

## Roles of “junk phosphorylation” in modulating biomolecular association of phosphorylated proteins?

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**P**rotein phosphorylation dynamically regulates cellular activities in response to environmental cues. Sequence conservation analysis of recent proteome-wide phosphorylation data revealed that many previously unidentified phosphorylation sites are not well-conserved leading to the proposal that many are non-functional. However, this is based on the assumption that protein phosphorylation modulates protein function through specific position on protein sequence. Based on emerging understanding on phosphoregulation of cellular activities, we argue, with examples, that non-positionally conserved phosphorylation sites can very well be functional. We previously identified phosphorylation events that need not be conserved at same positions across orthologous proteins but are likely maintained by evolutionary conserved signaling networks through orthologous kinases. We found that proteins with such conserved phosphorylation patterns are statistically over-represented with protein and DNA-binding annotation. Here, we further correlated these proteins with protein-protein interaction data from an independent systematic study and observed they indeed interact frequently with other proteins. Hence, we speculate that non-positionally conserved phosphorylation site could be modulating biomolecular association of phosphorylated proteins possibly through fine-tuning protein's bulk electrostatic charge and through creating binding sites for phospho-binding interaction

domains. We, therefore, advocate the development of complementary evolutionary approaches to interpret physiological important sites.

### Introduction

Protein phosphorylation is a prevalent reversible post-translational modification that influences proteins' structural conformation, enzymatic activities, molecular association and sub-cellular localization to modulate cellular activities in response to signaling cues. In eukaryotic cells, protein kinases transfer a phosphate group from ATP to the side-chain hydroxyl group of a specific set of serine, threonine and tyrosine in the proteome. Identifying these residues and the protein kinases that targeted them are crucial toward understanding the dynamic regulation of cellular activities by protein phosphorylation. Phospho-proteomic technologies now allow proteome-wide quantitative detection of proteins and residues phosphorylated under different physiological conditions,<sup>1-4</sup> and have been applied to unveil the phospho-proteomes of several model species.<sup>5,6</sup> The functional consequences of the majority of these phosphorylation events are largely unknown, and this calls for post-discovery endeavors to characterize their molecular functions and effects on cellular decision processes. Systematic approaches to categorize phosphorylation events, pinpoint their potential functions and generate testable hypothesis

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will be invaluable for prioritizing sites for experimental characterization.

Sequence conservation analysis is an inexpensive and readily deployable computational approach to interpret important phosphorylation events. Phosphorylation sites that exhibit strong sequence conservation, especially when across distantly related species, are presumably under evolutionary constraint, and hence likely to be functionally important. Conversely, the lack of conservation at similar positions across orthologous proteins for many previously unidentified phosphorylated residues (referred as non-conserved site hereafter) seemingly suggest that many recently discovered sites are of little or no functional importance,<sup>7</sup> and was used to estimate the portion of non-functional sites in existing datasets.<sup>8</sup> However, conservation at sequence position level is only one way to interpret the conservation and importance of phosphorylation events. Here, we argue, based on the emerging understanding of phospho-regulation of cellular activities, that non-conserved phosphorylation sites may well be functional, and advocate for complementary approaches to identify evolutionary conserved phosphorylation events.

### Functional Non-Conserved Phosphorylation Sites

Undoubtedly, a portion of the non-conserved phosphorylation sites are spuriously localized, in particular, in MS-identified phospho-peptides that have multiple phosphorylatable residues despite that this is somewhat proclaimed to be statistically controlled in MS experiments. In addition, the phospho-peptide enrichment techniques conventionally applied in phospho-proteomic studies might have identified low abundant sites that may or may not have cellular consequences. Otherwise, non-conserved phosphorylation sites could be mediating lineage- or species-specific cellular functions such as sites involved in cell-cell communication that are not expected to be conserved in unicellular organisms. Example of species-specific sites are the previously unidentified CDK phosphorylation sites on Mcm3 protein that were gained in *S. cerevisiae* lineage after divergence from *C. albicans*

but are involved in mediating nucleus export of MCM complex that is unique to *S. cerevisiae*.<sup>9,10</sup>

Identifying sites that only appear in a particular lineage and are selectively retained among its species can give insight to the unique cellular activities or development pertaining to that particular lineage. Monitoring the dynamics of phosphorylation sites (increased- or decreased-phosphorylation) under different physiological conditions or stimulus is an alternative to evolutionary approaches for interpreting the importance of lineage- or species-specific phosphorylation sites. Importantly, lineage- or species-specific sites can easily be falsely identified if they lie in disordered regions that, in general, are fast evolving,<sup>11-13</sup> and hence, easily missed by multiple-sequence-alignment (MSA) algorithms that had been optimized for conserved globular domains.<sup>14</sup> An arch example of functionally important but seemingly lineage-specific phosphorylation site is human p53's Ser46 which has been implicated in regulating apoptosis, cell growth and transcription by numerous studies, but was not found in mouse p53 based on sequence alignment.<sup>15</sup> However, non-alignment-based computational analysis, biochemical and functional assays suggest that Ser58 in mouse p53 is functional equivalent to Ser46 in human p53.<sup>15</sup> Hence, the development of specialized alignment algorithms, alternative computational approaches<sup>13,15</sup> and benchmarking datasets<sup>14</sup> will be crucial for minimizing identification of spurious non-conserved phosphorylation sites.

Other than affecting individual proteins, protein phosphorylation can target higher order molecular machineries at the level of protein complexes to regulate cellular activities.<sup>16,17</sup> In the case where protein phosphorylation serves to disable the activities of a protein complex by targeting its subunits for ubiquitination and subsequently degradation, phosphorylation of any subunit is presumably sufficient for the purpose, and hence need not be evolutionary conserved on orthologous protein.<sup>17</sup> Analysis of gene expression data across human (*Homo sapiens*), budding yeast (*Saccharomyces cerevisiae*), fission yeast (*Schizosaccharomyces pombe*) and *Arabidopsis thaliana* revealed that the

periodically expressed and constitutively expressed subunits in evolutionary conserved cell cycle protein complexes differ among the four species. Interestingly, combined experimental and computational data analysis divulged that the periodically expressed subunits are preferentially phosphorylated compared to constitutively expressed subunits in each species<sup>16</sup> although the periodically expressed subunits differ in each species. This observation indicates dynamic interplay between differentiated gene expression and protein phosphorylation for regulating cellular activities, and at a higher level suggests changes in another regulatory mechanism can relax the evolutionary constraint on a functional site. In support, a recent comparative analysis of phospho-proteomes across three yeast species (*Saccharomyces cerevisiae*, *Candida albicans* and *Schizosaccharomyces pombe*) revealed the intensity of phosphorylation is highly conserved among different cellular activities although the intensity vary considerably among individual proteins within each functional group across the three species which indicate prevalent global switching of proteins targeted by kinases during evolution.<sup>18</sup>

### Modulating Biomolecular Association by Protein Phosphorylation

An emerging view is protein phosphorylation can fine-tune the bulk electrostatic charge of targeted proteins to negatively regulate their association with other negatively charged biomolecules such as phospholipid membrane<sup>19</sup> and polynucleotide<sup>20</sup> by electrostatic interference/repulsion.<sup>21,22</sup> This mechanism does not require phosphorylation to occur at precise location but a region at large on substrates to create negatively charged protein surfaces to modulate molecular association (reviewed in ref. 22). Protein phosphorylation is also known to instigate protein-protein interaction by bulk electrostatics as observed for *S. cerevisiae*'s Sic1 which need to be phosphorylated on any six out of the nine poorly conserved CDK1 phosphorylation sites for binding to a single binding site on Cdc24 component of SCF ubiquitin ligase.<sup>23-25</sup>

**Table 1.** Relationship between protein phosphorylation and protein-protein interaction

| Protein Group                | Size | Average number of interacting partners per protein | Portion of proteins with $\geq 1$ interacting partners | Portion of proteins with $\geq 2$ interacting partners | Portion of proteins with $\geq 4$ interacting partners |
|------------------------------|------|--|--|--|--|
| <b>Global Set</b>            | 6982 | 0.77   | 21.6%  | 11.1%  | 5.0%   |
| <b>Phospho-protein</b>       | 2452 | 0.99   | 25.4%  | 14.1%  | 6.7%   |
| <b>Phospho-protein (HTP)</b> | 2227 | 0.95   | 25.3%  | 14.0%  | 6.8%   |
| <b>Core Site Protein</b>     | 145  | 0.92   | 29.0%  | 17.2%  | 6.9%   |
| <b>Core Net Protein</b>      | 340  | 1.17   | 29.4%  | 17.9%  | 8.5%   |

Protein phosphorylation data used is as reported in ref. 13, that was assembled from PhosphoSitePlus and PhosphoELM databases. As human protein-protein interaction dataset assembled from conventional gene-centric studies are likely biased toward popular proteins, we correlate protein phosphorylation data with human protein-protein interactions detected in a systematic yeast two-hybrid study involving ~7,200 human proteins.<sup>30</sup> We are able to map about 97% these human proteins to protein identifiers used in the phosphorylation dataset. Only the subset of phosphorylated proteins that had been assessed for protein-protein interaction in ref. 34 is included in analysis. *Phospho-proteins (HTP)* are those containing phosphorylation site detected in various high throughput studies. *Core site proteins* are those containing phosphorylation sites which corresponding positions across orthologous proteins in yeast, worm or fly (as determined by multiple sequence alignment) are also phosphorylated. *Core net proteins* are phosphorylated protein with orthologous phosphorylated proteins in yeast, worm or fly inferred to be targeted by orthologous kinases.

It is well established that a large portion of protein phosphorylation events dynamically promote protein-protein interaction by creating temporal binding sites for phospho-residue binding protein domains such as SH2, PTB, 14-3-3, WD40 and FHA for which phosphorylation sites need not be precisely located on the substrates. Many linear motifs bound by modular interaction domains, which include phospho-residue binding domains, need not be conserved at specific positions but a region at large across orthologous proteins<sup>26,27</sup> like those involved in mediating protein subcellular localization. In particular, co-operative binding has been observed where multiple linear motifs on a protein have additive effect on binding.<sup>27,28</sup> Similar effects had been observed for interactions modulated by protein phosphorylation through bulk electrostatic charge in disordered regions where each additional phosphorylation site progressively decrease or increase molecular associations.<sup>24</sup>

The presence of functionally redundant sites implies some can be lost during evolution in some lineages with minimum functional consequences, and can contribute to phenotypic diversity observed in a population.<sup>18</sup> This can provide the evolutionary plasticity needed to fine-tune conserved cellular activities for unique developmental and physiological needs of individual species. Given that the majority of known phosphorylation sites are located in disordered regions, it is likely that a portion of these sites is regulating biomolecular associations of proteins<sup>29,30</sup>

through electrostatic interference. In addition, non-conserved phosphorylation sites can serve as decoys to buffer against spurious phosphorylation of other sites. For example, Wee1 in *Xenopus* contains a set of poorly conserved CDK1 (Cyclin-dependent kinase 1) sites that soak up stochastic activation of CDK1.<sup>31</sup> These CDK sites vary in number and location in Wee1 across different species but nevertheless are important for the timely inactivation of Wee1 during mitosis.<sup>31</sup> Interestingly, many proteins are multi-phosphorylated by CDK on residues located close to each other on primary sequences, and had been exploited to improve prediction of bona fide CDK substrates.<sup>32,33</sup>

### Identifying Non-Positionally Conserved Phosphorylation Sites

To identify sites that are not positionally conserved but functional, we recently queried human phosphorylation sites detected by traditional biochemical studies and high throughputs MS experiments with phospho-proteomes of three model species (fly, worm and yeast) generated on a common experimental platform.<sup>13</sup> We adopted a computational approach to detect phosphorylation events that need not be conserved at same positions across orthologous proteins but are potentially instigated by evolutionary conserved signal processing networks through orthologous kinases. We termed proteins with such conserved phosphorylation pattern as core net proteins. The approach is partially motivated

by the observation that phosphorylation sites in Wee1 and Sic1 are not positionally observed across orthologs but, nevertheless, are targeted by orthologous kinases. Interestingly, core net proteins are statistically ( $p < 0.01$ , corrected for multiple hypothesis testing) associated with cellular activities of chromosome organization and biogenesis, DNA-dependent regulation of transcription, macromolecular complex assembly and protein targeting as based on gene ontology annotation. These proteins are also statistically enriched with protein- and DNA-binding annotation ( $p < 0.00001$  and  $p < 0.01$ , corrected for multiple hypothesis testing) as compared to the superset of human phospho-proteins with phosphorylated orthologs in query species, supporting the notion that some non-positionally conserved phosphorylation sites are implicated in the biomolecular association of the phosphorylated proteins. Integrative analysis with an independent systematic yeast two-hybrid study of interactions among ~7,200 human proteins<sup>34</sup> upon further investigation further validated that core net proteins interact frequently with other protein and likely to be protein interaction hub (see Table 1).

We found that core net proteins are statistically associated genes with mutation casually linked to cancer and other regulatory diseases. Highly important for future studies of cancer cell lines or in vivo systems is that we found that core-net proteins with increasing number of non-positionally conserved phosphorylation

sites in the conserved core regulatory networks are increasingly enriched in disease genes. This strongly suggests that we can deploy similar protocols for proteomic data generated in cancer cell lines to identify important sub-networks that drive the disease progression. For reasons to be worked out, these proteins are tightly regulated by protein phosphorylation throughout evolution, possibly to minimize promiscuous molecular interactions associated with proteins enriched in disordered regions<sup>35,36</sup> as it has been observed that cell evolves to minimize unspecific or deleterious interactions.<sup>37,38</sup> The clustering of most phosphorylation sites and the binding sites of modular interaction domains together in disordered regions<sup>27,39</sup> could either be independent events due to the structural nature of disordered regions that promote both types of interactions, or could be a result of evolutionary pressure to co-locate them near each other for co-regulation<sup>28,40,41</sup> or a combination of both. In addition, we found evidence that suppression of phosphorylation might be an important evolutionary aspect of phospho-regulation.

Through a sequence alignment approach, we also identified a set of human phosphorylation events that are strictly conserved at the same position (defined through multiple sequence alignment) across orthologous proteins in fly, worm or yeast. Protein phosphorylation partially regulates proteins' functions through allosteric regulation and conformational changes that largely require precise phosphorylation location to coordinate interactions and repositioning of amino acids. Therefore, we postulated that many of these sites are likely regulating proteins' function through allosteric regulation and conformational changes given they have been positionally conserved over long evolutionary distance. This is supported by the observation that a portion of the sites is found in activation loops implicated in allosteric regulation of kinases.<sup>13</sup> Although not observed to statistically enriched in protein-binding function based on gene ontology annotation, integrative analysis with the independent systematic yeast two-hybrid interaction study of ~7,200 human proteins<sup>34</sup> revealed that these pro-

teins are associated with protein-protein interaction functions (see Table 1).

### Concluding Remark

Prioritizing functionally important phosphorylation sites for experimental studies is paramount for unraveling the dynamic regulation of cellular activities by protein phosphorylation such as for understanding emergence of diseases arising from deregulation. Evolutionary and conservational analysis can help in prioritizing phosphorylation sites for experimental characterization but the dynamic mechanisms of cellular regulation by protein phosphorylation necessitate the development of non-alignment-based computational approaches to detect evolutionary conserved phosphorylation events. The possibility that multiple phosphorylation sites on a protein can have similar functions such as modulating biomolecular association implies sensitive quantitative assays are needed or multiple phosphorylation sites have to be mutated together to assay their functional roles.<sup>7,42</sup> Grouping phosphorylation sites according to their effector kinases and/or their dynamic profile under different stimulus can serve to organize sites for experimental studies if the possible combination of sites that can be mutated is big.

Experimental studies of phosphorylation sites can be complicated if the functional roles of phosphorylation sites depend on the physiological condition of the cell<sup>43</sup> as a site that has no observable phenotypic effect under one physiological condition assessed may have one in another condition. As phosphorylation states of signaling proteins are known to dictate different cellular response to similar stimulus,<sup>43</sup> the dynamics and contextual nature of phosphorylation sites in regulatory networks are important factors to consider in experimental studies. Like proving negative in many biological studies, validating that a phosphorylation site has no functional role is often inconclusive, as one could need to test a site under all possible physiological conditions. Hastily dismissing non-conserved sites to be non-functional will restrict advancement in understanding the phospho-regulation of cellular activities at both molecular and system levels.

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