Supplementary Data

Phosphorylation-dependent binding of 14-3-3 terminates signalling by the Gab2 docking protein

Tilman Brummer, Mark Larance, Maria Teresa Herrera Abreu, Ruth J. Lyons, Paul Timpson, Christoph H. Emmerich, Emmy D. G. Fleuren, Gillian M. Lehrbach, Daniel Schramek, Michael Guilhaus, David E. James and Roger J. Daly

I. Six Supplementary Figures

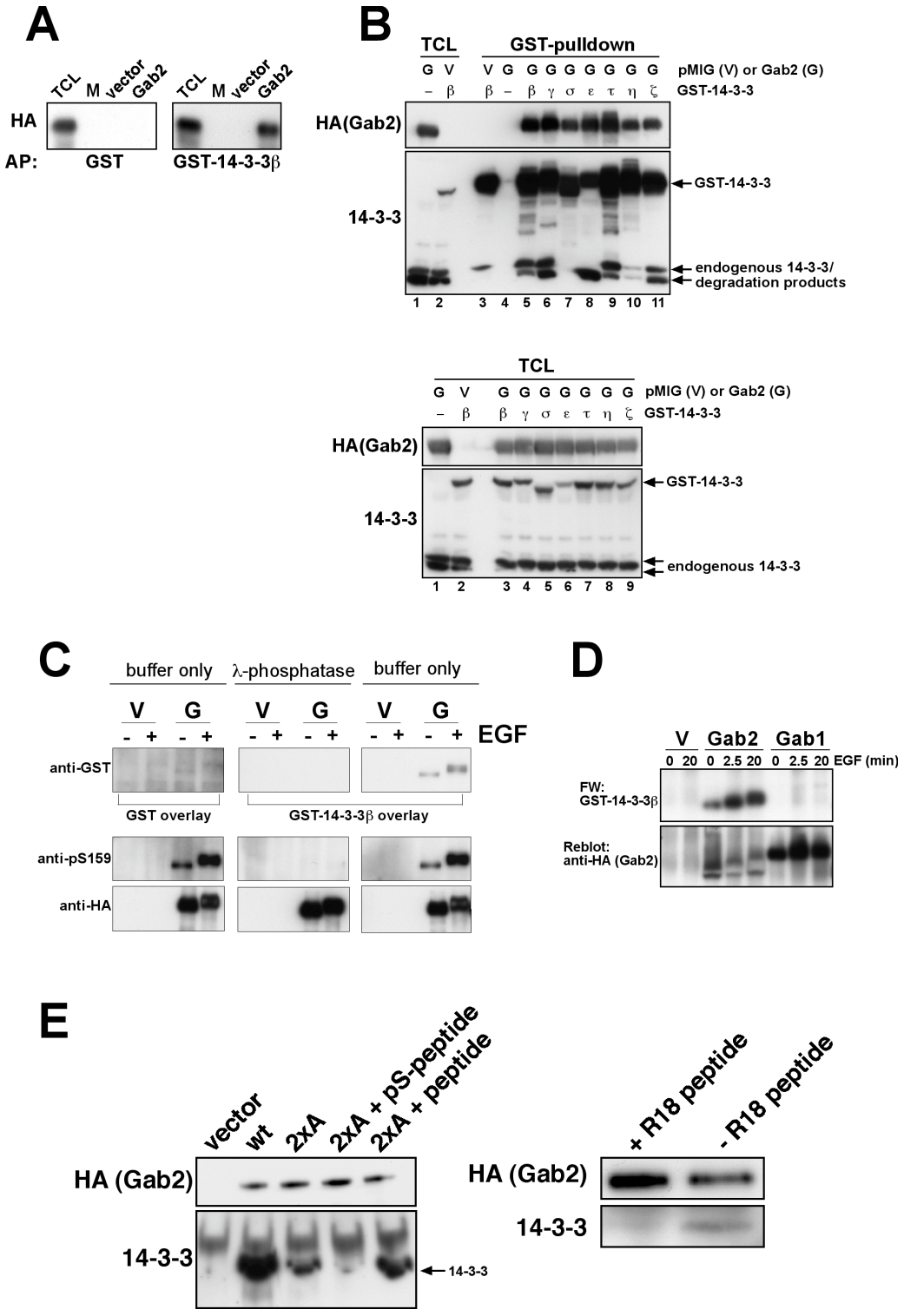

Figure S1. Binding of Gab2 to 14-3-3 proteins. (A) Phoenix-Eco cells were transfected with pMIG/Gab2 or empty pMIG. Cleared lysates were then subject to affinity purification (AP) with either GST or GST14-3-3β and isolated proteins analysed by Western blotting using an anti-HA antibody. Total cellular lysates (TCL) were used as a positive control for the detection of HA-tagged Gab2. M: protein marker lane. **(B)** Phoenix-Eco cells were transfected with either empty vector (V) or pMIG/Gab2 (G) and the indicated pSRα/GST-14-3-3 constructs corresponding to all seven human 14-3-3 proteins (Beguin et al., 2005). Expressed GST-14-3-3 fusion proteins were purified from the cell lysates using glutathionine-sepharose and subject to Western blot analysis (upper panel). The

expression level of HA-tagged Gab2 and the individual GST-14-3-3 proteins is shown in the Western blot analysis of cell lysates (lower panel). **(C)** Anti-HA immunoprecipitates from MCF-10A cells infected with either empty pMIG vector (V) or pMIG/Gab2 (G) were separated by SDS-PAGE and transferred to PVDF membranes. Following transfer, the membranes were treated with λ-phosphatase or buffer only as described previously (Brummer et al., 2003). The membranes were then incubated with GST-14-3-3β fusion protein or GST alone and bound GST proteins were detected with an anti-GST antibody. Successful dephosphorylation of Gab2 by λ-phosphatase was confirmed by Western blotting using anti-pS159 phospho-specific antibodies. **(D)** HA-tagged Gab2 or Gab1 was purified from EGF-stimulated MCF-10A cells and subject to Far Western Blot (FW) analysis using GST-14-3-3β. (**E**) Competition of residual 14-3-3 binding to $Gab2^{2xA}$ using two types of 14-3-3-binding synthetic peptides. $Gab2^{2xA}$-expressing cells were stimulated with EGF for 2.5 min and lysed. Left panel: Lysates were incubated with phosphorylated (pS-) or non-phosphorylated peptides containing the 14-3-3 binding motif around S585 from the GM-CSFR common β chain (Guthridge et al., 2000) prior to anti-HA immunoprecipitation and Western blot analysis. Right panel: Lysates were incubated with or without R18 peptide prior to anti-HA immunoprecipitation and Western blot analysis.

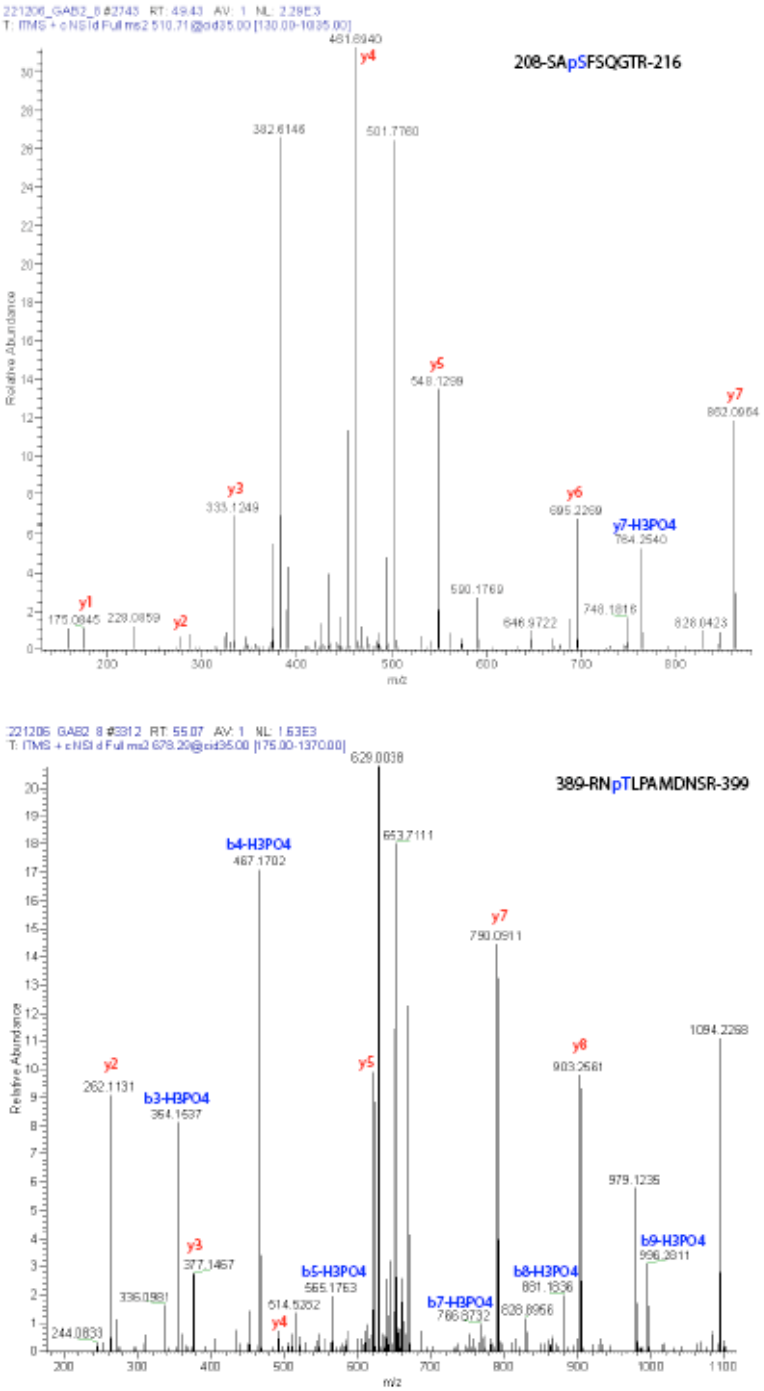

Figure S2. MS/MS spectra of peptides containing pS210 and pT391. Gab2 was isolated via anti-HA immunoprecipitation from EGF-stimulated MCF-10A cells and subjected to LC-MS/MS. Spectra generated by collision-induced dissociation in the LTQ linear ion trap are shown for phosphopeptides encompassing S210 (upper panel) and T391 (lower panel). Red labels indicate the y-ion series, blue labels indicate ions showing sequence specific loss of phosphoric acid. Representative spectra from more than three independent experiments are shown.

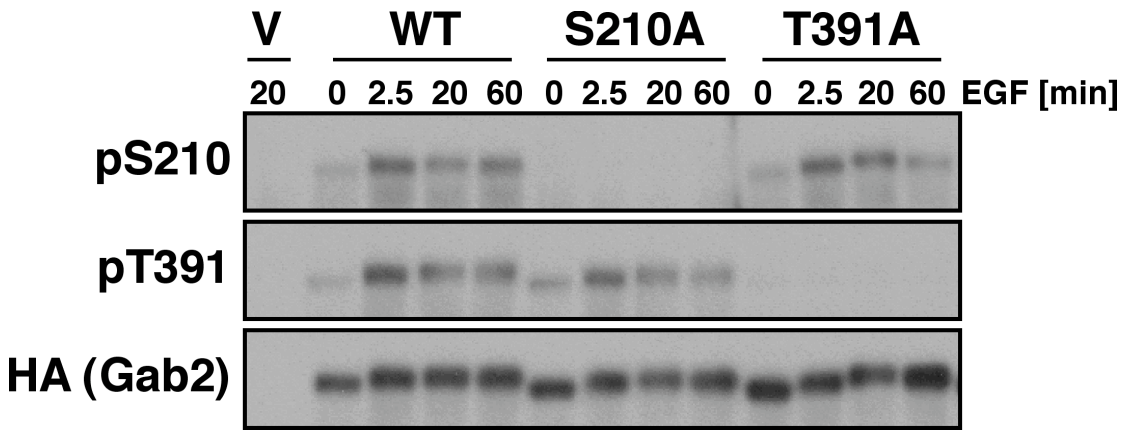

Figure S3. Validation of phosphospecific antibodies. MCF-10A cells expressing the indicated Gab2 proteins were stimulated with EGF for the indicated times. Total cellular lysates were then prepared and Western blotted with the indicated antibodies. V: vector control.

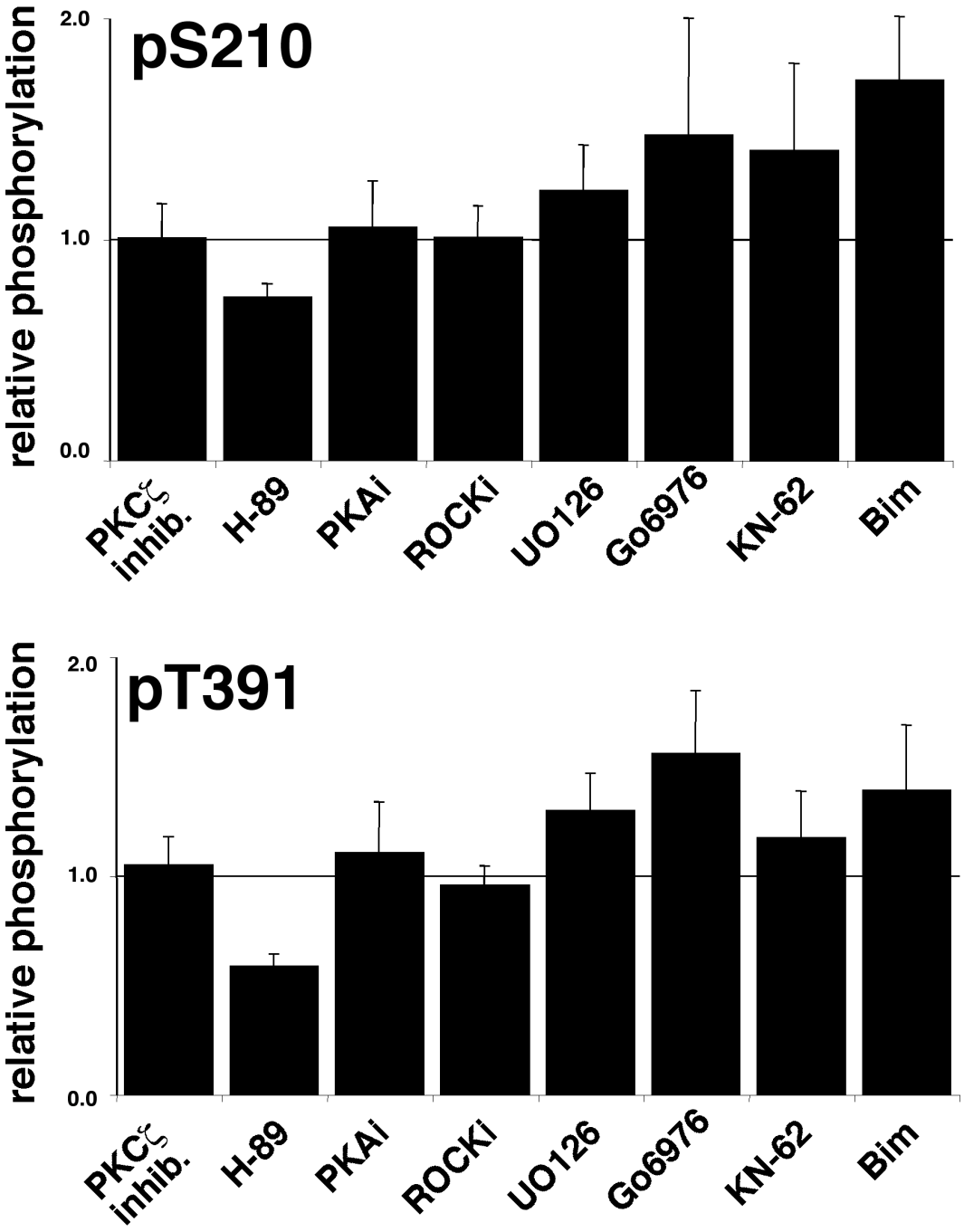

Figure S4. Effect of particular kinase inhibitors on S210 and T391 phosphorylation. MCF-10A/Gab2 cells were pre-incubated with the indicated inhibitors as described in Supplementary Methods and then stimulated with EGF (100 ng/ml, 2.5 min). Cell lysates were Western blotted with anti-pS210, -pT391 and –HA antibodies. Following normalization for Gab2 levels, EGF-induced phosphorylation on these sites was expressed relative to the appropriate vehicle control, which is arbitrarily set at 1.0. The values represent the mean of at least 3 determinations, and bars standard error. Abbreviations are as follows: PKAi, cell-permeable PKA inhibitory peptide; ROCKi, Y27632; Bim, bisindolylmaleimide I.

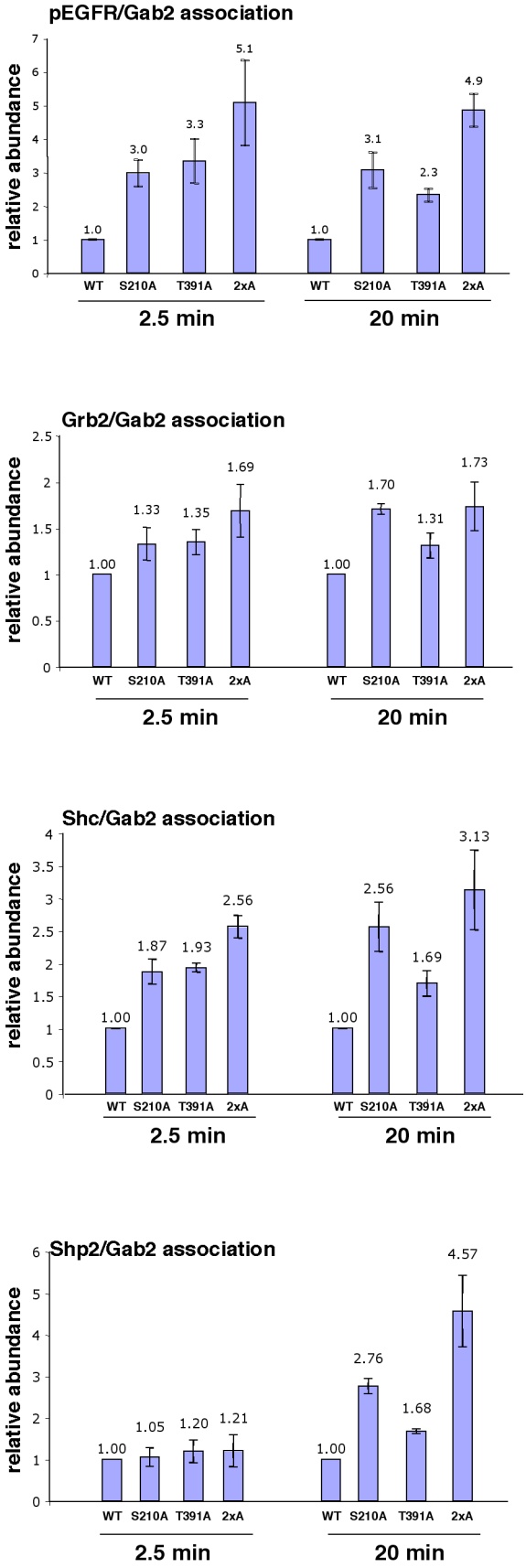

Figure S5. Effect of single and combined S210A and T391A substitutions on assembly of Gab2 signalling complexes. Data were analysed as for Figure 4C and are derived from three to four independent experiments.

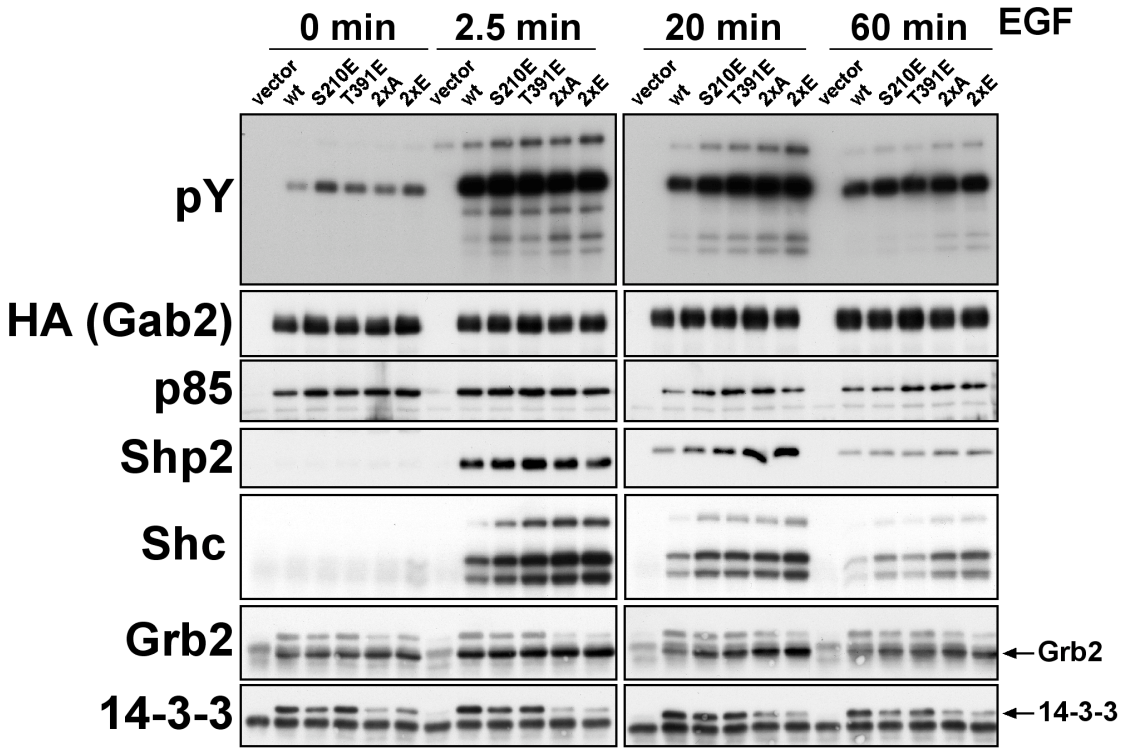

Figure S6. Mutation of S210 and T391 to either alanines or glutamates leads to sustained signalling. MCF-10A pools were stimulated with EGF for different times. Anti-HA immunoprecipitates were then Western blotted with the indicated antibodies.

II. Supplementary Table 1

List of phosphorylation sites identified in this study. Novel phosphorylation sites are shown in bold, potential or confirmed 14-3-3 binding sites are shown in red with the Scansite prediction score indicated. Phosphorylation sites present on the same proteolytic peptide are indicated by a particular number of asterisks. As quantitation was performed on singly-phosphorylated peptides, it represents the average response for each of the phosphorylated sites present on the peptide. A site was considered as EGF-inducible if the increase in phosphorylation was > 2-fold. Highly EGF-inducible indicates a >10-fold increase. Peptides were either generated by tryptic (T) or chymotryptic (C) digest. MS/WB indicates confirmation by both LC-MS/MS and Western blotting. Phosphorylation sites S159 and S623 have been described previously (Arnaud et al., 2004; Lynch and Daly, 2002). Phosphorylation of the S264 and Y293 equivalents in murine Gab2 was described while this manuscript was in preparation (Cao et al., 2007).

Residue	Protease	Sequence (putative 14-3-3 binding motif italicised)	Scansite prediction (score)	Phosphorylation status
S133	T/C	LRNVS**S**AGHGP		Highly EGF-inducible
S140	T/C	GH*GPR**S**SP*AEL	Low (0.6049)	EGF-inducible*
S141	T	HGPRS**S**PAELS		EGF-inducible*
S148	T	AELSS**S**SQHLL		EGF-inducible**
S149	T	ELSSS**S**QHLLR		EGF-inducible**
S159[#]	C	RE*RKSS**A**PS*H	**High** (0.3133)	EGF-inducible (MS/WB)
S164	C	SAPSH**S**SQP		Only Double Phosphopeptide Observed Not Quantifiable
S210	T	NA*RSA**S**FS*QGT	**Medium** (0.3821)	EGF-inducible (MS/WB); Insulin inducible (MS)
S218	T/C	QGTRA**S**FLMRS		Too Small m/z Not Quantifiable
S223	T	SFLMR**S**DTAVQ		EGF-inducible
S264	T	TEFRD**S**TYDLP		EGF-inducible
S278	C	ASHGH**T**KGSLT		Constitutively phosphorylated
S281	T	GHTKG**S**LTGSE		EGF-inducible***
T287	T/C	LTGSE**T**DNEDV		EGF-inducible***
Y293	T	DNEDV**Y**TFKTP		EGF-inducible***
T331	T	IP*RTF**T**LD*KNH	Low (0.5517)	EGF-inducible
T385	T	RSVAA**T**IPRRN		Constitutively phosphorylated
T391	**T**	IP*RRN**T**LP*AMD	**High** (0.2883)	EGF/insulin-inducible
S405	T	LH*RAS**S**CE*TYE	Low (0.5601)	Constitutively phosphorylated
S480	C	VYIPM**S**PGAHH		Constitutively phosphorylated
S543	T	ELPFK**S**PITKS		Highly EGF-inducible
S622	T	LDFQP**S**SPSPH		EGF-inducible****
S623[*]	T	DFQPS**S**PSPHR		EGF-inducible****

III. Supplementary Methods

Retrieval of Gab docking protein sequences and their alignments

Sequence alignments were performed using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) and a BLOSUM62 matrix.The following sequences were used (with accession numbers in brackets): human Gab2 (NM_080491.1), rodent Gab2 (*Mus musculus*; BC103524.1, *Rattus norvegicus*; NM_053417.1), Opossum Gab2 (*Monodelphis domestica*; XM_001362752.1), chicken Gab2 (*Gallus gallus*; XM_001234548.1), puffer fish Gab2 (*Tetraodon nigriviridis*; CAAE01015113.1), human Gab1 splice variant A (AAH64848.1) and Gab3 (AAL25825.1).

Protein purification and peptide preparation for LC-MS/MS analysis

For the analysis of Gab2 signalosomes using LC-MS/MS, Gab2 complexes were purified using 70 μl of a self-made anti-HA antibody/agarose beads conjugate using the monoclonal anti-HA 16B12 antibody from Covance. Following an overnight incubation with a total cell lysate from seven to ten 15 cm dishes of subconfluent MCF-10A cells (approx. 10^8 cell equivalents), the beads were washed 6 times with 1 ml of normal lysis buffer. After the final wash, the supernatant was removed and the beads were resuspended in 500 μl PBS and the suspension was transferred to Handee-Minispin columns (Pierce) and spun twice at 500 x g at 4ºC for 1 min to remove all fluid phase. Subsequently, the beads were overlaid with 20 μl of 1 x Sample buffer supplemented with 50 mM TCEP Bond Breaker Neutral pH solution (Pierce) and incubated at 65ºC for 5 min. Following a spin at 500 x g for 1 min, the fluid phase (approx. 20 μl) was recovered and loaded on a 10 % SDS-PAGE. Following electrophoresis, the gel was stained with Sypro-Ruby (Invitrogen) according to the manufacturer's instructions. For in-gel digestion, gel bands of interest were excised and de-stained twice in 1 ml of 50% acetonitrile, 250 mM ammonium bicarbonate at room temperature for 30 min with shaking. The gel slice was dehydrated by incubation in 1 ml of 100% acetonitrile for 10 min at room temperature with shaking. All solution was carefully removed prior to the addition of modified trypsin (12.5 ng/l) in 100 mM NH_4HCO_3 and incubation overnight at 37ºC. The digestion was stopped and peptides extracted by the addition of 0.1 ml of 5% formic acid and incubation at 37ºC for 1 h. Further extraction was performed by the addition of 0.1 ml

of 100% acetonitrile and incubation at 37ºC for 1 h. Complete dehydration of the gel slice was mediated by the addition of 0.5 ml of 100% acetonitrile and incubation at 37ºC for 1 h. The entire supernatant was then removed (0.75 ml) and placed into a fresh tube and vacuum dried. The peptides were re-dissolved in 20 μl of 5% formic acid for LC-MS/MS analysis. Peptide solutions (5 μl) were automatically injected using an auto-sampler and desalted on a capillary C18 cartridge. Peptides were subsequently resolved on a 100 mm x 75 μm C18 Magic reverse phase analytical column with a flow rate of 200 nl/min. Peptides were ionised by nano-electrospray ionisation at 2.8 kV from the end of the column which was pulled to an internal diameter of 5 μm by a P-2000 laser puller (Sutter Instruments Co). Tandem mass spectral analysis was carried out on a Thermo Electron Corporation (San Jose, CA) LTQ-FT Ultra mass spectrometer. A data-dependent acquisition method was used for all experiments where precursor ions needed to have intensity higher than 10 counts and be in the +2, +3, or +4 charge state.

LC-MS/MS based phosphopeptide analysis

In order to specifically identify phosphopeptides and their corresponding phosphorylation sites, data-dependent neutral loss MS^3 experiments were carried out using an LTQ-FT Ultra (Thermo Electron Corp, San Jose, CA) linear ion trap. The peptide digests used for the LC-MS/MS experiments described above were separated by reverse phase chromatography and directly electrosprayed into the LTQ-FT Ultra mass spectrometer for analysis. The LTQ-FT Ultra was setup to acquire an initial MS scan of all peptides currently eluting from the column and to conduct MS^2 experiments on the five most intense ions. If a neutral loss of H_3PO_4 was observed which is characteristic of peptides containing phospho-serine or phospho-threonine residues the instrument would conduct a subsequent MS^3 analysis of the neutral loss product ion. This extra analysis step dramatically improves sequence specific fragment ion formation from phosphopeptides. In order to quantify the ratio of non-phosphorylated to phosphorylated peptides extracted ion chromatograms were created for each phosphopeptide and its cognate non-phosphopeptide. The area under each peak was integrated and ratios calculated. The fold induction for each peptide was calculated by dividing the ratio of each phosphopeptide by the unstimulated or 0 min sample.

Mascot Database Searching

MS/MS spectra were searched against a metazoan database generated from Swiss-prot and Trembl containing 574734 sequences, using Mascot (Matrix Science Inc., Boston, MA). One missed cleavage per peptide was tolerated and peptides could be partially tryptic. Variable modifications included methionine oxidation, propionamide (acrylamide) modified cysteine and phosphorylated serine, threonine or tyrosine. Peptide tolerance was 5 ppm and the tolerance for peptide fragments was 1 Da. Peptides were counted as valid if they had a peptide score above 30. Proteins were counted as valid if identified by two or more different peptides.

Kinase inhibitor assays

Sources and final concentrations of kinase inhibitors were as follows: wortmannin (100 nM, Sigma); LY294002 (10 μM, Merck); Go6976 (5 μM, Biomol); Akt-I-1/2 (5 μM, a gift from Dr Peter Shepherd, Auckland); rapamycin (100 nM, Merck); bisindolylmaleimide I (Go6850) (10 μM, Alexis Biochemicals); cell-permeable PKCζ inhibitory peptide (5 μM, Merck); cell-permeable PKA inhibitory peptide (10 μM, Merck); UO126 (5 μM, Promega); H89 (10 μM, Merck); Y27632 (10 μM, Merck); KN62 (10 μM, Sigma). Cells were pretreated with each inhibitor, or the corresponding vehicle control, for 40 min (30 min for Akt-I-1/2) prior to EGF stimulation.

Supplementary References

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