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Supplementary Materials for

Dual phosphorylation of Ric-8A enhances its ability to mediate G protein α subunit folding and to stimulate guanine nucleotide exchange

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The PDF file includes:

Other Supplementary Material for this manuscript includes the following:
(available at www.sciencesignaling.org/cgi/content/full/11/532/eaap8113/DC1)

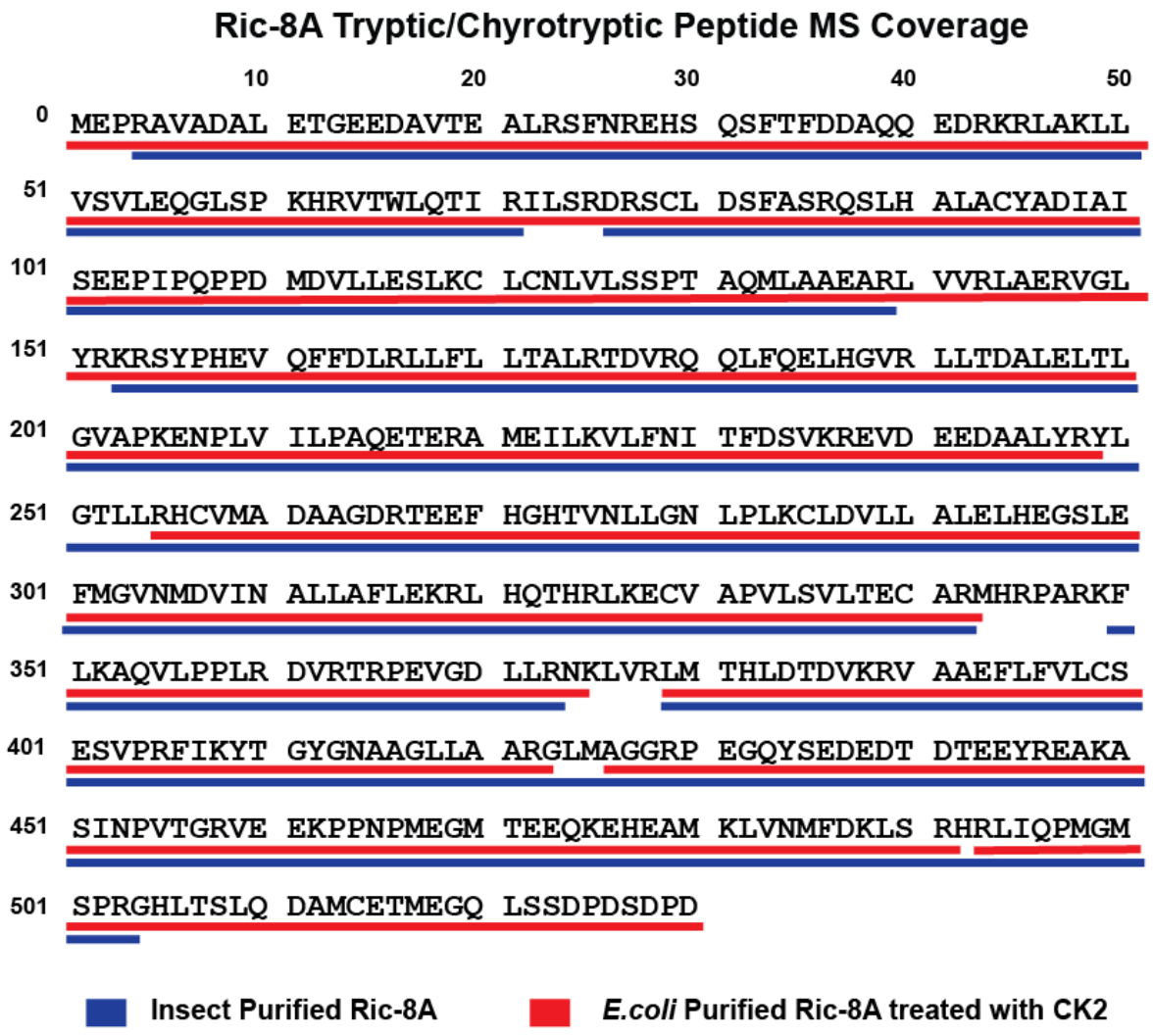

Fig. S1. Rat Ric-8A sequence and MS coverage of tryptic and chymotryptic peptides. Amino acid sequence of *Rattus norvegicus* Ric-8A, which is identical to GenBank Accession #BC160852.1 except for the probable polymorphism F232Y. Insect cell–purified recombinant rat Ric-8A was treated with trypsin before undergoing MS analysis. Fifty-eight tryptic peptides were identified that spanned the sequence underlined in blue. *E. coli*–purified recombinant rat Ric-8A was treated with CK2 and then was repurified to remove the kinase. The phosphorylated protein was subjected to tryptic and chymotryptic digestion and MS analysis. A combination of 344 peptides were identified that corresponded to the sequence underlined in red.

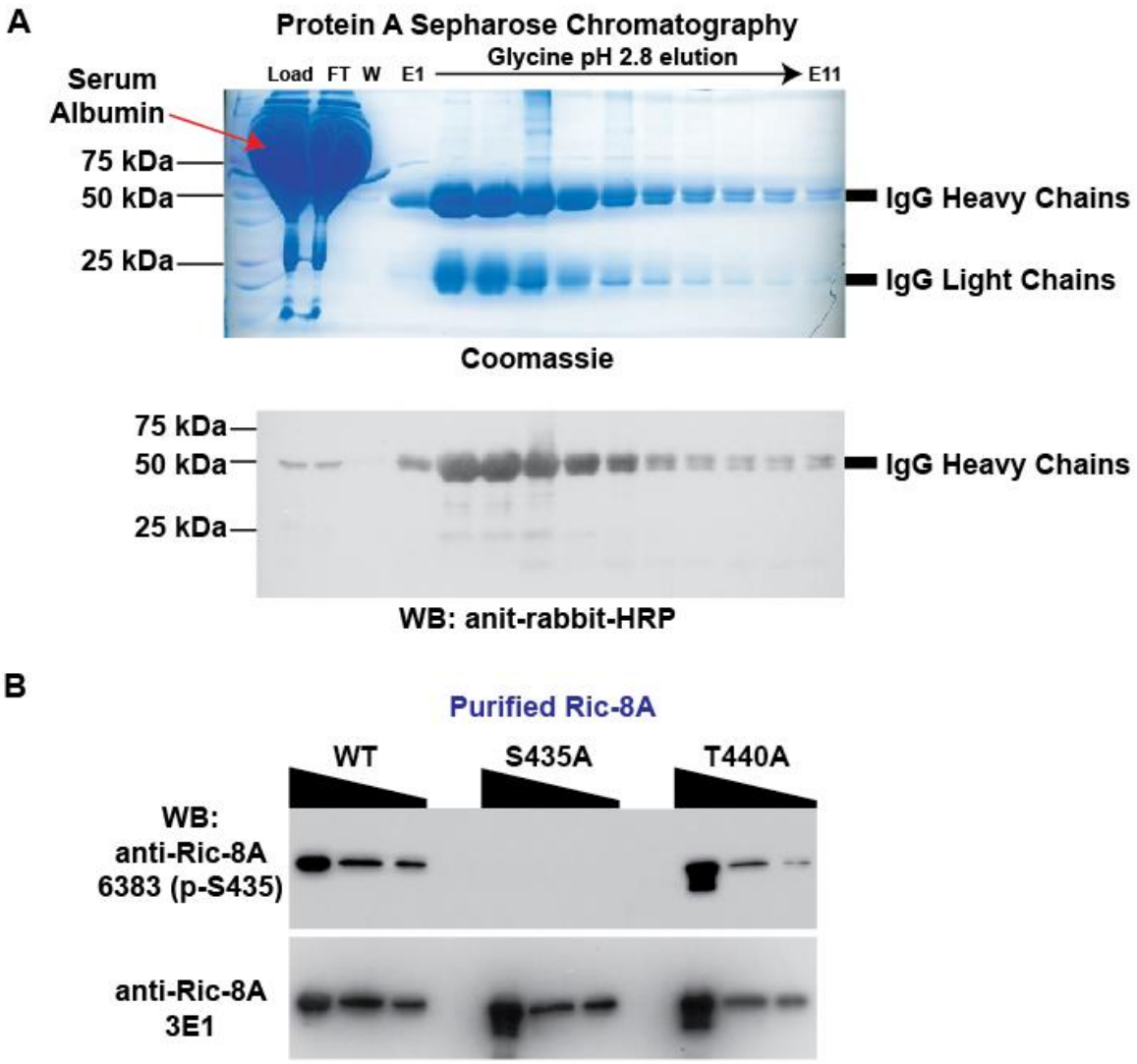

Fig. S2. Enrichment of the IgG fraction from rabbit Ric-8A p-Ser^{435} antiserum. (**A**) Clarified plasma from the rabbit antiserum was diluted 10-fold in PBS (Load) and passed over a Protein A Sepharose column. The column flow through (FT) was collected to evaluate proteins not bound to the resin. The column was washed with PBS (Wash, W) and eluted with 100 mM glycine-HCl (pH 2.8). Fractions (E1, E2, etc.) were collected and immediately neutralized with 100 mM Tris-HCl (pH 7.7). The IgG-containing fractions were pooled and passed over a PD-10 desalting column to exchange the buffer into PBS. Column fractions were resolved by SDS-PAGE and visualized by Coomassie staining (top) and direct Western blotting with a HRP-conjugated anti-rabbit antibody (bottom). (**B**) Purified WT, S435A, and T440A Ric-8A proteins produced in insect cells (15, 33, and 150 ng, as indicated) were analyzed by Western blotting with the enriched Ric-8A 6383 IgG pool and the Ric-8A monoclonal antibody 3E1 (*11*). Blots are representative of at least three independent experiments.

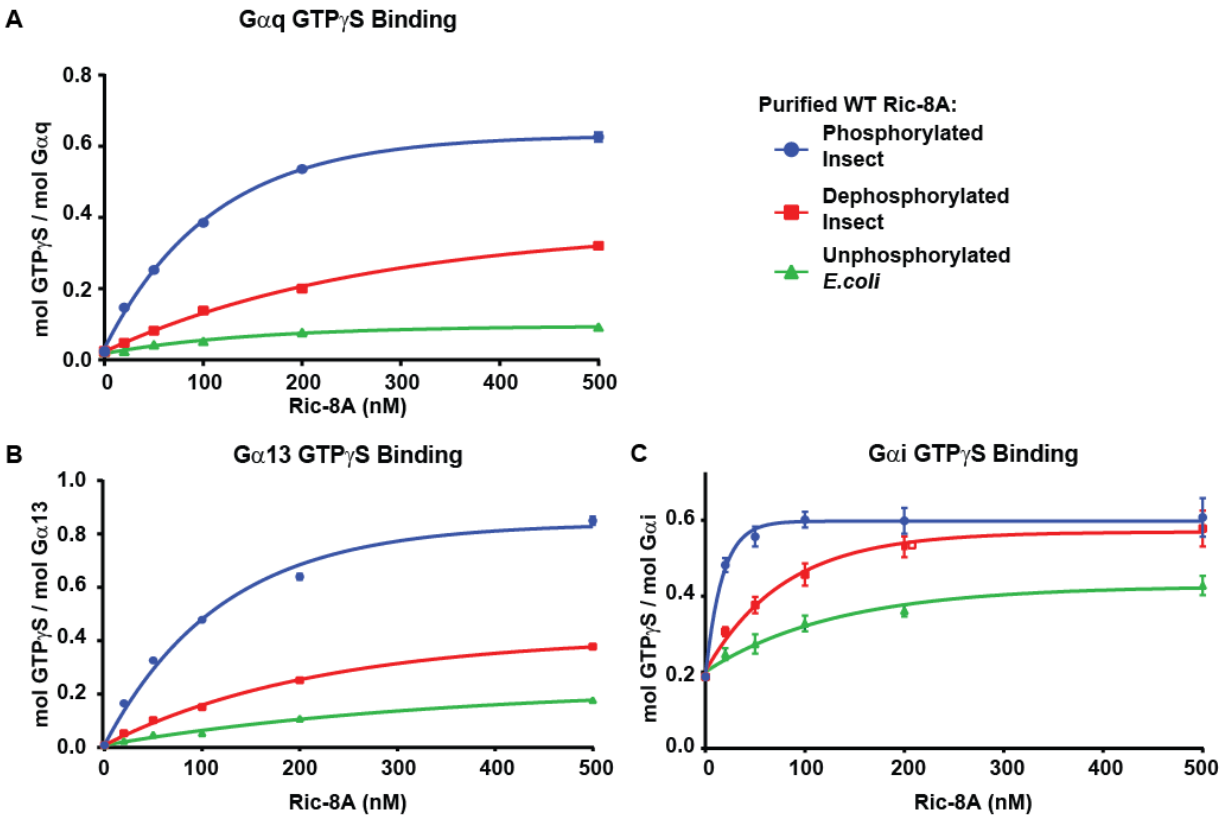

Fig. S3. Stimulation of GTPγS binding to $G\alpha_i$, $G\alpha_q$, and $G\alpha_{13}$ by phosphorylated and unphosphorylated Ric-8A. (**A** to **C**) The effect of phosphorylated (blue), enzymatically dephosphorylated (red), or unphosphorylated (green) Ric-8A proteins on the rate of GTPγS binding to (A) $G\alpha_q$, (B) $G\alpha_{13}$, and (C) $G\alpha_{i1}$. [^{35}S]-GTPγS binding assays were performed with 100 nM Gα protein and 0 to 500 nM purified recombinant Ric-8A protein, as indicated. Reactions were incubated for 8 min for $G\alpha_i$ or $G\alpha_{13}$ and for 10 min for $G\alpha_q$ and then were quenched and filtered onto BA85 nitrocellulose filters to capture the [^{35}S]-GTPγS–bound Gα protein. The filters were washed, dried, and subjected to scintillation counting to determine the fraction of GTPγS-bound Gα protein. Data are means ± SEM of three experiments. Curves were fit to one-phase exponential association functions with GraphPad Prism software.

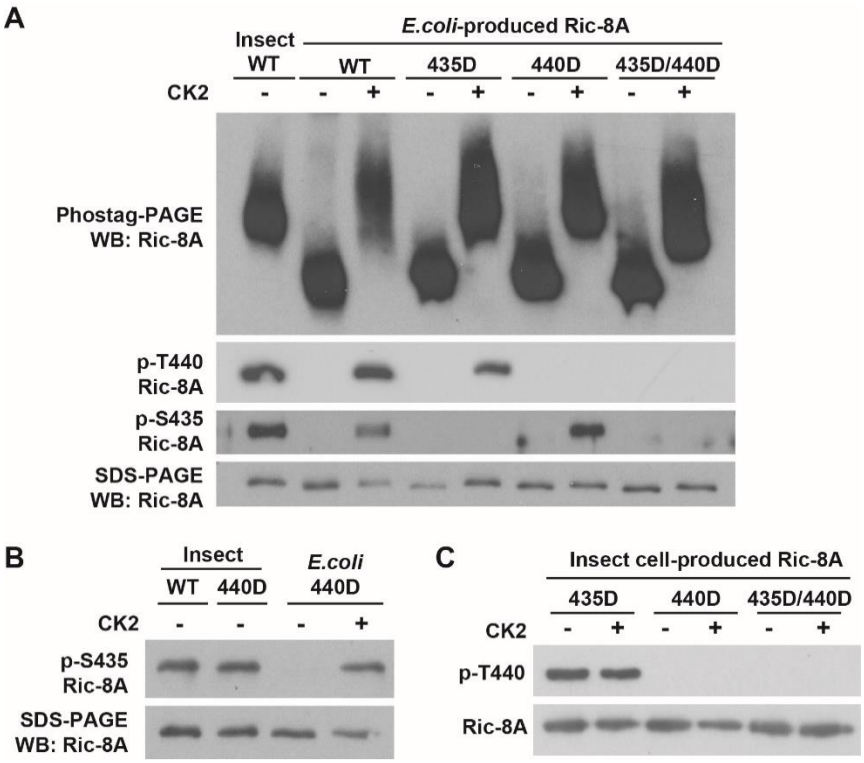

Fig. S4. Western blotting analysis of Ric-8A aspartic acid phosphorylation site mutants. (**A** to **C**) Ric-8A Thr^{440} or Ser^{435} with the indicated aspartic acid substitutions was purified from insect cells or *E. coli* and treated with or without CK2. (A) The proteins were resolved by standard SDS-PAGE or phostag-PAGE and analyzed by Western blotting with 1184 Ric-8A antiserum, the commercial CK2 consensus site antibody (pS/pT)-D-X-E (which recognizes Rat Ric-8A phosphorylated at Thr^{440}), and the 6383 Ric-8A antibody (which recognizes Ric-8A phosphorylated at Ser^{435}). (B) WT or T440D Ric-8A purified from insect cells and Ric-8A T440D treated with or without CK2 were resolved by SDS-PAGE and then analyzed by Western blotting with the 6383 Ric-8A IgG pool or the 1184 Ric-8A antiserum to assess phosphorylation of Ric-8A at Ser^{435} by CK2 in the T440D mutant background. (C) The indicated purified Ric-8A proteins were resolved by SDS-PAGE and analyzed by Western blotting with the pT440 commercial antibody to assess the effect of mutation of Ric-8A Thr^{440} to aspartic acid on recognition of that site by the phosphorylation-specific Thr^{440} antibody. Data are representative of at least three independent experiments.

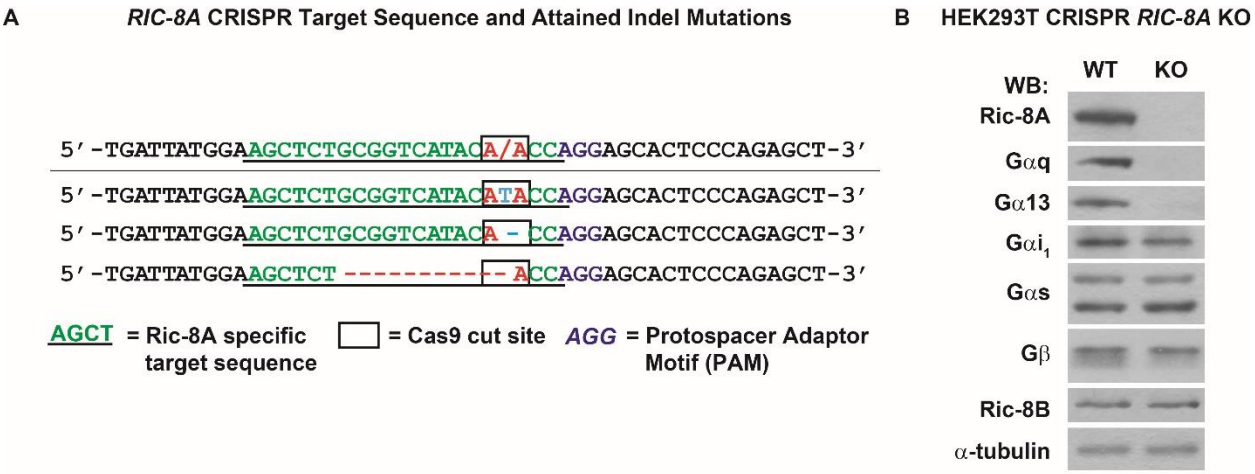

Fig. S5. Validation of the CRISPR-Cas9–generated *RIC-8A*–null HEK293T cell line. (**A** and **B**) *RIC-8A* knockout cell clones were isolated by dilution cloning, propagated, and *RIC-8A* knockout was verified by genomic sequencing and Western blotting analysis with the 1184 Ric-8A antiserum. (A) The indicated indel mutations were observed in the different copies of the *RIC-8A* gene: a single deletion of adenine, a single insertion of thymidine, and an 11-bp deletion. The presence of three copies of *RIC-8A* is reasonable because HEK 293T cells are hypotriploid, possessing two to three copies of each chromosome (*56*–*58*). All observed indel mutations caused premature termination. At least 10 isolates of a PCR amplicon of *RIC-8A* were sequenced. (B) Western blotting analysis of Ric-8A, Ric-8B, and G proteins in the HEK 293T cells and the *RIC-8A*-null HEK 293T cell line (KO). Western blots are representative of at least three independent experiments.

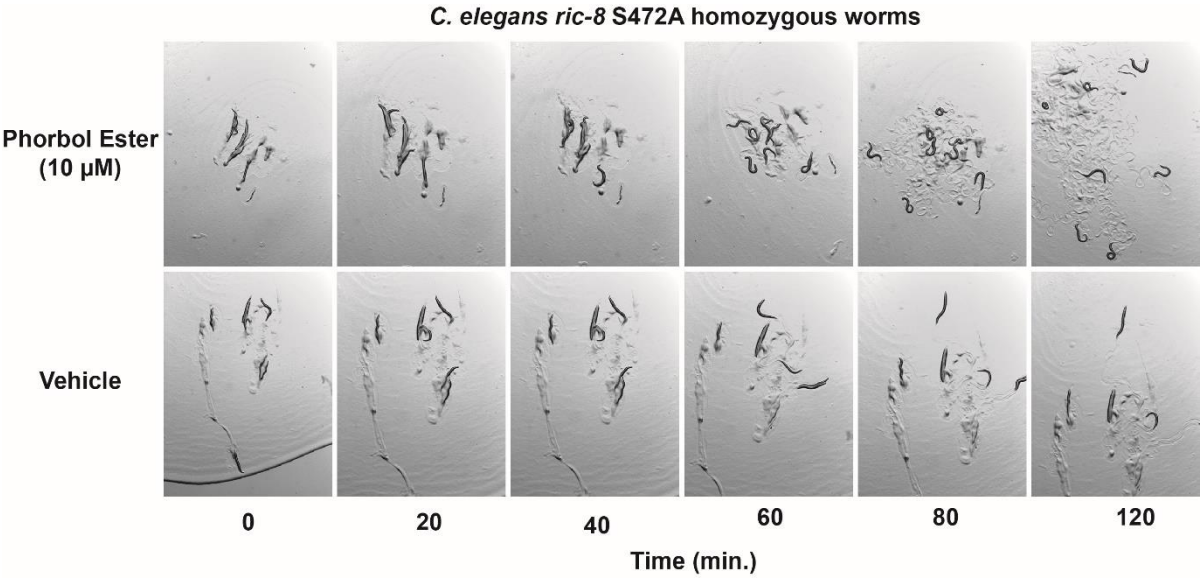

Fig. S6. Locomotion and body posture defects in *C. elegans ric-8A*–S472A mutants are rescued by phorbol ester. *C. elegans* strains modified by CRISPR/Cas9 to express *ric-8* with an alanine mutation at site Ser^{472} (equivalent to rat Ric-8A Thr^{440}) were treated with 10 µM phorbol ester or ethanol over 120 min. For movements of the mutants in the presence and absence of phorbol ester, see movies S1 and S2). Images show 6 or 7 independent worms for each condition.

Table S1. MS/MS analysis of purified recombinant Ric-8A. The relative ratios of the spectra were quantified by calculating the area under the curve for each peak, and phosphorylation assignments were made by determining the number of ~80-Dalton increases in molecular mass, which were attributed to phosphorylation. All proteins were purified from High-Five insect cells, except for WT (*E. coli*), which was purified from *E. coli* BL21 (DE3) cells. The *E. coli*–purified Ric-8A protein consisted of a single 100% unphosphorylated species, despite the presence of additional peaks that were determined to be adducts of the protein with HEPES buffer. The major peaks for each protein are highlighted in green. Instances in which mutation at the identified sites Ser^{435} and Thr^{440} resulted in shifts in the overall stoichiometry of phosphorylation are highlighted in blue.

	WT (Phos.)	WT (Dephos.)	S435A	S435D	T440A	T440D	WT (*E. coli*)
Ric-8A (0 PO_4^-)	-	40.6%	-	-	-	-	100%
+ 1 PO_4^-	-	46.0%	0.8%	-	0.2%	0.1%	-
+ 2 PO_4^-	0.7%	0.8%	12.6%	2.5%	2.8%	3.7%	-
+ 3 PO_4^-	6.9%	1.5%	34.2%	19.6%	16.8%	25.3%	-
+ 4 PO_4^-	34.4%	4.5%	18.9%	32.0%	27.5%	45.6%	-
+ 5 PO_4^-	38.6%	4.1%	6.0%	1.8%	2.7%	6.1%	-
+ 6 PO_4^-	5.1%	0.9%	15.5%	12.1%	13.1%	7.4%	-
+ 7 PO_4^-	9.3%	1.7%	7.8%	19.9%	21.2%	7.6%	-
+ 8 PO_4^-	5.0%	-	-	-	-	4.2%	-
+ 9 PO_4^-	-	-	4.2%	3.8%	5.3%	-	-
+10 PO_4^-	-	-	-	8.3%	10.5%	-	-

Table S2. Representative CID analysis of Ric-8A to identify specific phosphorylation sites after tryptic digest and MS analysis. Data are provided as a Microsoft Excel spreadsheet. The file includes two sheets of data. Spreadsheet 1, entitled "Hi5 Insect," contains pertinent information for the CID analysis of recombinant rat Ric-8A purified from High-Five insect cells, digested by trypsin, and subjected to ESI-MS/MS analysis. The second spreadsheet, entitled "*E. coli* + CK2," contains pertinent information for the CID analysis of recombinant rat Ric-8A purified from BL21 *E. coli* cells, phosphorylated in vitro with CK2, repurified to remove the kinase, digested with trypsin and chymotrypsin, and then subjected to ESI-MS/MS analysis. Each sheet contains a listing of identified phosphorylated residues and the tryptic or chymotryptic peptide from which the sites were identified. The total number of peptides is identified in the "# Peptide hits" column, with the number of peptides containing phosphorylated residues listed in the "# Phos-site hits" column. A representative CID analysis of b- and y-ions has been included to validate each identified phosphorylation site, as well as the actual and predicted peptide masses and the assigned posttranslational modifications from the representative CID spectra.

Movie S1. *C. elegans ric-8A*–S472A mutant movements. CRISPR/Cas9 was used to modify the *C. elegans* Bristol N2 strain to express *ric-8*-S472A. Worms were treated with ethanol (vehicle) for 60 min before the recording. A 2-min video was captured using WormLab system (MBF Biosciences). Frame rates are shown at 5× real time speed. The file is provided in mp4 format and is 13.8 MB.

Movie S2. *C. elegans ric-8A*–S472A mutant movements after phorbol ester treatment. CRISPR/Cas9 was used to modify the *C. elegans* Bristol N2 strain to express *ric-8*-S472A. Worms were treated 10 µM phorbol ester for 60 min before the recording. A 2-min video was captured using WormLab system (MBF Biosciences). Frame rates are shown at 5× real time speed. File is provided in mp4 format and is 13.1 MB.