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Reconstruction of global regulatory network from signaling to cellular functions using phosphoproteomic data

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Abstract

Cellular signaling regulates various cellular functions via protein phosphorylation. Phosphoproteomic data potentially include information for a global regulatory network from signaling to cellular functions, but a procedure to reconstruct this network using such data has yet to be established. In this paper, we provide a procedure to reconstruct a global regulatory network from signaling to cellular functions from phosphoproteomic data by integrating prior knowledge of cellular functions and inference of the kinase–substrate relationships (KSRs). We used phosphoproteomic data from insulin-stimulated Fao hepatoma cells and identified protein phosphorylation regulated by insulin specifically over-represented in cellular functions in the KEGG database. We inferred kinases for protein phosphorylation by KSRs, and connected the kinases in the insulin signaling layer to the phosphorylated proteins in the cellular functions, revealing that the insulin signal is selectively transmitted via the Pi3k-Akt and Erk signaling pathways to cellular adhesions and RNA maturation, respectively. Thus, we provide a method to reconstruct global regulatory network from signaling to cellular functions based on phosphoproteomic data.

KEYWORDS
cellular functions, insulin signaling, network integration, phosphoproteomic data, phosphorylation, systems biology, Trans-omics

*These authors contributed equally to this work.

1 | INTRODUCTION

Protein phosphorylation plays a pivotal role in signaling-dependent regulation of various cellular functions. The recent development of proteomic technologies has enabled us to quantify abundance of protein phosphorylation comprehensively (Humphrey, James, & Mann, 2015; Olsen et al., 2006). Phosphoproteomic data potentially include information about global regulatory networks from signaling to cellular functions via protein phosphorylation. Prior knowledge of cellular functions in databases such as KEGG which is a manually curated database for biological pathway and functions of genes (Kanehisa, Furumichi, Tanabe, Sato, & Morishima, 2017; Kanehisa, Goto, Sato, Furumichi, & Tanabe, 2012), reactome which is a manually curated database for biological pathways and processes of signaling and metabolic molecules (Croft et al., 2014), and Gene Ontology (GO) which is a set of functional terms related to biological process and localization of proteins represented as directed acyclic graph (Ashburner et al., 2000) has been used to identify specific cellular functions of protein phosphorylation from phosphoproteomic data. Several tools inferring kinase–substrate relationship (KSR) based on similarity of recognition motifs have also been developed, such as NetPhorest (Miller et al., 2008), KinomeXplorer (Horn et al., 2014), KinasePhos (Huang, Lee, Tzeng, & Horng, 2005) and iGPS (Song et al., 2012), and using such methods, association of kinases with protein phosphorylation can be inferred from phosphoproteomic data (Emdal et al., 2015; Giansanti et al., 2015; Kanshin, Kubiniok, Thattikota, D'Amours, & Thibault, 2015; Patella et al., 2015; Sacco et al., 2016; Yugi et al., 2014). Biological interpretation of phosphoproteomic data based on prior knowledge has addressed specific cellular functions regulated by protein phosphorylation, but not the signaling-dependent regulation of such cellular functions. KSR addresses the regulation of substrates by kinases in the signaling pathway, but not regulation of the cellular functions. Integrating these two approaches may enable the reconstruction of the global regulatory network from signaling to cellular functions based on phosphoproteomic data, but a method for this has not thus far been established.

Insulin regulates diverse cellular functions through phosphorylation of substrate proteins by protein kinases in the insulin signaling pathway, including Akt and extracellular signal-regulated kinase (Erk) (Lizcano & Alessi, 2002; Saltiel & Kahn, 2001); insulin-regulated cellular functions include cell adhesion, cytoskeletal organization, RNA transport and RNA splicing (Hartmann et al., 2009; Reiss et al., 2001; Tsakiridis et al., 1999; Wolf et al., 2013). It has been reported that mutation of substrate proteins for Akt and inhibition of phosphoinositide 3-kinase (Pi3k) reduce actin reorganization and intracellular adhesion in response to insulin in cancer cells (Reiss et al., 2001; Tsakiridis et al., 1999; Wolf et al., 2013). Erk, a member of insulin signaling, has also been reported to regulate multiple alternative splicing factors, as well as playing a role in transcriptional regulation (Al-Ayoubi, Zheng, Liu, Bai, & Eblen, 2012; Boulton et al., 1991; Eblen, 2018; Matter, Herrlich, & König, 2002). Thus, insulin regulates various cellular functions by phosphorylating multiple substrate proteins via various protein kinases. However, the global regulatory network of insulin action via protein phosphorylation has not thus far been elucidated (Buescher et al., 2012; Chiappino-Pepe, Pandey, Ataman, & Hatzimanikatis, 2017; Hatzimanikatis & Saez-Rodriguez, 2015; Hyduke, Lewis, & Palsson, 2013; Joyce & Palsson, 2006; Palsson & Zengler, 2010). Phosphoproteomic studies of insulin signaling have been performed with *Drosophila* cells (Friedman et al., 2011; Vinayagam et al., 2016), 3T3-L1 mouse adipocytes (Humphrey et al., 2013), mouse hepatoma cells (Monetti, Nagaraj, Sharma, & Mann, 2011), mouse brown preadipocytes (Krüger et al., 2008), rat primary hepatocytes (Zhang, Zhang, & Yu, 2017), rat Fao hepatoma cells (Yugi et al., 2014) and mouse hepatoma (Humphrey, Azimifar, & Mann, 2015). Among these phosphoproteomic studies, the cellular functions of insulin-dependent protein phosphorylation have been estimated using databases of prior knowledge such as GO (Friedman et al., 2011; Humphrey, Azimifar, et al., 2015; Vinayagam et al., 2016; Zhang et al., 2017), whereas insulin-dependent regulatory networks of protein phosphorylation by kinases have been estimated using KSR inference (Humphrey et al., 2013; Miller et al., 2008). However, none of these studies integrated both prior knowledge of cellular functions from the databases and KSR inference, so the global regulatory network from insulin signaling to cellular functions has not thus far been reconstructed.

This study integrates prior knowledge of cellular functions from the above-mentioned databases and KSR inference to provide a procedure to reconstruct the global regulatory network from signal to cellular function based on phosphoproteomic data. First, we identified the cellular functions of protein phosphorylation from phosphoproteomic data by over-representation analysis using the KEGG database. Next, we inferred the protein kinases regulating protein phosphorylation in the cellular functions by KSR using NetPhorest, along with the selective use of kinases for each cellular function. Finally, we mapped the inferred protein kinases in the insulin signaling pathway in the KEGG database, by connecting the protein kinases in the signaling pathway to the protein phosphorylation in the cellular functions layer and reconstructing global regulatory network from insulin signaling to the cellular functions via protein phosphorylation. The global regulatory network revealed that the insulin signal is selectively transmitted via the Pi3k-Akt pathway and the Erk pathway to cellular adhesions and RNA maturation such as RNA transport or splicing, respectively.

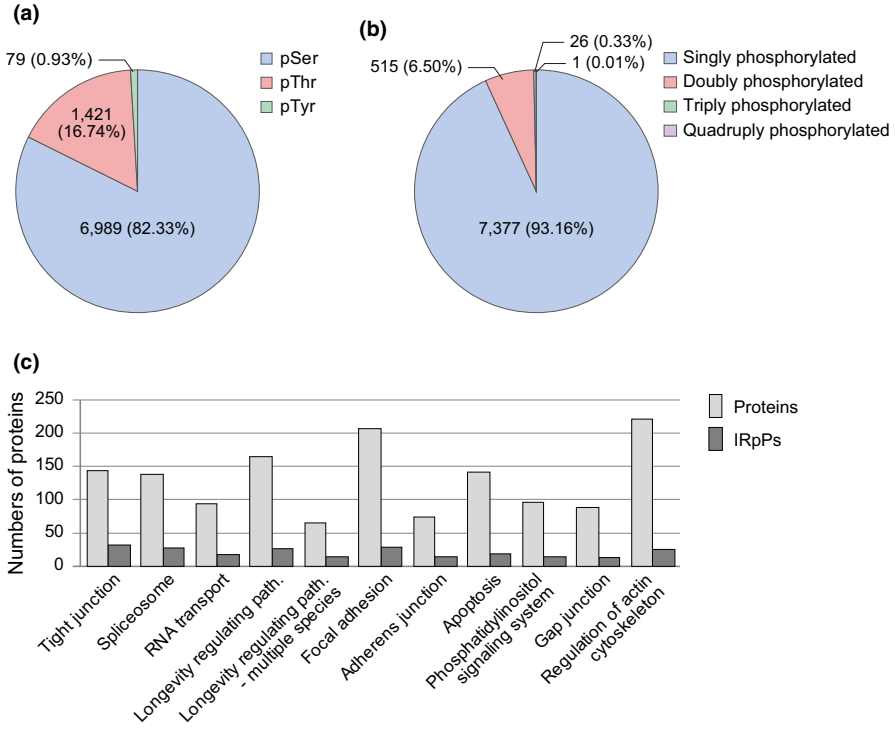


FIGURE 1 Characterization of phosphoproteomic data. Distribution of phosphorylation sites by (a) amino acid residues and (b) number of phosphorylated sites per peptide. Numbers and parentheses indicate the number of phosphorylation sites or phosphopeptides, and percentage relative to the total phosphorylation sites or phosphopeptides, respectively. (c) Number of proteins in the KEGG cellular functional pathways. The light gray bars indicate the total number of proteins, and the dark gray bars indicate the number of IRpP contained in each signaling pathway

2 | RESULTS

2.1 | Characterization of the phosphoproteomic data

Previously, we obtained phosphoproteomic data of rat Fao hepatoma cells in acute insulin action (<60 min; Yugi et al., 2014). We used the 199 phosphopeptides from the 49 metabolic enzymes in our previous work; the remaining phosphopeptides have yet to be analyzed. For this analysis, we use all 7,929 phosphopeptides from 3,468 proteins; the phosphopeptides included 6,989 phosphoserine (pSer), 1,421 phosphothreonine (pThr) and 79 phosphotyrosine (pTyr) (Figure 1a). Singly phosphorylated peptides represented 93% of the 7,929 phosphopeptides (Figure 1b). The distribution of phosphorylation sites by amino acid was consistent with the previous studies (Humphrey et al., 2013; Humphrey, Azimifar, et al., 2015; Olsen et al., 2006, 2010).

2.2 | Identification of cellular functions regulated by insulin

To identify the cellular functions regulated by insulin via protein phosphorylation, we identified quantitatively changed phosphopeptides from the phosphoproteomic data. We identified 3,288 quantitatively changed phosphopeptides exhibiting a change in phosphorylation intensity greater than a 1.5-fold increase or less than a 0.67-fold decrease at one or more time points in response to insulin stimulation; these sites were present on 1,947 proteins. Hereafter, we define a protein with at least one quantitatively changed phosphopeptide as an insulin-responsive phosphoprotein (IRpP).

To determine cellular functions related to the 1,947 IRpPs, we performed pathway over-representation analysis using KEGG pathways (that is, those pathways included in the KEGG database). We extracted pathways related to various cellular functions from KEGG (hereafter cellular functional pathways), except for those related to signaling and those that function specifically in tissues other than liver (see Section 4). The analysis with KEGG pathways related to signaling was performed in our previous study (Kawata et al., 2018). The pathways that function specifically in tissues other than liver were excluded because the phosphoproteomic data are from rat Fao hepatoma cells, which are a liver cell line. IRpPs were significantly over-represented in 11 out of 128 cellular functional pathways, including those regulating cell adhesion (Figure 1c), such as *Tight junction* (rno04530) and *Gap junction* (rno04540), and those regulating RNA maturation, such as *RNA transport* (rno03013) and *Spliceosome* (rno03040). We extracted the 11 pathways in which IRpPs were significantly over-represented and defined them as the cellular functional layer (Table 1, Supporting Information Table S1).

2.3 | Inference of protein kinases for protein phosphorylation in the cellular functional pathways

To infer the specific protein kinases regulating the IRpPs in the cellular functional pathways, we inferred protein kinases recognizing quantitatively changed phosphopeptides as

TABLE 1 Cellular functional pathways in which quantitatively changed phosphopeptides were over-represented

Pathway name	Odds ratio	*p* value	*FDR*
Tight junction	4.11	2.12×10^{-9}	2.41×10^{-7}
Spliceosome	3.40	7.64×10^{-7}	2.85×10^{-5}
Longevity regulating pathway	3.24	7.61×10^{-5}	1.16×10^{-3}
RNA transport	2.59	7.17×10^{-5}	1.16×10^{-3}
Longevity regulating pathway—multiple species	3.73	1.30×10^{-4}	1.77×10^{-3}
Adherens junction	3.16	5.45×10^{-4}	4.92×10^{-3}
Focal adhesion	2.17	5.07×10^{-4}	4.92×10^{-3}
Apoptosis	2.11	4.43×10^{-3}	3.15×10^{-2}
Phosphatidylinositol signaling system	2.30	6.94×10^{-3}	4.72×10^{-2}
Gap junction	2.33	8.20×10^{-3}	5.33×10^{-2}
Regulation of actin cytoskeleton	1.74	1.19×10^{-2}	7.40×10^{-2}

substrates in the cellular functional pathways, using the KSR prediction tool NetPhorest (Horn et al., 2014; Miller et al., 2008). The kinase classifier with the largest posterior probability value for each phosphopeptide was considered the responsible protein kinase. We inferred kinase–substrate relationships between a total of 26 responsible protein kinases and 489 out of 492 quantitatively changed phosphopeptides from the 216 IRpPs in the 11 cellular functional pathways (Supporting Information Table S2).

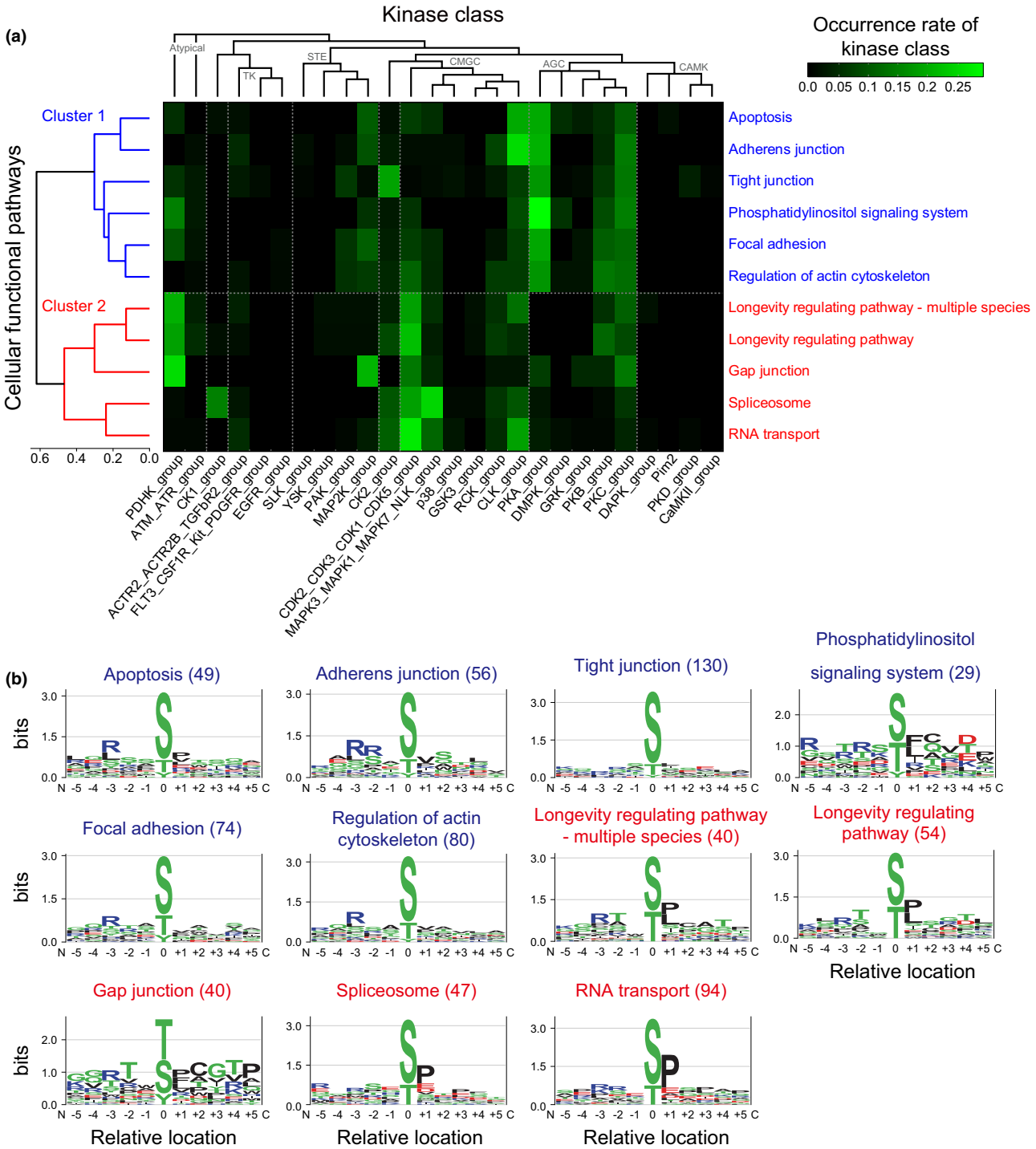
2.4 | Selective use of protein kinases according to cellular functions

To examine the selective use of the protein kinases regulating specific cellular functions, we calculated the occurrence rate of the responsible kinases of the phosphopeptides of IRpPs in each cellular functional pathway (Supporting Information Table S3). We performed cluster analysis using the occurrence rates, which resulted in two clusters (Figure 2a): Cluster 1 included pathways regulating cell adhesion and the cytoskeleton, such as *Tight junction* (rno04530), *Gap junction* (rno04540) and *Regulation of actin cytoskeleton* (rno4810) (Figure 2a, blue); Cluster 2 included pathways regulating RNA maturation, such as *RNA transport* (rno03013) and *Spliceosome* (rno03040) (Figure 2a, red).

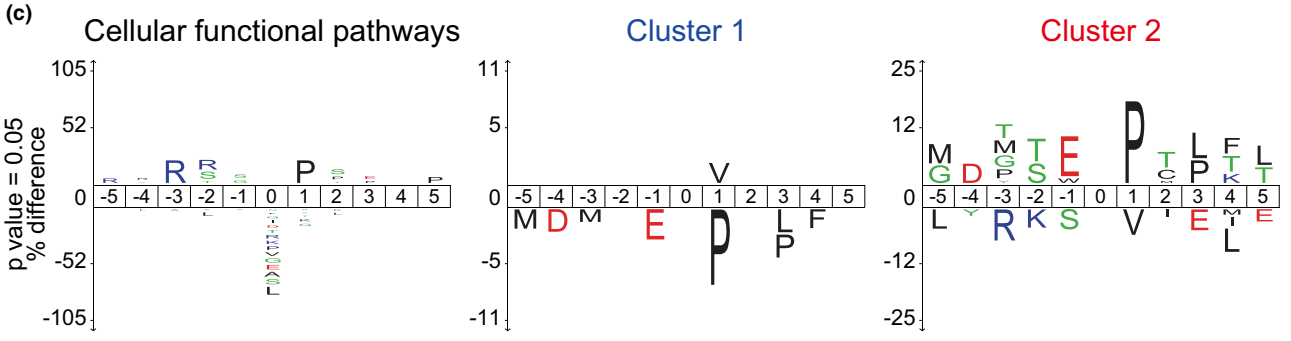
To investigate the protein kinase regulating each cellular function, we compared the motif logos of amino acid sequences (Colaert, Helsens, Martens, Vandekerckhove, & Gevaert, 2009) of the quantitatively changed phosphopeptides of IRpPs in each cellular functional pathway (Figure 2b). For many of the pathways in Cluster 1, arginine residue (R) at the −3 position was emphasized, whereas proline residue (P) at the +1 position was emphasized for many pathways in Cluster 2. R at the −3 position and P at the +1 position were known as target sequences of basophilic kinases such as Akt and proline-directed kinases such as Erk, respectively. The results of cluster analysis and motif logos indicate that cell adhesion and the cytoskeleton were selectively regulated mainly by Akt, whereas RNA maturation was selectively regulated mainly by Erk.

To confirm these findings, the representation of the amino acid residues at each position of the phosphopeptides (Workman et al., 2005) included in each cluster was tested statistically (Figure 2c). In phosphopeptides of IRpPs on the pathways in the cellular functional layer, P at the +1 position and R at the −3 position were significantly over-represented ($p < 0.05$) in comparison with the amino acid composition of rat proteins, indicating insulin regulates cellular functions via basophilic kinases such as Akt and proline-directed kinases such as Erk. In the phosphopeptides in Cluster 1, only a valine residue (V) at the +1 position was over-represented and P at the +1 position was significantly under-represented in comparison with all phosphopeptides in the phosphoproteomic data; contrary to the expectations, R at the −3 position was not significantly over-represented. For the phosphopeptides in Cluster 2, P at the +1 position was significantly over-represented. These results suggest selective regulation of cellular functions by two distinct type of protein kinases: Akt regulates both of the pathways in Cluster 1 (such as cell adhesion and the cytoskeleton) and those in Cluster 2 (such as RNA maturation), whereas Erk regulates the pathways in Cluster 2 specifically.

FIGURE 2 Protein kinase selectivity for protein phosphorylation in the cellular functional pathways. (a) Clustering of cellular functional pathways by occurrence rates for each kinase class. Green indicates the larger occurrence rate of the kinase class. (b) Motif logos of amino acid sequences of phosphopeptides in each of the indicated pathways. The height of each letter at each position is scaled relative to the information content, reflecting the frequency of the corresponding amino acid. Blue pathway names correspond to those in Cluster 1; red names correspond to those in Cluster 2. The number in parentheses attached to each pathway name represents the number of quantitatively changed peptides included in the pathway. Note that statistical differences of frequencies from any reference sequences are not considered for these motif logos. (c) Statistical amino acid motifs for phosphopeptides of IRpPs included in the cellular functional layer (left), Cluster 1 (middle) and Cluster 2 (right) are shown as logo plots. The characters above and below each horizontal line indicate amino acids showing significant over-representation and under-representation ($p < 0.05$), respectively. The height of the letter representing an amino acid at each position reflects the difference in the frequency of its occurrence in the sets of phosphopeptides and reference proteins


(a)
Kinase class
Atypical
STE
TK
CMGC
AGC
CAMK
Occurrence rate of kinase class
0.0 0.05 0.1 0.15 0.2 0.25
Cellular functional pathways
Cluster 1
Cluster 2
Apoptosis
Adherens junction
Tight junction
Phosphatidylinositol signaling system
Focal adhesion
Regulation of actin cytoskeleton
Longevity regulating pathway - multiple species
Longevity regulating pathway
Gap junction
Spliceosome
RNA transport
0.6 0.4 0.2 0.0
PDHK_group
ATM_ATR_group
CK1_group
ACTR2_ACTR2B_TGFbR2_group
FLT3_CSF1R_Kit_PDGFR_group
EGFR_group
SLK_group
YSK_group
PAK_group
MAP2K_group
CK2_group
CDK2_CDK3_CDK1_CDK5_group
MAPK3_MAPK1_MAPK7_NLK_group
p38_group
GSK3_group
RCK_group
CLK_group
PKA_group
DMPK_group
GRK_group
PKB_group
PKC_group
DAPK_group
Pim2
PKD_group
CaMKII_group

(b)
Apoptosis (49)
Adherens junction (56)
Tight junction (130)
Phosphatidylinositol signaling system (29)
Focal adhesion (74)
Regulation of actin cytoskeleton (80)
Longevity regulating pathway - multiple species (40)
Longevity regulating pathway (54)
Gap junction (40)
Spliceosome (47)
RNA transport (94)
bits
N -5 -4 -3 -2 -1 0 +1 +2 +3 +4 +5 C
Relative location


(c)
Cellular functional pathways
Cluster 1
Cluster 2
p value = 0.05
% difference
105
52
0
-52
-105
11
5
-5
-11
25
12
-12
-25
-5 -4 -3 -2 -1 0 1 2 3 4 5

2.5 | Reconstruction of global regulatory network from signaling to the cellular functions

Finally, we reconstructed the global regulatory network from signaling to the cellular functional layer via protein phosphorylation by connecting the inferred responsible kinases in the insulin signaling pathway to IRpPs in the cellular functional layer. As the insulin signaling pathway, we used *insulin signaling pathway* (rno04910) in the KEGG database (Figure 3a). Among the 61 molecules on the signaling pathway, 12 are registered as protein kinases in NetPhorest. Among the 26 kinase groups inferred in Figure 2a, Akt in the PKB_group, Erk1/2 in the MAPK3_MAPK1_MAPK7_NLK_group, Pka in the PKA_group, Pkc in the PKC_group, Mek1/2 in the MAP2K_group and Gsk3β in the GSK3_group were included in the insulin signaling pathway (Figure 3a, red). Mek1/2 was the only kinase in MAP2K_group involved in insulin signaling pathway. Given that Mek1/2 is a specific kinase for Erk family (Pearson et al., 2001), other kinases in the MAP2K_group not involved in insulin signaling pathway, rather than Mek1/2, may be the responsible kinase for the IRpPs regulated by MAP2K_group.

We connected the protein kinases in the insulin signaling pathway to the IRpPs in the cellular functional layer, resulting in a reconstructed global regulatory network (Figure 3b). Among the 489 phosphopeptides of IRpPs in the cellular functional layer for which responsible kinases were inferred, 193 phosphopeptides of IRpPs were connected to the signaling pathway via the kinases (Figure 3b).

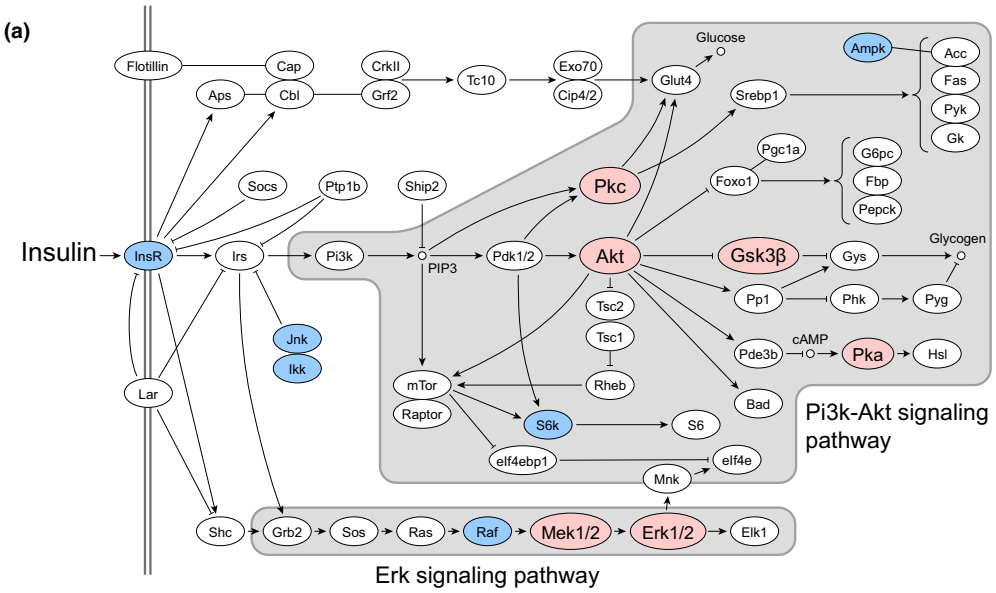
To examine the functional selectivity of the protein kinases, we counted the number of phosphopeptides of IRpPs in each cluster connected to the protein kinases (Table 2). Of the 193 phosphopeptides of IRpPs in the cellular functional layer, 122 peptides (63.2%) and 93 peptides (48.2%) were included in Clusters 1 and 2, respectively; 22 phosphopeptides (11.4%) were included in both clusters. Kinases in the Pi3k-Akt signaling pathway such as Pka, Pkc and Akt were connected to more phosphopeptides in Cluster 1 than Cluster 2, whereas the kinases in the Erk signaling pathway—such as Mek1/2 and Erk1/2—were connected to more phosphopeptides in Cluster 2 (Figure 3c). This indicates that the Pik3-Akt and Erk signaling pathways selectively regulate different cellular functions via protein phosphorylation; the former mainly regulates cell adhesion such as the adherens junction, and the latter mainly regulates RNA maturation such as RNA splicing and RNA transport.

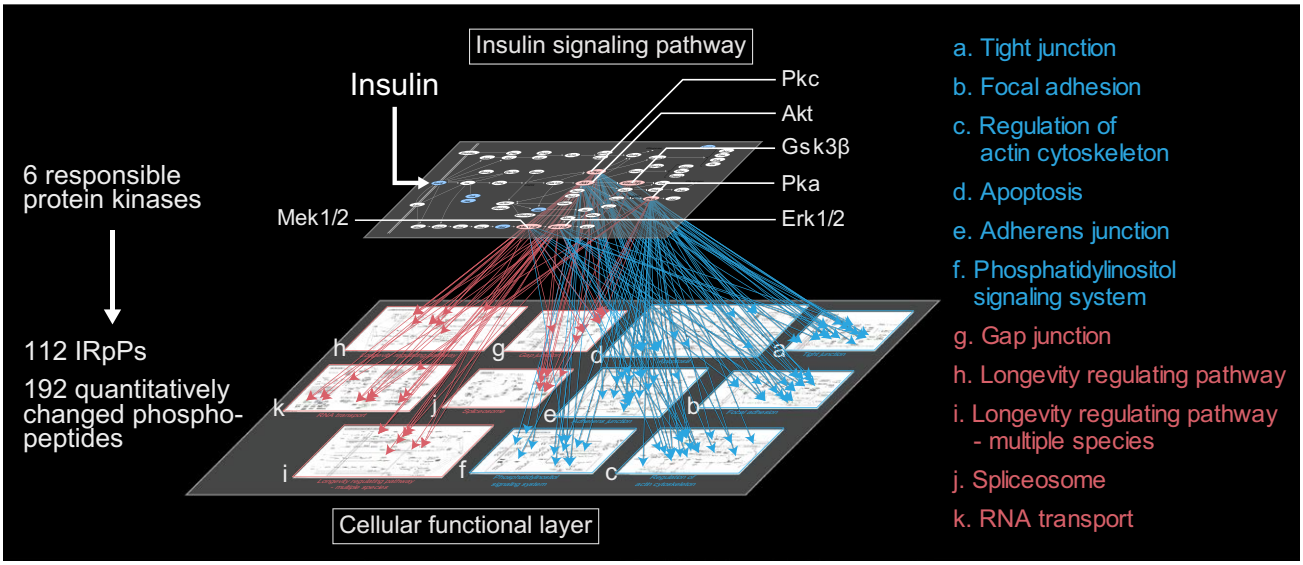
3 | DISCUSSION

We analyzed the phosphoproteomic data of insulin-stimulated Fao hepatoma cells by integrating prior knowledge of cellular functions and KSR inference and reconstructed a global regulatory network from signaling to cellular function. This reconstructed network of insulin action revealed that insulin signals are selectively transmitted via the Pi3k-Akt and Erk signaling pathways to cellular adhesions and RNA maturation, respectively. This result is consistent with the earlier finding that Akt and Erk play important roles in insulin signaling pathway in adipocytes using phosphoproteomic data (Humphrey et al., 2013). Blood insulin showed a transient high concentration during the fed state (induced insulin secretion), whereas a sustained low concentration of blood insulin (basal insulin secretion) was maintained during the fasting state (Lindsay et al., 2003; Polonsky, Given, & Van Cauter, 1988). We have reported that high and low doses of insulin selectively regulate different signaling molecules in the liver (Kubota et al., 2012). We also found that high and low doses of insulin regulate different cell functions through Erk and Akt, in Fao rat hepatoma cells and the rat liver (Kawata et al., 2018). Our finding suggests that high and low doses of insulin selectively regulate cellular adhesions via the Pi3k-Akt signaling pathway and RNA maturation via the Erk signaling pathway, respectively, in vivo. Elucidating such selective regulation in vivo is a subject for future study.

Previous studies combining phosphoproteomic data and NetPhorest indicated that Akt has an important role for cellular regulation in response to insulin (Alli Shaik et al., 2016; Vinayagam et al., 2016), consistent with this study. These studies did not focus on the kinase selectivity of the regulated cellular functions, and this study provides the first evidence for the kinase selectivity of cellular functions regulated by insulin. The similarities in phosphorylation site consensus motifs shared by many protein kinases may also have resulted in inaccurate predictions of KSR. Although approximately 500 protein kinases have been reported in humans (Manning, Whyte, Martinez, Hunter, & Sudarsanam, 2002), only approximately 200 kinases are registered in NetPhorest, which is the KSR prediction tool

FIGURE 3 Reconstruction of the global regulatory network from the insulin signaling pathway to the cellular functional layer. (a) Insulin signaling pathway based on KEGG database used as the signaling pathway in the global regulatory network. Red and blue indicate kinases connected to the phosphopeptides of the IRpPs, and other kinases, respectively. Pi3k-Akt and Erk signaling pathways are indicated by gray frames. (b) Constructed global regulatory network. The arrows (from the top to the bottom layer) indicate phosphorylation of the quantitatively changed phosphopeptides by the responsible protein kinases. (c) Number of quantitative changed phosphopeptides inferred as substrates for the kinases on the signaling pathway. Red and blue bars indicate the number of phosphopeptides on only the pathways in Clusters 1 and 2, respectively; the green bar indicates the number of phosphopeptides on pathways in both clusters


(a)
Insulin
InsR
Irs
Pi3k
PIP3
Pdk1/2
Akt
Pkc
Glucose
Glut4
Flotillin
Cap
Aps
Cbl
CrkII
Grf2
Tc10
Exo70
Cip4/2
Socs
Ptp1b
Ship2
Jnk
Ikk
Lar
Ampk
Acc
Fas
Pyk
Gk
Srebp1
Pgc1a
Foxo1
G6pc
Fbp
Pepck
Gsk3β
Gys
Glycogen
Pp1
Phk
Pyg
Tsc2
Tsc1
Rheb
mTor
Raptor
S6k
S6
elf4ebp1
elf4e
Mnk
Pde3b
cAMP
Pka
Hsl
Bad
Pi3k-Akt signaling pathway
Shc
Grb2
Sos
Ras
Raf
Mek1/2
Erk1/2
Elk1
Erk signaling pathway


(b)
Insulin signaling pathway
Insulin
Pkc
Akt
Gsk3β
Pka
Erk1/2
Mek1/2
6 responsible protein kinases
112 IRpPs
192 quantitatively changed phospho-peptides
Cellular functional layer
a. Tight junction
b. Focal adhesion
c. Regulation of actin cytoskeleton
d. Apoptosis
e. Adherens junction
f. Phosphatidylinositol signaling system
g. Gap junction
h. Longevity regulating pathway
i. Longevity regulating pathway - multiple species
j. Spliceosome
k. RNA transport

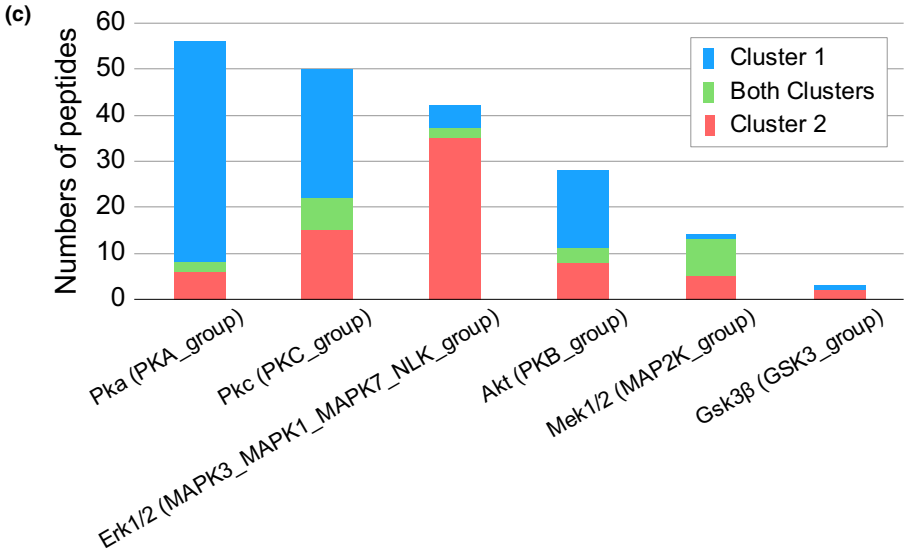

(c)
Numbers of peptides
60
50
40
30
20
10
0
Cluster 1
Both Clusters
Cluster 2
Pka (PKA_group)
Pkc (PKC_group)
Erk1/2 (MAPK3_MAPK1_MAPK7_NLK_group)
Akt (PKB_group)
Mek1/2 (MAP2K_group)
Gsk3β (GSK3_group)

TABLE 2 Number of quantitatively changed phosphopeptides inferred as substrates for kinases on the signaling pathway

Kinase	Cluster 1	Cluster 2	Both clusters	Total
Pka	48	6	2	56
Pkc	28	15	7	50
Erk1/2	5	35	2	42
Akt	17	8	3	28
Mek1/2	1	5	8	14
Gsk3β	1	2	0	3
Total	100	71	22	193

with the largest number of registered protein kinases. Moreover, the phosphoproteomic data in this study obtained with shotgun approach include missing KSRs and nonfunctional phosphorylation. We select the pathway in which IRpPs are concentrated using statistical over-representation analysis to reduce the influence of missing and nonfunctional phosphorylation on the landscape of insulin action. Although KSR prediction can find novel pathways between the signaling pathway and cellular function, it cannot find novel cellular functions. In this study, we used KEGG pathways to provide prior knowledge of cellular functions because they clearly distinguish signaling and cellular functions. In addition to KEGG pathways, GO (Ashburner et al., 2000) and pathway information included in reactome (Croft et al., 2014) are used to provide established pathway information. Depending on cellular function, the precision and completeness of prior knowledge may be quite different; it is possible that over-representation may not have resulted in the original cellular function. Despite these limitations, we obtained novel molecular insight into how cells interpret insulin stimulation through the integration of cellular functions available in the databases mentioned above to the signaling identified by KSR prediction.

Since metabolism did not indicate significant over-representation in the pathway analysis, it is not included in the targeted cellular functions. However, metabolic regulation is one of the well-known functions of insulin (Lizcano & Alessi, 2002; Saltiel & Kahn, 2001), and we have previously reconstructed a global regulatory network involved in metabolism (Yugi et al., 2014). We revealed the large contribution of Akt and Erk in regulating cellular functions via phosphorylation in response to acute insulin action. We propose that these reconstruction methods be applied to explore cellular responses to other stimuli.

4 | EXPERIMENTAL PROCEDURES

4.1 | Phosphoproteomic data

In this study, we used published datasets of the quantitative phosphoproteomic data of a time series of insulin stimulation of Fao cells (RRID:CVCL_0269, male) that we measured previously (Yugi et al., 2014). Rat hepatoma Fao cells were seeded at a density of 3×10^6 cells per dish on 6-cm dishes (Corning) and cultured in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum at 37°C under 5% CO2 for 2 days before deprivation of serum (starvation). The cells were washed twice with phosphate-buffered saline (PBS) and starved for 16 hr in serum-free medium including 0.01 nM insulin (Sigma-Aldrich) and 10 nM dexamethasone (Wako), which increases the expression of gluconeogenesis genes such as *G6pase* and *Pck1* (Lange et al., 1994). We continuously added 0.01 nM insulin before the stimulation, and 0.01 nM insulin was present throughout the experiments unless otherwise specified to mimic in vivo basal secretion during fasting (Polonsky et al., 1988). The medium was changed at 4 and 2 hr before the stimulation. Cells were stimulated with 1 nM insulin and collected at 0, 2, 5, 10, 30, 45 and 60 min after stimulation. Cell lysate digested with LysC and trypsin was subjected to Fe-IMAC and iTRAQ labeling for the enrichment of phosphopeptides and quantification by mass spectrometry. All samples were analyzed with a QSTAR Elite (AB Sciex) instrument equipped with a Paradigm MS4 HPLC pump and HTC-PAL autosampler (CTC Analytics AG). The peak lists were generated using Analyst Mascot.dll v1.6b27 (AB SCIEX). A MASCOT search was performed with the following parameter settings: trypsin as the enzyme used; the allowed number of missed cleavages as 2; iTRAQ label at the NH2-terminus, Lys and carbamidomethylation of Cys as fixed modifications; oxidized Met, iTRAQ label on Tyr, pyroglutamination of NH2-terminal Glu or Gln, and phosphorylation on Ser, Thr and Tyr as variable modifications; precursor mass tolerance as 100 ppm; and tolerance of MS/MS ions as 0.2 Da. Assigned rank 1 peptide sequences (MASCOT score >20) were extracted. Evaluation of phosphorylation sites was performed at a post-MASCOT search with in-house script.

4.2 | Identification of differential phosphorylation

Because the phosphoproteomic data consisted of two different time series from two separate experiments (0, 5, 10 and 45 min and 2, 10, 30 and 60 min), some of the phosphopeptides were identified and quantified in data from only one of the time series. Therefore, a fold change of phosphorylation intensity was calculated as a ratio of the phosphorylation intensity at each time point to the phosphorylation intensity at $t = 0$ or 2 min. A phosphopeptide with a phosphorylation intensity greater than a 1.5-fold increase or less than a 0.67-fold decrease at one or more time points was defined as a quantitatively changed phosphopeptide. Proteins including one or more quantitatively changed phosphopeptides were defined as insulin-responsive phosphoproteins

(IRpPs). The detailed procedure of identification of differential phosphorylation can be found in Yugi et al (Yugi et al., 2014).

4.3 | Construction of the cellular functional layer

We selected all rat pathways from the KEGG database for cellular functions with the following exceptions: (a) signaling pathways (43 pathways); (b) global pathways (rno01XXX) including *Metabolic pathways* (rno01100) (9 pathways); (c) disease-related pathways (rno05XXX) (63 pathways); (d) pathways that work in specific tissues other than liver or are not regulated by insulin signaling in the liver (52 pathways); and (e) pathways that include the character string of "diabetes," "NAFLD" or "Insulin resistance"; in their names (8 pathways). The reasons why we excluded the above five kinds of pathways are as follows. The signaling pathways were excluded from cellular functional pathways because these were analyzed in the previous study (Kawata et al., 2018). Global and disease-related pathways were excluded because of their redundancy with other cellular functional pathways as subsets. To identify the cellular functions regulated by insulin stimulation, we performed over-representation analysis of the IRpPs in the cellular functional pathways. The international protein index (IPI) (Kersey et al., 2004) IDs of phosphopeptides in the phosphoproteomic data were converted to KEGG gene ID using bioDBnet (Mudunuri, Che, Yi, & Stephens, 2009) to correspond with the pathway information in the KEGG database. Over-representation of the IRpPs for each pathway was determined using Fisher's exact test with FDR using Storey's procedure (Storey et al., 2004) (significant over-representation when FDR <0.1). The cellular functional pathways in which the IRpPs were significantly over-represented were defined as the cellular functional layer.

4.4 | Inference of protein kinases for protein phosphorylation

We inferred KSRs for amino acid sequences in the proteins with quantitatively changed phosphopeptides using a standalone version of NetPhorest (Horn et al., 2014; Miller et al., 2008) (http://netphorest.info/download/netphorest_human.tsv.xz). NetPhorest inputs are rat protein sequences associated with the IPI in FASTA format (ftp://ftp.ebi.ac.uk/pub/databases/IPI/last_release/current/ipi.RAT.fasta.gz). The outputs for NetPhorest are posterior probabilities of an amino acid residue being recognized by a protein kinase classifier (kinases with similar substrate recognition motifs). Among the candidate classifiers, we selected the classifier with the largest posterior probability value as the kinase classifier related to the amino acid sequence. A predicted KSR is represented as an edge between a kinase classifier as one node and a phosphorylation site of an IRpP phosphopeptide as the other node. We also extracted individual kinases within in each class from NetPhorest (view source: http://netphorest.info/download.shtml) and defined these kinases as responsible protein kinases.

4.5 | Clustering the cellular functional pathways by the occurrence rates of responsible protein kinases

We performed hierarchical cluster analysis on the cellular functional pathways in the cellular functional layer using the occurrence rate of the predicted responsible protein kinases. The occurrence rate of a specific kinase classifier (i) in a specific pathway (j) was calculated as the rate of the number of quantitatively changed phosphopeptides having the kinase classifier i (including the responsible protein kinases) to the total number of quantitatively changed phosphopeptides in the pathway j. The sum of occurrence rates of the kinase classifiers in pathway j is 1. We performed the hierarchical clustering of the cellular functions pathways using the Euclidean distance to calculate the intracluster distances and using Ward's method to calculate the intercluster distances (Ward, 1963).

4.6 | Generating motif logos

Motif logos of the quantitatively changed phosphopeptides included in each cellular functional pathway were generated using enoLOGOS (Workman et al., 2005; http://biodev.hgen.pitt.edu/enologos/) with relative entropy as the logo plot method. These logos are based on the frequency of each amino acid residue within the sequences of the phosphopeptides included in each cellular functional pathway. Ranges of motifs were provided from −5 to +5 residues from phosphorylated sites. Statistical tests of amino acid composition at each position of the quantitatively changed phosphopeptides included in the cellular functional layer or in each cluster were performed using iceLogo (Colaert et al., 2009; http://iomics.ugent.be/icelogoserver/index.html) with percentage difference as the scoring system and a p value cutoff of 0.05. These logos are based on the statistical significance of frequency of each amino acid residue within the sequences of the phosphopeptides included in each cluster against the reference compositions. The *Rattus norvegicus* amino acid compositions from Swiss-Prot and quantitatively changed phosphopeptides included in cellular functions were used as reference compositions for tests of quantitatively changed phosphopeptides included in the cellular functional layer and for tests of those included in each cluster.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest with the contents of this article.

DATA AVAILABILITY

The dataset used in this analysis including time series of phosphoproteomic data obtained from Fao cells in response to insulin stimulation can be obtained from *jPOST* (accession number: PXD005900).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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