SPECHT – Single-stage phosphopeptide enrichment and stable-isotope chemical tagging: Quantitative phosphoproteomics of insulin action in muscle

Arminja N. Kettenbach[1,2*], Hiroyuki Sano[1], Susanna R. Keller[3], Gustav E. Lienhard[1], Scott A. Gerber[1,2,4*]

1. Department of Biochemistry, Geisel School of Medicine at Dartmouth, Lebanon, NH 03756, USA
2. Norris Cotton Cancer Center, Lebanon, NH 03756, USA
3. Department of Medicine, Division of Endocrinology, University of Virginia, Charlottesville, VA 22903, USA
4. Department of Genetics, Geisel School of Medicine at Dartmouth, Lebanon, NH 03756, USA

* to whom correspondence should be addressed:
arminja.n.kettenbach@dartmouth.edu scott.a.gerber@dartmouth.edu

SUPPORTING FIGURES

Figure S1. Comparison of SILAC and reductive dimethyl-labeling strategies. Pearson correlation analysis of average SILAC and reductive dimethyl-labeling $\log_2$ ratios of HeLa cell-derived phosphopeptides identified and quantified by both labeling strategies confirmed the expected independent relationship of two normally distributed datasets with a certain degree of noisiness.

Figure S2. Experimental setup of insulin signaling in murine skeletal muscle tissue analysis. Mice were fasted for 6 h and then injected with either insulin in PBS or PBS only. Muscle tissue was homogenized in lysis buffer, reduced and alkylated, and trypsin digested. The muscle homogenates from the three control-treated mice were combined into a common standard to reduce mouse-to-mouse variation. Phosphopeptides were enriched and phosphopeptides from insulin-treated mice were reductive dimethyl-labeled heavy, while phosphopeptides from control-treated mice were reductive dimethyl-labeled light, mixed, and separated using strong-cation exchange (SCX) chromatography and analyzed by LC-MS/MS.

Figure S3. Assessment of quantification variability in mouse muscle. Pearson correlation analysis of phosphopeptide $\log_2$ ratios with a p-value < 0.1 (Students T-Test) quantified in the indicated mice.

Figure S4. **Analysis of protein changes in insulin-treated murine skeletal muscle tissue. A**, Log_2 ratio distribution of proteins from insulin versus control-treated mice. Total numbers of proteins identified in each experiment are indicated. **B.** Distribution of p-values (Students T-Test) of proteins quantified in the three analyses. **C**, Correlation analysis of protein $\log_2$ ratios quantified in all three analyses with a p-value (Students T-Test) of less than 0.1 identified in all three experiments.

Figure S5. **Volcano plot of quantified phosphopeptides in insulin-treated murine skeletal muscle tissue.** To determine **s**tatistical significance and fold-change cut-offs for the insulin-regulated murine muscle phosphoproteome the negative log10 of the p-value (Students T-Test) was plotted against the log2 ratio of the respective phosphopeptide. Green lines marked 1.5-fold ratio change cut-off. Red line indicates significance cut-off at p-value of 0.05.

Figure S6. **Motif analysis.** . Motif enrichment analysis on phosphopeptides significantly upregulated 1.5-fold or more with a p-value < 0.05 (Students T-Test) in insulin-treated murine skeletal muscle were performed using the Motif-x algorithm. In this analysis, RxxpS and pSP motifs were identified as significantly enriched, which are likely due to the activation of Akt and ERK signaling pathways by insulin stimulation.

Figure S7. **Canonical insulin signaling pathway.**
Early steps in insulin signaling. Binding of insulin to its receptor (INSR) results in its autophosphorylation and activation. The INSR then phosphorylates the insulin receptor substrates (IRS1, IRS2) and activation of the Akt and ERK kinase pathways ensues.

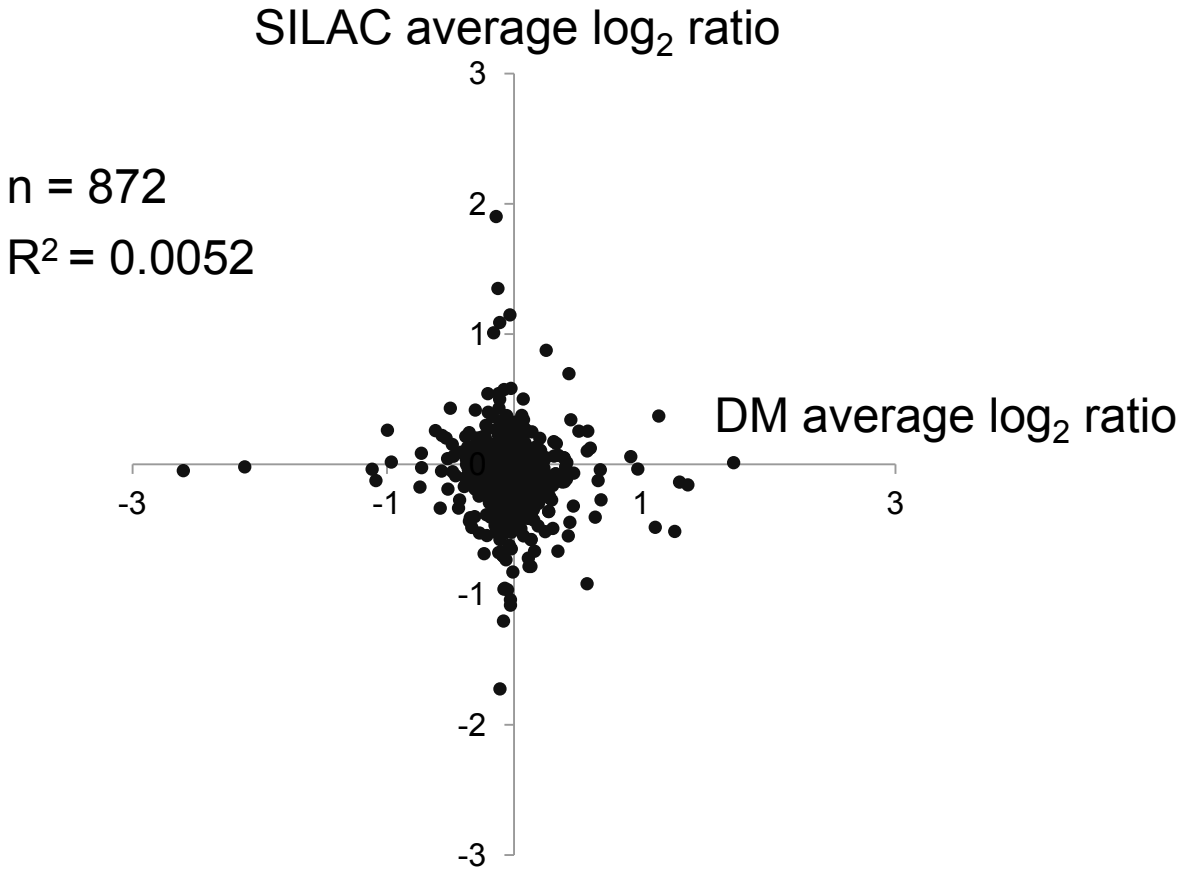

SILAC average log2 ratio
n = 872
R2 = 0.0052
DM average log2 ratio
3
2
1
0
-1
-2
-3
-3
-1
1
3

Figure S-1

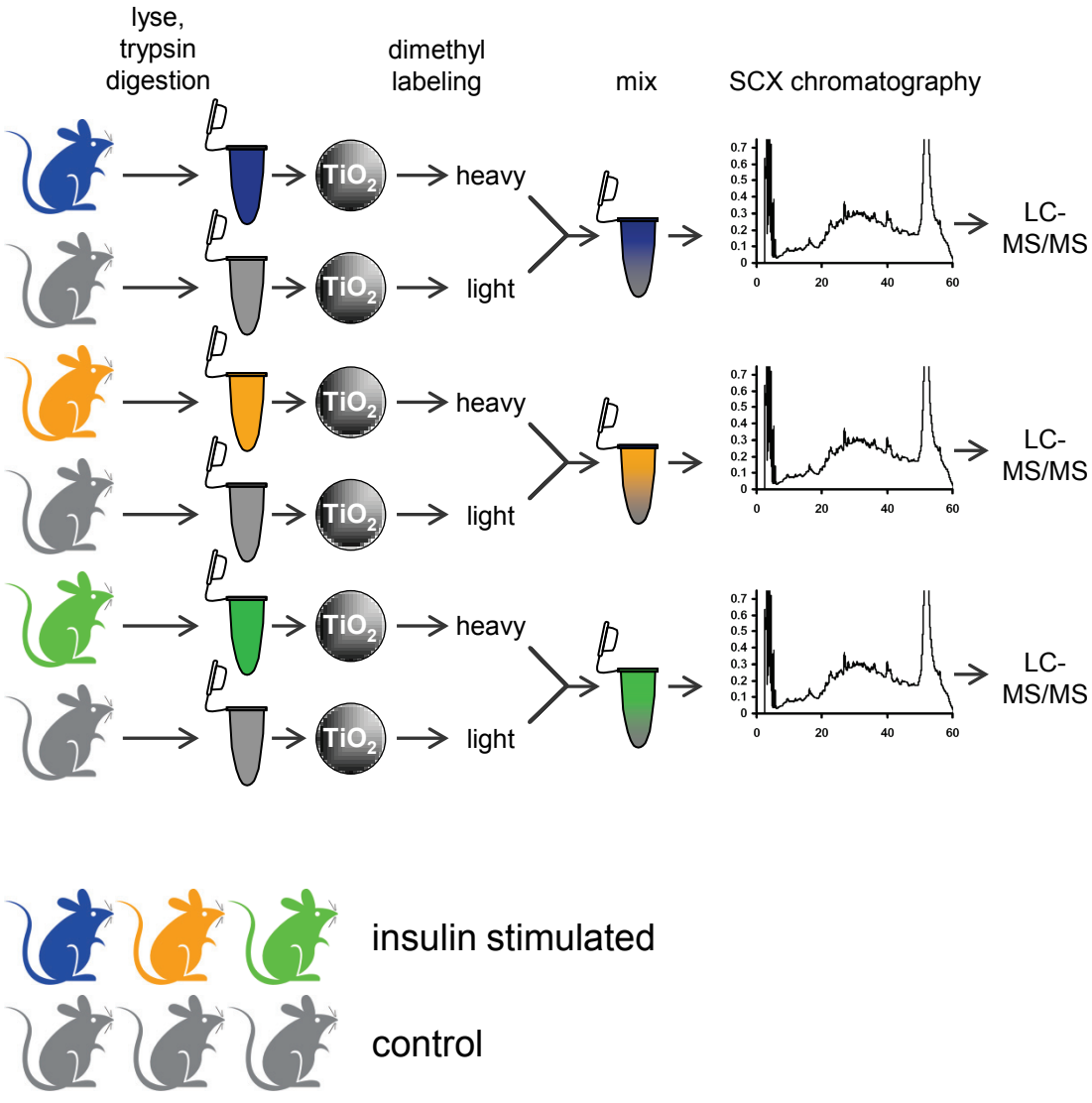
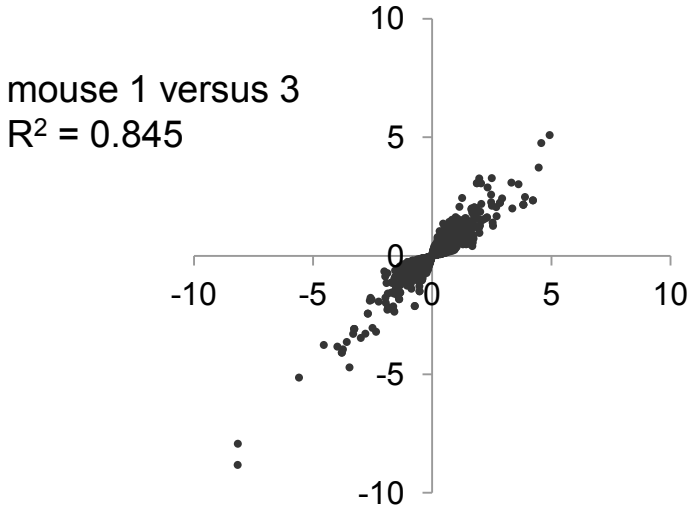

lyse,
trypsin
digestion
dimethyl
labeling
mix
SCX chromatography
TiO2
heavy
light
LC-
MS/MS
insulin stimulated
control

Figure S-2

A


mouse 1 versus 3
R2 = 0.845
10
5
0
-5
-10
-10
-5
0
5
10

B

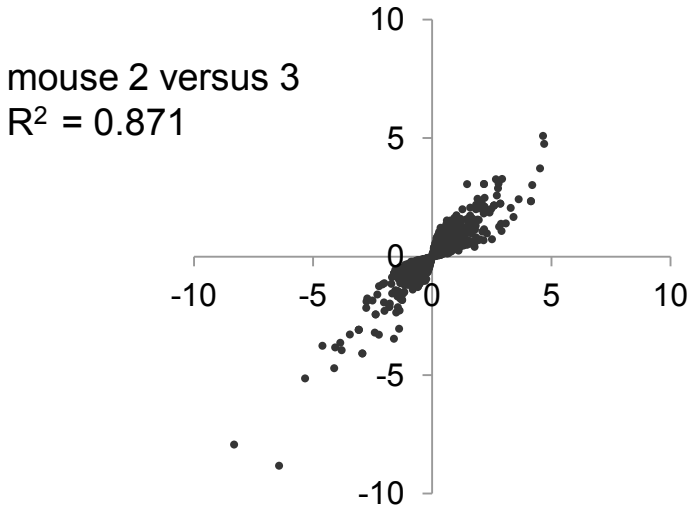
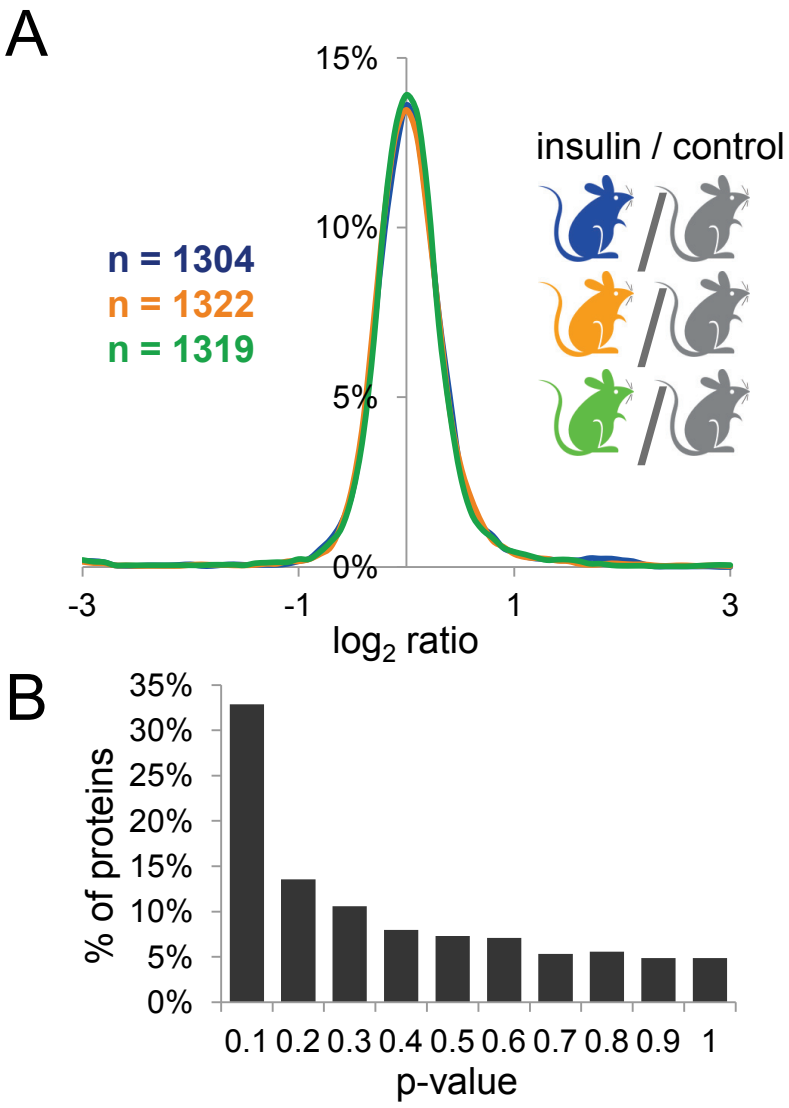

mouse 2 versus 3
R2 = 0.871
10
5
0
-5
-10
-10
-5
0
5
10

Figure S-3


A
15%
10%
5%
0%
-3
-1
1
3
log2 ratio
insulin / control
n = 1304
n = 1322
n = 1319
B
35%
30%
25%
20%
15%
10%
5%
0%
% of proteins
0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1
p-value

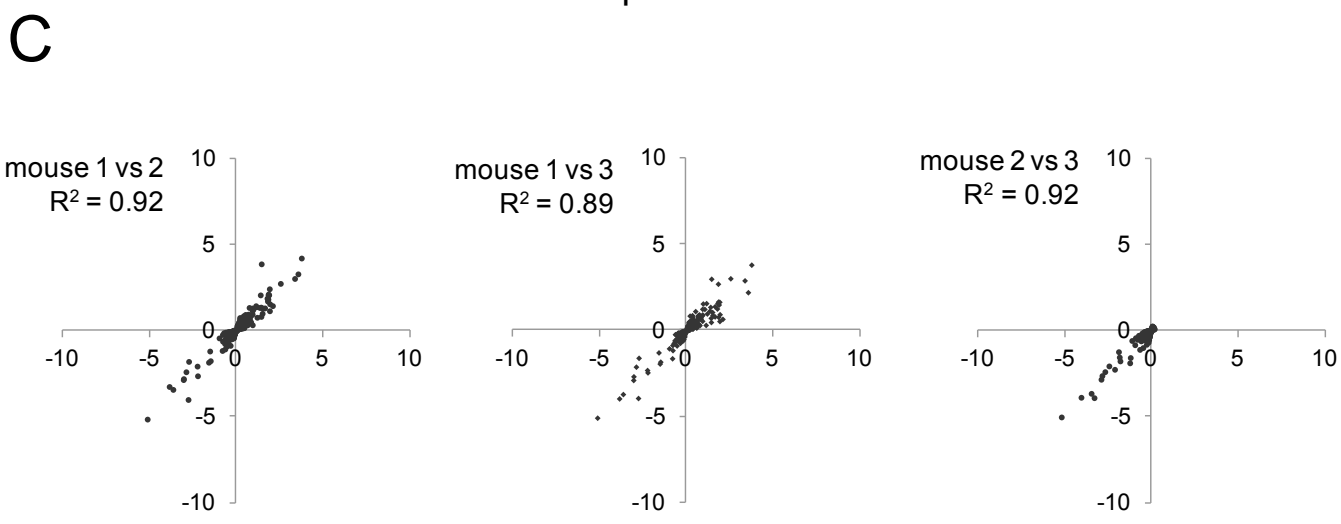

C
mouse 1 vs 2
R2 = 0.92
mouse 1 vs 3
R2 = 0.89
mouse 2 vs 3
R2 = 0.92

Figure S-4

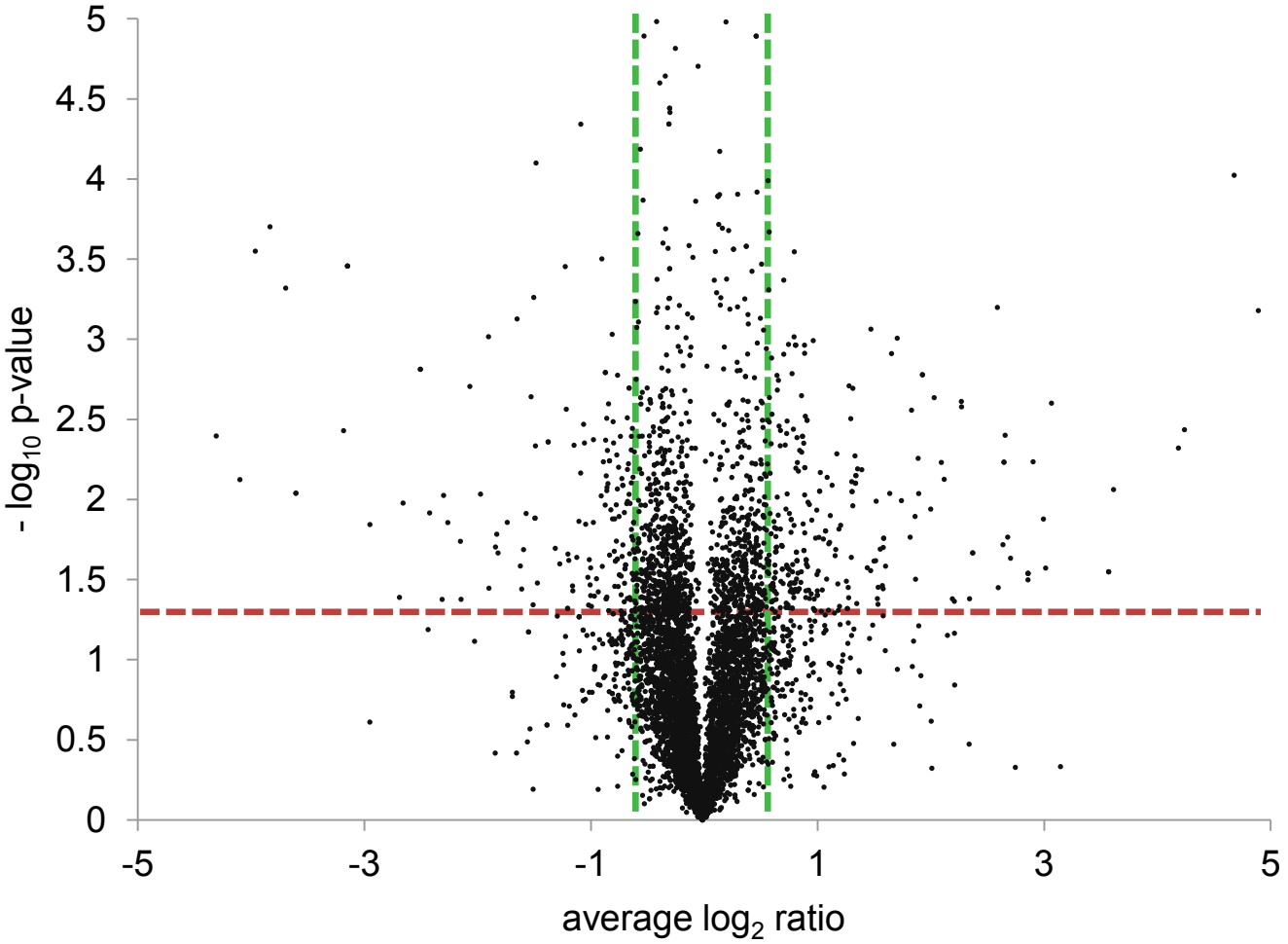
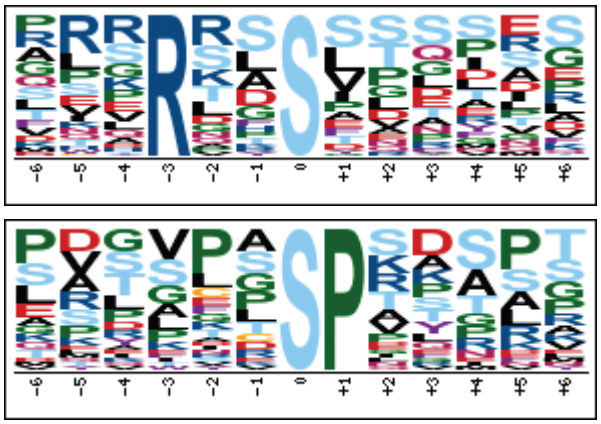


Figure S-5



Motifs enriched in peptides upregulated 1.5-fold or more and quantified with a p-value < 0.05

Figure S-6

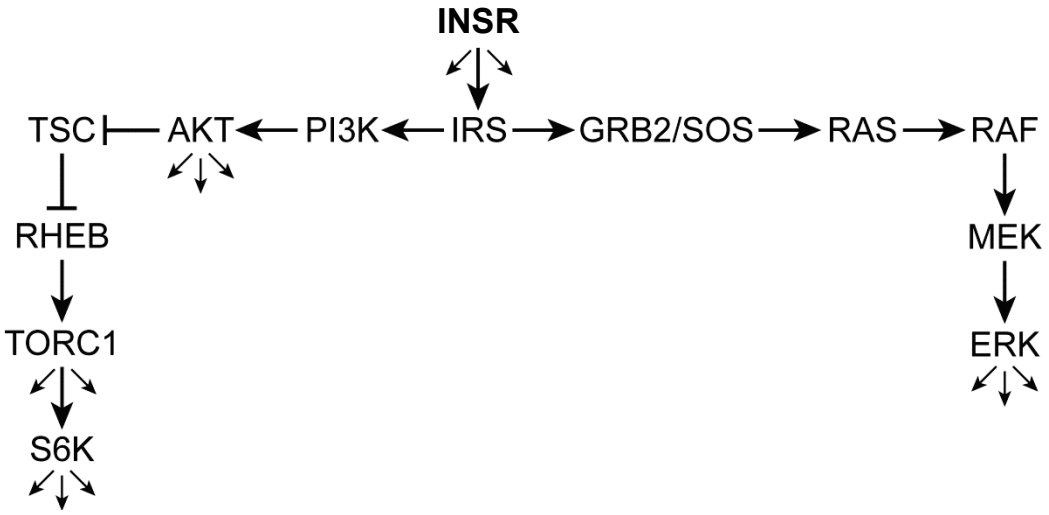


Figure S-7