

# Grb2 controls phosphorylation of FGFR2 by inhibiting receptor kinase and Shp2 phosphatase activity

Zamal Ahmed,<sup>1</sup> Chi-Chuan Lin,<sup>1</sup> Kin M. Suen,<sup>1</sup> Fernando A. Melo,<sup>1</sup> James A Levitt,<sup>2</sup> Klaus Suhling,<sup>2</sup> and John E. Ladbury<sup>1</sup>

<sup>1</sup>Department of Biochemistry and Molecular Biology and Center for Biomolecular Structure and Function, University of Texas MD Anderson Cancer Center, Houston, TX 77030

<sup>2</sup>Department of Physics, Kings College London, London WC2R 2LS, England, UK

**C**onstitutive receptor tyrosine kinase phosphorylation requires regulation of kinase and phosphatase activity to prevent aberrant signal transduction. A dynamic mechanism is described here in which the adaptor protein, growth factor receptor-bound protein 2 (Grb2), controls fibroblast growth factor receptor 2 (FGFR2) signaling by regulating receptor kinase and SH2 domain-containing protein tyrosine phosphatase 2 (Shp2) phosphatase activity in the absence of extracellular stimulation. FGFR2 cycles between its kinase-active, partially phosphorylated, nonsignaling state and its Shp2-dephosphorylated state. Concurrently, Shp2 cycles between its FGFR2-phosphorylated and dephosphorylated forms.

Both reciprocal activities of FGFR2 and Shp2 were inhibited by binding of Grb2 to the receptor. Phosphorylation of Grb2 by FGFR2 abrogated its binding to the receptor, resulting in up-regulation of both FGFR2's kinase and Shp2's phosphatase activity. Dephosphorylation of Grb2 by Shp2 rescued the FGFR2–Grb2 complex. This cycling of enzymatic activity results in a homeostatic, signaling-incompetent state. Growth factor binding perturbs this background cycling, promoting increased FGFR2 phosphorylation and kinase activity, Grb2 dissociation, and downstream signaling. Grb2 therefore exerts constitutive control over the mutually dependent activities of FGFR2 and Shp2.

## Introduction

Even in the absence of extracellular stimulation, receptor tyrosine kinase (RTK) phosphorylation is continuously turned over in eukaryotic cells (Kleiman et al., 2011). Uncontrolled kinase and/or phosphatase activity leads to aberrant signal transduction, thus regulation of the opposing catalytic functions is required to ensure that downstream response only occurs when an appropriate extracellular-stimulating ligand binds. Regulation of the RTK fibroblast growth factor receptor 2 (FGFR2) and the SH2 domain-containing protein tyrosine phosphatase 2 (Shp2) is controlled by the growth factor receptor-bound protein 2 (Grb2).

Previously, we observed that cells stably expressing FGFR2 exhibit elevated receptor phosphorylation in the basal state (Ahmed et al., 2008; Schüller et al., 2008). In the absence of extracellular growth factor stimulus, Grb2 binds to FGFR2

via its C-terminal SH3 domain (C-SH3; Ahmed et al., 2010; Lin et al., 2012). Grb2 is able to form a dimer and recruit two receptor molecules into a heterotetramer. In this state at least the two activation loop tyrosine residues (Y653 and Y654) of FGFR2 are phosphorylated, but no downstream mitogen-activated protein (MAP) kinase signaling is observed (Lin et al., 2012). On engagement of the growth factor by FGFR2, receptor dimerization is stabilized and autophosphorylation is up-regulated. Grb2 is phosphorylated on a tyrosine residue (Y209) by the fully active FGFR2, which results in dissociation from the complex with the receptor. Release of the interaction with Grb2 permits the FGFR2 kinase domain to access additional tyrosine residues on the receptor, and to recruit downstream effector proteins required for signal transduction. We also demonstrated *in vitro* that Grb2 was able to inhibit Shp2-mediated FGFR2 activation loop tyrosine dephosphorylation (Ahmed et al., 2010). Grb2 therefore exerts pivotal control of receptor

Correspondence to John E. Ladbury: jeladbury@mdanderson.org; or Zamal Ahmed: zahmed@mdanderson.org

Abbreviations used in this paper: C-SH3, C-terminal SH3 domain; ERK, extracellular signal-regulated kinase; FGFR2, fibroblast growth factor receptor 2; FLIM, fluorescence lifetime imaging microscopy; FRET, fluorescence resonance energy transfer; FRS2, FGFR substrate 2; Grb2, growth factor receptor-bound protein 2; MAP, mitogen-activated protein; RTK, receptor tyrosine kinase; Shp2, SH2 domain-containing protein tyrosine phosphatase 2.

© 2013 Ahmed et al. This article is distributed under the terms of an Attribution-Noncommercial-Share Alike-No Mirror Sites license for the first six months after the publication date (see <http://www.rupress.org/terms>). After six months it is available under a Creative Commons License (Attribution-Noncommercial-Share Alike 3.0 Unported license, as described at <http://creativecommons.org/licenses/by-nc-sa/3.0/>).

phosphorylation–dephosphorylation; however, the mechanistic details for this important, constitutive role remain elusive.

Ubiquitously expressed Grb2 forms a heterotetrameric complex with FGFR2 but plays a more familiar role in linking RTKs to the MAP kinase signaling pathway (Lowenstein et al., 1992; Chardin et al., 1993; Rozakis-Adcock et al., 1993). Grb2 largely consists of a central SH2 domain sandwiched between N- and C-terminal SH3 domains. Grb2 is a highly abundant protein and is able to interact with numerous cellular phospho- and nonphosphoproteins through its SH2 and SH3 domains, respectively.

Somatic mutations in FGFR2 have been associated with a number of human cancers (Jang et al., 2001; Pollock et al., 2007; Dutt et al., 2008; Byron et al., 2010), whereas missense germline mutations of the *fgfr2* gene are seen in congenital skeletal disorders (Wilkie et al., 1995; Johnson et al., 2000; Yu et al., 2000; Goriely et al., 2010; Turner and Grose, 2010). Alternative gene-splicing events provide numerous structural variants of FGFR2. C-terminal sequence splicing provides a major group of FGFR2 isoforms. Variants that result in deletions of the C-terminal sequence show enhanced transforming activity (Cha et al., 2008) and are expressed in increased amounts in gastric, bladder, and stomach cancer cell lines (Hattori et al., 1990, 1996; Itoh, et al., 1994; Ishii et al., 1995) and in a majority of human breast carcinoma cells (Cha et al., 2009). Furthermore, point mutations in the C-terminal region of FGFR2 have recently been linked with melanoma (Gartside et al., 2009). The C terminus of FGFR2 harbors numerous sites for the recruitment of downstream signaling effector proteins, thus perturbation of this region of FGFR2 can contribute to oncogenesis.

Shp2 (also known as protein tyrosine phosphatase non-receptor type 11 [PTPN11]) can be recruited by several RTKs either directly, or indirectly via auxiliary adaptor proteins (Freeman et al., 1992; Feng et al., 1993; Barford and Neel, 1998; Grossmann et al., 2010). Its phosphatase activity is thus important in regulation of intracellular signaling activity (Holgado-Madruga et al., 1996; Kouhara et al., 1997; Neel et al., 2010). Shp2 is auto-inhibited by an intramolecular SH2 domain-mediated interaction, displacement of which is necessary to promote catalytic activity (Hof et al., 1998). Although there is some conjecture in the field, it has been reported that activated RTKs phosphorylate tyrosine 542 (Y542) on Shp2, which appears to enhance phosphatase activity (Lu et al., 2001; Araki et al., 2003; Keilhack et al., 2005).

Here we provide the mechanism for the control by Grb2 of FGFR2 kinase and the Shp2 phosphatase activity in the absence of extracellular stimulation. Grb2 plays the pivotal role in regulating the level of FGFR2 phosphorylation through controlled inhibition of (1) the kinase activity of the receptor necessary for full autophosphorylation and signal transduction (Lin et al., 2012); (2) the kinase activity of the receptor required to phosphorylate Shp2; and (3) the phosphatase activity of Shp2 directed at FGFR2. Our data demonstrate the mutual dependency of FGFR2 and Shp2 on Grb2 to dictate their respective cellular activity and maintain homeostasis of receptor phosphorylation in the nonstimulated state. Perturbation of this control in the nonstimulated cell can disrupt appropriate FGFR2

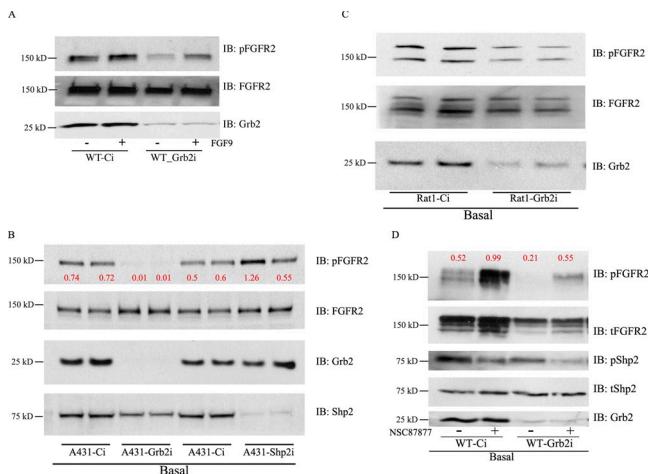
regulation and lead to abnormal receptor function and aberrant downstream signaling. Thus, rather than being a passive adaptor protein, Grb2 is an essential positive and negative regulator of receptor phosphorylation and hence ultimately downstream signal transduction.

## Results

### Shp2-mediated dephosphorylation of FGFR2 is controlled by Grb2

In an earlier *in vitro* experiment we demonstrated that Shp2-mediated dephosphorylation of the FGFR2 activation loop tyrosine residues occurs only in the absence of Grb2 (Ahmed et al., 2010). This finding was confirmed in a cellular context by showing that knocking down the Grb2 concentration in three different cell lines results in lower constitutive FGFR2 phosphorylation. Stable Grb2-depleted HEK293T cells (~80% knockdown) overexpressing wild-type FGFR2 with a C-terminal GFP fusion tag (<sup>WT</sup>FGFR2-GFP) were generated using Grb2 shRNA (Fig. 1 A). In addition, we also generated stable Grb2-depleted A431 (Fig. 1 B) and Rat-1 (Fig. 1 C) cells that express only endogenous FGFR2. The basal level of phosphorylated FGFR2 (pFGFR2) was compared between Grb2 knockdown cells (Grb2i) and those infected with a scrambled control shRNA (Ci). In HEK293T cells overexpressing <sup>WT</sup>FGFR2-GFP, Grb2 knockdown resulted in a decrease in the level of basal receptor phosphorylation (Fig. 1 A). Similarly, knockdown of Grb2 in A431 and Rat-1 cells also led to a measurable decrease in the level of endogenous FGFR2 phosphorylation (Fig. 1, B and C). It should be noted that in A431 cells the efficiency of Grb2 knockdown was much higher than that in Rat-1 cells, which is reflected in the respective levels of receptor dephosphorylation observed between the two cell lines. In addition to the experiments in the Grb2 knockdown background, we used a HEK293T cell line overexpressing the oncogenic S252W mutant FGFR2 (<sup>S252W</sup>FGFR2). This mutant receptor is incapable of binding to Grb2 (Fig. S1 C). In the absence of the Grb2 interaction, basal receptor phosphorylation is reduced in a similar way to that seen in the Grb2i cells (Fig. S2 A). The observation of decreasing pFGFR2 in all three Grb2 knockdown cell lines, and in the cells expressing <sup>S252W</sup>FGFR2 is consistent with (1) the increased access of active phosphatase to the receptor in the absence of the inhibitory effect of Grb2, and/or (2) the loss of Grb2–FGFR2 heterotetramer formation reducing the propensity for basal receptor phosphorylation.

Knocking down of Shp2 in A431 (Shp2i, Fig. 1 B) in the presence of Grb2 shows a small increase in receptor phosphorylation under nonstimulated conditions, suggesting that Shp2 is a phosphatase for FGFR2 and that Grb2 inhibition of Shp2 activity in normal cells is not 100% effective. However, to confirm that Shp2 is responsible for the dephosphorylation of FGFR2 in the absence of Grb2, we measured the effect of a Shp2 inhibitor (NSC87877) on the pFGFR2 level in HEK293T cells in which Grb2 had been knocked down (Fig. 1 D). NSC87877 is a potent inhibitor that is selective for Shp2 over other PTPs (except the highly homologous Shp1; Chen et al., 2006). In Grb2i cells the concentration of pFGFR2 was reduced, corroborating the data



**Figure 1. Knockdown of Grb2 and Shp2 reveal that the level of WT FGFR2 phosphorylation is controlled by Grb2.** (A) Total HEK293T cell lysates were immunoblotted with anti-pFGFR antibody (top), and reprobed for total FGFR (middle) and Grb2 (bottom). Anti-pFGFR antibody is specific for A loop residues Y653 and Y654. (B) Cell lysates of overnight serum-starved stable A431 cells containing control shRNA (Ci), Grb2-shRNA (Grb2i), or Shp2-shRNA (Shp2i) were analyzed for FGFR2 phosphorylation as above. Only the nonstimulated state is shown (i.e., each lane is duplicated). Numbers on pFGFR2 panel are normalized intensity pFGFR2/total FGFR2. (C) Analysis of FGFR2 phosphorylation in Rat1 fibroblast cells with control shRNA (Ci) and Grb2-shRNA (Grb2i) as above. Only the nonstimulated state was investigated (i.e., each lane is duplicated). (D) Inhibition of Shp2 in Grb2 knockdown cells restores basal receptor phosphorylation. Serum-starved WT-Ci and WT-Grb2i cells were incubated with 50  $\mu$ M NSC87877 for 4 h and the resultant cell lysates were analyzed by Western blotting with anti-pFGFR2 antibody, Shp2 pY542-specific antibody, and anti-Grb2 antibody. The immunoblot was stripped and reprobed for total FGFR2 and Shp2 as the loading control. The numbers on the pFGFR2 panel represent normalized intensity pFGFR2/total FGFR2.

from Fig. 1, A–C. Incubation with the Shp2 inhibitor resulted in an increase in pFGFR2 to levels similar to those seen in Ci cells (Fig. 1 D). Again in HEK293T cells overexpressing the S<sup>252</sup>W<sup>253</sup>FGFR2 mutant receptor, which is unable to bind Grb2, incubation with NSC87877 resulted in increased basal receptor phosphorylation (Fig. S2, B–D). These data strongly suggest that Grb2 constitutively inhibits the ability of Shp2 to dephosphorylate the receptor. Furthermore, it also infers that a basal level of FGFR2 phosphorylation prevails even in the absence of Grb2. Thus, it appears that in wild-type cells Grb2 serves to stabilize and control this FGFR2 kinase activity through heterotetramer formation.

#### Grb2 inhibits direct complex formation between FGFR2 and Shp2

Having demonstrated that dephosphorylation of FGFR2 by Shp2 is impeded in the presence of Grb2, we investigated whether Grb2 was inhibiting the formation of a direct equilibrium complex between the receptor and phosphatase. Formation of a stable complex is likely to be important in directing phosphatase activity toward FGFR2. To measure direct binding of Shp2 to FGFR2 we used fluorescence lifetime imaging microscopy (FLIM) to detect stable complexes through fluorescence resonance energy transfer (FRET) between fluorophore-tagged proteins. RFP-tagged Shp2 was transfected into HEK293T Ci and Grb2i cells that were stably overexpressing FGFR2-GFP.

Colocalization and binding were imaged under serum-starved conditions (Fig. 2, A–D). As expected, in nonstimulated cells FGFR2-GFP is localized primarily in the plasma membrane, whereas Shp2-RFP displays a diffused distribution (Fig. 2, A–D). In both the wild-type Ci control cells and Grb2i cells no significant interaction between FGFR2-GFP and Shp2-RFP was seen (Fig. 2, A and B, basal), as shown by the population distribution of the fluorescence lifetimes being largely at longer lifetimes (right shifted) than that of isolated GFP at 2.0 ns (indicated by the vertical line through the right-hand panels), i.e., there is no evidence of FRET between the GFP and RFP fluorophores. Because the FGFR2–Shp2 interaction might only occur on transient catalytic turnover of receptor substrate phosphotyrosine residues, we repeated the FLIM experiments in cells transfected with the Shp2 C459S “substrate-trapping” mutant, the catalytic activity of which is compromised, and the phosphatase domain interacts with the substrate in an equilibrium-binding mode only (Agazie and Hayman, 2003; Blanchetot et al., 2005). Again we see no significant evidence of binding between receptor and phosphatase in the presence of Grb2 (Fig. 2 C). However, in the absence of Grb2, binding of Shp2 C459S mutant to FGFR2 can be observed (Fig. 2 D; peak in right-hand panel shifts to left of the vertical line, i.e., to shorter lifetimes resulting from FRET between GFP and RFP). These data support a model in which the presence of Grb2 inhibits direct binding of Shp2 to the receptor. The presence of Grb2 bound to the C terminus of FGFR2 may sterically hinder access of the phosphatase to its cognate site, and/or inhibit the receptor kinase activity toward a tyrosine residue(s) required for Shp2 recognition.

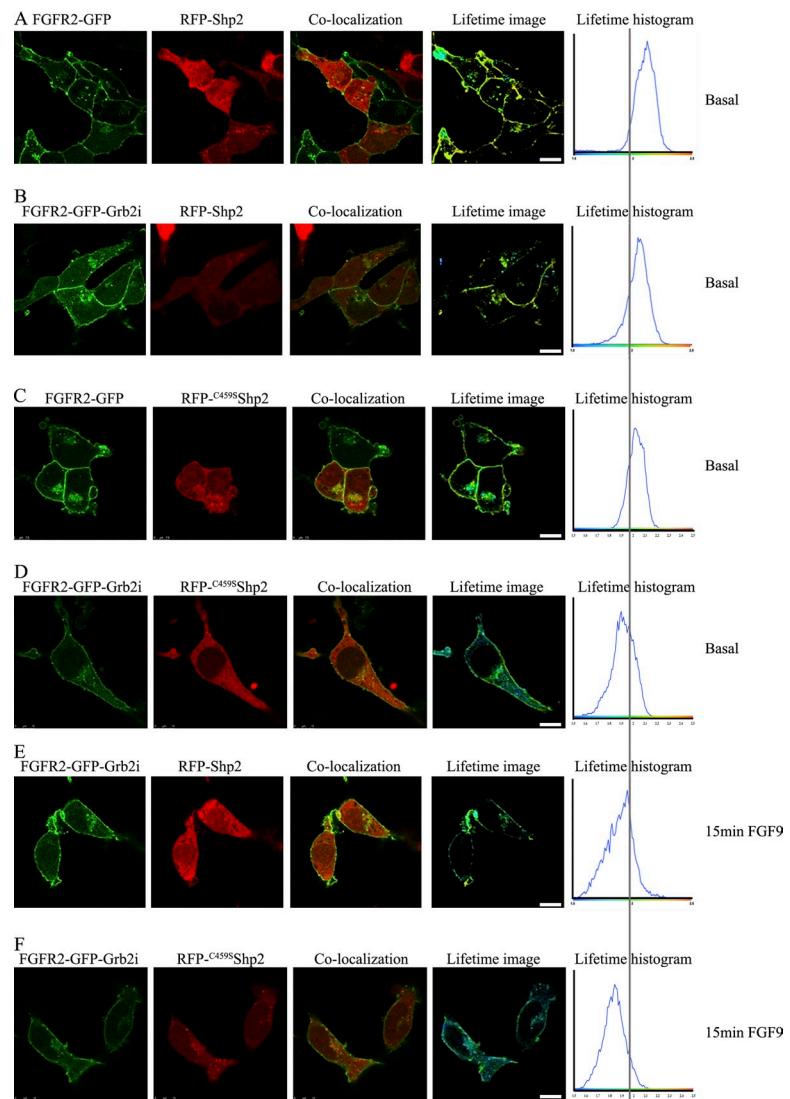
By way of comparison, we show that on stimulation of FGFR2 with FGF9 the population of Shp2 and FGFR2 complex increases significantly (Fig. 2, E and F; see population shifts to shorter lifetimes in right-hand panels). This is consistent with the model in which stimulation and up-regulation of kinase activity of FGFR2 results in dissociation of Grb2 (Lin et al., 2012), promoting formation of the Shp2–FGFR2 complex.

#### FGFR2-mediated phosphorylation of Shp2 is controlled by Grb2

Phosphatase activity has been reported to be enhanced by tyrosyl phosphorylation on Shp2. Shp2 has two C-terminally located tyrosine residues (Y542 and Y580) that have been implicated in the mechanism for release of the auto-inhibited state of the phosphatase (Lu et al., 2001). It has been suggested that phosphorylation of one (or both) of these tyrosines is important in Shp2 activation, although there has been no clear demonstration that tyrosyl phosphorylation of Shp2 is necessary to initiate signaling. However, phosphorylation of Shp2 does appear to be involved in effecting downstream extracellular signal-regulated kinase (ERK) activation (Araki et al., 2003).

RTKs have been shown to phosphorylate Shp2, although a role for FGFR2 in this activity has not been reported. To demonstrate that Shp2 is a substrate for FGFR2, HEK293T Ci serum-starved cells were incubated with and without FGFR2 kinase inhibitor (SU5402) for 2 h. Fig. 3 A shows in the Ci nonstimulated cells there is a background level of phosphorylated Y542

**Figure 2. Grb2 inhibits the interaction of Shp2 with FGFR2.** (A) FLIM analysis of the FRET between the FGFR2-GFP and RFP-Shp2. In the control (WT-Ci) serum-starved cells no interaction between FGFR2 and Shp2 was observed in the basal state. The mean FRET lifetime is  $\sim 2.0$  ns (line in right-hand panel), which corresponds to the mean lifetime for isolated GFP. No apparent interaction between FGFR2 and Shp2 in WT-Grb2i cells (B), or WT-Ci cells transfected with the RFP-tagged substrate-trapping C459S Shp2 mutant (C). Interaction between FGFR2 and Shp2 is observed in the Grb2i (the substrate-trapping C459S mutant) (D). Stimulating cells that contain <sup>WT</sup>Shp2 or C459S mutant Shp2 with FGF9 results in clear binding between FGFR2 and Shp2 after 15 min (E and F), respectively. Bar, 10  $\mu$ m.



(pY542) on Shp2. In the presence of the FGFR2 inhibitor the concentration of pY542 is reduced. This strongly suggests that Shp2 is a substrate for basal FGFR2 kinase activity and thus the receptor is able to enhance phosphatase activity. We have thus identified a catalytic cycle around Shp2 phosphatase dephosphorylating FGFR2, and FGFR2 kinase phosphorylating Shp2.

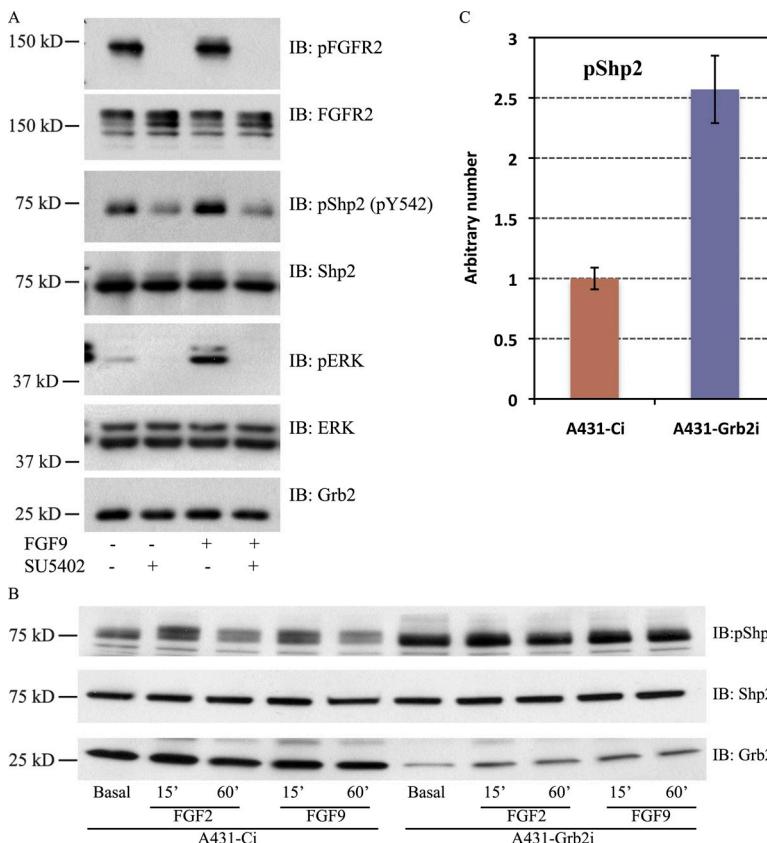
To assess whether constitutive FGFR2 phosphorylation of Shp2 is under the control Grb2, phosphorylation of Y542 on Shp2 was investigated in the presence or absence of Grb2. In A431-Grb2i cells the concentration of phosphorylated Shp2 is elevated by  $\sim 2.5$ -fold compared with A431-Ci cells in the nonstimulated state (Fig. 3, B and C). The presence of Grb2 therefore has an inhibitory effect on FGFR2-derived phosphorylation of the phosphatase.

#### Shp2 binds to and dephosphorylates Grb2

Previously we reported that Grb2 can be phosphorylated by FGFR2. Phosphorylation of Y209 in the C-terminal SH3 domain (C-SH3) of Grb2 in the interface with FGFR2 sterically and/or electrostatically hinders complex formation, resulting in the release of phosphorylated Grb2 (pGrb2; Lin et al., 2012).

FGFR2 kinase activity is switched on in the nonstimulated state, as demonstrated by phosphorylation of activation loop tyrosine residues Y653/Y654 (Fig. 1, A–C). Here we confirm the occurrence of this activity under nonstimulated conditions by the observation of a higher molecular weight band corresponding to phosphorylated Grb2 (also described by Lin et al., 2012) that is apparent in HEK293T cells overexpressing FGFR2 (Fig. 4 A). This phosphorylation of Grb2 can be attributed to FGFR2 kinase activity because the higher molecular weight band is absent in wild-type HEK293T cells, which do not express endogenous FGFR2 kinase. Thus, the phosphorylation and subsequent dissociation of Grb2 can occur in the nonstimulated state.

Accumulation of increasing cellular concentrations of pGrb2 (which cannot bind receptor) would result in release of the controlling influence of Grb2 on FGFR2 kinase activity; thus, dephosphorylation of Grb2 is required to maintain cellular homeostasis in the nonstimulated state. Using an in vitro experiment in which purified full-length wild-type Shp2 (<sup>WT</sup>Shp2) was incubated with phosphorylated C-SH3 domain of Grb2 we demonstrated that Shp2 can dephosphorylate Grb2 on residue



**Figure 3. Shp2 phosphorylation by FGFR2 is inhibited by Grb2.** (A) Serum-starved HEK293T cells were incubated with 30  $\mu$ M FGFR inhibitor (SU5402) for 2 h and then either stimulated with 10 ng/ml FGF9 for 15 min or left untreated. Cell lysates were prepared and analyzed by Western blotting with the indicated antibody. Anti-pFGFR and anti-Y542 on Shp2 antibodies were used to evaluate phosphorylation of proteins. The immunoblot was stripped and reprobed with a pan-antibody to determine total protein level. (B) Comparison of ligand-stimulated Shp2 phosphorylation between A431-Ci and A431-Grb2i cells in nonstimulated and on stimulation by FGF2 or FGF9 for 1 h. Shp2 phosphorylation was detected with anti-pY542 antibody (top). The immunoblot was reprobed for total Shp2 as a loading control (middle) and with Grb2 (bottom). (C) Densitometric quantification of basal state Shp2 phosphorylation levels in A431 cells in control shRNA (A431-Ci) and Grb2-shRNA (A431-Grb2i). Error bars represent SD,  $n = 7$ .

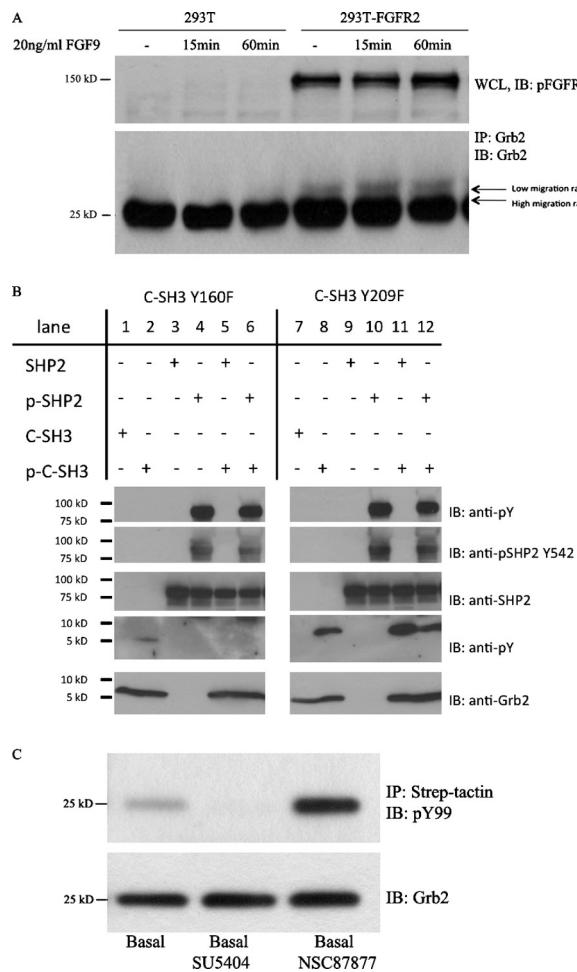
Y209 (Fig. 4 B). There are two phosphorylatable tyrosines on C-SH3 (Y160 and Y209; Lin et al., 2012). To assess whether Shp2 phosphatase activity was specific for either site we individually mutated each of these residues to a nonphosphorylatable phenylalanine. Fig. 4 B shows that the amount of pGrb2 of the Y160F decreases in the presence of Shp2, but not in the Y209F mutant. This result shows that Shp2 can dephosphorylate the C-SH3 domain of Grb2 and is specific for pY209.

To confirm phosphatase activity of Shp2 toward pGrb2 in a cellular context, HEK293T cells were cotransfected with GFP-FGFR2 and strep-tagged Grb2 (strep-Grb2) and incubated with either an FGFR2 kinase inhibitor (SU5402) or a Shp2 inhibitor (NSC87877), and the level of pGrb2 was determined (Fig. 4 C). Under nonstimulated conditions, in the absence of either inhibitor we observed the presence of a low level of pGrb2. pGrb2 is not observed in the presence of the kinase inhibitor, confirming that the basal FGFR2 kinase activity is required for Grb2 phosphorylation. Inhibition of the phosphatase resulted in increased concentrations of pGrb2 compared with when the enzyme is uninhibited.

Having established that Grb2 is a substrate for Shp2, we investigated the mechanism by which the phosphatase is recruited. Because Shp2 phosphorylated on Y542 has been previously reported to bind Grb2 and in doing so affect auto-inhibition of the phosphatase domain (Lu et al., 2001), we confirmed that this was important for complex formation between these two proteins in cells. FLIM data on A431 cells expressing N-terminal CFP-tagged Grb2 (CFP-Grb2) and N-terminal RFP-tagged Shp2 (RFP-Shp2) reveal that in the nonstimulated state a low

level of interaction between Shp2 and Grb2 is observed, as shown by slight left shift in peak position and overall distribution under the curve compared with CFP alone (line drawn at the lifetime of isolated CFP, 2.2 nm; see right-hand panels of Fig. 5, A and B). The shift to shorter lifetimes is more pronounced in the stimulated state, presumably due to the presence of increased concentrations of phosphorylated Shp2 (Fig. 5 B). The nonphosphorylatable Y542F Shp2 mutant showed less ability to bind Grb2, even under stimulated cellular conditions (Fig. 5 C). This demonstrates that binding to, and hence dephosphorylation of Grb2 by Shp2 is, at least in part, mediated by the binding of pY542. Interestingly, the substrate-trapping C459S mutant shows significant binding in the basal state (almost the entire population of tagged protein is left shifted; Fig. 5 D). Because the substrate-trapping mutant binds to the protein in an equilibrium mode, the observed enhanced population of complex, compared with that in the presence of <sup>WT</sup>Shp2, suggests that wild-type phosphatase is turning over pGrb2.

The above observations are important because they confirm the cycling of Grb2 between its FGFR2-phosphorylated state and Shp2-dephosphorylated state. This cycling of Grb2 is fundamental to the maintenance of homeostasis of FGFR2 kinase activity. The rate of cycling maintains a steady-state level of the phosphorylation of Grb2 and ensures that the phosphorylated receptor population is controlled and incapable of effecting a downstream response. On exposure to an extracellular ligand this cycling is presumably pushed toward increased concentrations of pGrb2 and hence expanding the population of up-regulated FGFR2 molecules.

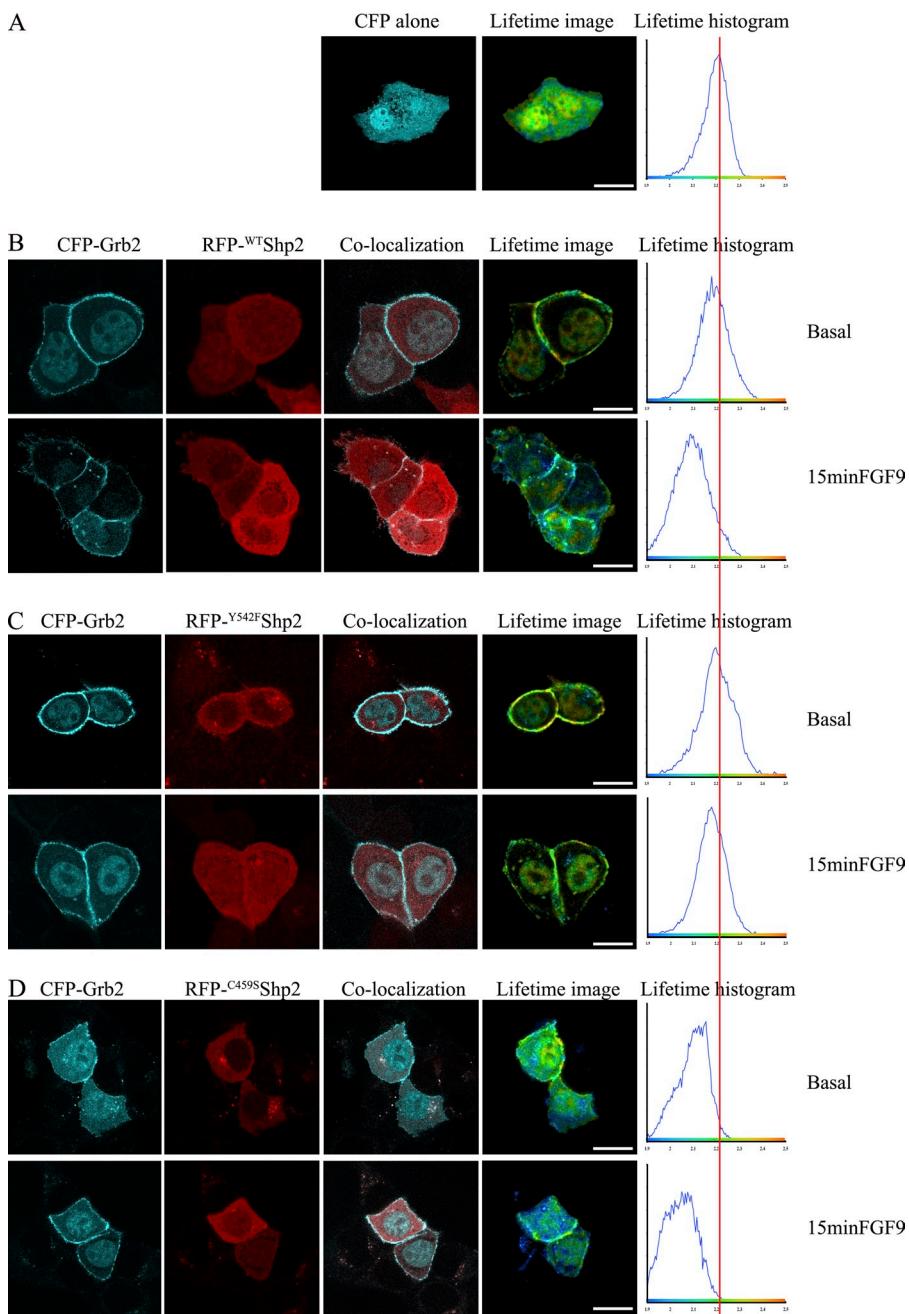


**Figure 4. Shp2 dephosphorylates Grb2.** (A) Wild-type or FGFR2 stably transfected HEK293T were starved overnight, then stimulated using 10 ng/ml FGF9 for either 15 or 60 min. Cells were lysed in the presence of protease and phosphatase inhibitors. 50 µg of total cell lysate were used for immunoblotting studies. Phosphorylation of FGFR2 was examined using anti-pFGFR2 (first panel). To examine the Grb2 phosphorylation states in the absence or presence of FGFR2 expression, 1 mg of total cell lysates were used for immunoprecipitation using an anti-Grb2 antibody, and probed with an anti-Grb2 antibody. The immunoprecipitated Grb2 from FGFR2-overexpressing cells show multiple bands (both serum starved and FGF9 stimulated), suggesting the high molecular weight species is tyrosine-phosphorylated Grb2, which is only phosphorylated in the presence of FGFR2. (B) Recombinant Grb2 C-SH3 mutants (Y160F, left; Y209F, right) were expressed and purified from *E. coli* and incubated with pure FGFR2 cytoplasmic domain in a 1:1 molar ratio in the presence of ATP and MgCl<sub>2</sub> at room temperature for 1 h. Recombinant GST-fused pShp2 was obtained via the same protocol. A general anti-pY antibody was used to examine the phosphorylation state of FGFR2-phosphorylated Shp2 (lanes 4, 6, 10, and 12; panel 1) and Grb2 C-SH3 domains (lanes 2 and 8; panel 4). A specific anti-pY542 Shp2 antibody was also used to confirm that Y542 of Shp2 is phosphorylated. A pool of both proteins was dephosphorylated by mixing phosphatase (either pShp2 or Shp2) with phosphorylated protein substrates (either pGrb2 C-SH3 Y160F or phospho-Grb2 C-SH3 Y209F) at 4°C overnight. The anti-pY blot shows only the pGrb2 C-SH3 Y160F can be dephosphorylated by both pShp2 and Shp2 (lanes 5 and 6; panel 4). However, the phosphorylation state of pGrb2 C-SH3 Y209F is not affected by Shp2, suggesting that the Y209 is the target of Shp2. A total Shp2 antibody (panel 3) and total Grb2 antibody (panel 5) were used to confirm equal protein loading. (C) HEK293T cells were cotransfected with FGFR2-GFP and Grb2-strep-tag. After 48 h cells were starved for 4 h and incubated with either FGF-specific inhibitor (50 µM SU5402) or Shp2-specific inhibitor (100 µM NSC87877) for 1 h. Cell lysates were subjected to affinity purification using strep-tactin agarose beads and immunoblotting with anti-pY antibody (top) followed by anti-Grb2 antibody (bottom).

To demonstrate the cycling of Grb2 between the receptor-bound/dephosphorylated state and the unbound/phosphorylated state we used an *in vitro* FRET-based spectroscopic assay (Fig. 6 A). The cytoplasmic region of FGFR2 (FGFR2<sub>cyto</sub> residues 400–821) and full-length Grb2 N-terminally labeled with GFP and RFP fusion proteins, respectively, were expressed and purified from *Escherichia coli*. Initially the fluorescence lifetime of isolated GFP-FGFR2 was measured (~2.64 ns; Fig. 6 B). On adding RFP-Grb2 the fluorescence lifetime in solution was measured as they formed the complex in which two receptor molecules were recruited to the Grb2 dimer (Fig. 6 A and Fig. S3; Lin et al., 2012). The formation of the complex results in FRET between the GFP and RFP fluorophores, and hence, a decrease in the fluorescence lifetime. ATP/Mg<sup>2+</sup> was added to activate the FGFR2 kinase and the fluorescence lifetimes were measured at given time points. The lifetime was seen to increase as a consequence of the reduction in direct interaction between receptor and adaptor protein as Grb2 is phosphorylated by the activated RTK (Fig. 6 B). The lifetime reaches a steady state as the concentration of pGrb2 maximizes (~2.64 ns). Shp2 was then added to the solution of FGFR2<sub>cyto</sub> and Grb2 (Fig. 6 A) and the lifetime was recorded. The presence of the phosphatase results in the decrease in the fluorescence lifetime of GFP-FGFR2<sub>cyto</sub> due to FRET as the concentration of pGrb2 is reduced and the resulting dephosphorylated Grb2 binds to the receptor (Fig. 6, A and B). These *in vitro* data mirror the observations in cells described in Fig. 4 by reproducing the interactions and turnover of Grb2 phosphorylation in the presence of kinase and phosphatase.

By way of additional confirmation that Grb2 was a substrate for Shp2 we used the substrate-trapping C459S mutant of Shp2 in the fluorescence spectroscopic assay. This mutant has no inherent catalytic activity, and hence Grb2 should not be dephosphorylated in its presence. The addition of this mutant results in no reduction in the fluorescence lifetime, consistent with no recovery of the Grb2–FGFR2 complex (Fig. 6 B). Furthermore, to confirm the requirement of pY542 to promote phosphatase activity against Grb2 we used the nonphosphorylatable Y542F mutant Shp2 in place of <sup>WT</sup>Shp2. In this case the recovery of the Grb2–FGFR2 complex was abrogated (the fluorescence lifetime remains at ~2.64 ns; Fig. 6 C), reflecting the reduction in the binding of the mutant Shp2 to Grb2 and hence reduced dephosphorylation of Grb2.

We confirmed these observations using the independent approach of time-correlated single-photon counting (TCSPC) to measure the GFP-FGFR2<sub>cyto</sub> lifetime. This experiment measures FRET between the RFP-Grb2 and FGFR2-GFP fluorophore fusion tags in a similar way to the previous experiment; however, rather than measuring one lifetime at a specific wavelength, this method uses a dicrotic filter to detect fluorescence emission over a range of wavelengths. The data are measured and presented as a percentage of the total population of interactions (Fig. 6 C and Fig. S3). The FLIM data for the interaction between FGFR2<sub>cyto</sub> and Grb2 in the presence of <sup>WT</sup>Shp2 and the C459S and Y542F mutants was entirely consistent with the spectroscopic data. Interestingly, after an extended period (18 h) in the presence of the Y542F Shp2 mutant, the percentage of



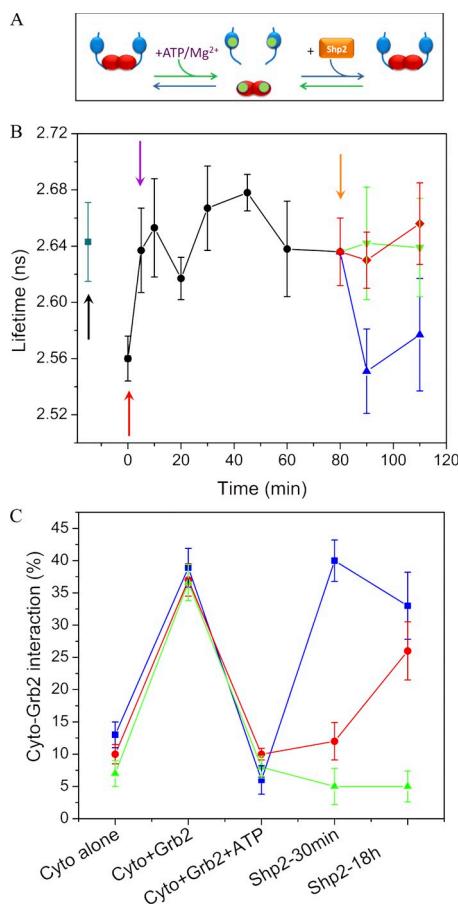
**Figure 5. Interaction between Grb2 and Shp2.** CFP-Grb2 and RFP-Shp2 colocalization and direct interaction measurement using FLIM in A431 cells. (A) Control lifetime measurement for CFP alone. (B) Interaction of CFP-Grb2 with RFP-tagged wild-type Shp2 (RFP<sup>WT</sup>Shp2) at basal and after 20 ng/ml FGF9 stimulation. (C) Co-localization and direct interaction of Y542F mutant Shp2 with CFP-Grb2 at basal and after FGF9 stimulation. (D) Constitutive interaction of the C459S substrate-trapping Shp2 mutant with Grb2. A left-shifted peak relative to the line drawn along 2.2 ns indicates a binding. A peak centered on the 2.2 ns line indicates nonbinding. Bar, 20  $\mu$ M.

interactions increases. This is presumably the result of phosphatase activity that still occurs, but the rate is significantly reduced in the absence of the amplifying effect of the pY542 interaction with Grb2.

#### Shp2 inhibition restores MAP kinase activity in Grb2 knockdown cells

The focus of this work is on the cycling of kinase and phosphatase activity under the control of Grb2 in the nonstimulated state. However, this control could ultimately have an effect on signal transduction once the cells are exposed to growth factor. The general viability of Grb2 knockdown cells indicates some of the primary functions generally associated with the presence of Grb2 in these cells are still intact (e.g., the reduction in overall Grb2 concentration does not abrogate signaling through the

MAP kinase pathway once cells are stimulated). However, Grb2-depleted HEK293T and A431 cells do display lower levels of growth factor-stimulated MAP kinase activity as reflected in levels of phosphorylated ERK (pERK; Fig. 7, A, C, and D; Fig. S4, A and C). One explanation for this could be that the binding of Grb2 to FGFR2 stabilizes receptor dimers, increasing the probability of active signaling on growth factor stimulation. This obviously would not occur in the absence of Grb2. Another possibility is that in the absence of Grb2, ligand-activated receptors are rapidly and concomitantly deactivated by Shp2, rendering them signaling incompetent. This would result in reduced FGF-stimulated MAP kinase activation. We investigated this latter hypothesis using the Shp2-specific inhibitor to demonstrate that, if Shp2 is responsible for deactivating FGFR2 in Grb2i cells, then its inhibition would allow MAP



**Figure 6. In vitro demonstration of catalytic cycling of FGFR2 and Shp2 in the presence of Grb2.** (A) Schematic of interactions performed in vitro to demonstrate catalytic activity of FGFR2 and Shp2 on Grb2. Mixing FGFR2<sub>cyto</sub> (blue) with Grb2 (red) promotes the formation of a heterotetrameric complex (Lin et al., 2012). Addition of ATP and MgCl<sub>2</sub> to this results in phosphorylation of FGFR2 and Grb2 (green circle). Addition of Shp2 (orange) results in dephosphorylation of FGFR2 and Grb2 (blue line). The heterotetrameric complex is recovered under these conditions. (B) Fluorescence lifetime measurement between GFP-FGFR2<sub>cyto</sub> and RFP-Grb2 as a function of time. The first point corresponds to the fluorescence lifetime for isolated GFP-FGFR2 (black arrow). On addition of Grb2 (red arrow) a heterotetrameric complex between Grb2 and FGFR2 forms. This results in FRET between the GFP and RFP and the concomitant reduction in fluorescence lifetime. On addition of ATP/Mg<sup>2+</sup> (purple arrow) up-regulation of the RTK ensues and Y209 on Grb2 becomes phosphorylated and the FGFR2-Grb2 complex dissociates. The lifetime increases, reflecting reduction in complex concentration and the accumulation of pGrb2. After 80 min Shp2 was added (orange arrow). At this point clear reassociation of Grb2 and FGFR2 is observed as Grb2 is dephosphorylated in the presence of Shp2 and consequently the fluorescence lifetime decreases (blue line on graph). Replacing <sup>WT</sup>Shp2 with the Y542F (red line) or C459S (green line) mutant results in no immediate reduction in lifetime, confirming that the FGFR2-Grb2 complex is not rescued by adding these compromised phosphatases. (C) Measurement of FRET between GFP-FGFR2<sub>cyto</sub> (Cyto) and RFP-Grb2 in solution using FLIM. Cyto alone is GFP-FGFR2<sub>cyto</sub> and represents the background false-positive percentage FRET readout. Cyto+Grb2 is the population of molecules undergoing FRET when RFP-Grb2 is present. Cyto+Grb2+ATP is the population of GFP-FGFR2<sub>cyto</sub> undergoing FRET with RFP-Grb2 when the FGFR2 kinase was activated. Shp2 30 min and 18 h represent the reestablishment of GFP-FGFR2<sub>cyto</sub>/RFP-Grb2 complex in the presence of wild-type (blue line), Y542F (red line), and C459S (green line) mutant Shp2 as a function of time.

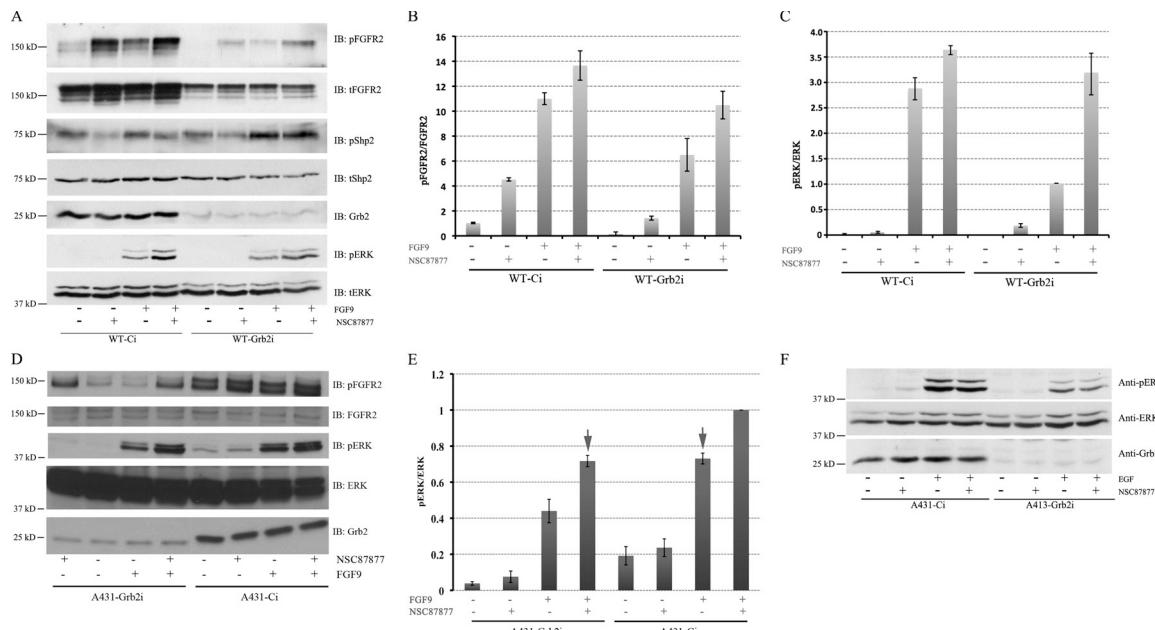
kinase pathway activation to be restored to its normal level. The control Ci and Grb2i HEK293T cells were treated with the Shp2 inhibitor (NSC87877) and receptor phosphorylation level

and MAP kinase activation, as manifested by phosphorylation ERK (Fig. 2, A and C), were monitored under basal conditions and after FGF9 stimulation. The results of these experiments show that inhibition of Shp2 in Grb2 knockdown cells does indeed restore FGF9-stimulated pERK (Fig. 7, A and C). Furthermore, decreased <sup>WT</sup>FGFR2 phosphorylation in Grb2i cells can be rescued by Shp2 inhibition (Fig. 7, A and B). Shp2 inhibition in Grb2-depleted A431 cells also leads to increased receptor phosphorylation and restoration of the FGF-stimulated MAP kinase response (Fig. 7, D and E). However, Shp2 inhibition elicits little effect in EGF-stimulated MAP kinase, which remains unchanged (Fig. 7 F and Fig. S4 B). This therefore provides further evidence that Grb2 is an inhibitor of Shp2 in FGFR2 signaling, and in the absence of Grb2, Shp2 inhibition limits receptor activation and downstream signaling. Importantly, the Shp2 inhibitor pretreatment increases basal receptor phosphorylation, eliciting a significant downstream MAP kinase response. This increase in receptor phosphorylation appears significantly higher than the specific FGF-induced receptor phosphorylation that restored the downstream MAP kinase response (Fig. 7). This observation is important because it emphasizes that receptor phosphorylation alone is not sufficient for signal transduction.

## Discussion

The constitutive turnover of RTK phosphorylation requires control mechanisms to abrogate the possibility of aberrant signal transduction. This can be achieved by controlling kinase activity, blocking the attainment of the fully active state, and inhibiting recruitment of downstream effector proteins to the RTK. The data reported herein establish a role for Grb2 in which it applies a controlling influence over the basal kinase activity of FGFR2 and inhibits Shp2-mediated receptor dephosphorylation. This central control exerted by Grb2 can be represented by the cycle of contributing catalytic activity shown in Fig. 8 and summarized below.

Our recent findings revealed that under nonstimulated conditions, dimeric Grb2 can bind to two receptor molecules resulting in partial phosphorylation of FGFR2 including activation loop tyrosine residues (Lin et al., 2012; Fig. 8 A). In forming this heterotetrameric complex Grb2 inhibits both the dephosphorylation of FGFR2 by Shp2 and the phosphorylation of Shp2 by FGFR2 (Fig. 8, B and C, respectively). The inhibition of the dephosphorylation of partially phosphorylated FGFR2 (Fig. 8 B) can be demonstrated in cells in which Grb2 expression has been knocked down which show negligible basal receptor phosphorylation (Fig. 1). In the absence of Grb2, Shp2 is able to interact with FGFR2. This results in receptor dephosphorylation. As a result, inhibition of Shp2 under these conditions results in the recovery of the phosphorylated receptor (Fig. 1 D and Fig. 7). Not only does this confirm an inhibitory role for Grb2 on phosphatase activity, but it also suggests that an inherent background phosphorylation of FGFR2 occurs even in the absence of Grb2 in nonstimulated cells. We show that inhibition of Shp2 activity is likely to result from the inability of Shp2 to bind to the receptor in the presence of the Grb2-FGFR2



**Figure 7. Grb2 inhibits Shp2 activity toward FGFR2.** (A) WT-Ci and WT-Grb2i cells were incubated with serum-free media with or without 50  $\mu$ M Shp2 inhibitor NSC87877 overnight, and then either stimulated with 10 ng/ml FGF9 for 15 min or left untreated. Total cell lysates were analyzed by Western blotting with the indicated antibody. (B and C) Densitometric quantification of bands from experiments as described in A. Histogram values correspond to normalized bands for pFGFR2 against total FGFR2 (B) and pErk against total Erk (C) of three independent experiments. Error bars represent SD. (D) A431-Ci and A431-Grb2i cells were serum starved with 50  $\mu$ M Shp2 inhibitor overnight, then either stimulated with FGF9 or left untreated. 50  $\mu$ g total cell lysates were immunoblotted with indicated antibody. (E) Densitometric quantification of bands from experiments as described in D, where the ratio of pERK/total Erk is plotted from three independent experiments. Error bars represent SD. The A431-Ci FGF9/NSC87877 ratio was fixed as 1.0 for each experiment. Arrows highlight the comparison of the level of pErk in the control cells after FGF-ligand stimulation and the recovery of this level in the Grb2 knockdown cells only when stimulated in the presence of Shp2 inhibitor. (F) Shp2 inhibition does not affect EGF-stimulated MAP kinase response in A431 cells. The experimental procedure is as above except 50 ng/ml EGF was used to stimulate cells for 5 min.

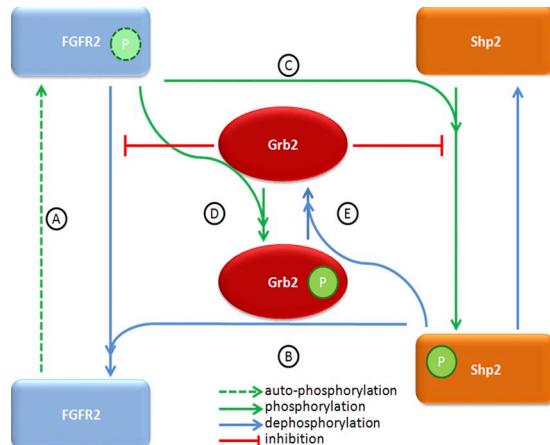
complex. Knocking down of Grb2 in cells allows Shp2 to interact with the receptor (Fig. 2).

Grb2 also has an effect on Shp2 activity inhibiting the phosphorylation of Shp2 by FGFR2 (Fig. 8 C). Knockdown of Grb2 elevates Shp2 phosphorylation (Fig. 3, B and C), strongly suggesting that the inability of Shp2 to interact directly with the receptor in the presence of Grb2 prevents FGFR2 kinase activity toward Shp2. The role of Grb2 therefore appears to be as the pivotal regulator of the phosphorylation of FGFR2 and the recruitment of Shp2 to its receptor substrate in the non-stimulated state.

The controlling effect of Grb2 can be relieved by phosphorylation of Y209 by FGFR2 (Fig. 8 D). Removal of the influence of Grb2 on FGFR2 kinase activity promotes Shp2 phosphorylation. The active phosphatase can then dephosphorylate the receptor, and importantly, rescue the inhibitory complex with Grb2 by dephosphorylating Y209 (Figs. 5 and 6). We confirmed that the phosphorylation of Y542 on Shp2 is important for binding to, and dephosphorylation of Grb2 (Fig. 6). This interlocking cycle of catalytic activity occurs constitutively, strongly suggesting a dynamic mechanism whereby there is continuous cycling of kinase (positive feedback) and phosphatase (negative feedback) activity toward FGFR2 phosphorylation. This “background cycling” is centrally controlled by Grb2 based on the relative apparent concentrations of dephosphorylated/phosphorylated Grb2, respectively. Under basal conditions, the pool of unphosphorylated Grb2 is large and the probability of finding it bound to the receptor is high, thus Shp2 activity is

down-regulated. This mutually dependent network of enzymatic activity is in homeostatic state, which is incapable of signal transduction. Addition of extracellular growth factor perturbs this state, promoting receptor kinase activity leading to the dissociation of Grb2 and resulting increased concentration of unfettered FGFR2 capable of recruitment of early signaling complex proteins and downstream response (O’Rourke and Ladbury, 2003). An additional outcome of up-regulated receptor activity is phosphorylation of Shp2, increasing its activity toward both FGFR2 and Grb2. Increased populations of dephosphorylated Grb2 and hence Grb2–FGFR2 complex restores the system to its constitutive homeostatic state.

In a Grb2 knockdown background loss of the Grb2–FGFR2 interaction leads to reduced receptor phosphorylation. This might mimic the effect of oncogenic mutations/deletions of FGFR2, which potentially abrogate Grb2–FGFR2 complex formation. Although knockdown of Grb2 concentration is likely to impinge on its recruitment to the son-of-sevenless (Sos) protein and hence affect MAP kinase pathway activation, Grb2 knockdown cells are still capable of eliciting a response on growth factor stimulation (Fig. S4). This implies that the FGFR2–Grb2 complex does not abrogate signaling in the nonstimulated state simply because it sequesters Grb2 and thus prevents it from being recruited to Sos. This finding is particularly important because, for example, a large number of FGFR2 mutations in melanoma are described as loss-of-function (Gartside et al., 2009), yet our data suggest that this apparent loss-of-function could still be oncogenic due to the mutation leading to the loss of receptor



**Figure 8. Schematic diagram of cycle of enzymatic activity under the control of Grb2 in the absence of extracellular stimulation.** (A) FGFR2 (blue) is stabilized in a basally phosphorylated state in the form of a heterotetramer in which a dimer of Grb2 recruits two receptor molecules. In this complex the receptors are able to autophosphorylate the activation loop tyrosines. The partially phosphorylated, nonsignaling state is represented by inclusion of the green circle with dashed border. (B) Active Shp2 is able to dephosphorylate FGFR2. This phosphatase activity is inhibited by Grb2 (red oval) when it is bound to the receptor. (C) Basally activated FGFR2 is able to phosphorylate Shp2 phosphatase (orange). The phosphorylation of Shp2 is represented by a solid green line. On phosphorylation of Y542 Shp2 is enhanced. This catalytic activity is inhibited in the presence of Grb2 bound to FGFR2. The phosphorylated Shp2 is represented by inclusion of the green circle. (D) Grb2 is phosphorylated by FGFR2. In this phosphorylated state Grb2 is no longer able to bind to the receptor and hence its inhibitory properties are lost. (E) Shp2 is able to dephosphorylate Grb2. This restores the adaptor protein to a state competent of binding FGFR2. Key: straight lines between proteins represent the change from one state of that protein to another. Green lines, phosphorylation. Blue lines, dephosphorylation. Green dashed line, autoprophosphorylation. Red lines, inhibition. Curved arrows, enzymatic activity, e.g., blue line from Shp2 intercepting dephosphorylation blue line between pFGFR and FGFR indicates that Shp2 is the active enzyme for that change of state.

regulation by Grb2. The major role of Grb2 with respect to FGFR2 therefore appears to be to stabilize the receptor molecules in a predimerized state in which the prevailing phosphorylation state can be kept in check through restraining kinase activity toward downstream effector recruitment sites on the receptor and inhibiting recruitment of Shp2.

The control by Grb2 of FGFR2 and Shp2 activity is quite different to that reported for FGFR1, where Grb2 and Shp2 are downstream of FGFR substrate 2 (FRS2) and their recruitment only occurs after receptor activation and is dependent on FRS2 phosphorylation (Hadari et al., 1998; Sato et al., 2010). To confirm this, FLIM was used to assess whether Grb2 was able to interact with FGFR1 in the same way observed for FGFR2 (Ahmed et al., 2010). As expected, Grb2 interacts with FGFR2 in HEK293T cells under nonstimulated conditions (Fig. S1 A), but has no discernible interaction with FGFR1 under the same conditions (Fig. S1 B). This differential recruitment of early signaling protein suggests that the complex generated by FGFR2 is significantly different from that which accompanies FGFR1. A further distinction between FGFR2 and FGFR1 can be observed in the recruitment of the key downstream effector FRS2. We had previously shown under nonstimulated conditions that unlike FGFR1, FGFR2 does not form a complex with FRS2

(Ahmed et al., 2008). Co-immunoprecipitation experiments confirm that under nonstimulated conditions FRS2 does not bind to FGFR2 (Fig. S1 D). Furthermore, no discernible interaction between Shp2 or Grb2 with FRS2 can be observed (Fig. S1, E and F), as has been reported for FGFR1 (Hadari et al., 1998). In contrast, ligand-induced signal transduction from FGFR2 and FGFR1 appears to occur predominantly through formation of an FGFR–FRS2–Shp2 complex (Fig S1, D–F). These distinctions between protein recruitment by the receptors could partially explain the differences in oncogenic properties of the receptors because in some cancers signaling through FGFR2 and FGFR1 has opposing outcomes for cancer progression and metastasis (Korah et al., 2000; Freeman et al., 2003; Xian et al., 2007).

This study raises important questions regarding the future design of therapeutic agents directed against FGFR2-initiated signaling because it highlights the balance between kinase and phosphatase activity (see schematic in Fig. S5), which can be readily perturbed by inhibitors. Disturbing one aspect of this balance through therapeutic interaction could influence regulation of another (Fig. 8). Indeed, understanding the mechanism of control of the balance between kinase and phosphatase activity could suggest novel targets for pharmaceutical intervention. For example, synthetic molecules that influence the control function of Grb2 might provide innovative drug compounds.

## Materials and methods

### Reagents

Recombinant human FGF9, FGF2, and EGF were purchased from PeproTech or R&D Systems. Antibodies against FGFR2 (C-17), Shp2 (C-18), Grb2 (C-23) and phosphotyrosine (pY99), as well as Shp2 inhibitor NSC87877 and FGFR2 inhibitor SU5402, shRNA, and also lentivirus particles for control (catalog no. sc-108080), Grb2 (catalog no. sc-29335-v), and Shp2 (catalog no. sc-36488-v) were all purchased from Santa Cruz Biotechnology, Inc. The following antibodies were purchased from Cell Signaling Technology: pFGFR2 (anti-pY653/654), Shp2 (pY542), pERK, and ERK. Anti-GFP goat antibody was purchased from Rockland Immunochemicals. Metafectin cell transfection reagents were purchased from Biontex-USA.

### Cell culture

HEK-293T, A431, and Rat-1 fibroblast cells were maintained in Dulbecco's modified Eagle's high glucose medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum (FBS) and 1% antibiotic/antimycotic (Lonza) in a humidified incubator with 10% CO<sub>2</sub>. Stable HEK293T cells containing the <sup>WT</sup>FGFR2-GFP (GFP fused to the C terminus of <sup>WT</sup>FGFR2) were produced as described previously (Ahmed et al., 2008). In brief, the wild-type FGFR2 gene was cloned into a pEGFP-N2 vector, and transfection of the FGFR2 containing plasmid was done using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Stable cells were generated by G418 selection (1 mg/ml) for 2 wk with a change of medium every 4 d. The presence of the GFP tag has no discernible effect on FGFR2 function (Lin et al., 2012). PC12 cells (European Collection of Cell Cultures accession no. 88022401) were cultured in DMEM supplemented with 5% FBS and 10% horse serum and 1% antibiotic/antimycotic (Lonza) in a humidified incubator with 10% CO<sub>2</sub> (Schüller et al., 2008). Control (Ci), Grb2, and Shp2 knockdown cells were generated by infecting designated cells with respective lentiviral particles. After 24 h infection, 7 µg/ml puromycin was added and antibiotic-resistant cells were further expanded and used for experimentation. Mammalian cell transfection was performed in a 6-well plate using Metafectin reagents according to the manufacturer's instructions.

### Western blots

Cells were grown in 10-cm dishes, serum starved overnight, and stimulated with either 10 ng/ml FGF2 or FGF9 for the indicated time period.

Cells were lysed with buffer containing 50 mM Hepes, pH 7.5, 1% (vol/vol) igepal-C630, 1 mg/ml bacitracin, 1 mM EDTA, 10 mM NaF, 1 mM sodium orthovanadate, 10% (vol/vol) glycerol, 50 mM NaCl, 1 mM PMSF, and Protease Inhibitor Cocktail Set III (EMD Millipore). The detergent-soluble fraction was used for Western blotting. Quantification of Western blots was done using ImageJ software.

#### Expression and purification of proteins from bacteria

Histidine-tagged fusion proteins were purified from BL21 (DE) cells. 10 ml of cells grown overnight were used to inoculate 1 liter of LB media loaded with 50 µg/ml kanamycin. The culture was allowed to grow with constant shaking (180 rpm) at 37°C until the OD<sub>600</sub> = 0.6. At this point the culture was cooled down to 20°C before being induced by the addition of 300 µM IPTG. The culture was allowed to grow for a further 12 h before harvesting by centrifugation. Cells were resuspended in 50 mM Tris buffer at pH 8.0, containing 100 mM NaCl and 1 mM β-ME, in the presence of protease inhibitors and lysed by sonication. Cell debris was removed by centrifugation (20,000 g at 4°C for 45 min). The soluble fraction was applied to an affinity column previously loaded with the above buffer followed by washing in 20 mM imidazole. Protein was eluted from the column with 200 mM imidazole and then concentrated to 5 ml before being applied to a Superdex SD75 gel filtration column loaded with 50 mM Tris buffer at pH 8.0, containing 100 mM NaCl and 1 mM β-ME. Analysis of pure proteins on SDS-PAGE showed greater than 95% purity. N-terminal GFP-tagged FGFR2<sub>cyto</sub>, N-terminal RFP-tagged Grb2, Shp2 wild-type, and Shp2 mutants were expressed and purified using this protocol.

#### Fluorescence lifetime measurement

FRET fluorescence lifetime experiments to measure the formation of the complex between Grb2 and FGFR2 in the absence/presence of ATP/Mg<sup>2+</sup> and Shp2 were performed in a spectrofluorometer (QuantaMaster-4-CW; Photon Technology International) at 23°C in 50 mM Tris-HCl at pH 8.5 containing 100 mM NaCl, 1 mM β-ME, and 5% glycerol. Phosphorylated C-terminal GFP-FGFR2<sub>cyto</sub> (donor) and N-terminal RFP-tagged Grb2 (acceptor) were mixed together in a 1:1 molar ratio to a final concentration of 1 µM for each protein. Donor lifetime decay (FRET fluorescence lifetime) was recorded with the pulsed nitrogen laser operating under the following parameters: excitation, 488 nm; emission, 510 nm; start delay, 135 ns; end delay, 150 ns; channels, 200; integration time, 50 µs; averages, 3; shots, 5; frequency, 8 Hz. The fluorescence lifetime is based on the average time that a fluorophore takes to return to the ground state from its excited state after absorption of light. When the acceptor fluorophore is proximal to the donor such that FRET occurs, the lifetime of the fluorescing donor is shortened. As they dissociate the donor fluorescence lifetime increases. 500 µM of ATP/Mg<sup>2+</sup> was added to the solution and lifetime measurements were taken at 20-min intervals up to 80 min. At the end of each time point EDTA was added to quench the reaction. Over this period the Grb2-FGFR2 complex dissociated. At the 80-min time point Shp2 was added to the system to a final concentration of 1 µM. At this point the molar ratio of FGFR2<sub>cyto</sub>/Grb2/Shp2 was ~1:1:1 because Grb2 and FGFR2 were diluted less than 3% after adding Shp2 to the solution. The fluorescence lifetime was then measured at 20-min intervals for 40 min to monitor the binding of Grb2 and FGFR2. Lifetime constants were calculated by fitting the decays using FeliX32 analysis software (Photon Technology International). A sample of raw data is shown in Fig. S3.

#### Cell imaging and FLIM

Cells were cotransfected with fluorescent protein-tagged proteins. Cells were grown on glass coverslips for 24–48 h before experimentation. Serum-starved cells were either left untreated as basal or stimulated with 20 ng/ml FGF9 for the indicated time periods. Stimulation was stopped by addition of 4% (wt/vol) paraformaldehyde, pH 8.0. Cells were washed 6–7 times with PBS, pH 8.0, and then mounted onto a slide with mounting medium (0.1% p-phenylenediamine/75% glycerol in PBS at pH 7.5–8.0). Cells were imaged using a confocal microscope (model SP5; Leica) at 20°C. FLIM experiments were performed using an inverted advanced confocal microscope system (TCP SP5; Leica) with internal photomultiplier tube (PMT) detector for time-correlated single-photon counting (TCSPC). The sample was excited with a tunable femtosecond titanium-sapphire pumped laser (Mai Tai BB; Spectra-Physics). The wavelength used for two-photon excitation was 930 nm. Images were obtained with an oil-immersion objective (NA 1.4) and a line scan speed of 400 Hz, with image size of 512 × 512 pixels. For FLIM analysis the pixels were reduced to 256 × 256. FLIM data were collected using an SPC830 data and image acquisition card (Becker & Hickl) for TCSPC. The fluorescence decays were fitted

with a single exponential decay model using SPCImage software (Becker & Hickl) and the GFP fluorescence lifetimes were displayed in a false-color map. The data shown are representative of three slides prepared from three independent experiments. For each image shown,  $n > 10$  cells analyzed from each slide

#### In vitro FLIM

GFP-FGFR2 cytoplasmic domain (Cyto), RFP-Grb2, and histidine-tagged Shp2 were bacterially expressed and purified. 300 µl of Cyto at the final concentration of 2 µM in PBS buffer containing 10% glycerol was loaded onto a 35-mm glass-bottom dish (MatTek Corporation). The fluorescence lifetime was measured using an oil immersion objective at 20°C as described above for FLIM, except the acquisition period was reduced to 120 s. RFP-Grb2 at final concentration of 2 µM was added to the Cyto mixture and allowed to equilibrate for 30 min (Fig. S4). The fluorescence lifetime of GFP-Cyto/RFP-Grb2 was recorded. 1 mM ATP/MgCl<sub>2</sub> was then added to the reaction mixture, and after 30 min another recording of the fluorescence lifetime was performed. Shp2 was then added to the mixture at a final concentration of 2 µM and allowed to equilibrate for 20 min before adding 2 mM EDTA to the reaction. After 30 min of EDTA addition GFP-Cyto lifetime was measured again. The reaction was then placed at 4°C overnight (18 h) and a further GFP-Cyto lifetime was recorded. This procedure was repeated for the Y542F and C459S Shp2 mutants. The lifetime data were used to calculate the percentage of interacting molecules (see Fig. S3).

#### Online supplemental material

Fig. S1 (A–C) shows FLIM data to demonstrate that Grb2 binds to FGFR2, but not FGFR1 or the oncogenic mutant S<sup>252W</sup>FGFR2. Fig. S1 (D–F) demonstrates that FRS2 does not interact with FGFR2 in the nonstimulated state. Fig. S2 shows that the S<sup>252W</sup>FGFR2 mutant receptor is not constitutively phosphorylated consistent with the absence of Grb2 inhibition. It also shows that when Shp2 is inhibited the phosphorylation of the receptor is restored to <sup>WT</sup>FGFR2 levels, suggesting that FGFR2 is a substrate the phosphatase, and in the background of <sup>WT</sup>FGFR2, Grb2 inhibits Shp2 activity. Fig. S3 (A) shows the fluorescence lifetime decay for GFP-FGFR2 and RFP-Grb2. Fig. S3 (B–E) shows the FLIM fluorescence lifetime plots for the in vitro interaction of FGFR2 Grb2 and Shp2. These raw data were used to produce Fig. 7 C. Fig. S4 shows reduced FGF- and EGF-activated MAP kinase activation in cells with reduced Grb2 protein levels. Fig. S5 is a schematic representation of the enzymatic cycle of FGFR2 kinase and Shp2 phosphatase activity influenced by Grb2 under nonstimulated (Fig. S5 A) and ligand-stimulated (Fig. S5 B) conditions.

This work was supported by the G. Harold and Leila Y. Mathers Charitable Foundation and by the MD Anderson Cancer Center Trust.

Submitted: 19 April 2012

Accepted: 18 January 2013

## References

- Agazie, Y.M., and M.J. Hayman. 2003. Development of an efficient “substrate-trapping” mutant of Src homology phosphotyrosine phosphatase 2 and identification of the epidermal growth factor receptor, Gab1, and three other proteins as target substrates. *J. Biol. Chem.* 278:13952–13958. <http://dx.doi.org/10.1074/jbc.M210670200>
- Ahmed, Z., A.C. Schüller, K. Suhling, C. Tregidgo, and J.E. Ladbury. 2008. Extracellular point mutations in FGFR2 elicit unexpected changes in intracellular signalling. *Biochem. J.* 413:37–49. <http://dx.doi.org/10.1042/BJ20071594>
- Ahmed, Z., R. George, C.C. Lin, K.M. Suen, J.A. Levitt, K. Suhling, and J.E. Ladbury. 2010. Direct binding of Grb2 SH3 domain to FGFR2 regulates SHP2 function. *Cell. Signal.* 22:23–33. <http://dx.doi.org/10.1016/j.cellsig.2009.08.011>
- Araki, T., H. Nawa, and B.G. Neel. 2003. Tyrosyl phosphorylation of Shp2 is required for normal ERK activation in response to some, but not all, growth factors. *J. Biol. Chem.* 278:41677–41684. <http://dx.doi.org/10.1074/jbc.M306461200>
- Barford, D., and B.G. Neel. 1998. Revealing mechanisms for SH2 domain mediated regulation of the protein tyrosine phosphatase SHP-2. *Structure.* 6:249–254. [http://dx.doi.org/10.1016/S0969-2126\(98\)00027-6](http://dx.doi.org/10.1016/S0969-2126(98)00027-6)
- Blanchetot, C., M. Chagnon, N. Dubé, M. Hallé, and M.L. Tremblay. 2005. Substrate-trapping techniques in the identification of cellular PTP targets. *Methods.* 35:44–53. <http://dx.doi.org/10.1016/j.jymeth.2004.07.007>

Byron, S.A., M.G. Gartside, C.L. Wellens, P.J. Goodfellow, M.J. Birrer, I.G. Campbell, and P.M. Pollock. 2010. FGFR2 mutations are rare across histologic subtypes of ovarian cancer. *Gynecol. Oncol.* 117:125–129. <http://dx.doi.org/10.1016/j.ygyno.2009.12.002>

Cha, J.Y., Q.T. Lambert, G.W. Reuther, and C.J. Der. 2008. Involvement of fibroblast growth factor receptor 2 isoform switching in mammary oncogenesis. *Mol. Cancer Res.* 6:435–445. <http://dx.doi.org/10.1158/1541-7786.MCR-07-0187>

Cha, J.Y., S. Maddileti, N. Mitin, T.K. Harden, and C.J. Der. 2009. Aberrant receptor internalization and enhanced FRS2-dependent signaling contribute to the transforming activity of the fibroblast growth factor receptor 2 IIIb C3 isoform. *J. Biol. Chem.* 284:6227–6240. <http://dx.doi.org/10.1074/jbc.M803998200>

Chardin, P., J.H. Camonis, N.W. Gale, L. van Aelst, J. Schlessinger, M.H. Wigler, and D. Bar-Sagi. 1993. Human Sos1: a guanine nucleotide exchange factor for Ras that binds to GRB2. *Science*. 260:1338–1343. <http://dx.doi.org/10.1126/science.8493579>

Chen, L., S.S. Sung, M.L.R. Yip, H.R. Lawrence, Y. Ren, W.C. Guida, S.M. Sefti, N.J. Lawrence, and J. Wu. 2006. Discovery of a novel shp2 protein tyrosine phosphatase inhibitor. *Mol. Pharmacol.* 70:562–570. <http://dx.doi.org/10.1124/mol.106.025536>

Dutt, A., H.B. Salvesen, T.H. Chen, A.H. Ramos, R.C. Onofrio, C. Hatton, R. Nicoletti, W. Winckler, R. Grewal, M. Hanna, et al. 2008. Drug-sensitive FGFR2 mutations in endometrial carcinoma. *Proc. Natl. Acad. Sci. USA*. 105:8713–8717. <http://dx.doi.org/10.1073/pnas.0803379105>

Feng, G.S., C.C. Hui, and T. Pawson. 1993. SH2-containing phosphotyrosine phosphatase as a target of protein-tyrosine kinases. *Science*. 259:1607–1611. <http://dx.doi.org/10.1126/science.8096088>

Freeman, R.M. Jr., J. Plutzky, and B.G. Neel. 1992. Identification of a human src homology 2-containing protein-tyrosine-phosphatase: a putative homolog of *Drosophila* corkscrew. *Proc. Natl. Acad. Sci. USA*. 89:11239–11243. <http://dx.doi.org/10.1073/pnas.89.23.11239>

Freeman, K.W., R.D. Gangula, B.E. Welm, M. Ozen, B.A. Foster, J.M. Rosen, M. Ittmann, N.M. Greenberg, and D.M. Spencer. 2003. Conditional activation of fibroblast growth factor receptor (FGFR) 1, but not FGFR2, in prostate cancer cells leads to increased osteopontin induction, extracellular signal-regulated kinase activation, and in vivo proliferation. *Cancer Res.* 63:6237–6243.

Gartside, M.G., H. Chen, O.A. Ibrahimi, S.A. Byron, A.V. Curtis, C.L. Wellens, A. Bengtson, L.M. Yudt, A.V. Eliseenkova, J. Ma, et al. 2009. Loss-of-function fibroblast growth factor receptor-2 mutations in melanoma. *Mol. Cancer Res.* 7:41–54. <http://dx.doi.org/10.1158/1541-7786.MCR-08-0021>

Goriely, A., H. Lord, J. Lim, D. Johnson, T. Lester, H.V. Firth, and A.O. Wilkie. 2010. Germline and somatic mosaicism for FGFR2 mutation in the mother of a child with Crouzon syndrome: Implications for genetic testing in “paternal age-effect” syndromes. *Am. J. Med. Genet. A*. 152A:2067–2073. <http://dx.doi.org/10.1002/ajmg.a.33513>

Grossmann, K.S., M. Rosário, C. Birchmeier, and W. Birchmeier. 2010. The tyrosine phosphatase Shp2 in development and cancer. *Adv. Cancer Res.* 106:53–89. [http://dx.doi.org/10.1016/S0065-230X\(10\)06002-1](http://dx.doi.org/10.1016/S0065-230X(10)06002-1)

Hadari, Y.R., H. Kouhara, I. Lax, and J. Schlessinger. 1998. Binding of Shp2 tyrosine phosphatase to FRS2 is essential for fibroblast growth factor-induced PC12 cell differentiation. *Mol. Cell. Biol.* 18:3966–3973.

Hattori, Y., H. Odagiri, H. Nakatani, K. Miyagawa, K. Naito, H. Sakamoto, O. Katoh, T. Yoshida, T. Sugimura, and M. Terada. 1990. K-sam, an amplified gene in stomach cancer, is a member of the heparin-binding growth factor receptor genes. *Proc. Natl. Acad. Sci. USA*. 87:5983–5987. <http://dx.doi.org/10.1073/pnas.87.15.5983>

Hattori, Y., H. Itoh, S. Uchino, K. Hosokawa, A. Ochiai, Y. Ino, H. Ishii, H. Sakamoto, N. Yamaguchi, K. Yanagihara, et al. 1996. Immunohistochemical detection of K-sam protein in stomach cancer. *Clin. Cancer Res.* 2:1373–1381.

Hof, P., S. Pluskey, S. Dhe-Paganon, M.J. Eck, and S.E. Shoelson. 1998. Crystal structure of the tyrosine phosphatase SHP-2. *Cell*. 92:441–450. [http://dx.doi.org/10.1016/S0092-8674\(00\)80938-1](http://dx.doi.org/10.1016/S0092-8674(00)80938-1)

Holgado-Madruga, M., D.R. Emlet, D.K. Moscatello, A.K. Godwin, and A.J. Wong. 1996. A Grb2-associated docking protein in EGF- and insulin-receptor signalling. *Nature*. 379:560–564. <http://dx.doi.org/10.1038/379560a0>

Ishii, H., T. Yoshida, H. Oh, S. Yoshida, and M. Terada. 1995. A truncated K-sam product lacking the distal carboxyl-terminal portion provides a reduced level of autophosphorylation and greater resistance against induction of differentiation. *Mol. Cell. Biol.* 15:3664–3671.

Itoh, H., Y. Hattori, H. Sakamoto, H. Ishii, T. Kishi, H. Sasaki, T. Yoshida, M. Kono, T. Sugimura, and M. Terada. 1994. Preferential alternative splicing in cancer generates a K-sam messenger RNA with higher transforming activity. *Cancer Res.* 54:3237–3241.

Jang, J.H., K.H. Shin, and J.G. Park. 2001. Mutations in fibroblast growth factor receptor 2 and fibroblast growth factor receptor 3 genes associated with human gastric and colorectal cancers. *Cancer Res.* 61:3541–3543.

Johnson, D., S.A. Wall, S. Mann, and A.O. Wilkie. 2000. A novel mutation, Ala315Ser, in FGFR2: a gene-environment interaction leading to craniostenosis? *Eur. J. Hum. Genet.* 8:571–577. <http://dx.doi.org/10.1038/sj.ejhg.5200499>

Keilhack, H., F.S. David, M. McGregor, L.C. Cantley, and B.G. Neel. 2005. Diverse biochemical properties of Shp2 mutants. Implications for disease phenotypes. *J. Biol. Chem.* 280:30984–30993. <http://dx.doi.org/10.1074/jbc.M504699200>

Kleiman, L.B., T. Maiwald, H. Conzelmann, D.A. Lauffenburger, and P.K. Sorger. 2011. Rapid phospho-turnover by receptor tyrosine kinases impacts downstream signaling and drug binding. *Mol. Cell.* 43:723–737. <http://dx.doi.org/10.1016/j.molcel.2011.07.014>

Korah, R.M., V. Sysouthone, Y. Golowa, and R. Wieder. 2000. Basic fibroblast growth factor confers a less malignant phenotype in MDA-MB-231 human breast cancer cells. *Cancer Res.* 60:733–740.

Kouhara, H., Y.R. Hadari, T. Spivak-Kroizman, J. Schilling, D. Bar-Sagi, I. Lax, and J. Schlessinger. 1997. A lipid-anchored Grb2-binding protein that links FGF-receptor activation to the Ras/MAPK signaling pathway. *Cell*. 89:693–702. [http://dx.doi.org/10.1016/S0092-8674\(00\)80252-4](http://dx.doi.org/10.1016/S0092-8674(00)80252-4)

Lin, C.-C., F.A. Melo, R. Ghosh, K.M. Suen, L.J. Stagg, J. Kirkpatrick, S.T. Arold, Z. Ahmed, and J.E. Ladbury. 2012. Inhibition of basal FGF receptor signaling by dimeric Grb2. *Cell*. 149:1514–1524. <http://dx.doi.org/10.1016/j.cell.2012.04.033>

Lowenstein, E.J., R.J. Daly, A.G. Batzer, W. Li, B. Margolis, R. Lammers, A. Ullrich, E.Y. Skolnik, D. Bar-Sagi, and J. Schlessinger. 1992. The SH2 and SH3 domain-containing protein GRB2 links receptor tyrosine kinases to Ras signaling. *Cell*. 70:431–442. [http://dx.doi.org/10.1016/0092-8674\(92\)90167-B](http://dx.doi.org/10.1016/0092-8674(92)90167-B)

Lu, W., D. Gong, D. Bar-Sagi, and P.A. Cole. 2001. Site-specific incorporation of a phosphotyrosine mimetic reveals a role for tyrosine phosphorylation of SHP-2 in cell signaling. *Mol. Cell.* 8:759–769. [http://dx.doi.org/10.1016/S1097-2765\(01\)00369-0](http://dx.doi.org/10.1016/S1097-2765(01)00369-0)

Neel, B.G., G. Chan, and S. Dhanji. 2010. SH2 domain-containing protein-tyrosine phosphatases. *Handbook of Cell Signaling* 2:771–809.

O’Rourke, L., and J.E. Ladbury. 2003. Specificity is complex and time consuming: mutual exclusivity in tyrosine kinase-mediated signaling. *Acc. Chem. Res.* 36:410–416. <http://dx.doi.org/10.1021/ar020167s>

Pollock, P.M., M.G. Gartside, L.C. Dejeza, M.A. Powell, M.A. Mallon, H. Davies, M. Mohammadi, P.A. Futreal, M.R. Stratton, J.M. Trent, and P.J. Goodfellow. 2007. Frequent activating FGFR2 mutations in endometrial carcinomas parallel germline mutations associated with craniostenosis and skeletal dysplasia syndromes. *Oncogene*. 26:7158–7162. <http://dx.doi.org/10.1038/sj.onc.1210529>

Rozakis-Adcock, M., R. Fernley, J. Wade, T. Pawson, and D. Bowtell. 1993. The SH2 and SH3 domains of mammalian Grb2 couple the EGF receptor to the Ras activator mSos1. *Nature*. 363:83–85. <http://dx.doi.org/10.1038/363083a0>

Sato, T., T. Shimazaki, H. Naka, S. Fukami, Y. Satoh, H. Okano, I. Lax, J. Schlessinger, and N. Gotoh. 2010. FRS2 $\alpha$  regulates Erk levels to control a self-renewal target Hes1 and proliferation of FGF-responsive neural stem/progenitor cells. *Stem Cells*. 28:1661–1673. <http://dx.doi.org/10.1002/stem.488>

Schüller, A.C., Z. Ahmed, and J.E. Ladbury. 2008. Extracellular point mutations in FGFR2 result in elevated ERK1/2 activation and perturbation of neuronal differentiation. *Biochem. J.* 410:205–211. <http://dx.doi.org/10.1042/BJ20070859>

Turner, N., and R. Grose. 2010. Fibroblast growth factor signalling: from development to cancer. *Nat. Rev. Cancer*. 10:116–129. <http://dx.doi.org/10.1038/nrc2780>

Wilkie, A.O., S.F. Slaney, M. Oldridge, M.D. Poole, G.J. Ashworth, A.D. Hockley, R.D. Hayward, D.J. David, L.J. Pulley, P. Rutland, et al. 1995. Apert syndrome results from localized mutations of FGFR2 and is allelic with Crouzon syndrome. *Nat. Genet.* 9:165–172. <http://dx.doi.org/10.1038/ng0295-165>

Xian, W., K.L. Schwertfeger, and J.M. Rosen. 2007. Distinct roles of fibroblast growth factor receptor 1 and 2 in regulating cell survival and epithelial-mesenchymal transition. *Mol. Endocrinol.* 21:987–1000. <http://dx.doi.org/10.1210/me.2006-0518>

Yu, K., A.B. Herr, G. Waksman, and D.M. Ornitz. 2000. Loss of fibroblast growth factor receptor 2 ligand-binding specificity in Apert syndrome. *Proc. Natl. Acad. Sci. USA*. 97:14536–14541. <http://dx.doi.org/10.1073/pnas.97.26.14536>