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

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Grb2-associated binder (Gab)2 functions downstream of a variety of receptor and cytoplasmic tyrosine kinases as a docking platform for specific signal transducers and performs important functions in both normal physiology and oncogenesis. Gab2 signalling is promoted by its association with specific receptors through the adaptor Grb2. However, the molecular mechanisms that attenuate Gab2 signals have remained unclear. We now demonstrate that growth factor-induced phosphorylation of Gab2 on two residues, S210 and T391, leads to recruitment of 14-3-3 proteins. Together, these events mediate negative-feedback regulation, as Gab2$^{S210A/T391A}$ exhibits sustained receptor association and signalling and promotes cell proliferation and transformation. Importantly, introduction of constitutive 14-3-3-binding sites into Gab2 renders it refractory to receptor activation, demonstrating that site-selective binding of 14-3-3 proteins is sufficient to terminate Gab2 signalling. Furthermore, this is associated with reduced binding of Grb2. This leads to a model where signal attenuation occurs because 14-3-3 promotes dissociation of Gab2 from Grb2, and thereby uncouples Gab2 from the receptor complex. This represents a novel regulatory mechanism with implications for diverse tyrosine kinase signalling systems.

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Introduction

Docking proteins, which in mammals include the Grb2-associated binder (Gab) and insulin receptor substrate (IRS) families, represent key regulatory nodes in tyrosine kinase signalling networks (Schlessinger, 2000; Gu and Neel, 2003; Araujo *et al*, 2007). Following tyrosine phosphorylation, they function as assembly platforms at the plasma membrane for specific SH2 domain-containing effectors, leading to activation or attenuation of particular downstream pathways. In addition, they may be subject to complex patterns of positive and negative feedback regulation that modulate the amplitude, kinetics and frequency of signal output and allow integration of different receptor signalling systems.

There are three Gab proteins in mammals, Gab1-3. These exhibit a conserved architecture, containing an N-terminal pleckstrin homology (PH) domain, one canonical and one atypical Grb2 SH3 domain-binding site and multiple tyrosine phosphorylation sites (Gu and Neel, 2003). Following tyrosine phosphorylation, all Gab proteins recruit the protein tyrosine phosphatase Shp2 and the p85 subunit of phosphatidylinositol (PI)3-kinase, leading to potentiation of the Ras/Erk and PI3-kinase/Akt pathways (Gu and Neel, 2003). Gab2 is tyrosine-phosphorylated upon activation of a variety of growth factor, hormone, cytokine, antigen and cell matrix receptors (Gu and Neel, 2003) and performs important functions in normal physiology. It is required for haematopoiesis (Zhang *et al*, 2007), allergic responses (Gu *et al*, 2001), osteoclast differentiation (Wada *et al*, 2005) and, in combination with Gab1, for cardiac function (Nakaoka *et al*, 2007). In addition, Gab2 exhibits transforming activity when relieved of negative feedback control (Lynch and Daly, 2002) and is strongly implicated in several human malignancies. For example, Gab2 is required for transformation of myeloid cells by the Bcr-Abl oncoprotein that drives chronic myelogenous leukaemia (Sattler *et al*, 2002). In addition, Gab2 is overexpressed in breast cancer (Daly *et al*, 2002) and promotes the growth and metastasis of erbB2-induced mouse mammary tumours (Bentires-Alj *et al*, 2006; Ke *et al*, 2007).

The important functions of Gab2 in signalling systems that underpin both normal physiological responses and oncogenesis highlight the importance of defining its control mechanisms. Tyrosine phosphorylation of Gab proteins is promoted by their association with particular receptors. Whereas Gab1 can bind MET directly, other recruitment mechanisms are indirect and require Grb2, which binds the Gab protein through its C-terminal SH3 domain and specific tyrosine-phosphorylated targets through its SH2 domain (Gu and Neel, 2003). In some cases, for example, binding of Gab2 to ErbB2 (Bentires-Alj *et al*, 2006), the adaptor 'bridge' consists of only Grb2. In others, the Grb2 SH2 domain binds an intermediary protein. For example, a Grb2/Shc complex mediates Gab2 binding to the 'common β chain' (βc) of various cytokine receptors (Gu *et al*, 2000). In addition, binding of the Gab PH domain to plasma membrane-localized

PI3-kinase products also promotes receptor coupling (Rodrigues *et al*, 2000), and this may be particularly important at low levels of receptor activation (Sampaio *et al*, 2008). However, Gab proteins are also subject to negative regulation. For example, Akt and Erk attenuate Gab2 signalling by phosphorylation of S159 and S623, respectively (Lynch and Daly, 2002; Arnaud *et al*, 2004), although the detailed molecular mechanisms are unclear.

In this study, we have addressed negative-feedback regulation of Gab2 using an integrated approach that combines a proteomics-based definition of the Gab2 phosphomap with bioinformatics, biochemistry and cell biology. This has identified a novel, 14-3-3-mediated mechanism for termination of Gab2 signalling that promotes dissociation of the Gab2/receptor complex.

Results

Gab2, but not Gab1, recruits 14-3-3 proteins

As specific protein sequence motifs containing phosphorylated serine and threonine residues can function as binding sites for regulatory proteins, such as members of the 14-3-3 family (Mackintosh, 2004; Aitken, 2006) or those containing WW domains (Seet *et al*, 2006), we hypothesized that a proteomic approach to identify novel interaction partners of Gab2 may provide an insight into negative-feedback control mechanisms. To this end, we utilized the MCF-10A mammary epithelial cell line, which represents a powerful model for studying the function of Gab2 in growth factor signalling and cell transformation (Bentires-Alj *et al*, 2006; Brummer *et al*, 2006b). MCF-10A cells expressing high levels of HA-tagged Gab2 (Brummer *et al*, 2006b) were stimulated with EGF and Gab2 complexes purified by immunoprecipitation. Protein bands specific to Gab2 immunoprecipitates were excised and identified by liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS). This identified several known interaction partners of Gab2, including the EGF receptor (EGFR) (Figure 1A), and the PI3-kinase subunits p85α and β, which co-migrated with Gab2. In addition, we detected two prominent bands at approximately 30 kDa (Figure 1A). These contained all seven members of the 14-3-3 protein family (Mackintosh, 2004; Aitken, 2006) (Figure 1A). To confirm that these interactions were physiologically relevant, we utilized a separate pool of MCF-10A cells expressing lower levels of Gab2 similar to those observed in human breast cancer (Brummer *et al*, 2006b). Anti-HA immunoprecipitations from this pool confirmed the Gab2/14-3-3 interaction and revealed that it was EGF dependent (Figure 1B). Recruitment of 14-3-3 also exhibited selectivity among Gab family members, as only a low basal level of 14-3-3 associated with Gab1. An interaction between endogenous Gab2 and 14-3-3 proteins was also observed in MCF-7 breast cancer cells, where β-heregulin simulation led to a 1.5-fold increase in Gab2-bound 14-3-3 (Figure 1C), and upon cross-linking of FcεRI antigen receptors on RBL-2H3 rat basophilic leukemia cells (Figure 1D). Co-expression of each 14-3-3 isoform as a glutathionine-*S*-transferase (GST) fusion protein with HA-Gab2 followed by GST-pull-downs and anti-HA blotting confirmed that all 14-3-3 isoforms are capable of interacting specifically with Gab2 (Supplementary Figure S1). Far-western blotting confirmed the specific, direct and phosphorylation-dependent nature of the Gab2/14-3-3 interaction and lack of 14-3-3 binding to Gab1 (Supplementary Figure S1C and D). Taken together, these findings establish Gab2 as a novel 14-3-3 client protein.

Determination of the 14-3-3-binding sites on Gab2

Next, we addressed the mechanism underlying the Gab2/14-3-3 interaction. A Scansite analysis (Obenauer *et al*, 2003) of the human Gab2 amino-acid sequence for predicted 14-3-3-binding sites revealed three high- and three medium-scoring sites corresponding to the mode 1 motif RXXpS/TXP (Figure 2A). These motifs are highly conserved in all known vertebrate Gab2 orthologues, but not in the human Gab1 and Gab3 paralogues, consistent with the lack of direct Gab1/14-3-3 binding. To test the function of the six high/medium-scoring sites, we performed systematic site-directed mutagenesis, substituting non-phosphorylatable alanine for the serine or threonine residue. Sites were altered singly or in combination, and association of the mutant Gab2 proteins with 14-3-3 assayed by co-immunoprecipitation and Far-western blot analysis (Figure 2B and C). Mutation of S159 and S668, two high-scoring sites, did not attenuate 14-3-3 binding. However, the S210A and T391A mutants exhibited reduced activity in these assays, whereas 14-3-3 binding by the S210A, T391A ($2 \times A$) double mutant was markedly impaired. Indeed, Gab2$^{2 \times A}$ was as deficient in recruiting 14-3-3 proteins as mutants with mutations in four (Figure 2B and C) or six consensus sites (data not shown). Of note, as the residual 14-3-3 binding to Gab2$^{2 \times A}$ immunoprecipitates was competed by two synthetic 14-3-3 peptide ligands, but not a control peptide (Supplementary Figure S1E), it reflects specific interactions mediated by the 14-3-3 peptide-binding groove. This residual binding may be conferred by other 14-3-3 client proteins in the signalling complex, such as the EGFR and Shc (Foschi *et al*, 2001; Oksvold *et al*, 2004) and/or putative low-affinity 14-3-3-binding sites on Gab2 (Supplementary Table S1).

Characterization of the Gab2 'phosphomap'

To confirm that S210 and T391 are phosphorylated *in vivo*, mass spectrometry was then used to map Gab2 post-translational modifications. This revealed that Gab2 is subject to a complex pattern of serine/threonine phosphorylation events that differ in their temporal regulation as well as growth factor and pathway dependency. Nineteen novel serine/threonine phosphorylation sites were identified, including S210 and T391 (Supplementary Figure S2 and Supplementary Table S1). Phosphorylated peptides corresponding to the low-scoring 14-3-3-binding sites S140, T331 and S405 were detected, but we did not detect phosphorylation of the medium/high-scoring binding sites T259, S445 and S668.

To characterize the regulation of S210 and T391 phosphorylation in more detail, phosphospecific antibodies were generated against these sites (Supplementary Figure S3). Western blotting with these antibodies revealed that S210 and T391 exhibited basal phosphorylation in serum-starved cells that was enhanced two- to threefold following EGF stimulation (Figure 3A and B), consistent with the increased 14-3-3 binding induced by EGF (Figure 1B). Maximal phosphorylation at S210 was slightly delayed compared with that at T391, and the increased phosphorylation on S210 was also more sustained (Figure 3B).

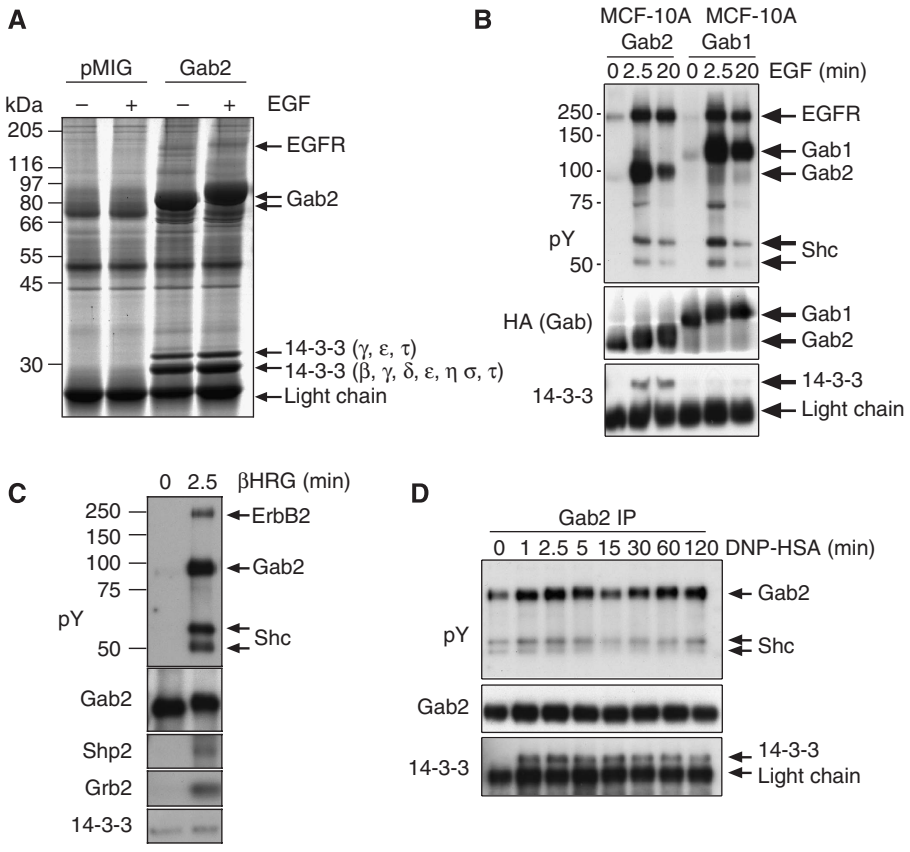

Figure 1 Gab2 is a novel 14-3-3 client protein. (**A**) MCF-10A cells stably expressing HA-tagged Gab2 (Gab2) or vector controls (pMIG) were stimulated with EGF for 10 min. Anti-HA immunoprecipitates were subjected to SDS–PAGE. Protein bands were visualized with Sypro-Ruby, excised and identified by LC-MS/MS. (**B**) MCF-10A cells expressing HA-tagged Gab1 or Gab2 were serum-starved and stimulated with EGF. Anti-HA immunoprecipitates were subjected to western blot analysis. (**C**) MCF-7 cells were serum-starved and then stimulated with 7.5 nM β-heregulin. Gab2 immunoprecipitates were subjected to western blotting. (**D**) FcεRI receptors on RBL-2H3 cells were cross-linked for the indicated times, and anti-Gab2 immunoprecipitates were western blotted as specified.

To identify the kinases that phosphorylate these sites, we used a variety of pharmacological inhibitors, taking into account published guidelines regarding their specificity (Davies *et al*, 2000; Bain *et al*, 2007). A semi-quantitative analysis by LC-MS/MS revealed that in MCF-10A cells, EGF-induced phosphorylation on both S210 and T391 was partially inhibited by the structurally unrelated PI3-kinase inhibitors wortmannin and LY249002 (data not shown) and the effect of wortmannin on phosphorylation on these sites was confirmed by Western blotting (Figure 3C and D). Upon testing candidate kinases downstream of PI3-kinase (Akt, PKCζ, TORC1), only the selective Akt inhibitor Akt-I-1/2 (Bain *et al*, 2007) significantly inhibited S210 and T391 phosphorylation ($P=0.01$ for both sites) (Figure 3C, D and Supplementary Figure S4). With regard to the former site, the magnitude of inhibition by Akt-I-1/2 (~25%) was less than that achieved with wortmannin (~50%), whereas in the case of T391, the effect of the two inhibitors was similar (~30% inhibition). By themselves, these data indicate that either Akt1 or 2, or a kinase downstream of these enzymes, partly mediates EGF-induced S210 and T391 phosphorylation. However, as we previously reported that S159 is the only site on Gab2 phosphorylated by Akt1 *in vitro* (Lynch and Daly, 2002), Akt2 and/or a kinase downstream of Akt1/2 is involved. As, according to Scansite, S210 and T391 are low- and medium-scoring Akt consensus sites, respectively, a function of a kinase downstream of Akt1/2 is particularly likely for S210. Interestingly, there was a trend for rapamycin to enhance phosphorylation on S210 (Figure 3C and D), indicating that phosphorylation on this site is subject to negative regulation by a TORC1-dependent pathway.

The identity of the other kinases that contribute to S210 and T391 phosphorylation are currently unclear. EGF-induced phosphorylation on these sites was not inhibited by UO126, Go6976, bisindolylmaleimide I (Go6850), KN62 or Y27632, indicating that it does not require activation of MEK, conventional or novel PKCs, calmodulin-dependent protein kinase 2 or Rho-dependent protein kinase (Supplementary Figure S4). However, phosphorylation on both S210 and T391 was significantly inhibited by H89, which is marketed as a protein kinase A (PKA) inhibitor (Supplementary Figure S4). Although this is consistent with T391 being a high stringency site for PKA, EGF-induced phosphorylation on both S210 and T391 was unaffected by a PKA-inhibitory peptide (Supplementary Figure S4). These data indicate that PKA is unlikely to be involved in phosphorylation of these sites and that the effect of H89 is mediated through other kinases. Indeed, recent reports indicate that H89 exhibits poor selectivity, inhibiting a variety of kinases including Akt1 and 2 (Davies *et al*, 2000; Bain *et al*, 2007).

An additional mechanism that may explain the sensitivity of S210 and T391 phosphorylation to PI3-kinase inhibition is

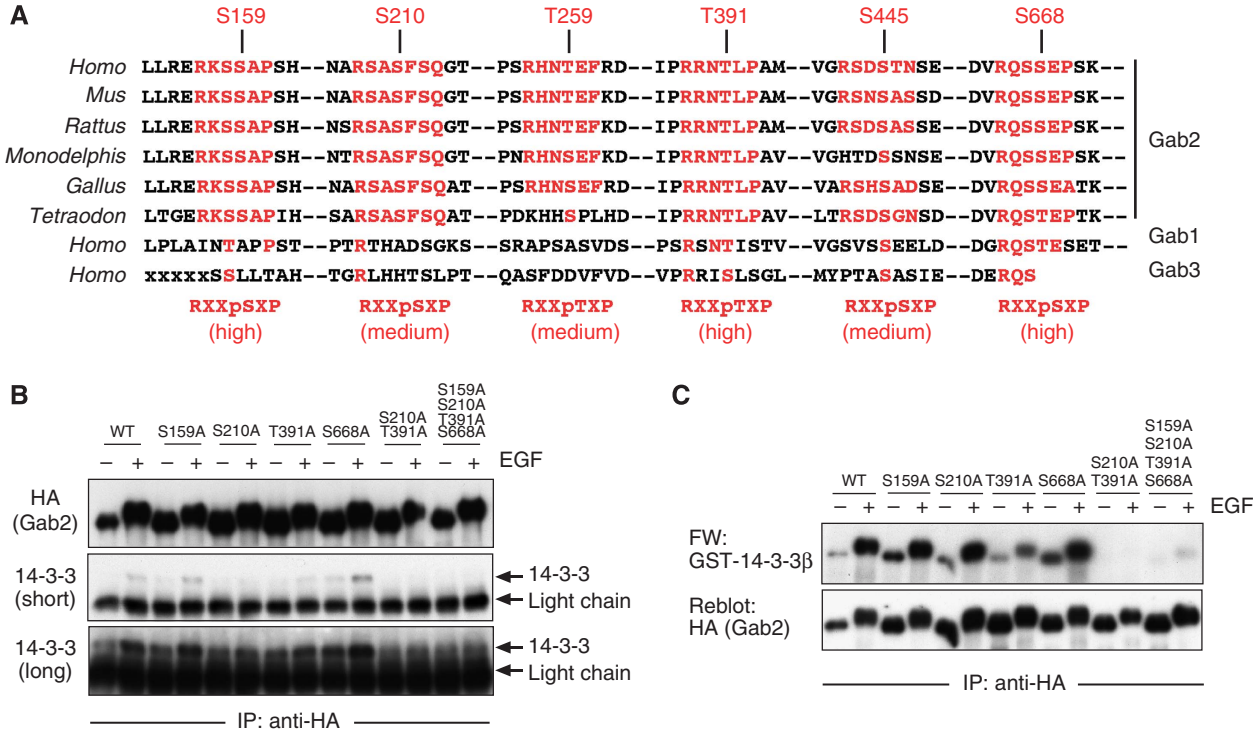

Figure 2 Identification of the 14-3-3-binding sites on Gab2. (**A**) The translated cDNA sequences of vertebrate Gab2 orthologues were aligned with those of human Gab1 and Gab3 (see Supplementary data for details). The mode I 14-3-3-binding motif RXXpS/TXP is highlighted in red along with the Scansite ranking, and the corresponding potential phosphorylation site is indicated on the top. (**B**, **C**) The specified Gab2 constructs were expressed in MCF-10A cells and encoded proteins purified by anti-HA immunoprecipitation. Immunocomplexes were separated by SDS–PAGE and subject to western blotting (B) or Far-western blot analysis with GST-14-3-3β (C).

recruitment of Gab2 to the plasma membrane by binding of its PH domain to PI3-kinase products. To address this possibility, we utilized a Gab2 PH domain mutant, R32C, that is defective in binding of PI(3,4)P2 and PI(3,4,5)P3 (Yu *et al*, 2006a). In COS-7 cells, EGF-induced phosphorylation of S210 and T391 was inhibited by wortmannin and impaired by introduction of the R32C mutation (Figure 3E). Therefore, efficient phosphorylation on these sites was dependent on PI3-kinase activation and required a functional PH domain. To test whether activation of PI3-kinase is sufficient to induce phosphorylation of S210 and T391, and if so, the PH domain-dependency of this effect, we co-expressed wild-type Gab2 or $Gab2^{R32C}$ with a constitutively active form of PI3-kinase (Hu *et al*, 1995). Expression of active PI3-kinase robustly activated Akt, and also induced S210 and T391 phosphorylation on wild-type Gab2. However, PI3-kinase-induced phosphorylation of these sites on $Gab2^{R32C}$ was not detected (Figure 3E). Consequently, maximal EGF-induced phosphorylation on both S210 and T391 requires PI3-kinase activation for two reasons: first, phosphorylation is partly dependent on activation of Akt, a PI3-kinase effector; and secondly, it requires PH domain-mediated binding of Gab2 to the products of PI3-kinase, which may juxtapose Gab2 with the S210/T391 kinases at the plasma membrane and/or induce a conformational change required for phosphorylation.

S210 and T391 mediate negative-feedback regulation of Gab2

To determine the function of S210 and T391 phosphorylation, we characterized the tyrosine phosphorylation status and composition of signalling complexes assembled by Gab2 and the $2\times A$ mutant. Upon EGF stimulation of MCF-10A cells, Gab2 associates with the EGFR and Shc through Grb2, and directly with the effectors Shp2 and PI3-kinase. Under basal conditions, $Gab2^{2\times A}$ exhibited a significant increase in association with Shc, and a trend for enhanced Grb2, EGFR and p85 association, when compared with the wild-type protein (Figure 4A–C). Following EGF stimulation, increased association of $Gab2^{2\times A}$ with Grb2, Shc and the EGFR was observed at all time points examined. Also, commensurate with more sustained tyrosine phosphorylation, $Gab2^{2\times A}$ exhibited a marked enhancement of Shp2 association and a modest increase in p85 binding at late time points (Figure 4A–C). Quantitative analysis of the signalling complexes assembled by the $Gab2^{S210A}$, $Gab2^{T391A}$ and $Gab2^{2\times A}$ mutants revealed that at most time points examined, the effects of mutation of both sites on Gab2/Grb2, Gab2/Shc, Gab2/Shp2 and Gab2/pEGFR interactions were additive (Supplementary Figure S5). Therefore, phosphorylation of either S210 or T391 can partially inhibit signalling, but maximal attenuation requires phosphorylation of both sites.

Of note, $Gab2^{4\times A}$ contains alanine substitutions at S159, S210, T391 and S668. Although our mass spectrometric analysis did not detect phosphorylation at S668, we have previously characterized S159 as a site of Akt-mediated negative feedback (Lynch and Daly, 2002). As $Gab2^{4\times A}$ did not exhibit enhanced complex formation compared with $Gab2^{2\times A}$ at a high EGF concentration (100 ng/ml) (Figure 4A), we repeated the comparison of $Gab2^{2\times A}$ and $Gab2^{4\times A}$ under two different conditions: at a lower concentration of EGF (10 ng/ml) and upon HRG stimulation. The latter condition was utilized in our previous study (Lynch and

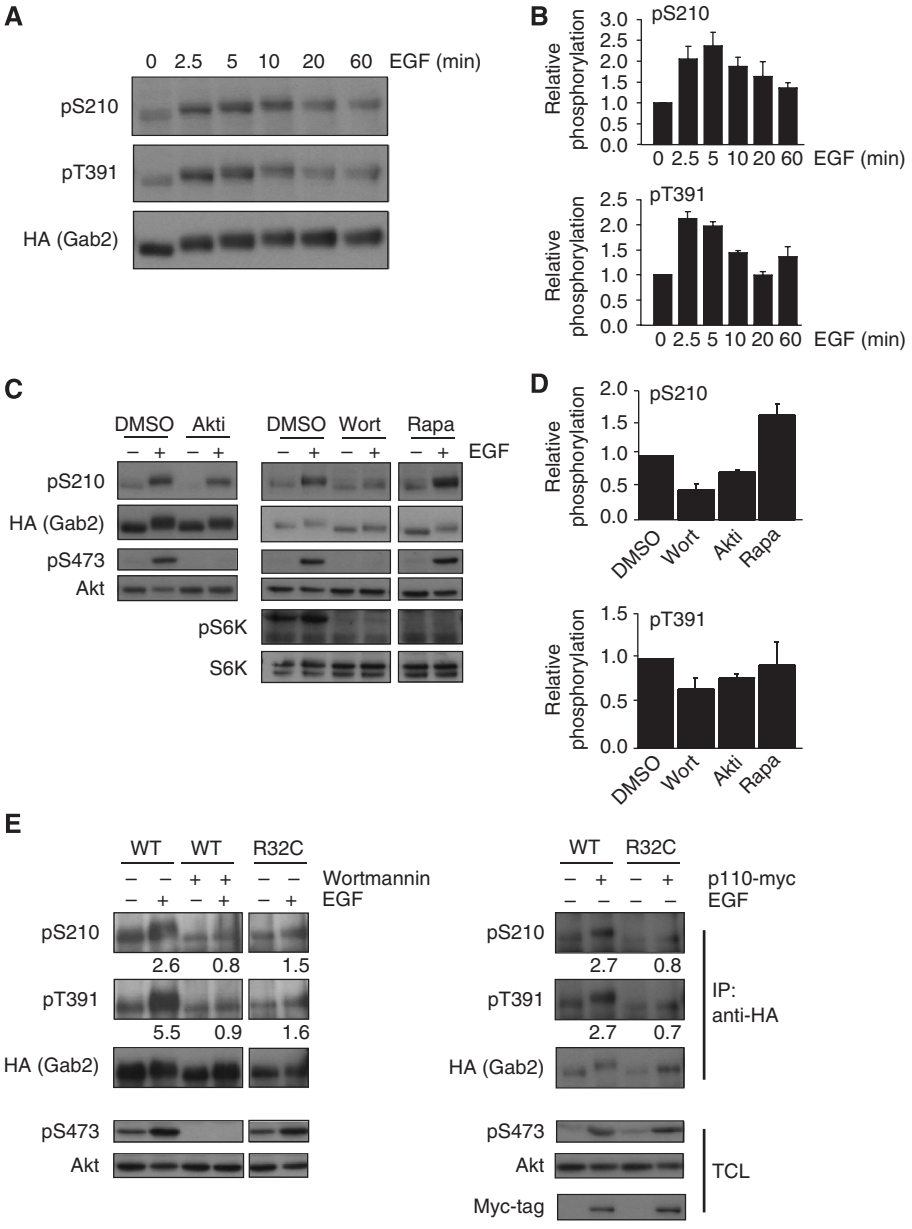

Figure 3 Regulation of S210 and T391 phosphorylation. (**A**) Lysates from MCF-10A/Gab2 cells stimulated with EGF for different times were western blotted with the indicated antibodies. (**B**) Phosphorylation on S210 and T391 was normalized for Gab2 expression and plotted against time. The values and error bars represent the mean and standard error from three independent experiments, respectively. (**C**) MCF-10A/Gab2 cells were pre-incubated with the indicated inhibitors or vehicle controls and then stimulated with EGF for 2.5 min. Lysates were western blotted as indicated. (**D**) EGF-induced phosphorylation on S210 and T391 was normalized for Gab2 expression and is expressed relative to the corresponding vehicle control, which is arbitrarily set at 1.0. The values and error bars represent the mean and standard error from three independent experiments, respectively. (**E**) COS cells were transfected with the indicated expression constructs and treated with wortmannin and/or EGF. EGF stimulations were for 2.5 min. Anti-HA immunoprecipitates were western blotted with anti-pS210, anti-pT391 and anti-HA antibodies. Cell lysates were also western blotted as indicated. Following densitometry, phosphorylation on S210 and T391 was normalized for Gab2 expression, enabling calculation of the fold induction of S210 and T391 phosphorylation upon EGF treatment or expression of active PI3-kinase. The fold induction for each treatment is indicated below the appropriate panel.

Daly, 2002). Under both conditions, growth factor-induced tyrosine phosphorylation of Gab2$^{4\times A}$ was enhanced relative to Gab2$^{2\times A}$ (1.6-fold for EGF and 1.5-fold for HRG, both at 20 min, mean of replicate experiments). This indicates that S159 can exert an inhibitory effect in the presence of combined S210A and T391A substitutions, but this can be masked at high growth factor concentrations.

Next, we characterized the subcellular localization of Gab2 and Gab2$^{2\times A}$. Upon EGF stimulation, both proteins rapidly translocated from the cytoplasm to the plasma membrane. Although the majority of wild-type Gab2 molecules returned to the cytoplasm between 20 and 60 min, this process was drastically delayed with the Gab2$^{2\times A}$ mutant (Figure 4D). As, in diverse signalling systems, association of Gab2 with Grb2 is essential for receptor and plasma membrane recruitment, as well as maximal and sustained Gab2 tyrosine phosphorylation (Gu *et al*, 2000; Edmead *et al*, 2006; Brummer *et al*, 2006b; Yu *et al*, 2006a), these data are consistent with a

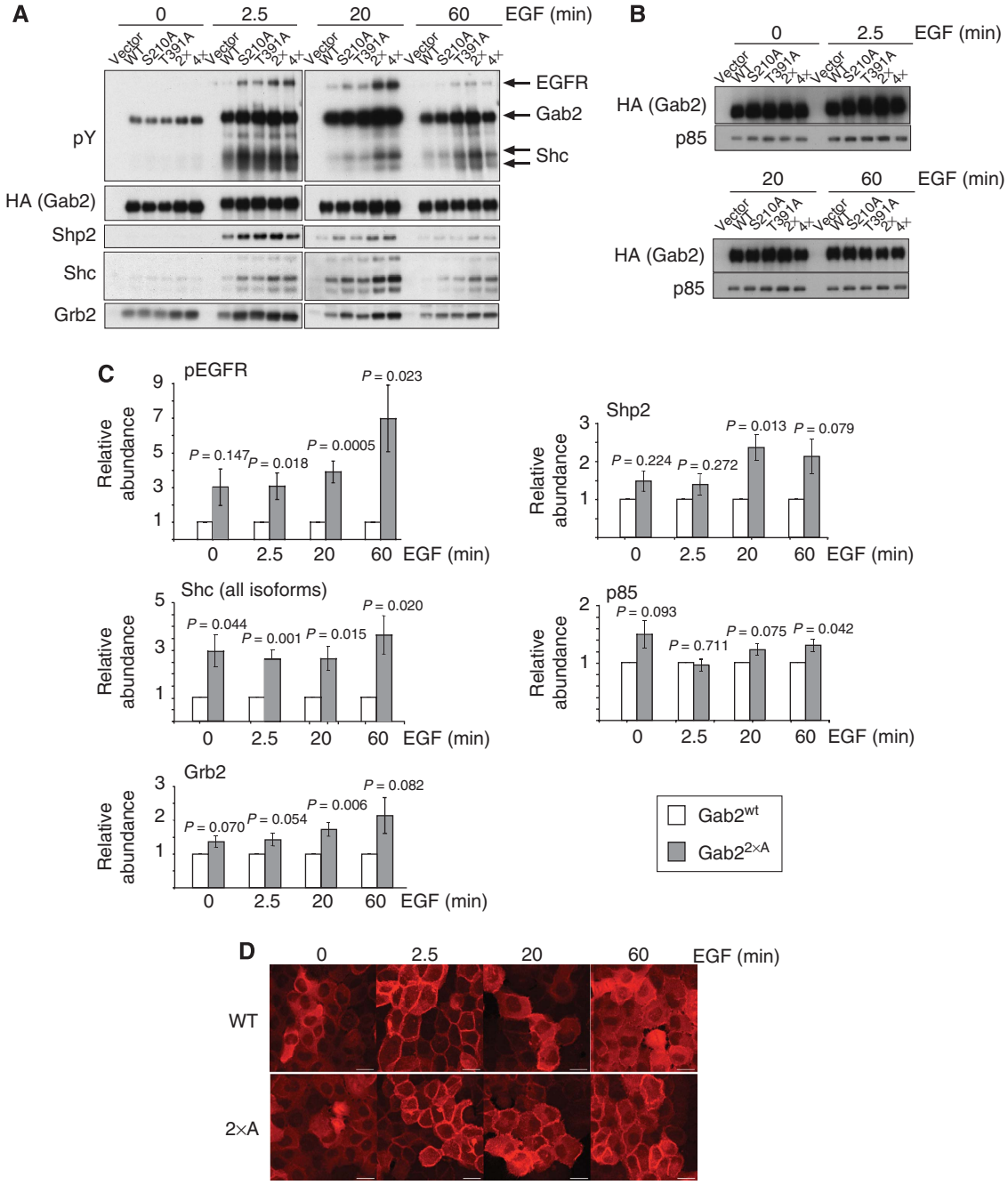

Figure 4 Phosphorylation of S210 and T391 limits assembly of the Gab2 signalling complex. (**A**, **B**) MCF-10A cells expressing the indicated Gab2 mutants were serum-starved, stimulated with EGF for the indicated time points and subsequently lysed. Gab2 was isolated using anti-HA antibodies and subject to western blot analysis using the indicated antibodies. (**C**) Densitometric analysis was performed on experiments described in (A) and (B). For each time point, the amount of a particular interaction partner in a Gab2 complex was normalized for the amount of Gab2 present, and data for $Gab2^{2\times A}$ complexes were expressed relative to those for $Gab2^{wt}$, with the value for the latter arbitrarily set at 1.0. Data were derived from 3–8 independent experiments. Error bars represent standard error of the mean. The *P*-values were calculated by an ANOVA single factor analysis. (**D**) MCF-10A cells expressing the indicated HA-tagged Gab2 proteins were stimulated with EGF for the indicated time points, then fixed and stained with anti-HA antibodies to visualize the subcellular localization of Gab2. The scale bar represents 20 μm.

model in which S210 and T391 phosphorylation impairs Grb2 binding, leading to attenuation of Gab2 signalling.

These findings prompted us to determine the effects of S210 and T391 phosphorylation on signal output and biological responses downstream of Gab2. As described previously (Brummer *et al*, 2006b), Gab2 expression in MCF-10A cells enhanced EGF-induced Akt activation. Expression of $Gab2^{2\times A}$ at similar levels resulted in further signal potentiation (Figure 5A and B), and analysis of the data by two-way ANOVA indicated that there was a significant effect of $Gab2^{2\times A}$ versus wild-type Gab2 when the 20 and 60 min time points were taken into account ($P=0.029$ and $P=0.013$ for S473 and T308 phosphorylation, respectively). However, despite the prolonged retention of Shp2 molecules in the $Gab2^{2\times A}$ signalling complex, we did not detect a significant increase in Erk phosphorylation (Figure 5A). As pretreatment

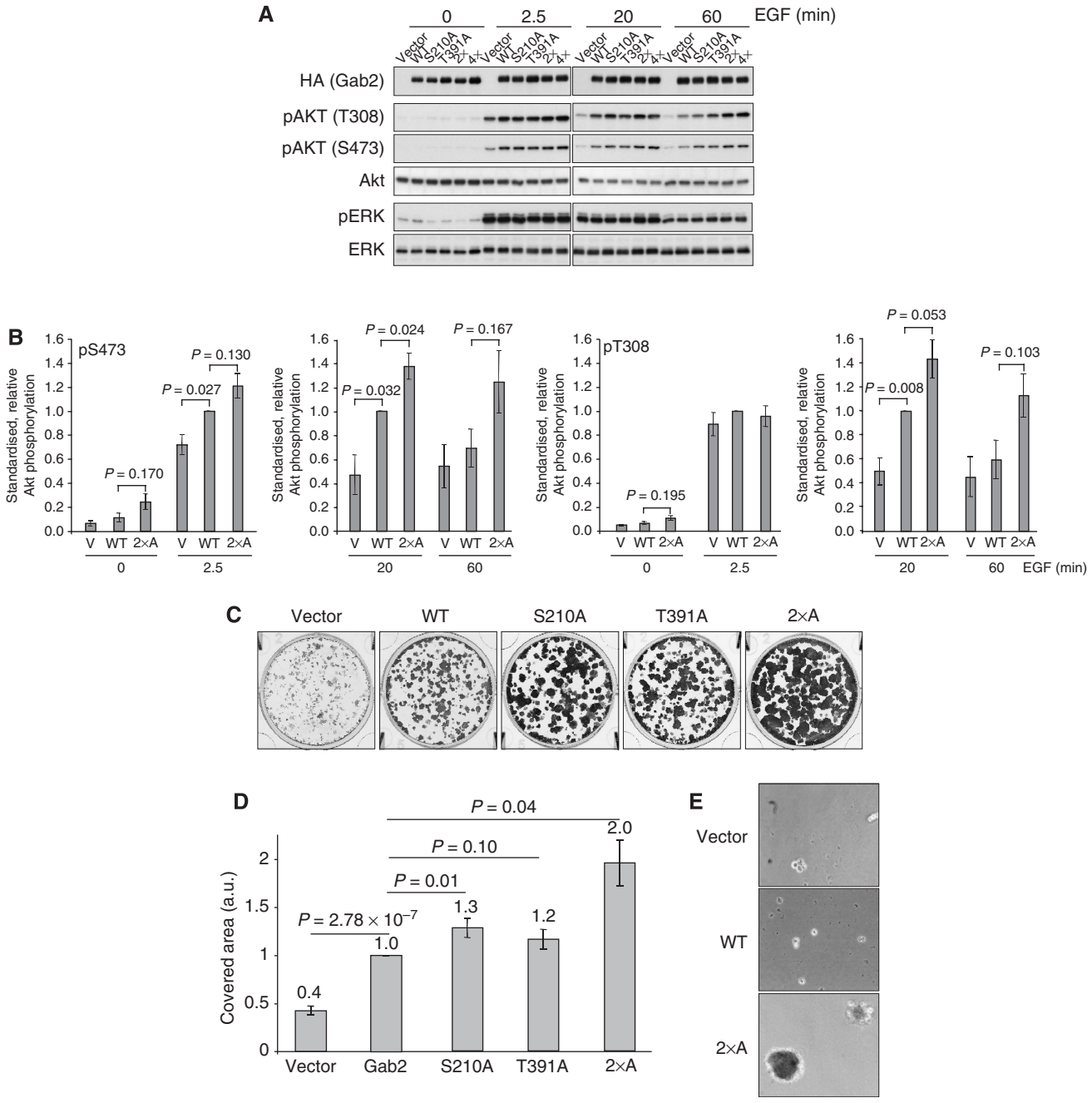

Figure 5 Uncoupling S210 and T391 from negative-feedback phosphorylation enhances downstream signalling and proliferative responses. (**A**) MCF-10A cells expressing the indicated Gab2 mutants were serum-starved and then stimulated with EGF for the indicated time points. Total cellular lysates were subject to western blot analysis using the indicated antibodies. Data are representative of four independent experiments. (**B**) Following densitometric analysis, Akt phosphorylation at each time point was normalized for total Akt protein. As the data for 0/2.5 and 20/60 min were obtained from separate gels, the Akt phosphorylation in cells expressing wild-type Gab2 at either 2.5 min (left panels) or 20/60 min (right panels) was arbitrarily set at 1.0 and other values expressed relative to this. Data are derived from four independent experiments including the experiment shown in (A). Error bars represent standard error of the mean. The *P*-values were calculated by an ANOVA single-factor analysis. (**C**) MCF-10A cells expressing the indicated Gab2 proteins were seeded at low density and grown in medium supplemented with only insulin for 14–16 days. Cells were then fixed and stained with Giemsa solution. (**D**) Densitometric analysis of the clonogenic assays. Plates were scanned and the staining intensity of the plate area was quantified with IPLabgel H software. The mean value from at least four independent experiments (each performed in triplicate) is shown. Error bars represent standard error of the mean. The *P*-values were calculated by an ANOVA single-factor analysis. (**E**) The indicated MCF-10A pools were seeded into soft-agar cultures and maintained as described previously (Lynch and Daly, 2002). Representative photographs of one out of three independent experiments are shown.

of the Gab2$^{2\times A}$-expressing cells with the PI3-kinase inhibitor wortmannin did not result in more sustained Erk activation (data not shown), the lack of an effect of Gab2$^{2\times A}$ on Erk does not appear to reflect negative regulatory cross talk between the Akt and the Raf/Erk pathways in this cell type. This suggests that an alternative mechanism must suppress Erk activation in the Gab2$^{2\times A}$-expressing cells.

The effects of Gab2$^{2\times A}$ on cell proliferation were assayed by two approaches. First, as Gab2 promotes EGF-independent proliferation of MCF-10A cells (Brummer *et al*, 2006b), we

performed monolayer colony formation assays in the absence of this growth factor. Although single alanine substitutions of S210 and T391 had only a weak effect, simultaneous mutation of both sites led to a robust increase in EGF-independent proliferation (Figure 5C and D). Second, as MCF-10A cells are immortalized but non-transformed, we determined the ability of the cells to form colonies in soft agar. Expression of wild-type Gab2 did not confer this property. However, cells expressing Gab2$^{2\times A}$ exhibited prominent colony formation (Figure 5E), demonstrating that uncoupling from S210, S391-mediated negative feedback confers a potent transforming activity upon Gab2.

14-3-3 binding is necessary and sufficient to terminate Gab2 signalling

The data presented so far demonstrate that alanine substitutions of S210 and T391 confer an increased signalling potential to Gab2, while abrogating its 14-3-3 binding capacity. However, they do not address whether phosphorylation at S210 and T391 mediates negative regulation of Gab2 (with 14-3-3 acting as a bystander) or whether recruitment of 14-3-3 is the critical event. To discriminate between these possibilities, we designed two experimental approaches (Figure 6a). First, we replaced the S210 and T391 residues by phosphomimetic glutamate residues. This introduces a constitutive negative charge at these sites, but does not permit 14-3-3 binding (Maudoux *et al*, 2000; Dhillon *et al*, 2002b). When expressed in MCF-10A cells, Gab2$^{2\times E}$ exhibited a marked reduction in 14-3-3 binding, prolonged tyrosine phosphorylation and sustained association with Grb2, Shc and other binding partners. These effects were all comparable with that observed with the $2\times A$ mutant (Supplementary Figure S6). Furthermore, cells expressing Gab2$^{2\times E}$ phenocopied the hyperproliferative behaviour of Gab2$^{2\times A}$ cells (Figure 6B and C). These data suggested that 14-3-3 binding is required to confer negative regulation. To test whether this is sufficient, we engineered a Gab2 protein where the 14-3-3-binding motifs around S210 and T391 were replaced by two R18 peptide sequences that mediate phosphorylation-independent binding of all 14-3-3 isoforms (Wang *et al*, 1999; Light *et al*, 2002; Ramm *et al*, 2006) (Figure 6A). To control for the effect of R18 peptide insertion, we also generated an expression vector for a Gab2$^{2\times KK}$ protein in which the aspartate and glutamate residues of the R18 core motif (WLDLE) were replaced by lysines, a mutation known to abrogate 14-3-3 binding (Ramm *et al*, 2006). When expressed in MCF-10A cells, Gab2$^{2\times R18}$ exhibited strong, constitutive 14-3-3 association, whereas Gab2$^{2\times KK}$ was as impaired for 14-3-3 binding as Gab2$^{2\times A}$ (Figure 6D). Strikingly, association with Grb2, Shc and the EGFR was abolished for Gab2$^{2\times R18}$. Furthermore, this mutant exhibited only a weak and transient tyrosine phosphorylation, resulting in reduced binding to Shp2 and p85. In all respects, this behaviour is identical to the Gab2$^{\Delta Grb2}$ mutant, with both the canonical and atypical Grb2-binding sites mutated (Brummer *et al*, 2006b). Consistent with its effect on 14-3-3 association, mutation of the R18 motifs in Gab2$^{2\times KK}$ restored Grb2 binding and assembly of Gab2 signalling complexes to a level comparable with the Gab2$^{2\times A}$ mutant. These results confirm that 14-3-3 binding to specific sites is sufficient to restrain Gab2 signalling and demonstrate that recruitment of 14-3-3 and Grb2 are mutually exclusive events.

Discussion

In this study, we have identified 14-3-3 proteins as novel interaction partners of Gab2 that limit its mitogenic signalling potential by binding to S210 and T391 in a phosphorylation-dependent manner. Of note, as the closely related Gab1 does not bind 14-3-3 proteins, and Gab3 lacks corresponding consensus sequences for 14-3-3 recruitment, this regulatory mechanism appears unique to Gab2. This may reflect a requirement for Gab2 signalling to be tightly regulated for it to execute its specific cellular and developmental functions.

A schematic representation of how 14-3-3 regulates Gab2 is presented in Figure 7. A key aspect of this model is that binding of 14-3-3 proteins promotes disassembly of the Gab2/Grb2 complex. This is based on several lines of evidence. First, the signalosomes assembled by the 14-3-3-binding-deficient Gab2 mutants $2\times A$ and $2\times E$ contain increased amounts of not only Grb2 but also Shc and the EGFR, which are both recruited in a Grb2-dependent manner (Brummer *et al*, 2006b). Second, signalling complexes assembled by the constitutively 14-3-3-bound Gab2$^{2\times R18}$ mutant are devoid of Grb2, Shc and EGFR, whereas its 14-3-3-binding-deficient counterpart Gab2$^{2\times KK}$ recruits these molecules to a similar extent as the Gab2$^{2\times A}$ mutant. Finally, the Gab2$^{2\times R18}$ mutant phenocopies the weak and transient tyrosine phosphorylation of the Gab2$^{\Delta Grb2}$ mutant (Brummer *et al*, 2006b), which is probably dependent on PH domain-mediated plasma membrane recruitment. Although we have characterized this mechanism in the context of EGFR signalling, Gab2 requires Grb2 for recruitment to other signal initiators, including the Met (Lock *et al*, 2002), erbB2 (Bentires-Alj *et al*, 2006), FGF (Gu and Neel, 2003), SF-STK (Teal *et al*, 2006) and Kit (Yu *et al*, 2006b) tyrosine kinase receptors, FcεRI (Yu *et al*, 2006a), βc-containing cytokine receptors (Gu *et al*, 2000) as well as the Bcr-Abl and Tel-Abl oncoproteins (Sattler *et al*, 2002; Million *et al*, 2004). Consequently, 14-3-3 binding is likely to impact upon Gab2 signalling in a variety of settings and in both normal and pathological states. Of note, another example of negative-feedback regulation within the EGF signalling network that regulates Grb2 interactions is Erk-mediated phosphorylation of Sos (Rozakis-Adcock *et al*, 1995; Porfiri and McCormick, 1996). However, in this case, Sos phosphorylation results in dissociation of Grb2 from its SH2 domain-binding partners Shc and the EGFR.

At present, it is not clear how binding of 14-3-3 proteins attenuate Grb2 binding to Gab2. One possibility is that they shield the Grb2 binding sites. Alternatively, 14-3-3 binding may induce a conformational change in Gab2 that restricts access to these sites. The latter mechanism is more likely if a 14-3-3 dimer bridges S210 and T391 as proposed in the 'molecular anvil' model for 14-3-3 function (Yaffe, 2002). However, if bridging of the two sites by 14-3-3 induces a conformational change, then mutation of a single site might be expected to be as effective in enhancing signalling as the double site mutation, and our analysis (Supplementary Figure S5) indicates that this is not the case. Instead, the effects of the S210 and T391 substitutions on the assembly of Gab2 signalling complexes are largely additive. Also, there is a trend for the individual substitutions to enhance signalling approximately equally at 2.5 min, but for the S210A substitution to have a larger effect, relative to T391A, at 20 min (Supplementary Figure S5). This reflects the kinetics of the

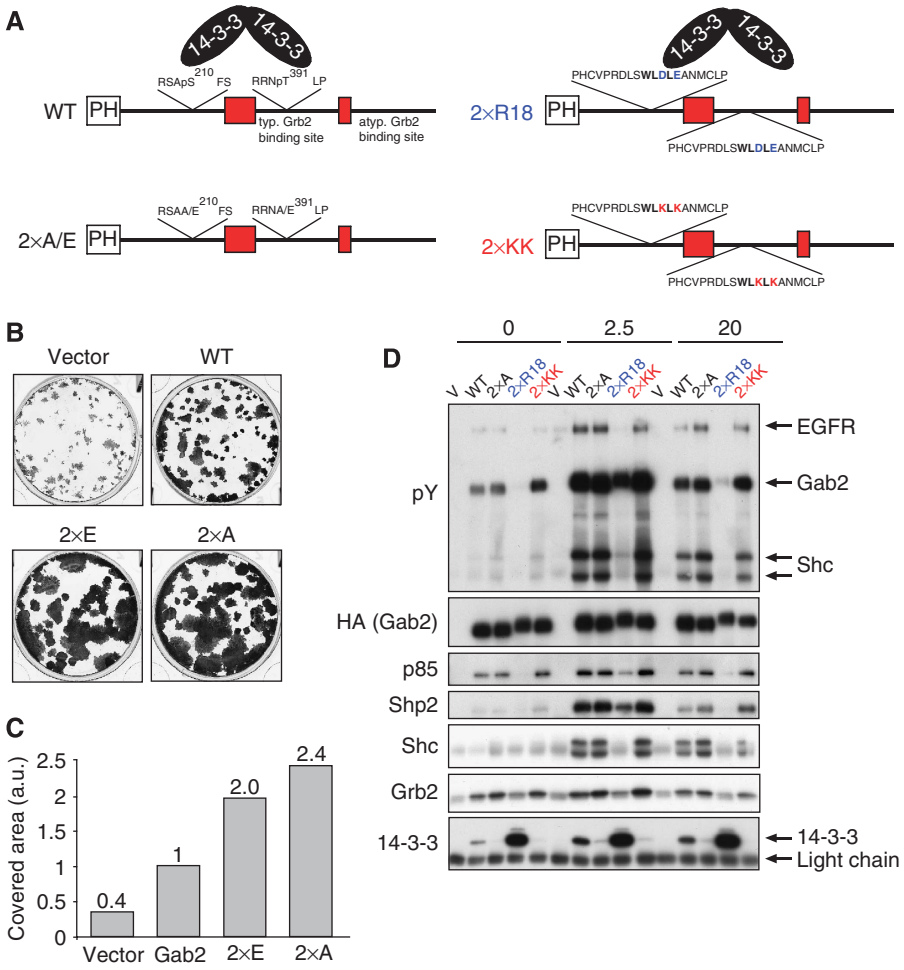

Figure 6 14-3-3 binding to S210 and T391 is necessary and sufficient to attenuate Gab2 signalling. (**A**) Schematic representation of the Gab2 proteins used in this experiment. (**B**) Colony-formation assays were performed as shown in Figure 5C. (**C**) Densitometric analysis of the clonogenic assays was performed as shown in Figure 5D. The values represent the mean of two independent experiments performed in triplicate. (**D**) MCF-10A cells expressing the indicated Gab2 proteins were stimulated with EGF for different times. Tyrosine phosphorylation and composition of Gab2 complexes were analysed as described in Figure 4A.

corresponding phosphorylation events (Figure 3). These data lead us to favour a model where 14-3-3 shields the Grb2-binding sites, and binding of 14-3-3 to one site is sufficient to partly inhibit signalling. When both sites are phosphorylated, the enhanced signal attenuation occurs either as a consequence of binding of separate 14-3-3 dimers to S210 or T391 or bridging of the two sites by a single dimer (Figure 7). It is also possible that the stoichiometry of 14-3-3 binding changes over time, reflecting the relative phosphorylation of S210 and T391. Further resolution of this issue will require determination of the stoichiometry of the Gab2/14-3-3 complex.

The 14-3-3-mediated uncoupling of Gab2 from a receptor complex at the plasma membrane exemplifies a recurrent theme in 14-3-3/client protein interactions, where 14-3-3 proteins regulate subcellular localization of their target. Indeed, it is interesting to compare this mechanism with 14-3-3-mediated regulation of the Raf serine/threonine kinases, where 14-3-3 binding to the CR2 domain inhibits stable interaction with plasma membrane-localized Ras and Raf activation (Dhillon *et al*, 2002a; Light *et al*, 2002; Brummer *et al*, 2006a; Rodriguez-Viciana *et al*, 2006). Furthermore, 14-3-3 proteins block the translocation of the KSR scaffold protein to the plasma membrane by preventing the interaction of the C1 domain with membrane phospholipids (Ory *et al*, 2003). In both cases, growth factor stimulation leads to dephosphorylation of a specific 14-3-3-binding site, plasma membrane translocation and signal transmission. However, in the case of Gab2, 14-3-3 proteins associate following growth factor treatment and negatively regulate binding to an adaptor 'bridge' that mediates recruitment to cell surface receptors, resulting in signal attenuation.

Maximal phosphorylation on both S210 and T391 requires PI3-kinase for two reasons: first, phosphorylation on both sites is partially dependent on the activation of Akt1/2; and second, it requires PH domain-mediated recruitment of Gab2 to the plasma membrane (Figure 3). In the case of S210, it appears unlikely that this site is directly phosphorylated by Akt, implicating a downstream kinase. One possibility is IκB kinase α (IKKα), as this is activated by Akt (Ozes *et al*, 1999), and the related kinase IKKβ negatively regulates the docking protein IRS-1 (Herschkovitz *et al*, 2007). The additional kinases that phosphorylate S210 and T391 under both basal and EGF-stimulated conditions are currently under

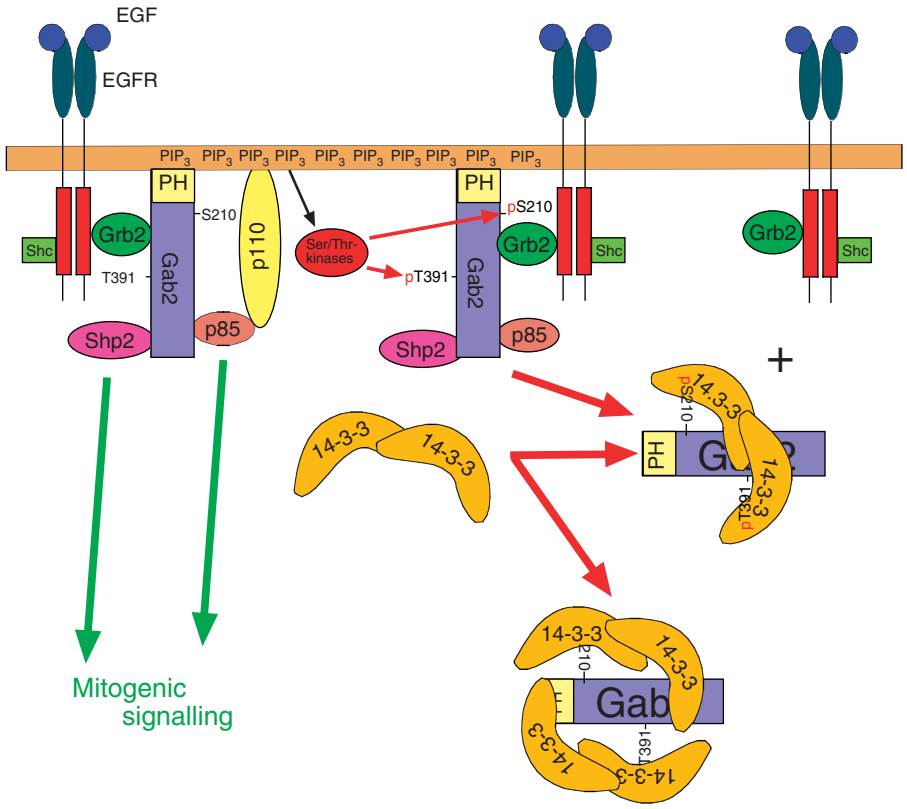

Figure 7 A model for negative-feedback regulation of Gab2 by 14-3-3. Gab2 is recruited to the activated EGFR through a Grb2 'bridge', and the complex is stabilized by binding of the Gab2 PH domain to PI3,4P2 and PI3,4,5P3 generated by PI3-kinase (Yu *et al*, 2006a). The latter enzyme also activates downstream kinases that phosphorylate S210 and T391. Binding of 14-3-3 to these phosphorylated sites promotes dissociation of Gab2 from Grb2 and termination of Gab2 signalling. Two possible modes of 14-3-3 binding are presented.

investigation. A further interesting question is how 14-3-3-bound Gab2 is recycled to an activation-competent state. Structural studies have revealed that the phosphate group of the 14-3-3-binding peptide is deeply buried in the 14-3-3-binding pocket and is consequently protected against phosphatases (Yaffe *et al*, 1997). Consequently, 14-3-3 proteins need to be actively displaced before dephosphorylation can occur. For example, a recent study has revealed an intricate cooperation between M-Ras and the Shoc2/PP1c holophosphatase complex in the displacement of 14-3-3 from Raf-kinases (Rodriguez-Viciana *et al*, 2006).

Given the important functions of Gab2 in normal physiology and oncogenesis, it will be important to determine whether the 14-3-3-dependent negative-feedback mechanism is subverted in human disease and can be exploited therapeutically. For example, the 14-3-3-binding motifs at S210 and T391 may be subject to germline or somatic mutation in developmental syndromes or cancer, respectively. Also, as phosphorylation of three critical regulatory sites on Gab2 (S159, S210 and T391) is PI3-kinase dependent, the effect of uncoupling Gab2 from negative-feedback control should be considered in the design of drug regimens that include PI3-kinase inhibitors. An example of how release of a feedback mechanism can impact upon drug sensitivity is provided by the amplification of IRS-1 signalling induced by treatment of cancer cells with the anti-mTOR drug rapamycin (O'Reilly *et al*, 2006). Finally, as 14-3-3 represents an isoform-specific regulator of Gab2, a strategy aimed at stabilization of the Gab2/14-3-3 complex, or allosteric modulation of Gab2 in a manner that mimics 14-3-3 binding, could be utilized to downregulate Gab2 signalling, for example, in Gab2-dependent cancers (Daly *et al*, 2002; Sattler *et al*, 2002; Bentires-Alj *et al*, 2006; Scherr *et al*, 2006) or inflammatory diseases (Gu *et al*, 2001; Batliwalla *et al*, 2005).

Materials and methods

Plasmids

The bicistronic retroviral pMIG and pMIG/Gab2-HA vectors have been described previously (Brummer *et al*, 2006b). The pMIG/Gab2-HA constructs used in this study were generated by sequential site-directed mutagenesis, overlap extension PCR or by swapping restriction endonuclease-generated fragments between individual plasmids. For the generation of pMIG/Gab1-HA, the full-length human Gab1 cDNA (splice variant isoform A) was amplified by PCR using the Sanger Centre IMAGE clone 303869555 as a template. Similar to our previous approach to generate an expression vector for HA-tagged Gab2 (Brummer *et al*, 2006b), the original STOP codon of *GAB1* was replaced by an HA-tag encoding sequence followed by a STOP codon. This modified Gab1-HA cDNA was subcloned between the *Eco*RI and *Xho*I-sites of pMIG. The cDNAs of the pMIG/Gab1-HA vector and of all pMIG/Gab2-HA mutants were confirmed by DNA sequencing. Oligonucleotide sequences and detailed cloning procedures are available on request. The pGEX2T-14-3-3β and the various pSRα/GST-14-3-3 expression plasmids were kind gifts from Drs John Hancock (Brisbane) and Walter Hunzicker (Singapore), respectively. The construct encoding a constitutively active form of PI3-kinase (a chimera consisting of the iSH2 domain of p85 fused to the N-terminus of p110) (Hu *et al*, 1995) was generously provided by Dr Morris Birnbaum (Philadelphia). Plasmids encoding HA-tagged mouse wild type and Gab2^{R32C} (Yu *et al*, 2006a) were kind gifts from Dr Haihua Gu (Denver).

Antibodies and recombinant GST proteins

Antibodies against phospho-Gab2 (S159), phospho-Akt (T308), phospho-Akt (S473), total Akt as well as phosphorylated and total S6 kinase and ERK were purchased from Cell Signaling Technology. The rat anti-HA monoclonal antibody 3F10 and rabbit anti-HA antibodies were obtained from Roche Molecular Diagnostics and Covance, respectively. The horseradish peroxidase-conjugated anti-phosphotyrosine antibody PY20 and antibodies against p85, Shc, Grb2 and Shp2 were obtained from BD Transduction Laboratories. Antibodies against 14-3-3β (rabbit polyclonal K-19 or mouse monoclonal H-8) and Gab2 (M-19) were purchased from Santa Cruz Biotechnology. The anti-myc antibody 9E10 was isolated from the corresponding hybridoma cells. Polyclonal anti-phospho-S210 and anti-phospho-T391 antibodies were generated in sheep using standard techniques by Symansis (Auckland, New Zealand). Specificity of the antibodies was confirmed by western blotting of lysates derived from cells expressing wild-type Gab2 and Gab2^{S210A} and Gab2^{T391A}. SDS–PAGE and western blotting were performed as described previously (Brummer *et al*, 2006b). GST and GST-14-3-3β were expressed and purified from *Escherichia coli* BL21Lys as described previously (Brummer *et al*, 2006a).

Cells, tissue culture, growth factor and antigen receptor stimulation

MCF-10A/ecoR cells (kindly provided by Drs D Lynch and J Brugge), their infection with ecotropic retroviruses and stimulation with EGF have been described previously (Brummer *et al*, 2006b). Retroviral supernatants were generated in either Phoenix-eco or Plat-E cells (a kind gift from Dr T Kitamura) using a published protocol (Brummer *et al*, 2006b). For clonogenic assays, 500 cells were seeded in each well of a 6-well plate and grown for 14 days. Medium was changed every 2–3 days. Cells were then fixed and stained with Giemsa solution. For the stimulation of MCF-10A cells with EGF, cells were starved in DMEM/F12 supplemented with 0.4% horse serum for approximately 18 h and then stimulated with 100 ng/ml recombinant human EGF (R&D systems) for the indicated time points. Sources of kinase inhibitors and their use are described in Supplementary data. Soft agar assays for the assessment of anchorage-independent growth were performed as described previously (Lynch and Daly, 2002). Culture conditions for MCF-7 cells have been published (Daly *et al*, 2002). Maintenance and transfection of COS-7 cells was as previously described (Lynch and Daly, 2002). RBL-2H3 cells were cultivated in RPMI 1640 supplemented with 10% heat-inactivated foetal calf serum (FCS), 100 U penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 10 mM HEPES and 50 μM β-mercaptoethanol. To cross-link FcεRI receptors, cells were preloaded with monoclonal anti-dinitrophenol (DNP) IgE antibody (75 ng/ml) for 18 h and then stimulated with DNP-HSA (75 ng/ml). Mouse monoclonal anti-DNP IgE and DNP-HSA were purchased from Sigma.

Cell lysis, immunoprecipitation and affinity purification

The preparation of cell lysates and the immunoprecipitation of Gab2 signalling complexes were performed as described previously (Brummer *et al*, 2006b). Affinity purifications using GST or GST-14-3-3β were performed according to a published protocol (Brummer *et al*, 2006a). Synthetic R18 peptide was synthesized by Auspep. Phosphorylated and non-phosphorylated peptides corresponding to S585 of the GM-CSF receptor-β common chain (Guthridge *et al*, 2000) were generously provided by Dr Mark Guthridge (Adelaide). Competition of 14-3-3 binding to Gab2$^{2\times A}$ was assayed by preincubating lysates with the synthetic peptide (250 μM) for 2 h at 4 °C before anti-HA immunoprecipitation.

LC-MS/MS analysis

A detailed description is provided in Supplementary data.

Immunofluorescence

A total of 500 MCF-10A cells infected with the indicated pMIG/Gab2 retroviruses were plated in 400 μl growth medium onto 4-well culture slides (BD Falcon) and grown to sub-confluency. Cells were then starved and stimulated as described above. Following stimulation, cells were immediately fixed by replacing the culture medium with 400 μl of 2% formaldehyde solution (2% v/v formaldehyde in PBS) at room temperature for 20 min. Subsequently, the formaldehyde solution was removed and the cell lawn was washed once with 400 μl of PBS and then incubated with permeabilization solution PBT (0.5% Triton-X 100 in PBS) for 1 h. Subsequently, unspecific binding sites were blocked by incubation in blocking buffer (10% v/v FCS in PBT) for 1 h followed by incubation with primary rabbit anti-HA antibody (Covance; diluted 1:200 in blocking buffer) at 4 °C overnight. Following three washes with 500 μl of PBS, specifically bound anti-HA antibodies were detected by incubation with Cy3-labelled goat anti-rabbit IgG antibodies (Jackson ImmunoResearch Laboratories Inc.). Following four washes with PBS, the cells were incubated with TOPRO-3 (Molecular Probes) solution (1:500 dilution in PBS) for 15 min, sealed with coverslips using Prolong Antifade Gold (Molecular Probes) and analysed using Confocal Laser Scanning microscopy.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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