cell. Proteomics 4 (2005) 762–772.
[60] R.E. Higgs, M.D. Knierman, A.B. Freeman, L.M. Gelbert, S.T. Patil, J.E. Hale, Estimating the statistical significance of peptide identifications from shotgun proteomics experiments, J. Proteome Res. 6 (2007) 1758–1767.
[61] L.J. Foster, Interpretation of data underlying the link between colony collapse disorder (CCD) and an invertebrate iridescent virus, Mol. Cell. Proteomics 10 (2011) M110 006387.
[62] G.M. Knudsen, R.J. Chalkley, The effect of using an inappropriate protein database for proteomic data analysis, PLoS One 6 (2011) e20873.
[63] D. Locke, S. Bian, H. Li, A.L. Harris, Post-translational modifications of connexin26 revealed by mass spectrometry, Biochem. J. 424 (2009) 385–398.
[64] V. Lange, P. Picotti, B. Domon, R. Aebersold, Selected reaction monitoring for quantitative proteomics: a tutorial, Mol. Syst. Biol. 4 (2008) 222.
[65] T.A. Addona, S.E. Abbatiello, B. Schilling, S.J. Skates, D.R. Mani, D.M. Bunk, C.H. Spiegelman, L.J. Zimmerman, A.J. Ham, H. Keshishian, S.C. Hall, S. Allen, R.K. Blackman, C.H. Borchers, C. Buck, H.L. Cardasis, M.P. Cusack, N.G. Dodder, B.W. Gibson, J.M. Held, T. Hiltke, A. Jackson, E.B. Johansen, C.R. Kinsinger, J. Li, M. Mesri, T.A. Neubert, R.K. Niles, T.C. Pulsipher, D. Ransohoff, H. Rodriguez, P.A. Rudnick, D. Smith, D.L. Tabb, T.J. Tegeler, A.M. Variyath, L.J. Vega-Montoto, A. Wahlander, S. Waldemarson, M. Wang, J.R. Whiteaker, L. Zhao, N.L. Anderson, S.J. Fisher, D.C. Liebler, A.G. Paulovich, F.E. Regnier, P. Tempst, S.A. Carr, Multi--site assessment of the precision and reproducibility of multiple reaction monitoring-based measurements of proteins in plasma, Nat. Biotechnol. 27 (2009) 633–641.
[66] L. Anderson, C.L. Hunter, Quantitative mass spectrometric multiple reaction monitoring assays for major plasma proteins, Mol. Cell. Proteomics 5 (2006) 573–588.
[67] E.W. Deutsch, H. Lam, R. Aebersold, PeptideAtlas: a resource for target selection for emerging targeted proteomics workflows, EMBO Rep. 9 (2008) 429–434.
[68] J.A. Vizcaino, R. Cote, F. Reisinger, H. Barsnes, J.M. Foster, J. Rameseder, H. Hermjakob, L. Martens, The Proteomics Identifications database: 2010 update, Nucleic Acids Res. 38 (2010) D736–D742.
[69] J.A. Vizcaino, R. Cote, F. Reisinger, J.M. Foster, M. Mueller, J. Rameseder, H. Hermjakob, L. Martens, A guide to the Proteomics Identifications Database proteomics data repository, Proteomics 9 (2009) 4276–4283.
[70] O.V. Krokhin, Sequence-specific retention calculator. Algorithm for peptide retention prediction in ion-pair RP-HPLC: application to 300- and 100-A pore size C18 sorbents, Anal. Chem. 78 (2006) 7785–7795.
[71] The call of the human proteome, Nat. Methods 7 (2010) 661.
[72] N. Bisson, D.A. James, G. Ivosev, S.A. Tate, R. Bonner, L. Taylor, T. Pawson, Selected reaction monitoring mass spectrometry reveals the dynamics of signaling through the GRB2 adaptor, Nat. Biotechnol. 29 (2011) 653–658.
[73] A. Wolf-Yadlin, S. Hautaniemi, D.A. Lauffenburger, F.M. White, Multiple reaction monitoring for robust quantitative proteomic analysis of cellular signaling networks, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 5860–5865.
[74] D. Domanski, L.C. Murphy, C.H. Borchers, Assay development for the determination of phosphorylation stoichiometry using multiple reaction monitoring methods with and without phosphatase treatment: application to breast cancer signaling pathways, Anal. Chem. 82 (2010) 5610–5620.
[75] A.F. Lau, W.E. Kurata, M.Y. Kanemitsu, L.W. Loo, B.J. Warn-Cramer, W. Eckhart, P.D. Lampe, Regulation of connexin43 function by activated tyrosine protein kinases, J. Bioenerg. Biomembr. 28 (1996) 359–368.
[76] B.J. Warn-Cramer, P.D. Lampe, W.E. Kurata, M.Y. Kanemitsu, L.W. Loo, W. Eckhart, A.F. Lau, Characterization of the mitogen-activated protein kinase phosphorylation sites on the connexin-43 gap junction protein, J. Biol. Chem. 271 (1996) 3779–3786.
[77] B.J. Warn-Cramer, A.F. Lau, Regulation of gap junctions by tyrosine protein kinases, Biochim. Biophys. Acta 1662 (2004) 81–95.
[78] G.T. Cottrell, R. Lin, B.J. Warn-Cramer, A.F. Lau, J.M. Burt, Mechanism of v-Src- and mitogen-activated protein kinase-induced reduction of gap junction communication, Am. J. Physiol. Cell Physiol. 284 (2003) C511–C520.
[79] J.R. Wisniewski, N. Nagaraj, A. Zougman, F. Gnad, M. Mann, Brain phosphoproteome obtained by a FASP-based method reveals plasma membrane protein topology, J. Proteome Res. 9 (2010) 3280–3289.
[80] D.W. Laird, The gap junction proteome and its relationship to disease, Trends Cell Biol. 20 (2010) 92–101.
[81] Z. Ai, A. Fischer, D.C. Spray, A.M. Brown, G.I. Fishman, Wnt-1 regulation of connexin43 in cardiac myocytes, J. Clin. Invest. 105 (2000) 161–171.
[82] M.Y. Kanemitsu, L.W. Loo, S. Simon, A.F. Lau, W. Eckhart, Tyrosine phosphorylation of connexin 43 by v-Src is mediated by SH2 and SH3 domain interactions, J. Biol. Chem. 272 (1997) 22824–22831.
[83] B.N. Giepmans, I. Verlaan, W.H. Moolenaar, Connexin-43 interactions with ZO-1 and alpha- and beta-tubulin, Cell Commun. Adhes. 8 (2001) 219–223.
[84] M.L. Vitale, C.D. Akpovi, R.M. Pelletier, Cortactin/tyrosine-phosphorylated cortactin interaction with connexin 43 in mouse seminiferous tubules, Microsc. Res. Tech. 72 (2009) 856–867.
[85] X. Li, M. Penes, B. Odermatt, K. Willecke, J.I. Nagy, Ablation of Cx47 in transgenic mice leads to the loss of MUPP1, ZONAB and multiple connexins at oligodendrocyte-astrocyte gap junctions, Eur. J. Neurosci. 28 (2008) 1503–1517.
[86] E. Butkevich, S. Hulsmann, D. Wenzel, T. Shirao, R. Duden, I. Majoul, Drebrin is a novel connexin-43 binding partner that links gap junctions to the submembrane cytoskeleton, Curr. Biol. 14 (2004) 650–658.
[87] B.N. Giepmans, I. Verlaan, T. Hengeveld, H. Janssen, J. Calafat, M.M. Falk, W.H. Moolenaar, Gap junction protein connexin-43 interacts directly with microtubules, Curr. Biol. 11 (2001) 1364–1368.
[88] S. Langlois, K.N. Cowan, Q. Shao, B.J. Cowan, D.W. Laird, Caveolin-1 and -2 interact with connexin43 and regulate gap junctional intercellular communication in keratinocytes, Mol. Biol. Cell 19 (2008) 912–928.
[89] C.T. Fu, J.F. Bechberger, M.A. Ozog, B. Perbal, C.C. Naus, CCN3 (NOV) interacts with connexin43 in C6 glioma cells: possible mechanism of connexin-mediated growth suppression, J. Biol. Chem. 279 (2004) 36943–36950.
[90] M.C. Penes, X. Li, J.I. Nagy, Expression of zonula occludens-1 (ZO-1) and the transcription factor ZO-1-associated nucleic acid-binding protein (ZONAB)-MsY3 in glial cells and colocalization at oligodendrocyte and astrocyte gap junctions in mouse brain, Eur. J. Neurosci. 22 (2005) 404–418.
[91] J.L. Cheng, M.J. Sweredoski, P. Baldi, Accurate prediction of protein disordered regions by mining protein structure data, Data Min. Knowl. Discov. 11 (2005) 213–222.
[92] L.M. Iakoucheva, P. Radivojac, C.J. Brown, T.R. O'Connor, J.G. Sikes, Z. Obradovic, A.K. Dunker, The importance of intrinsic disorder for protein phosphorylation, Nucleic Acids Res. 32 (2004) 1037–1049.
[93] E. Kardami, X. Dang, D.A. Iacobas, B.E. Nickel, M. Jeyaraman, W. Srisakuldee, J. Makazan, S. Tanguy, D.C. Spray, The role of connexins in controlling cell growth and gene expression, Prog. Biophys. Mol. Biol. 94 (2007) 245–264.
[94] X. Dang, M. Jeyaraman, E. Kardami, Regulation of connexin-43-mediated growth inhibition by a phosphorylatable amino-acid is independent of gap junction-forming ability, Mol. Cell. Biochem. 289 (2006) 201–207.
[95] D.C. Bates, W.C. Sin, Q. Aftab, C.C. Naus, Connexin43 enhances glioma invasion by a mechanism involving the carboxy terminus, Glia 55 (2007) 1554–1564.
[96] J. Behrens, P. Kameritsch, S. Wallner, U. Pohl, K. Pogoda, The carboxyl tail of Cx43 augments p38 mediated cell migration in a gap junction-independent manner, Eur. J. Cell Biol. 89 (2010) 828–838.
[97] S. Machtaler, M. Dang-Lawson, K. Choi, C. Jang, C.C. Naus, L. Matsuuchi, The gap junction protein Cx43 regulates B-lymphocyte spreading and adhesion, J. Cell Sci. 124 (2011) 2611–2621.
[98] B.W. Doble, J.R. Woodgett, GSK-3: tricks of the trade for a multi-tasking kinase, J. Cell Sci. 116 (2003) 1175–1186.
[99] B.J. Warn-Cramer, G.T. Cottrell, J.M. Burt, A.F. Lau, Regulation of connexin-43 gap junctional intercellular communication by mitogen-activated protein kinase, J. Biol. Chem. 273 (1998) 9188–9196.
[100] E. Leithe, E. Rivedal, Epidermal growth factor regulates ubiquitination, internalization and proteasome-dependent degradation of connexin43, J. Cell Sci. 117 (2004) 1211–1220.
[101] M.Y. Kanemitsu, W. Jiang, W. Eckhart, Cdc2-mediated phosphorylation of the gap junction protein, connexin43, during mitosis, Cell Growth Differ. 9 (1998) 13–21.
[102] P.D. Lampe, W.E. Kurata, B.J. Warn-Cramer, A.F. Lau, Formation of a distinct connexin43 phosphoisoform in mitotic cells is dependent upon p34cdc2 kinase, J. Cell Sci. 111 (Pt 6) (1998) 833–841.
[103] M.A. Beardslee, D.L. Lerner, P.N. Tadros, J.G. Laing, E.C. Beyer, K.A. Yamada, A.G. Kleber, R.B. Schuessler, J.E. Saffitz, Dephosphorylation and intracellular redistribution of ventricular connexin43 during electrical uncoupling induced by ischemia, Circ. Res. 87 (2000) 656–662.
[104] F. Duthe, I. Plaisance, D. Sarrouilhe, J.C. Herve, Endogenous protein phosphatase 1 runs down gap junctional communication of rat ventricular myocytes, Am. J. Physiol. Cell Physiol. 281 (2001) C1648–C1656.
[105] M.S. Turner, G.A. Haywood, P. Andreka, L. You, P.E. Martin, W.H. Evans, K.A. Webster, N.H. Bishopric, Reversible connexin 43 dephosphorylation during hypoxia and reoxygenation is linked to cellular ATP levels, Circ. Res. 95 (2004) 726–733.
[106] M. Jeyaraman, S. Tanguy, R.R. Fandrich, A. Lukas, E. Kardami, Ischemia-induced dephosphorylation of cardiomyocyte connexin-43 is reduced by

okadaic acid and calyculin A but not fostriecin, Mol. Cell. Biochem. 242 (2003) 129–134.
[107] P.D. Lampe, C.D. Cooper, T.J. King, J.M. Burt, Analysis of Connexin43 phosphorylated at S325, S328 and S330 in normoxic and ischemic heart, J. Cell Sci. 119 (2006) 3435–3442.
[108] O.V. Krokhin, R. Craig, V. Spicer, W. Ens, K.G. Standing, R.C. Beavis, J.A. Wilkins, An improved model for prediction of retention times of tryptic peptides in ion pair reversed-phase HPLC: its application to protein peptide mapping by off-line HPLC-MALDI MS, Mol. Cell. Proteomics 3 (2004) 908–919.
[109] R. Lin, B.J. Warn-Cramer, W.E. Kurata, A.F. Lau, v-Src-mediated phosphorylation of connexin43 on tyrosine disrupts gap junctional communication in mammalian cells, Cell Commun. Adhes. 8 (2001) 265–269.
[110] B.W. Doble, X. Dang, P. Ping, R.R. Fandrich, B.E. Nickel, Y. Jin, P.A. Cattini, E. Kardami, Phosphorylation of serine 262 in the gap junction protein connexin-43 regulates DNA synthesis in cell–cell contact forming cardiomyocytes, J. Cell Sci. 117 (2004) 507–514.
[111] T. Toyofuku, Y. Akamatsu, H. Zhang, T. Kuzuya, M. Tada, M. Hori, c-Src regulates the interaction between connexin-43 and ZO-1 in cardiac myocytes, J. Biol. Chem. 276 (2001) 1780–1788.
[112] K. Procida, L. Jorgensen, N. Schmitt, M. Delmar, S.M. Taffet, N.H. Holstein-Rathlou, M.S. Nielsen, T.H. Braunstein, Phosphorylation of connexin43 on serine 306 regulates electrical coupling, Heart Rhythm 6 (2009) 1632–1638.
[113] C.D. Cooper, P.D. Lampe, Casein kinase 1 regulates connexin-43 gap junction assembly, J. Biol. Chem. 277 (2002) 44962–44968.
[114] E.M. TenBroek, P.D. Lampe, J.L. Solan, J.K. Reynhout, R.G. Johnson, Ser364 of connexin43 and the upregulation of gap junction assembly by cAMP, J. Cell Biol. 155 (2001) 1307–1318.
[115] K. Yogo, T. Ogawa, M. Akiyama, N. Ishida, T. Takeya, Identification and functional analysis of novel phosphorylation sites in Cx43 in rat primary granulosa cells, FEBS Lett. 531 (2002) 132–136.
[116] P.D. Lampe, E.M. TenBroek, J.M. Burt, W.E. Kurata, R.G. Johnson, A.F. Lau, Phosphorylation of connexin43 on serine368 by protein kinase C regulates gap junctional communication, J. Cell Biol. 149 (2000) 1503–1512.
[117] L.N. Axelsen, M. Stahlhut, S. Mohammed, B.D. Larsen, M.S. Nielsen, N.H. Holstein-Rathlou, S. Andersen, O.N. Jensen, J.K. Hennan, A.L. Kjolbye, Identification of ischemia-regulated phosphorylation sites in connexin43: a possible target for the antiarrhythmic peptide analogue rotigaptide (ZP123), J. Mol. Cell. Cardiol. 40 (2006) 790–798.
[118] L.M. Brill, W. Xiong, K.B. Lee, S.B. Ficarro, A. Crain, Y. Xu, A. Terskikh, E.Y. Snyder, S. Ding, Phosphoproteomic analysis of human embryonic stem cells, Cell Stem Cell 5 (2009) 204–213.
[119] K. Rikova, A. Guo, Q. Zeng, A. Possemato, J. Yu, H. Haack, J. Nardone, K. Lee, C. Reeves, Y. Li, Y. Hu, Z. Tan, M. Stokes, L. Sullivan, J. Mitchell, R. Wetzel, J. Macneill, J.M. Ren, J. Yuan, C.E. Bakalarski, J. Villen, J.M. Kornhauser, B. Smith, D. Li, X. Zhou, S.P. Gygi, T.L. Gu, R.D. Polakiewicz, J. Rush, M.J. Comb, Global survey of phosphotyrosine signaling identifies oncogenic kinases in lung cancer, Cell 131 (2007) 1190–1203.
[120] N. Blom, T. Sicheritz-Ponten, R. Gupta, S. Gammeltoft, S. Brunak, Prediction of post-translational glycosylation and phosphorylation of proteins from the amino acid sequence, Proteomics 4 (2004) 1633–1649.