Co-Phosphorylation Networks Reveal Subtype-Specific Signaling Modules in Breast Cancer

Marzieh Ayati [1,*], Mark R Chance [2,3,4] and Mehmet Koyuturk [3,4,5,*]

[1]Department of Computer Science, University of Texas Rio Grande Valley, Edinburg, TX, 78531, USA and
[2] Department of Nutrition, Case Western Reserve University, Cleveland, OH and
[3] Center for Proteomics and Bioinformatics, Case Western Reserve University, Cleveland, OH and
[4] Case Comprehensive Cancer Center, Case Western Reserve University, Cleveland, OH and
[5]Department of Computer and Data Sciences, Case Western Reserve University, Cleveland, OH, 44106, USA.

Abstract

Motivation: Protein phosphorylation is a ubiquitous mechanism of post-translational modification that plays a central role in cellular signaling. Phosphorylation is particularly important in the context of cancer, as down-regulation of tumor suppressors and up-regulation of oncogenes by the dysregulation of associated kinase and phosphatase networks are shown to have key roles in tumor growth and progression. Despite recent advances that enable large-scale monitoring of protein phosphorylation, these data are not fully incorporated into such computational tasks as phenotyping and subtyping of cancers.

Results: We develop a network-based algorithm, CoPPNet, to enable unsupervised subtyping of cancers using phosphorylation data. For this purpose, we integrate prior knowledge on evolutionary, structural, and functional association of phosphosites, kinase-substrate associations, and protein-protein interactions with the correlation of phosphorylation of phosphosites across different tumor samples (a.k.a co-phosphorylation) to construct a dynamically weighted network of phosphosites. We then mine these networks to identify subnetworks with coherent phosphorylation patterns. We apply the proposed framework to two mass-spectrometry based phosphorylation datasets for breast cancer, and observe that (i) the phosphorylation pattern of the identified subnetworks are highly correlated with clinically identified subtypes, and (ii) the identified subnetworks are highly reproducible across datasets that are derived from different studies. Our results show that integration of quantitative phosphorylation data with network frameworks can provide mechanistic insights into the differences between the signaling mechanisms that drive breast cancer subtypes. Furthermore, the reproducibility of the identified subnetworks suggests that phosphorylation can provide robust classification of disease response and markers.

Availability: CoPPNet is available at http://compbio.case.edu/coppnet/

1 Introduction

Protein phosphorylation is a ubiquitous mechanism of post-translational modification observed across cell types and species, and plays a central role in cellular signaling. Phosphorylation is regulated by networks composed of kinases, phosphatases, and their substrates. Phosphorylation is particularly important in the context of cancer, as down-regulation of tumor suppressors and up-regulation of oncogenes (often kinases themselves) by dys-regulation of the associated kinase and phosphatase networks are shown to have key roles in tumor growth and progression [1, 2]. To this end, characterization of signaling networks enables exploration of the interconnected targets leading to the development of kinase inhibitors to treat a variety of cancers [3, 4]. In response to the growing need for large-scale monitoring of phosphorylation, advanced mass spectrometry (MS)-based phospho-proteomics technologies have exploded. These technologies enable simultaneous identification and quantification of thousands of phosphopeptides and phosphosites from a given sample [5].

MS-based phospho-proteomics screens create a great opportunity to discover biology that may not be observed in transcriptomic and proteomic data [6]. Indeed, recent research shows that, as compared to gene expression, data on post-transcriptional modifications can be more useful in subtyping cancers. As a striking example, monitoring of the specific phosphorylation pathways reveals a novel breast cancer subtype that is unique to the phospho-proteomics and cannot be captured based on DNA mutations, mRNA-level expression, or protein expression [7].

Although phospho-proteomics provides a critical data source to model signaling pathways, systematic methods for network analysis of phospho-proteins and phosphosites are relatively scarce. Since most of the methods designed for genomics and general proteomics are not designed to handle the complexity of phospho-proteomics, phospho-proteomic analyses are often are centralized at the protein level. However, due to the *many-to-one* mapping from phosphosites to proteins (i.e. each protein may have multiple phosphorylation sites), and also multi-layer annotations (e.g. regulatory function of phosphosites and kinase-phosphosite associations), novel approaches are needed to fully leverage the richness of the data. To enable analysis of phospho-proteomic data at the level of phosphorylation sites and the relationships between these sites, we propose CoPPNet, a network-based algorithm for the analysis of phospho-proteomic data, which offers the following innovations: (i) Construction of a

PhosphoSite Functional Association (PSFA) network that represents the functional relationship among individual phosphosites. In order to create PSFA network, we incorporate known structural, evolutionary, and functional associations between phosphosites, protein-protein interactions, and kinase-substrate associations. (ii) Utilization of the PFSA network in the identification of phosphorylation modules in breast cancer, through filtering of phosphosite pairs that are potentially functionally associated. CoPPNet accomplishes this by assigning co-phosphorylation (Co-P) based weights to the edges in PFSA network, where Co-P quantifies the similarity of the phosphorylation patterns of phosphosites across different breast cancer samples. We have recently introduced the notion of co-phosphorylation and used it in the context of predicting kinase-substrate associations, showing that it significantly enhances the coverage and accuracy of prediction methods over those that utilize static data such as sequences, structures, and generic networks [8]. Conceptually, Co-P is similar to gene co-expression, which has been shown to be effective in many biomedical applications [9, 10]. (iii) Development of a scoring scheme accompanied by an algorithm to identify co-phosphorylated signaling modules from this weighted PSFA network.

We test the proposed framework in the context of *unsupervised* identification of subtype-specific signaling modules in breast cancer. For this purpose, we apply CoPPNet on two independent public phospho-protoemics datasets for breast cancer (BC). Breast cancer is categorized into 4 molecular subtypes: Luminal A, Luminal B, HER2-enriched and triple-negative (basal-like). Among the subtypes, Luminal A has the greatest survival, and basal has the poorest survival [11]. While constructing the weighted PSFA network and identifying co-phosphorylation modules on this network, we do not use any information on the samples' clinically determined subtypes.

Our results show that the statistically significant modules identified by CoPPNet are reproducible between the two independent datasets and can capture the differential phosphorylation between breast cancer subtypes. The identified subtype-specific signaling modules have the potential to provide significant insights into the disruption of signaling processes in different cancer subtypes, and can be employed in developing subtype specific therapeutic targeting strategies for breast cancer.

2 MATERIALS AND METHODS

The workflow of the proposed framework for unsupervised identification of co-phosphorylation (Co-P) modules is shown in Figure 1. As seen in the figure, we first construct a network to model the functional relationship between phosphorylation sites. For this purpose, we incorporate available knowledge on functional associations between phosphosites, kinase-substrate associations and protein-protein interactions, and integrate these knowledge into a PhosphoSite Functional Association (PSFA) network. Subsequently, we utilize a module identification algorithm to identify sub-networks of the PSFA network that are

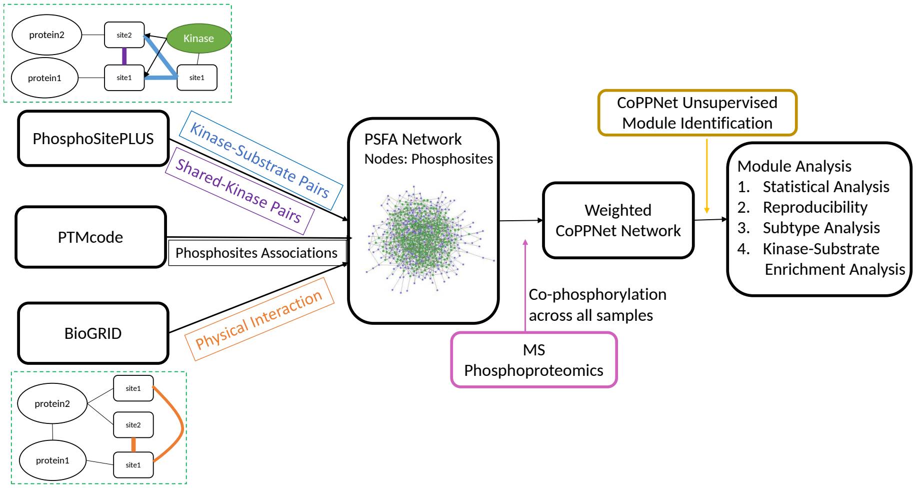


Figure 1: **Workflow of** CoPPNet. We first construct a PSFA network to represents the functional relationship among phosphosites, by utilizing generic kinase-substrate association, phosphosites associations and protein-protein interaction data. The nodes of the PSFA network represent phosphosites and the edges represent (1) kinase-substrate association, 2) phosphosites targeted by a common kinase, (3) functional associations between phosphosites, (4) physical interaction between proteins harboring the sites. For a given phosphorylation dataset collected from multiple cancer samples, we weigh the edges of the PSFA network based on the co-phosphorylation (Co-P) of pairs of sites across these samples. Then, we identify Co-P modules as sub-networks composed of heavy edges in this weighted network. Finally, we comprehensively assess the significance, reproducibility, subtype-specificity, and biological relevance of the Co-P modules.

composed of highly co-phosphorylated phosphosites (called *Co-P modules*). The premise of this approach is that, pairs of phosphosites whose phosphorylation is related to a specific cancer subtype will exhibit co-variation across different samples. For this reason, we expect that Co-P can highlight subtype-specific signaling modules even if subtype information is not available for the samples that are used to compute Co-P.

To assess the biological significance of the identified significant modules, we comprehensively evaluate their statistical significance and investigate the reproducibility of significant modules by utilizing a dataset that comes from a different patient cohort. Subsequently, we assess the differential phosphorylation of the sites in the signaling modules between different subtypes and perform pathway enrichment analysis and kinase enrichment analysis on these modules to annotate the modules.

PhosphoSite Functional Association (PSFA) Network. We define a PhosphoSite Functional Association (PSFA) network as a network that represents *potential* functional relationships between pairs of phosphosites. This network serves the purpose of filtering out the search space for pairs of phosphosites whose co-phosphorylation may reveal their functional relationship in the context of a specific process (e.g., dysregulation of a signaling pathway in the progression of a certain cancer subtype). In PSFA network $G(V, E)$, V denotes the set of nodes in the network, each of which represents a phosphosite; thus a protein is represented by multiple nodes in the PSFA network. The edge set E denotes the set of pairwise functional relationships between phosphosites, where an edge $s_i s_j \in E$ between phosphosites $s_i, s_j \in V$ may represent one of the following relationships:

- **Functional, Evolutionary, and Structural Association between Phosphosites (FES).** PTMCode is a database of known and predicted functional associations between phosphorylation and other post-translational modification sites [12]. The associations included in PTMCode are curated from the literature, inferred from residue co-evolution, or are based on the structural distances between phosphosites. We utilize PTMcode as a direct source of functional, evolutionary, and structural associations between phosphorylation sites.

- **Kinase-Substrate Association (KSA).** If phosphosite s_i is a target of kinase p_k and s_j is a phosphosite on kinase p_k, then there is an edge between s_i and s_j in the PFSA network. We call these edges *KSA edges*. This relationship indicates potential functional association between s_i and s_j since the regulation of kinase p_k through phosphorylation of s_j may influence p_k's action on s_i. In our experiments, we use PhosphositePLUS as the main source of information for kinase-substrate association [13].

- **Phosphosites Targeted by Common Kinase (TCK).** If phosphosites s_i and s_j (which may be on the same protein or on different proteins) are targeted by kinase p_k, then we call them a *shared-kinase pair* and include an edge between s_i and s_j in the PSFA network. We call these edges TCK edges. We include TCK edges in the PSFA network since the activity of p_k in a specific process may influence the phosphorylation of both s_i and s_j, which may be captured by their co-phosphorylation. Indeed, studies have shown that the substrates of a protein kinase can have significant similarity in terms of their biological functions [14].

- **Protein-Protein Interaction (PPI)**. If two proteins p_ℓ and p_r physically interact, site s_i is on p_ℓ, and site s_j is on protein p_r, then there is an edge between s_i and s_j in the PSFA network. We call these edges *PPI edges*. We include PPI edges in the PSFA network, since these edges may capture functional relationships and post-transcriptional modifications beyond phosphorylation, and may remedy the sparse and incomplete nature of existing kinase-substrate annotations. In our experiments, we use the PPIs that are annotated as ”physical” in the BIOGRID PPI database [15] to infer the PPI edges in the PFSA network.

The PSFA network is a generic network of potential functional associations between pairs of phosphosites. In the next section, we discuss how to assign weights to the edges of the PSFA network to represent the co-phosphorylation of pairs of phosphosites in a specific context.

Assessment of Co-Phosphorylation . As with gene co-expression, correlated phosphorylation of phosphosites on proteins may be indicative of their functional relationship in a specific biological context [8]. Based on this premise, we use context-specific phosphorylation data, obtained from mass spectrometry based phospho-proteomics assays, to assess the co-phosphorylation (Co-P) of all pairs of phosphosites that are connected in the PSFA network. In gene co-expression analysis, Pearson's correlation and mutual information are commonly used to assess linear and non-linear relations between the expression profiles of genes [16, 17]. Recognizing the benefits and shortcomings of each method, Song et al. [18] developed bi-weight mid-correlation as an alternative, and showed that it outperforms mutual information in terms capturing biologically relevant relationships between genes. while being more robust to outliers than Pearson's correlation. Motivated by these results, we use bi-weight mid-correlation to assess the Co-P of pairs of phosphosites.

Identification of Co-Phosphorylation Modules. Given a weighted PSFA network $G(V, E, w)$ associated with a specific phosho-proteomic dataset, our objective is to identify sub-networks of the PSFA network that are enriched in highly co-phosphorylated (positively or negatively) pairs of phosphosites. This problem is similar to the well-studied problem of identifying altered sub-networks, in which the nodes are scored based on their dysregulation (e.g., z-score indicating differential gene expression) in a given condition [19] or association with a disease (e.g., $-\log$ of the p-value of association) [20]. In this network, one or more connected sub-networks composed of high-scoring nodes are sought. In contrast, in our problem, scores are associated with edges, thus the problem is also similar to the infamous community detection problem in network analysis.

As with the altered sub-network identification problem, the key component of a solution to the problem is the definition of an objective function for scoring a given sub-network. Inspired by Newman and Girman's definition of network modularity [21] and our adaptation of this measure to the identification of disease-associated modules [20], we here propose a modularity-based approach to scoring co-phosphorylation modules. In this approach, subnetworks are scored based on the difference between their total edge weight and their expected total edge weight under a reference model that takes into account the degree distribution of the network (in our case, the distribution of Co-P across the network). Namely, for a given set of phosphosites $Q \subseteq V$, we define the Co-P score of Q according to $\mathcal{M}$ as

$$\sigma(Q) = \sum_{s_i, s_j \in Q} w(s_i, s_j) - \bar{w} \tag{1}$$

where $\bar{w}$ is the mean of the absolute values of Co-P across all pairs of phosphosites.

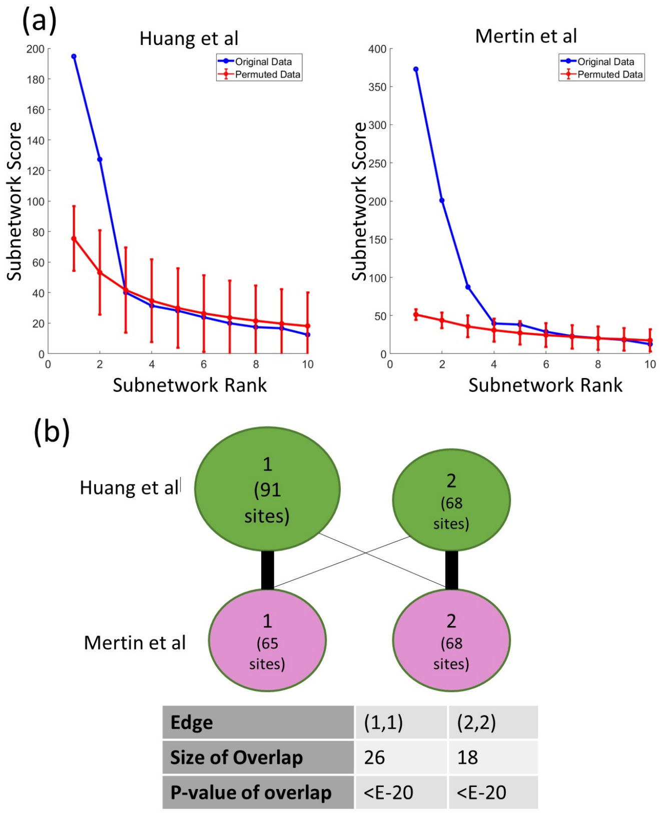
Having defined the Co-P score of a subnetwork as in Equation 1, given weighted PSFA network $G(V, E, w)$, we search for subnetworks of G that maximize $\sigma(Q)$. Since the *maximum-weight induced subgraph problem* is NP-hard [22], we use a greedy algorithm for this purpose. Once we identify a subnetwork with locally maximal Co-P score, we remove this subnetwork from G and use the greedy algorithm again to identify the next subnetwork with locally maximal Co-P score. We repeat this procedure until the entire network is exhausted, and sort all of the identified subnetworks (called Co-P modules) in decreasing order of their Co-P score.

Assessment of Statistical Significance To assess the statistical significance of all identified Co-P modules, we use permutation tests. For this purpose, we randomize the weights of the edges of the PSFA network while preserving the topology of the network (thereby preserving the degree distribution of the phosphosites) to generate N permuted networks (we use $N = 100$ in the experimental results reported in the next section). On each of the permuted networks, we identify and rank Co-P modules using the algorithm described in the previous section. We then assess the statistical significance of each module identified on the original network by comparing its score against the scores of the subnetworks that are ranked at least as high as itself on the permuted networks. We also visualize the scores of the identified modules in the context of these cumulative empirical distributions.

Assessment of Subtype Specificity. Although the weights of edges in the PSFA network are computed using co-phosphorylation (Co-P), which is agnostic to the subtypes of the samples, Co-P captures the co-variation of phosphorylation levels of phosphosites across different samples. Therefore, the identified modules have the potential to be associated with subtype-relevant mechanisms. Motivated by this insight, we investigate if the identified Co-P modules are composed of phosphosites that exhibit differential phosphorylation between cancer subtypes. For this purpose, we assess the differential phosphorylation of each phosphosite in a module between different subtypes. We use standard t-tests to compare the distribution of relative phosphorylation level (with respect to the common reference) in different subtypes.

Assessment of Reproducibility. We assess the reproducibility of identified co-P modules by investigating the overlap between significant modules identified on independent datasets. To assess the overlap between two Co-P modules that are identified in two independent datasets, we use standard hypergeometric test.

Kinase Substrate Enrichment Analysis. Kinase Substrate Enrichment Analysis (KSEA) seeks to identify kinases whose targets exhibit significantly altered phosphorylation levels in a given condition. KSEA scores each kinase based on the relative phosphorylation and dephosphorylation of its substrates (i.e fold change). In order to assess the value added by Co-P modules, we perform kinase enrichment analysis by restricting KSEA to the substrates that are in the significant modules as opposed to all phosphosites that are identified in the study. To infer the differential activity of kinases between subtypes, we compare the score of kinases which are computed using the fold change of



Edge	(1,1)	(2,2)
Size of Overlap	26	18
P-value of overlap	<E-20	<E-20

Figure 2: CoPPNet **identifies highly significant and reproducible co-phosphorylation (Co-P) modules**. (a) Statistical significance of identified sub-networks in two breast cancer datasets. For each dataset, the blue curve shows Co-P scores (y-axis) of the highest scoring 10 sub-networks in decreasing order (rank shown on x-axis). For each rank i on the *x-axis*, the red curve and error bar show the distribution of the scores of i highest scoring sub-networks in 100 randomized networks obtained by permuting the edge weights. (b) Reproducibility of significant Co-P modules between two independent dataset Huang *et al.* and Mertin *et al.*. The size of the circles indicates the number of phosphosites in each Co-P module, the number in the circle shows its rank among all identified sub-networks. The thickness of the edges represents the significance of the overlap between the two Co-P modules based on hypergeometric test.

target phosphosites across samples in different subtypes. We then investigate the reproducibility of these inferred kinase activities across independent studies.

Protein Expression Analysis. We also investigate if protein phosphorylation data provide information on cancer substypes beyond what can be captured by protein expression. For this purpose, we utilize mass-spectrometry based protein expression data that is obtained from the samples that are used to obtain the phospho-proteomic data used in our computational experiments. We utilize protein expression data in the following way: Using the phospho-proteomic data, we identify phosphosites in Co-P modules that are significantly differentially expressed ($p < 0.05$) between different subtypes. Subsequently, using proteomic data, we assess the differential expression of the proteins that harbor these significant phosphosites between different subtypes. If the protein that harbor the site is not identified in the protein expression data, we exclude them from the analysis.

3 Results and Discussion

Datasets

Phosphoproteomics Data. We use two independent public quantitative mass spectrometry (MS) based phospho-proteomics datasets obtained from breast cancer (BC) Patient-Derived Xenografts (PDX).

- **Huang** *et al.* **data:** Huang *et al.* [23] used isobaric tags for relative and absolute quantification (iTRAQ) to identify 56874 phosphosites in 24 breast cancer PDX models. The clinically determined subtypes for the samples in this dataset are *Basal* for 10 samples, *Luminal* for 9 samples and *HER2-enriched* for 5 samples. We remove phosphosites with missing intensity values in any sample. This results in intensity data for 15780 phosphosites from 4539 proteins, where 13840 serines, 2280 threonines and 67 tyrosines are phosphorylated. Protein expression data for all of these samples is also available.

- **Mertin** *et al.* **data:** The NCI Clinical Proteomic Tumor Analysis Consortium (CPTAC) conducted an extensive MS based phospho-proteomics of TCGA breast cancer samples [7]. After selecting the subset of samples that have the highest coverage and filtering the phosphosites with missing intensity values in those tumors, the remaining data contained intensity values for 11018 phosphosites mapping to 8304 phosphoproteins in 20 tumors. This dataset contains 4 *Basal*, 9 *Luminal* and 7 *HER2-enriched* samples.

Functional, Evolutionary, and Structural Association between Phosphosites (FES). We use PTMcode, a database for functional associations of post-translational modifications within and between proteins [12]. The functional association between PTM sites have been reported based on the literature survey, co-evolution of sites, structural proximity and if PTMs at the same residue and location are within PTM highly enriched protein regions. For our analysis, we just focus on the functional associations between phosphorylation sites of different proteins.

Kinase-Substrate Associations (KSAs). We use PhosphoSitePLUS as a reference dataset for kinase-substrate associations [13]. PhosphoSitePLUS reported

9699 kinase-substrate association over 347 kinases.

Protein-Protein Interaction (PPI) Data. We use a generic human PPI network downloaded from BioGRID database at *https://thebiogrid.org* [15]. This network contains 194639 interactions among 18719 proteins.

The resulting weighted PSFA networks for two datasets are as following: Huang *et al.* network contains 9472 phosphosites, 15209 FES edges, 1115 KSA edges, 37220 TCK edges and 133536 PPI edges. Mertin *et al.* network contains 8271 phosphosites, 8283 FES edges, 595 KSA edges, 17112 TCK edges and 55903 PPI edges.

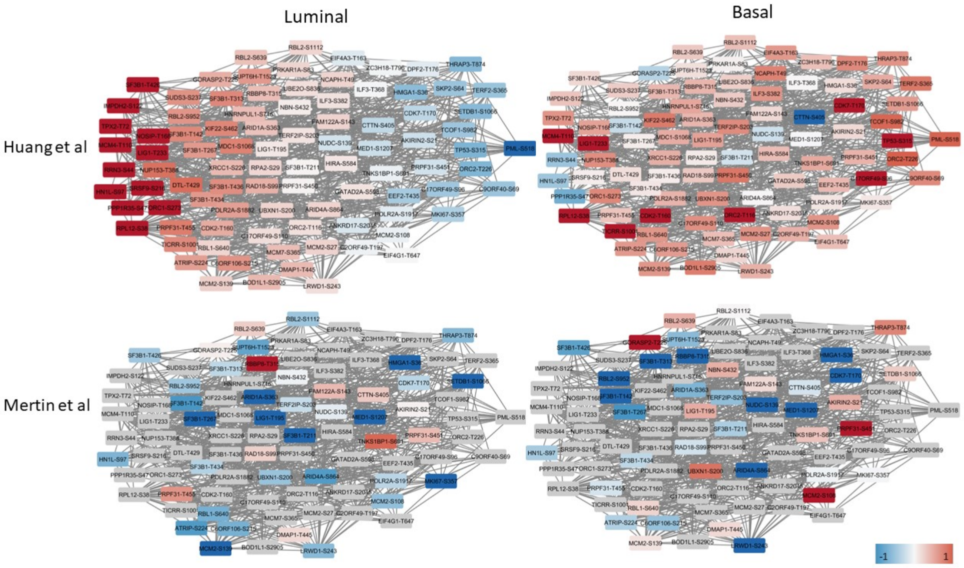


Figure 3: **Top Co-P module identified in via unsupervised analysis are associated with breast cancer subtypes.** The layout of the module is fixed where the nodes are sorted in decreasing order of average relative phosphorylation in Luminal samples with respect to the common reference. Subtype-specific phosphorylation is shown by node colors. On the left (right) panel, the color of each node indicates the direction of average relative phosphorylation of the phosphosite in Luminal (Basal) samples with respect to the common reference sample, where red indicates hyper-phosphorylation and blue indicates de-phosphorylation. Gray color indicates that the phosphosite has not been identified in the Mertin et al. The intensity of the color is identical on the left and on the right, and it indicates the significance of the differential phosphorylation of the site between Luminal and Basal samples.

CoPPNet identifies co-phosphorylation (Co-P) modules that are statistically significant and reproducible.

We identify co-phosphorylated sub-networks on each of the two datasets using CoPPNet. We investigate the statistical significance of these subnetworks and visualize the results of this analysis in Figure 2(a). As seen in the figure, the two top-scoring subnetworks identified on both datasets have scores at least two standard deviation above the mean of the top subnetworks identified on 100 randomized networks. At a q-value threshold of 0.01, two of these subnetworks are detected to be statistically significant for each dataset.

We also investigate the reproducibility of the significant modules identified on Huang *et al.* and Mertin *et al.* datasets. In Figure 2(b), the green circles represent the Co-P modules identified on Huang *et al.* dataset and the pink circles represent the Co-P modules identified on Mertin *et al.* dataset. As seen in the figure, there is considerable overlap between the top Co-P modules identified on each dataset; 26 out of the 91 sites in the top Huang *et al.* module and 65 sites in the top Mertin *et al.* module are identical. This overlap is highly statistically significant according to hypergeometric test and is particularly impressive considering that some phosphosites may not be present in a dataset because of the limited coverage of mass spectrometry based phosphoproteomics. Indeed, only 41 of 91 sites in the top Huang *et al.* module are identified in the Mertin *et al.* study, and only 54 of the 65 sites in the top Mertin *et al.* module are identified in the Huang *et al.* study. Many of these phospho-proteins such as *THRAP3 [24],* NBN [25], *RAD18 [26] and* CDK7 [27] are playing important role in different cancers.

The second top-scoring Co-P modules identified in the two datasets, which are both highly significant ($q < 0.01$), also exhibit significant overlap. Namely, 18 out of the 68 sites in the Huang *et al.* module (of which 33 are present in the Mertin *et al.* dataset) and 68 sites in the Mertin *et al.* module (of which 49 are present in the Huang *et al.* dataset) are identical. Note also that two of the sites in the top Huang *et al.* module are in the second Mertin *et al.* module, and one of the sites in the top Mertin *et al.* module is in the second-ranked Huang *et al.* module. The significant overlap and concordance between the top identified modules across two datasets show that the identified modules are highly reproducible and thus likely to be highly relevant to the dysregulation of signaling processes in breast cancer.

Co-P modules identified via unsupervised analysis are associated with breast cancer subtypes.

Since the subtype information is not used in the construction of the PSFA network and the assessment of co-phosphorylation, the identification of the Co-P modules is agnostic to the clinically determined subtypes of the samples; i.e. CoPPNet is an *unsupervised* method for the identification of breast-cancer associated signaling modules. However, since the Co-P modules capture co-variation across different samples and this variation can be associated with subtypes, these modules can be informative on subtypes. Motivated by this consideration, we investigate if the phosphorylation levels of phosphosites in the identified modules can differentiate breast cancer subtypes. The results of this analysis for the Huang *et al.* dataset are shown in Figure 3 and S1. Subtype-specific differential phosphorylation of Co-P modules identified on the Mertin *et al.* dataset are presented in Figure S2.

As seen in Figure 3, top significant Co-P module identified on the Huang *et al.* dataset are highly enriched in phosphosites with significant differential phosphorylation between Luminal and Basal subtypes. It is visually striking that the blue (denoting de-phosphorylation) and red (denoting hyper-phosphorylation) colors are clustered in opposite horizontal directions in the visualizations that correspond to Luminal and Basal subtypes. Indeed, there are 14 phosphosites in the top Huang *et al.* module with significant differential phosphorylation between Luminal and Basal subtypes ($p < 0.05$). Eight (*DPF2-T176, THRAP3-T874, TERF2-S365, EIF4A3-T163, SETDB1-S1066, TCOF1-S982, PRPF31-S451, PML-S518*) out of 14 of these sites are hyper-phosphorylated in Basal samples and de-phosphorylated in Luminal samples. For some of the proteins harboring these sites, the differentiation between breast cancer subtypes also has been captured at the level of mRNA expression. For example, *PML* (promyelocytic leukemia) and *SETDB1* (SET Domain Bifurcated 1) are significantly up-regulated in Basal cancers as compared to Luminal cancers, and their expression is related to the survival rate of the patients [28, 29]. Note that 50 out of 91 phosphosites in the first module are not identified in Mertin *et al.* (i.e. gray nodes), hence development of predictive model using incomplete data is challenging and needs further exploration.

Co-P modules provide a focal point for kinase activity inference.

To further understand the contribution of PSFA network and co-phosphorylation analysis, we assess the value added by the Co-P modules to the inference of the differential activity of kinases between Basal and Luminal subtypes. For this purpose, we use the Kinase-Substrate Enrichment Analysis (KSEA) tool, which infers the differential activity of a kinase based on the differential phosphorylation of its substrates [30]. The results of this analysis is shown in supplementary materials (Figure S3 - S4). This analysis infers several kinases with significantly altered activity between the two subtypes. Three of these kinases (*PAK1, UHMK1,CDC7*) are identified as significant on both datasets and they show the same activity pattern in the two datasets. This result suggests that focusing on Co-P modules has the potential to bring forward the kinases and phospho-proteins that are of interest, that might be missed if we consider all phosphosites in kinase activity inference.

Effective and direct utilization of phosphorylation data enhances the identification of subtype-associated proteins over protein expression.

In this section, taking advantage of the availability of mass spectrometry based protein expression data from the samples we use in our experiments, we investigate whether the subtype-specific phosphorylation signatures we identify can be explained by changes in protein expression. For this purpose, we first identify the phosphosites in the top two Co-P modules of the Huang *et al.* dataset with significant differential phosphorylation ($p < 0.05$) between Luminal and Basal subtypes. For each of these sites, we assess the differential expression of the protein harboring the site (if the protein is identified in the protein expression data). The results of this analysis are shown in Figure 4. As seen in the figure, while the phosphorylation of these phosphosites is significantly different between two subtypes, most of the the proteins that harbor these sites do not exhibit significant differential protein expression between the two subtypes.

4 CONCLUSION

In this study, we present CoPPNet a computational method that utilizes large scale phospho-proteomic data for unsupervised identification of phenotype-associated signaling modules in cancer. One important contribution of the proposed method is the construction of the phosphosite functional association (PSFA) network which is a site-centric network that comprehensively incorporates available functional information on phosphorylation sites to enable network-based analysis of phosphorylation data. Our systematic results on two breast cancer datasets show that CoPPNet identifies reproducible subtype-specific signaling modules without requiring knowledge of the sample subtypes. These results suggest that CoPPNet can be used to identify new subtypes for different cancers and also the identification and prioritization of subtype-specific signaling pathways. Overall, this study represents one of the first attempts on utilizing phospho-proteomics to generate reproducible functional readouts of cellular signaling that can be used to characterize the dysregulation of cellular signaling in cancers and development of future therapeutic strategies.

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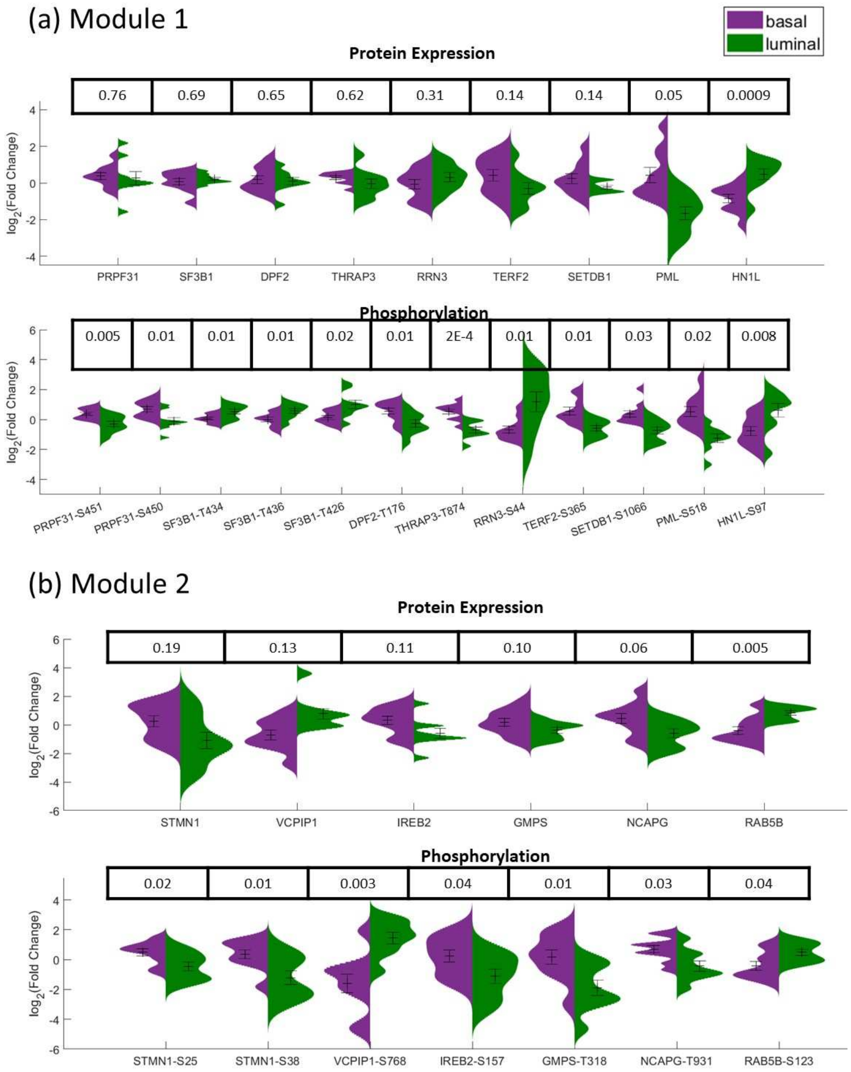

Figure 4: **Effective and direct utilization of phosphorylation data enhances the identification of subtype-associated proteins over protein expression.** For the significant phosphosites of module 1 (a) and module 2 (b) identified on the BC I dataset, the violin plots show the distribution of protein expression and phosphorylation for Luminal (green) versus Basal (purple) samples. The p-value of t-test between these distributions is shown above the plot for each protein and phosphosite.

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