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Global analysis of phosphorylation networks in humans

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Abstract

Phosphorylation-mediated signaling plays a crucial role in nearly every aspect of cellular physiology. A recent study based on protein microarray experiments identified a large number of kinase-substrate relationships (KSRs), and built a comprehensive and reliable phosphorylation network in humans. Analysis of this network, in conjunction with additional resources, revealed several key features. First, comparison of the human and yeast phosphorylation networks uncovered an evolutionarily conserved signaling backbone dominated by kinase-to-kinase relationships. Second, although most of the KSRs themselves are not conserved, the functions enriched in the substrates for a given kinase are often conserved. Third, the prevalence of kinase-transcription factor regulatory modules suggests that phosphorylation and transcriptional regulatory networks are inherently wired together to form integrated regulatory circuits. Overall, the phosphorylation networks described in this work promise to offer new insights into the properties of kinase signaling pathways, at both the global and the protein levels.

Keywords

phosphorylation network; kinase-substrate relationships (KSRs); network module; conservation

1. INTRODUCTION

Cells respond to a variety of signals, many of which are relayed from the cell membrane to specific intracellular targets. Protein phosphorylation, mediated by kinases, is one of the most important regulatory mechanisms during signal transduction. A kinase can be activated

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by incoming signals, and it subsequently alters the cellular function of other proteins through phosphorylation, which ultimately turn on genes that are responsive to the signals from their surroundings. Disruption in the signaling pathways has profound implications in a variety of human diseases [1–3]. For instance, mutations in kinase genes are frequently associated with human cancer [4, 5]. Therefore, identifying and characterizing kinase-mediated signal transduction pathways are critical for many aspects of cellular biology and also have potential clinical applications.

The model that depicts signaling pathways as a linear wiring diagram have been extremely useful to explain the properties of some biological systems; however, a large amount of genetic and biochemical data suggests that the network signaling model is a more complex one. For instance, a genome-wide RNAi screen of ERK/MAPK activity revealed that the contribution of each gene to the signaling output is continuous. In other words, various proteins participate in signal transduction with different levels of contribution [6]. Likewise, signaling networks underlying animal development are known to be resistant to both extrinsic and intrinsic perturbations; this robustness can be at least partially explained by a network model but not by a linear signaling model [7]. Finally, cancer genome sequencing projects have identified an average of 93 mutated genes per breast or colorectal tumor [8]. Because mutations in a few genes should sufficiently disrupt a signaling pathway, the finding of a large number of mutations per tumor suggests that signals are propagated through a signaling network, which needs many mutations to collapse the system. Together, this evidence strongly supports a network view of signal transduction rather than the canonical linear view. Constructing a phosphorylation-based signaling network will greatly help researchers perform a series of global analyses to gain new insights into the organization, specificity, and function of signaling networks.

The global analysis of phosphorylation networks in humans is limited due to our limited knowledge about KSRs in humans. Indeed, only ~2,000 human KSRs have been experimentally identified. In contrast, a large number of phosphorylation sites (>70,000) have been determined by MS/MS technology. Therefore, most of analysis of the phosphorylation events often focused on these sites, rather on the KSRs themselves. For example, evolutionarily conserved phosphorylation sites and their potential physiological functions were investigated by analyzing the phosphorylation sites in the human genome [9].

Recently, Newman et al published a study in which a large number of KSRs were determined by protein microarray technology and further refined by bioinformatics analysis [10]. This study resulted in 3,656 refined KSRs (refKSRs) that are likely to occur under physiological conditions, more than all KSRs generated from previous studies combined. In the present work, we used this comprehensive dataset to perform a series of global analyses of human phosphorylation networks.

2. METHODS

2.1 Datasets

In our previous study, we identified 24,046 kinase-substrate relationships (KSRs) using a protein microarray approach [10]. The complete set of *in vitro* KSRs were termed rawKSRs. The rawKSR dataset represents the biochemical relationships between kinases and their substrates. To enrich for physiologically relevant KSRs, we performed a Bayesian analysis by integrating other genomic and proteomic information such as sub-cellular localization, protein-protein interactions and tissue specificity [10, 11]. The resulting 3,656 KSRs were termed refined KSRs (refKSRs). We also integrated 744 known KSRs curated from the literature into the refKSR dataset to generate a combined KSR dataset (comKSR). The

datasets are available in supplementary information as well as on a searchable web site (phosphonetworks.org).

2.2 Overlap of substrate sets of kinases

Supposing there are N candidate substrates in total, kinase A and B recognize M_A and M_B substrates, respectively, M_{AB} of substrates are recognized by both kinases, the overlap rate (OR) of the two substrate sets can be computed by the Jaccard Index,

$$OR=\frac{A\cap B}{A\cup B}=\frac{M_{AB}}{M_A+M_B-M_{AB}} \quad (1)$$

The significance of the overlap can be computed by cumulative Hypergeometric distribution (supposing $M_B \geq M_A$),

$$p(M_{AB},M_A,M_B,N)=\sum_{i=M_{AB}}^{M_A}\frac{C_{M_B}^{i}\times C_{N-M_B}^{M_A-i}}{C_N^{M_A}} \quad (2)$$

Here, N is equal to 4,191, the total number of proteins on our protein microarray.

2.3 Evolutionally conserved network modules

To investigate the properties of the phosphorylation network more thoroughly, we constructed network modules and analyzed their properties. Once all size-3 network modules including at least one kinase were enumerated, the statistical significance of each module was evaluated using the same method as described in [12]. In a similar manner, network module conservation analysis was carried out by comparing the modules identified in the human and yeast comKSR networks that involved homologs between these two organisms. Here, two proteins were considered to be homologous if the e-value of their primary amino acid sequence alignment (BLASTP) was lower than 10^{-20}.

To obtain a dataset of yeast refined KSRs, we performed the same Bayesian analysis to the yeast KSRs obtained from the literature [13]. The gene expression is based on an integrated microarray expression dataset covering 255 different conditions [14], the cellular localization is based on GO annotation, and the protein-protein interactions are from databases and a recently published yeast kinase network [15]. We obtained 374 known yeast KSRs from the literature and various databases as positive controls and 20,000 protein pairs devoid of kinases as negative controls. After applying the Bayesian approach, we obtained 797 refKSR for yeast which, when combined with the 374 known KSRs, formed a comKSR dataset composed of 1167 yeast KSRs of high quality.

2.4 Comparison with conserved protein-protein interactions

To examine the relative conservation level of KSRs between human and yeast, we compared the conservation levels between KSR and protein-protein interactions (PPIs). We used the same standard to analyze conservation of the 55,048 human PPI that we collected before [11] relative to the 36,505 yeast PPI downloaded from the Intact database (http://www.ebi.ac.uk/intact). This analysis revealed that 1,068 of 55,048 human PPIs (1.94%) have orthologs in yeast, approximately three times lower than the KSR conservation rate (265/4375=6.06%).

2.5 Functional enrichment of a substrate set

If N is the number of proteins on our protein microarray, then M of them are annotated to have certain function by Gene Ontology. Likewise, if a kinase recognized N_k substrates and M_k of them are annotated to have that function by Gene Ontology, then the fold enrichment (FE) of the function in the substrate set can be computed by following formula,

$$FE=\frac{(M_k/N_k)}{(M/N)}=\frac{M_k \times N}{M \times N_k} \quad (3)$$

The significance of the enrichment can be computed by cumulative Hypergeometric distribution,

$$p(M_k, N_k, M, N)=\sum_{i=M_k}^{N_k} \frac{C_{N_k}^{i} \times C_{N-M}^{N_k-i}}{C_{N}^{N_k}} \quad (4)$$

2.6 Identification of network motifs with kinases and transcription factors

To examine the interplay between kinase-mediated phosphorylation and transcriptional regulation, ChIP-chip and ChIP-seq datasets were collected for a total of 94 transcription factors. CisGenome was then applied to each dataset in order to identify peaks in the genome [16]. The phosphorylation networks and the transcriptional regulatory networks were then superimposed and all size-2 and size-3 network modules which included at least one transcription factor and one kinase were evaluated. Finally, the statistical significance of 70 network modules was evaluated by comparing the occurrence of each module in the real and the randomized networks. The randomized network is a degree-preserving network in which the incoming degree (number of regulators on the node) and the outgoing degree (number of targets of the node) for each node are preserved relative to the real network [12].

3. RESULTS

3.1 General properties of kinase-substrate relationships

We identified KSRs in humans using protein microarray approach in our previous study [17]. The resulting dataset (comKSR) covers 4,375 KSRs. Using this dataset, we performed a series of global analyses to gain new insights into the organization, specificity, and function of human KSRs.

Based on this KSR dataset, we found that each kinase phosphorylated a distinct set of substrates, with the set sizes ranging from 1 to 134. On average, each kinase phosphorylated 14 substrates (Fig. 1A). Similarly, most substrates (87%) were recognized by fewer than 10 kinases, with each substrate being targeted by an average of 4.9 kinases (Fig. 1B). In general, there was little to no overlap between the specific substrates recognized by any given pair of kinases (Fig. 1C). Nonetheless, in some cases, we did observe substantial substrate overlap between a pair of kinases. For example, Erk2 and CHK2 checkpoint homolog (CHEK2), two kinases involved in the DNA damage response [18, 19], shared a subset of their substrates. Indeed, of the 30 and 15 substrates phosphorylated by Erk2 and CHEK2, respectively, six are shared between them ($P<10^{-9}$). Overall, we found that, while the majority of kinases occupy distinct territories in the phosphorylation landscape, about 20% of kinase pairs among all possible kinase pairs (255×254/2=32,385) exert combinatorial regulation on certain substrates. These overlapped substrates tend to have

various enriched functions, including “cellular response to stimulus”, “regulation of protein transport”, and “DNA metabolic process”.

This dataset also allowed us to determine whether a particular kinase group tends to recognize more substrates than other groups. While these analyses uncovered no obvious associations between the average number of substrates per kinase and the groups to which these kinases belong (p=0.24, ANOVA; Fig. 1D), they did reveal that the distribution of substrate number within a given kinase group can be quite broad. For example, whereas the CAMK group member, STK17A/Drak1, recognized 134 substrates, its fellow group member, STK33, phosphorylated only 3 substrates. These findings imply that, although the sequence similarity of the kinase domain within a particular kinase group is high, the mechanism of achieving substrate specificity may be unique for most kinases.

In contrast, when the KSRs were examined from the substrate perspective, we observed considerable variability in the percentage of phosphoproteins identified across different protein classes (Fig. 1E). For instance, while 34% (32/95) of the chromatin proteins tested were found in the KSRs, only 2% (2/96) of the ribosomal proteins in the dataset were phosphorylated. Interestingly, 22% (75/343) of kinases themselves were phosphorylated by other kinases. Note that the percentages of phosphoproteins in different protein classes correlate well with those identified by the MS/MS approach (PCC=0.6; Fig. 1F). These findings suggest that some protein families are more prone to kinase-mediated regulation than others.

3.2 Conserved kinase-to-kinase backbone

The construction of an extensive human phosphorylation network allowed us to explore the extent to which kinase-dependent signaling networks have been maintained throughout evolution. For instance, by comparing the human KSR dataset with an activity-based yeast KSR dataset [13] refined using our Bayesian approach [15], we discovered that, in general, KSRs are not well-conserved between yeast and humans. Indeed, of the 4,375 human comKSRs, only 265 (6.1%) are conserved between these two species (despite this relatively low degree conservation, the conservation rate among KSRs is still 3-fold higher than that of the protein-protein interactions between human and yeast (1.9%)).

Strikingly, however, among the conserved KSRs, kinase-to-kinase relationships are much more highly represented than kinase-to-non-kinase relationships. In fact, 75% of the conserved KSRs correspond to kinase-to-kinase relationships (Fig. 2A). Moreover, the conservation rate observed for kinase-to-kinase relationships (34%, 198/581) is 19 times higher than that observed for kinase-to-non-kinase relationships (1.8%, 67/3794) (P=6.18×10^{-128}). Similar results were also obtained when we compared our human phosphorylation network with another yeast network constructed using an orthogonal MS/MS-based approach [20]. According to this comparison, the fraction of conserved kinase-to-kinase relationships (38.6%; 224/581) is 15.6 times higher than that of kinase-to-non-kinase relationships (2.48%; 94/3794) (P=1.93×10^{-137}). Together, these findings strongly suggest that a kinase backbone (i.e., kinase-to-kinase connections) within the phosphorylation networks has been highly conserved during evolution, whereas other KSRs are under less evolutionary constraint (Fig. 2A).

These observations can be attributed to conservation at the levels of both the protein sequence and the phosphorylation relationship (Fig. 2B and C). For instance, we examined the protein sequence conservation and found that the kinases are more conserved than other proteins. 83% of kinase proteins have orthologs in yeast, whereas only 41% of non-kinases proteins have orthologs in yeast. We also investigated whether the kinase-to-kinase relationships are also more conserved than kinase-to-non-kinase relationships beyond the

protein sequence level. To exclude the contribution from protein sequence conservation, we examined the conservation level only for KSRs in which the two proteins (kinase and substrate) have homologs in yeast. For this comparison, we still found that kinase-to-kinase relationships are more conserved than kinase-to-non-kinase relationships (Fig. 2C), suggesting that the observed conservation level difference results from both protein sequences and phosphorylation relationships. The observation is not sensitive to the cutoff or methods used to define the ortholog, such as Ensembl (http://ensembl.org) and Inparanoid (http://inparanoid.sbc.su.se). For example, different BLAST E-values ranging from 1e–5 to 1e–40 were used to define homologs and the same trend was observed (Fig. 2B, C).

3.3 Functional conservation of kinase substrates

Among the kinases in the group composed of kinase-to-non-kinase relationships, some were found to be functionally conserved between humans and yeast, despite the fact that their identified substrates show little conservation. For example, 14 of the 71 substrates phosphorylated by PKA are known to be involved in cell differentiation ($P<3\times10^{-5}$); meanwhile, 12 of the 112 substrates phosphorylated by its yeast homolog, Tpk1, also belong to the same functional category ($P<2\times10^{-4}$) (Fig. 3). This observation suggests that the two homologous kinases are functionally conserved. However, there is little conservation between the two substrate groups. Indeed, PKA and Tpk1 each recognized distinct substrates involved in cell differentiation. In fact, many of the substrates phosphorylated by PKA have no homolog in yeast and vice versa. For instance, while three yeast-specific substrates of Tpk1 are not conserved in higher eukaryotes, most (9 of 12) human-specific substrates are only found in vertebrates. Consistently, many of the proteins phosphorylated by human PKA are involved in biological processes specific to multi-cellular organisms, such as regulation of tissue-specific gene expression (ID2), myogenesis (CSRP3), and neuronal development (GAS7 and NEUROD1). It is plausible that these proteins have evolved independently in higher eukaryotes to accommodate more complex processes of cell differentiation. Overall, 19 of the 27 orthologous kinase pairs defined by the Ensembl database were found to have conserved enriched function in their substrate sets, despite the fact that their identified substrates show little conservation (Table 1).

In summary, our analysis suggests that conserved phosphorylation regulation can be found both at the molecular level in the form of direct kinase-to-kinase relationships, as well as at higher functional levels.

3.4 Conserved network modules

We next examined which types of interaction patterns (i.e., modules) are more conserved in the phosphorylation networks (see method for details). Among the modules that are widely used in the human KSR network, several are highly conserved (these conserved modules are termed “modulogs”) [21, 22]. The top three conserved modulogs are kinase cascades, single input (i.e., one kinase phosphorylates multiple proteins), and co-regulation (i.e., two kinases co-regulate one protein). Indeed, these three modulogs are conserved in 22.2%, 18.8%, and 18.5% of the cases, respectively (Fig. 4). The prevalence of conserved single input and co-regulation modulogs suggests that parallel or compensatory interactions are a conserved and widely used design principle in eukaryotic phosphorylation networks [23]. In addition, cascade and co-regulation motifs were found to be enriched in exogenous processes while single input and feedforward loops were enriched in endogenous processes [24]. Interestingly, feedforward and feedback modules, which play important roles in transcriptional regulatory networks, are utilized to a lesser degree in the phosphorylation network and are also less well-conserved [25, 26]. Together, our network module analysis not only offers important insights into the organization of intracellular signaling networks, but it also provides clues about the context and relative frequency with which different

signaling architectures are utilized within these networks. Using this approach, we identified several significantly conserved modules between human and yeast which, based on their high degree of conservation, are likely to play an important role in the organization of signaling networks in eukaryotes.

3.5 Wired phosphorylation and transcription regulatory networks

Next, we examined the interplay between protein phosphorylation and other biological processes, in particular transcriptional regulation. We first assembled a transcriptional regulatory network based upon genome-wide transcription factor binding data (see method for details) and then integrated these findings into our phosphorylation network. Searching this integrated network for common regulatory modules, we found several statistically significant recurring modules that include both kinase-substrate and transcriptional regulatory interactions (Fig. 5). For example, we found 55 kinase-transcription factor feedback modules in which the kinase phosphorylates a transcription factor and the transcription factor binds to the promoter of the kinase gene. This type of feedback module is of high statistical significance, as only 18 cases would be expected in a randomized network ($P<10^{-20}$). In one such example, CHEK2 phosphorylates and activates E2F1 in response to DNA damage [27], while E2F1 targets the promoter of *CHEK2* [28]. These results suggest that phosphorylation and transcriptional regulatory networks are inherently wired together to integrate controls at different macromolecular levels and distinct temporal scales.

4. DISCUSSION

An exciting finding in this study is the discovery that an evolutionarily conserved kinase backbone exists between yeast and humans. The conserved kinase backbone is enriched with proteins involved in certain biological functions, such as the MAPKK and MAPK isoforms that are known to participate in some of the most fundamental biological processes, including protein metabolism and cellular responses to stress (Table 2). Although one may argue that protein kinases tend to be more conserved at the sequence level, we note that the conservation level of kinase-to-kinase relationships can be attributed to conservation at the levels of both the protein sequence and the phosphorylation relationship, itself (Figure 2 B and C). On the other hand, we also found that kinase-to-non-kinase relationships are much less conserved. It is generally believed that linear motifs possess more evolutionary plasticity than protein domains because the former are more likely to appear or disappear via single point mutations [29]. In fact, many phosphorylation events have been mapped to unstructured loop regions [30], suggesting that KSRs might be subject to rapid evolution. However, we found that kinase homologs from different species often retain the same functions, despite the fact that their respective substrates are largely distinct. This may suggest that rewiring in the KSR subnetworks composed of proteins with the same biological function is subject to less evolutionary constraint, in analogy to synonymous substitution in biological sequence evolution.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

A conserved kinase-to-kinase backbone exists in the phosphorylation networks;

Functions of homologous kinases are often conserved, even with distinct substrates;

Phosphorylation networks are inherently wired with gene regulatory networks.

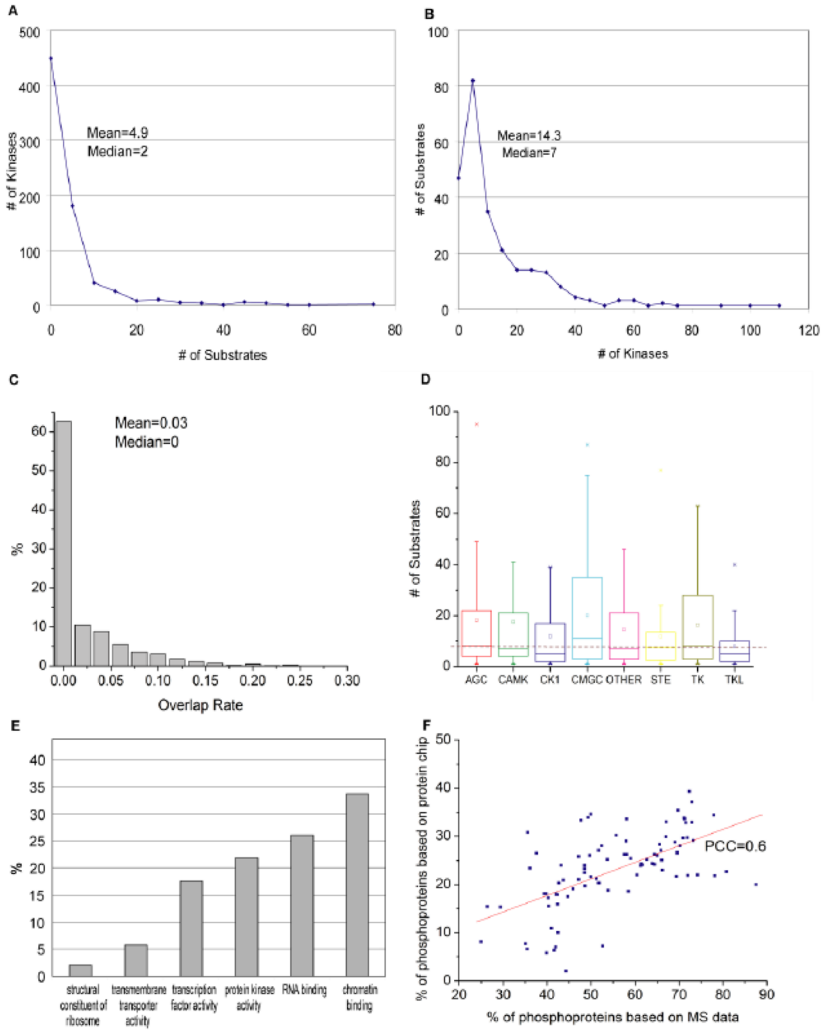


Fig. 1. Statistics of human phosphorylation network

(A) The number of kinases per substrate and (B) the number of substrates per kinase are plotted. Mean and median values are shown. (C) The distribution of substrate overlap rate among all possible kinase pairs. Supposing A and B are the substrate sets of a pair of kinases, the overlap rate is defined as Jaccard Index (A∩B)/(A∪B). (D) The distribution of substrate numbers per kinase among different kinase groups. The bottom and top of the boxes represent the 25th and 75th percentile of the distribution, respectively. The line near the middle of the box represents the 50th percentile. The empty squares in each box indicate the mean. The whiskers represent the 9th percentile and the 91st percentile. The dashed line across the figure indicates the overall average number of substrates per kinase identified in this study. (E) The distribution of phosphoproteins across various protein families. The Y-axis represents the fraction of proteins identified as kinase substrates in each of the six major protein families indicated. (F) The phosphoprotein percentage for different functional groups. Each point corresponds to one functional group (i.e., a GO term, such as “RNA-binding proteins”). The X-axis is the percentage calculated based on MS/MS data while the Y-axis is based on our protein microarray data. A good correlation between these two independent studies is observed. PCC, Pearson correlation coefficient.

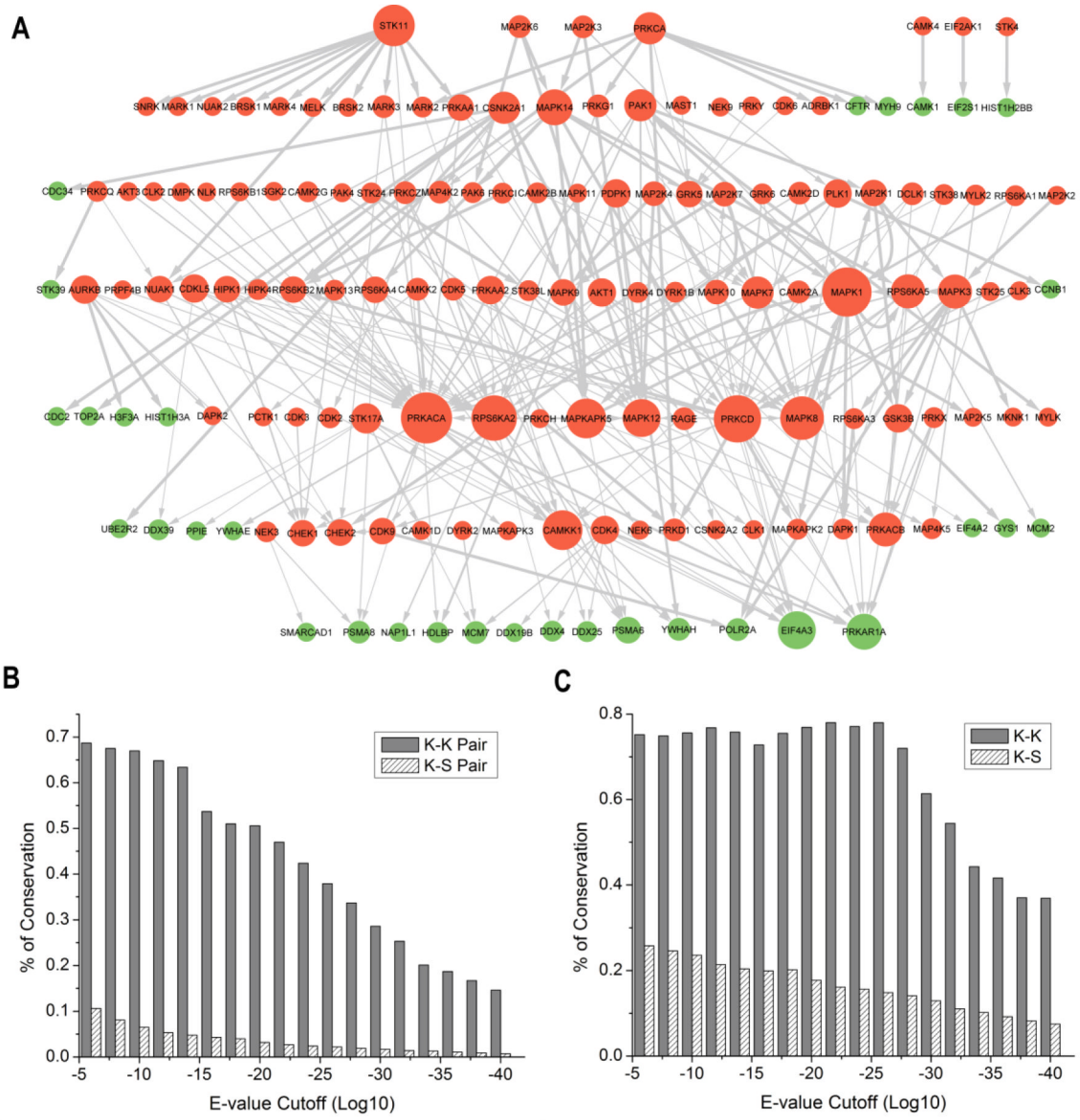


Fig. 2. Evolutionarily conserved core

(A) Conserved core of phosphorylation networks identified by comparing the human and yeast KSR networks. Kinases and non-kinase proteins are represented in orange and green nodes, respectively. The size of the nodes is proportional to the number of associated interactions. (B) The overall conservation of K–K and K–S relationships. All human KSRs were compared with yeast and the percentage of conserved human KSRs was calculated for each cutoff. The conservation was defined as that the two proteins (kinase and substrate) have homologs in yeast and the homologous pairs also have the same KSR relationship in both species. (C) The conservation of human K–K and K–S in which the two proteins (kinase and substrate) both have homologs in yeast. Using this approach, we essentially removed the contribution from the protein sequence conservation and only considered the contribution from the kinase-substrate interaction conservation.

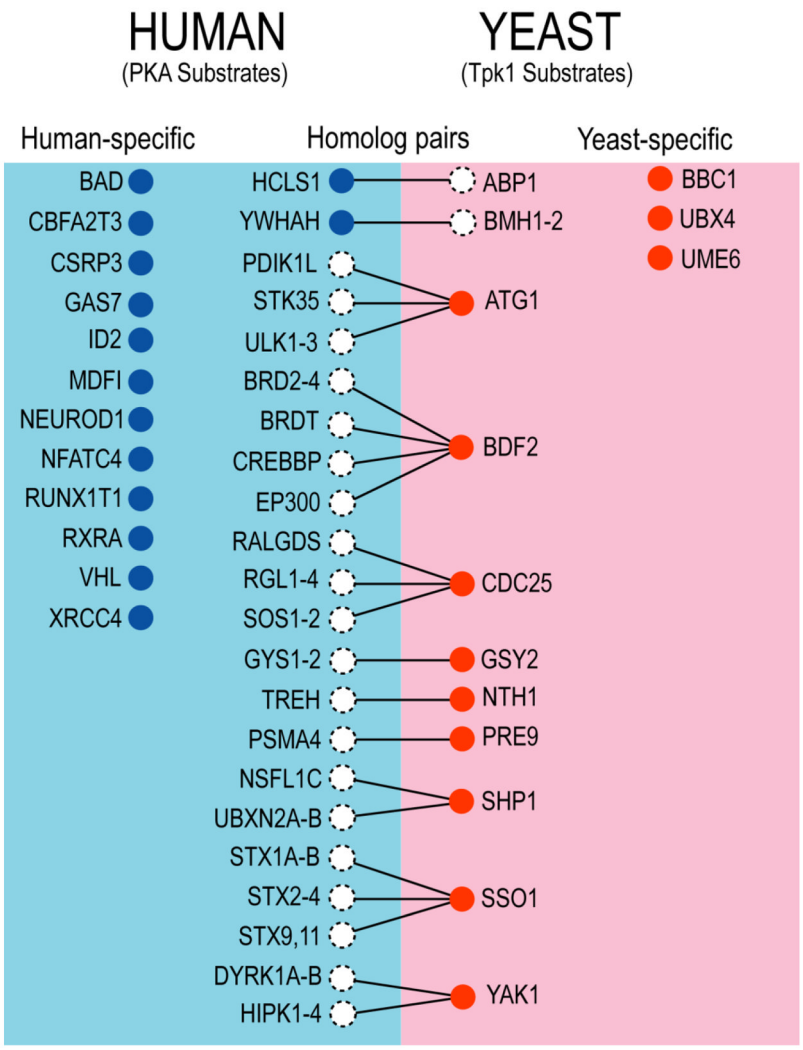


Fig. 3. Functional conservation between human PKA and yeast Tpk1

The substrates involved in cell differentiation are shown for these two kinases. Short lines indicate for homologous relationships between proteins from human and yeast.

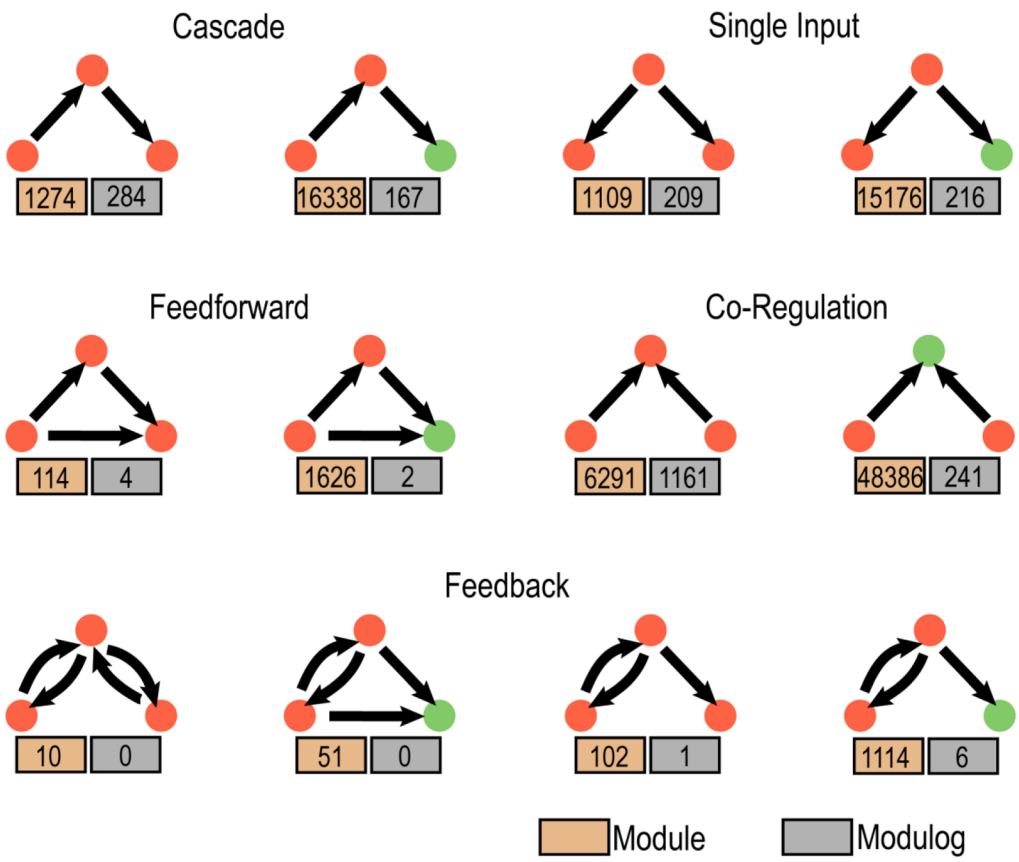
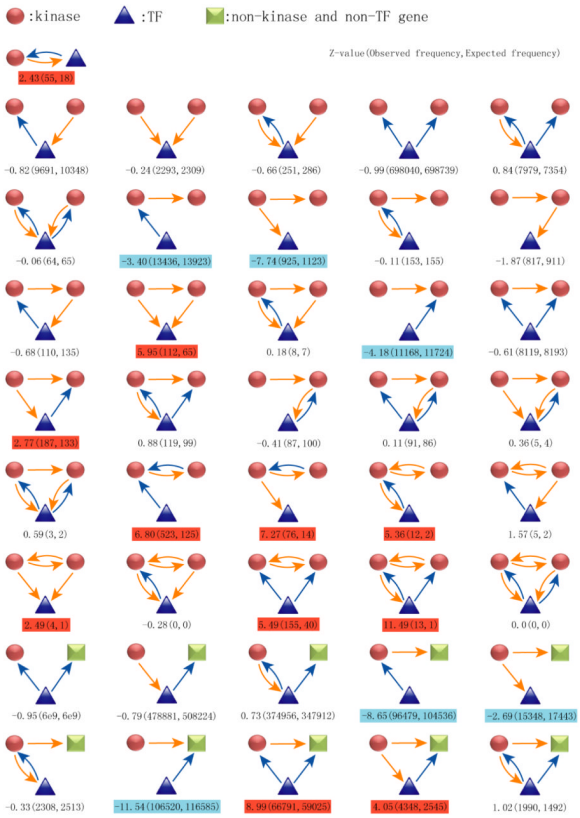


Fig. 4. Representative conserved modulogs

A modulog is defined as evolutionarily conserved regulatory modules (see text for more details). The two numbers under each module represent the number of this module found in the human comKSRs (orange box) and that found in the conserved core KSR networks (grey boxes), respectively.



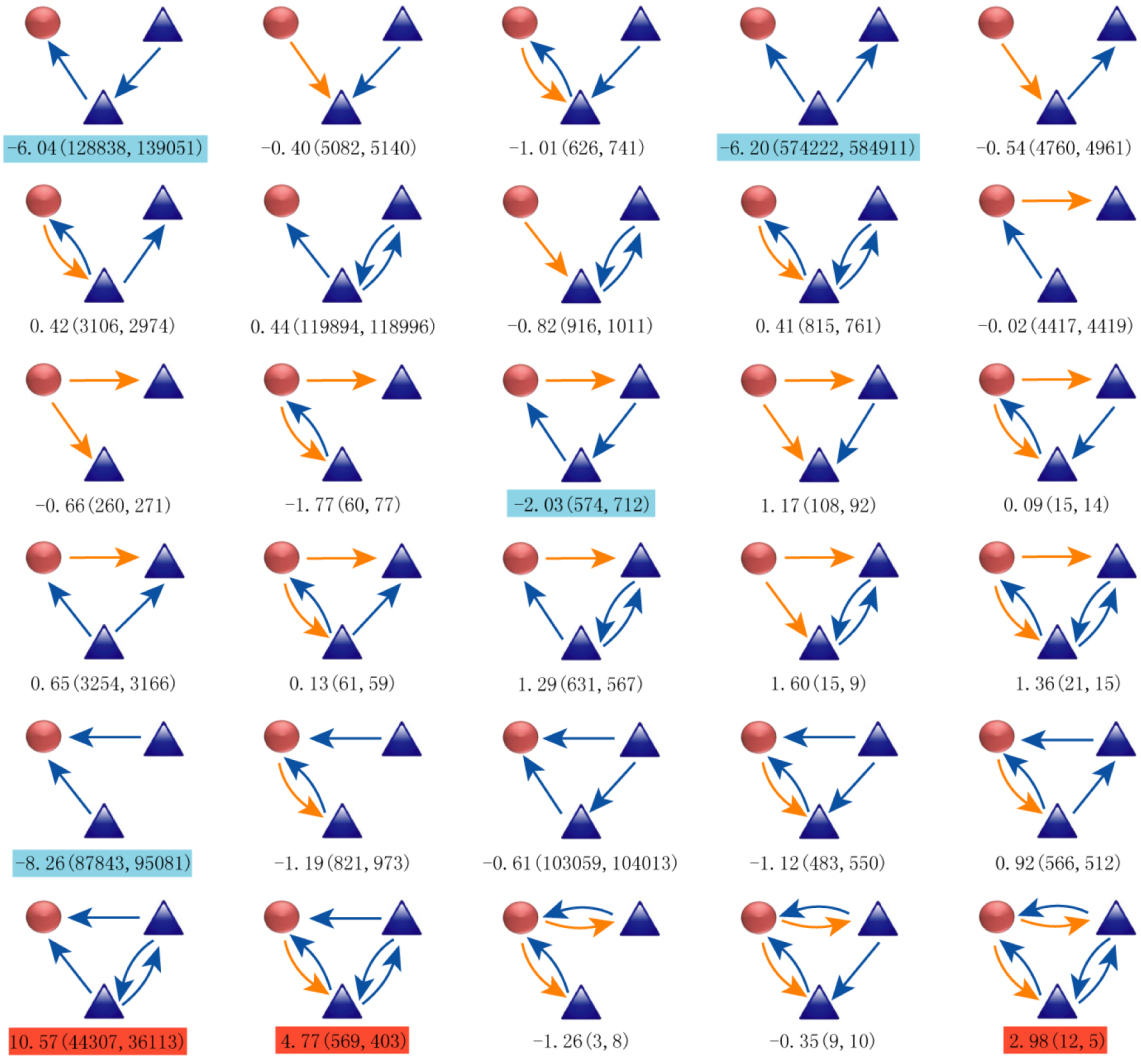


Fig. 5. List of 71 modules that include at least one transcription factor (TF) and one kinase

Arrows starting from kinases (orange) represent phosphorylation events, while arrows starting from TFs (blue) represent transcriptional activity. The three numbers below each module are the Z-score, the observed and expected numbers of the module in the network, respectively. The over- and under-represented modules are highlighted in red and blue, respectively.

Table 1

Ortholog kinase pairs with conserved enriched function in their substrate set.

Human Kinase	Yeast Ortholog	Conserved enriched function in substrate set
CDK4	Cdc28	DNA binding; Protein binding
CDK5	Cdc28	Protein binding; DNA metabolism
CDKL5	Ime2	RNA metabolism
CSNK2A2	Cka1	Protein binding
MAPK1	Fus3	DNA metabolism; Chromosome Organization
MAPK1	Kss1	Protein modification; Macromolecule metabolism
MAPK3	Fus3	Macromolecule metabolism
MAPK3	Kss1	Protein modification; Macromolecule metabolism
MAPK7	Fus3	Macromolecule metabolism
MAPK7	Kss1	Protein modification; Macromolecule metabolism
PAK1	Ste20	Regulation of transport
PDPK1	Pkh1	Protein binding
PLK1	YMR001C-A	Biological regulation
PRKAA1	Snf1	Development
PRKACA	Tpk1	Cell differentiation; Transcriptional regulation
PRKACA	Tpk3	Transcriptional regulation; Response to stimulus
PRKY	Tpk1	Transcriptional regulation
PRKY	Tpk3	Transcriptional regulation; regulation of RNA metabolism
WEE1	Swe1	Transcriptional regulation; Macromolecular metabolism

Table 2

Representative GO terms enriched in the conserved KSR network.

GO Term	# Observed	# Expected	P-value	GO annotation
GO:0006468	146	16.99	1.16E-122	Protein amino acid phosphorylation
GO:0019538	158	36.52	1.79E-82	Protein metabolic process
GO:0000165	15	1.34	2.45E-10	MAPKKK cascade
GO:0007254	8	0.51	3.26E-06	JNK cascade
GO:0007265	12	1.39	4.07E-06	Ras protein signal transduction
GO:0006950	52	22.88	5.16E-06	Response to stress