Grb2 binding induces phosphorylation-independent activation of Shp2

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The regulation of phosphatase activity is fundamental to the control of intracellular signalling and in particular the tyrosine kinase-mediated mitogen-activated protein kinase (MAPK) pathway. Shp2 is a ubiquitously expressed protein tyrosine phosphatase and its kinase-induced hyperactivity is associated with many cancer types. In non-stimulated cells we find that binding of the adaptor protein Grb2, in its monomeric state, initiates Shp2 activity independent of phosphatase phosphorylation. Grb2 forms a bidentate interaction with both the N-terminal SH2 and the catalytic domains of Shp2, releasing the phosphatase from its auto-inhibited conformation. Grb2 typically exists as a dimer in the cytoplasm. However, its monomeric state prevails under basal conditions when it is expressed at low concentration, or when it is constitutively phosphorylated on a specific tyrosine residue (Y160). Thus, Grb2 can activate Shp2 and downstream signal transduction, in the absence of extracellular growth factor stimulation or kinase-activating mutations, in response to defined cellular conditions. Therefore, direct binding of Grb2 activates Shp2 phosphatase in the absence of receptor tyrosine kinase up-regulation.
The reciprocal process of phosphorylation by kinases, and dephosphorylation by phosphatases, of selected residues regulates the intensity and longevity of intracellular tyrosine kinase-mediated signal transduction. The SH2 domain-containing tyrosine phosphate 2, Shp2, (aka. PTPN11) plays a prominent role in this process in a multitude of receptor tyrosine kinase (RTK)-mediated signalling pathways, including activation of the extracellular signal-regulated kinase Erk1/2 (aka. mitogen-activated protein kinase, MAPK) pathways. Shp2 is ubiquitously expressed in vertebrate cells and consists largely of, in sequential order: two Src homology 2 (SH2) domains (NSH2 and CSH2, respectively); a protein tyrosine phosphatase (PTP) domain; and a C-terminal tail with two tyrosyl phosphorylation sites (Y542 and Y580) and a proline-rich sequence (residues 559–568).

Shp2 was the first phosphatase to be identified as a human oncoprotein, and a large body of experimental and clinical approaches involving downregulation of Shp2 has shown interest in anticancer therapeutic activity because of the highly conserved amino-acid sequences of phosphatase domains. However, more recently an approach based on small molecule stabilisation of the auto-inhibited conformation of Shp2 has shown efficacy.

Crystal structural detail revealed that Shp2 utilises an auto-inhibitory mechanism that prevails under basal conditions. NSH2 forms an intramolecular interaction with the PTP domain, directly blocking access to the catalytic site, resulting in a ‘closed’ state. In this state, the NSH2 domain adopts a conformation that contains its phosphopeptide binding cleft. Gain-of-function somatic mutations that result in the abrogation of the interaction between NSH2 and the PTP domain have been shown to be activating. Auto-inhibition is released through one of two mechanisms involving the SH2 domains of Shp2. In the first, both N- and CSH2 interact with a binding partner including a phosphorylated bisphosphoryl tyrosine-based activation motif.

The second, more controversial, mechanism occurs under conditions where Shp2 has been phosphorylated, typically by an RTK. This induces an intramolecular, bidentate interaction between the two phosphorylated tyrosine residues in the C-terminus of Shp2 and both NSH2 and CSH2.

We have previously investigated the constitutive control that the adaptor protein growth factor receptor-bound protein 2, Grb2, exerts over Shp2 in non-stimulated cells. Grb2 consists of an SH2 domain sandwiched between two SH3 domains and is integral to several RTK-mediated signalling pathways. Non-phosphorylated Grb2 exists in a concentration-dependent dimer-monomer equilibrium. Depletion of intracellular expression of Grb2 results in increased concentrations of monomer. In addition, monomeric Grb2 (mGrb2) will also prevail when it is phosphorylated on tyrosine 160 (Y160) in the dimer interface. Under basal conditions, in the absence of growth factor stimulation, Grb2 cycles between the phosphorylated mGrb2, and the non-phosphorylated, typically dimeric state. The former is dependent on constitutive, background RTK activity, e.g., from fibroblast growth factor receptor 2 (FGFR2), whereas the latter results from concomitant Shp2 activity.

In this work, we provide molecular mechanistic detail on the activation of Shp2 in the absence of the two phosphorylation-dependent mechanisms highlighted above. Using the monomeric phosphorylation charge mimetic Grb2 mutant Y160E (Grb2Y160E), we show that in non-stimulated cells Grb2Y160E is able to greatly enhance Shp2 phosphatase activity via a bidentate interaction involving two discrete interfaces; (1) between the NSH2 domain of Shp2 and the SH2 of Grb2, and (2) between the PTP domain of Shp2 and the CSH3 of Grb2. The binding of Grb2Y160E releases Shp2 from its auto-inhibited state and results in an increase in the phosphatase activity independent of kinase-induced stimulation.

RTK-mediated signal transduction in cells that are not exposed to activating concentrations of extracellular stimuli is fundamental in maintaining homoeostasis and metabolic regulation. Aberrancies in this form of signalling can evoke cancer outcomes. However, our appreciation of this form of signalling remains limited. This work provides a valuable example of how in the absence of growth factor stimulation a key enzyme can be upregulated and control MAPK pathway response.

Results

Shp2 interacts with monomeric Grb2 in the absence of growth factor

Mutation of Y160 to glutamate (a phosphotyrosine charge mimic) in the Grb2 dimer interface abrogates self-association of the adaptor protein. To characterise the interaction between full-length Shp2 (Shp2WT) and monomeric Grb2 (including Y160E mutation, Grb2Y160E) we initially used microscale thermophoresis (MST) to measure the affinity of the interaction of full-length proteins (Kd = 0.33 ± 0.04 µM; Fig. 1a, Table 1 and Supplementary Data 1). We demonstrated that Grb2Y160E can bind at two discrete sites on Shp2 using biolayer interferometry (BLI) on the following four GST-tagged phosphatase constructs: Shp2WT, the tandem SH2 domains (residues 1–220: Shp2ΔSH2), the PTP domain (251–524: Shp2ΔPTP) and a peptide corresponding to the C-terminal 69 amino acids (525–593: Shp2Δ593). Both Shp2WT as well as Shp2ΔSH2 polypeptides were able to interact with Grb2Y160E. The truncated Shp2ΔPTP bound to Grb2Y160E weakly, whereas the C-terminal tail failed to interact (Fig. 1b and Supplementary Data 1). These two binding sites were confirmed in an in vitro pull-down experiment in which Grb2Y160E was precipitated by both GST-Shp2ΔSH2 and GST-Shp2ΔPTP (Fig. 1c, Supplementary Fig. 4a and Supplementary Data 1). The interaction with GST-Shp2ΔPTP was less pronounced. Interaction between Grb2WT and the Shp2 constructs appears to be negligible suggesting that, under the experimental conditions, the prevailing dimeric Grb2 is unable to interact. The limited complex formation seen with extended exposure of the blot is again presumed to be with the low population of monomeric protein at equilibrium (Fig. 1c inset, Supplementary Fig. 4a and Supplementary Data 1).

Upon phosphorylation, tyrosines 542 and 580 on the C-terminal tail of Shp2 are known binding sites for Grb2 SH2 domains. To confirm our in vitro observation in a cellular context, we measured intracellular binding of Shp2Δ99 (deleted of 69 residues 525–593 to eliminate SH2-phosphotyrosine mediated interactions) to Grb2ΔY160E. We used fluorescence lifetime imaging microscopy (FLIM) to detect stable complexes through fluorescence resonance energy transfer (FRET) between fluorophore-tagged proteins transfected into HEK293T cells under serum-starved conditions. FRET was confirmed by the left-shift of the fluorescence lifetime from the control lifetime measurements of donor (CFP-Grb2) in the presence of non-specific RFP-alone acceptor (2.2 nsec; Fig. 1d). No interaction between CFP-Grb2WT and RFP-Shp2Δ99 was observed since the fluorescent lifetime remains largely unaffected. Grb2WT exists in a monomeric-dimer equilibrium and CFP-Grb2WT overexpression would favour dimer at the elevated adaptor protein concentration, hence limiting the availability of Grb2 to interact with Shp2Δ99. To circumvent concentration-dependent dimerisation, we used the monomeric CFP-Grb2ΔY160E in the FLIM-binding assay. FRET between CFP-Grb2ΔY160E and Shp2Δ99 shows a left-shifted fluorescence lifetime by 100 psec shorter than the RFP-alone control or...
CFP-Grb2\textsubscript{WT} (Fig. 1d lower panel). This clearly demonstrates that only the monomeric Grb2\textsubscript{Y160E} interacts with Shp2. Immunoprecipitation further revealed that Grb2\textsubscript{Y160E} constitutively associates with Shp2\textsubscript{WT} in the absence of ligand stimulation (Supplementary Fig. 1). To further confirm the bidentate interaction in a cellular context, we co-expressed CFP-Grb2\textsubscript{Y160E} with RFP-Shp2\textsubscript{2SH2}, RFP-Shp2PTP, or RFP alone in HEK293T cells. RFP-Trap immunoprecipitation demonstrates that both RFP-Shp2\textsubscript{2SH2} and RFP-Shp2PTP can precipitate CFP-Grb2\textsubscript{Y160E} (Fig. 1e, Supplementary Fig. 4b and Supplementary Data 1). Again, the interaction with GST-Shp2PTP was less pronounced. This result is consistent with our in vitro data shown in Figs. 1b and 1c.
Fig. 1 Shp2 interacts with monomeric Grb2 in a phosphorylation-independent manner. 

a MST measurement of full-length Shp2 binding to fluorescent labelled monomeric Grb2. Data are presented as (mean ± SD) of technical triplicates. For further details, see Table 1 and Methods. 

b BLI characterisation of individual Shp2 domains binding to GST-Grb2Y160E immobilised on a GST sensor. GST-Grb2Y160E was captured and the sensor and 10 μM of each Shp2 protein was used to test the interaction. Black: Shp2WT, Red: Shp2NSH2, Green: Shp2PTP, Blue: Shp2CSH2. The BLI sensogram indicates that the Shp2NSH2 and Shp2PTP mediate the interaction with Grb2. 

c GST pull-down experiment using GST-tagged Shp2SH2 and GST-tagged Shp2PTP to precipitate monomeric or dimeric Grb2 (Grb2Y160E or Grb2Y160E, respectively). The pull-down results clearly demonstrate the interaction of Shp2 with monomeric Grb2. The blot represents three independent experiments. Inset: densitometric analysis of RFP-trap immunoprecipitation. Results are represented as mean ± SD. Statistics were determined using an unpaired Student’s t test. 

d Fluorescence lifetime imaging microscopy (FLIM) displaying fluorescence resonance energy transfer (FRET) between CFP-Grb2WT and RFP-alone control (top); CFP-Grb2WT and RFP-Shp2wt (middle); Grb2Y160E and RFP-Shp2wt (bottom). The lifetime image was generated using a false colour range pixel-by-pixel lifetime value corresponding to the average lifetime shown in the histograms. Two independent experiments were performed and each time 5–10 cells were counted. 

Table 1 Binding affinities (Kd, μM) obtained from this study using MST.

| Target protein | Ligand | Kd (μM) |
|---------------|--------|---------|
| Grb2Y160E     | Shp2WT | 0.33 ± 0.04 |
| Grb2Y160E     | Shp2SH2| 0.28 ± 0.03 |
| Grb2R86A/Y160E| Shp2SH2| 0.15 ± 0.01 |
| Shp2NSH2      | Grb2SH2| 33.8 ± 4.5  |
| Shp2NSH2      | Grb2Y160E| 422 ± 21.5 |
| Shp2PTP       | Grb2SH2| 207 ± 16.8  |
| Shp2PTP       | Grb2Y160E| 30.0 ± 0.3 |
| Grb2CSH3      | VLHGDG297PNEP300VSDYIN| N.B. |
| Grb2CSH3      | TKCNNS122PKPK352KTYATQ| 173 ± 6.89 |
| Grb2CSH3      | VERGK366KCVK369YWPDEY| 122 ± 7.24 |
| Grb2CSH3      | YGVMRV388MRV389YEAH| N.B. |
| Grb2CSH3      | AHDYTL399REKL5K405GQ| N.B. |
| Grb2CSH3      | WDHPHGV422PSPD432GGVLDF| 18.1 ± 0.188 |

Table 1 binding affinities were determined by MST. 

The SH2 domain of Grb2 binds to the N-terminal SH2 domain of Shp2. The affinity of Shp2SH2 binding to monomeric Grb2Y160E was determined by MST (Kd = 0.28 ± 0.03 μM; Fig. 2a, Table 1 and Supplementary Data 2). The possibility of binding of the pY-mimetic glutamate of Grb2Y160E to either of the SH2 domains of both proteins (on Shp2, R32A/R138A; Shp2SH2 R32A/R138A and on Grb2 R86A; Grb2R86A/Y160E), Grb2R86A/Y160E was still capable of binding to Shp2SH2 R32A/R138A with similar affinity to the wild-type Shp2SH2 construct (Kd = 0.15 ± 0.01 μM; Fig. 2b, Table 1 and Supplementary Data 2). Having observed the Shp2SH2 interaction with Grb2, we sought to identify whether an individual domain was responsible for critical contact. GST-tagged Shp2SH2 R32A/R138A as well as the individual SH2 domains (N-terminal SH2 domain: Shp2NSH2 and C-terminal SH2 domain: Shp2CSH2), were used in pull-down experiments in which the NSH2, and not the CSH2, domain of Shp2 was shown to be sufficient for Grb2 binding (Fig. 2c, Supplementary Fig. 4c and Supplementary Data 2). Re-probing the blot with an anti-pY antibody revealed that the interaction was not mediated through phosphorylated tyrosine(s) or glutamate on Grb2Y160E. This was further confirmed through in vitro binding assays using two phosphopeptides corresponding to the phosphorylatable tyrosine residues on Grb2 (pY160 and pY209), which show negligible interaction with Shp2SH2 (Supplementary Fig. 2a and Supplementary Data 5). Our data, therefore are consistent with a non-canonical, phosphorylation-independent interaction between Shp2NSH2 and Grb2Y160E.

Having established the role of Shp2NSH2 in binding to unphosphorylated Grb2Y160E, we attempted to establish which domain(s) of Grb2 was required for recognition of the phosphatase. GST-tagged Shp2NSH2 was captured on a GST BLI sensor and was exposed to the isolated SH2, as well as NSH3 and CSH3 domains of Grb2 (Grb2SH2, Grb2NSH3 and Grb2CSH3, respectively). The data indicate that Grb2SH2 binds to Shp2NSH2 (Supplementary Fig. 2b), accompanied by a weaker interaction of Grb2CSH3. MST experiments on the same isolated domains of Grb2 binding to Shp2NSH2 confirmed that the interaction with Grb2SH2 was dominant (Kd = 33.8 ± 4.5 μM). Interactions with both the SH3 domains of Grb2 were substantially weaker (Grb2NSH3: Kd = 422 ± 21.5 μM and Grb2CSH3: Kd = 207 ± 16.8 μM (Fig. 2d, Table 1 and Supplementary Data 2). To assess the impact of the Grb2SH2 domain binding to Shp2NSH2 domain, we carried out NMR titration experiments based on 2D (1H, 15N) HSQC spectra of 15N-Shp2NSH2 domain in the absence, and the presence of an increasing concentration of unlabelled Grb2SH2 domain. Peak assignments of Shp2NSH2 backbone 1H-15N resonances were derived from a suite of triple-resonance experiments (81% assignment coverage; Supplementary Fig. 3). The addition of Grb2SH2 led to chemical shift perturbations (CSPs) for several resonances, indicating disruption of local chemical environment of specific amino acids as a result of complex formation (Fig. 2e). Comparison of the spectra of the free Shp2NSH2 and the Shp2NSH2-Grb2SH2 complex showed that the average CSPs are relatively small. However, a limited number of residues show pronounced changes (Fig. 2f). The CSPs (>0.0075 ppm) were mapped onto a crystal structural representation of Shp2NSH2 (PDB code: 2SHP; Fig. 2g, h). From this, it is possible to interpret a mechanism for Grb2SH2-domain binding. The reported crystal structural data of Shp2 (PDB: 2SHP) reveal a non-phosphorylated auto-inhibited structure in which NSH2 directly interacts with the PTP domain, blocking the access of phosphorylated substrates. We see that a number of residues that are not within the Shp2NSH2-PTP interface are exposed to the isolated SH2, as well as NSH3 and CSH3 domains of Grb2 (Grb2SH2, Grb2NSH3 and Grb2CSH3, respectively). The data indicate that Grb2SH2 binds to Shp2NSH2 (Supplementary Fig. 2b), accompanied by a weaker interaction of Grb2CSH3. MST experiments on the same isolated domains of Shp2 binding to Shp2NSH2 confirmed that the interaction with Grb2SH2 was dominant (Kd = 33.8 ± 4.5 μM). Interactions with both the SH3 domains of Shp2 were substantially weaker (Grb2NSH3: Kd = 422 ± 21.5 μM and Grb2CSH3: Kd = 207 ± 16.8 μM (Fig. 2d, Table 1 and Supplementary Data 2). To assess the impact of the Grb2SH2 domain binding to Shp2NSH2 domain, we carried out NMR titration experiments based on 2D (1H, 15N) HSQC spectra of 15N-Shp2NSH2 domain in the absence, and the presence of an increasing concentration of unlabelled Grb2SH2 domain. Peak assignments of Shp2NSH2 backbone 1H-15N resonances were derived from a suite of triple-resonance experiments (81% assignment coverage; Supplementary Fig. 3). The addition of Grb2SH2 led to chemical shift perturbations (CSPs) for several resonances, indicating disruption of local chemical environment of specific amino acids as a result of complex formation (Fig. 2e). Comparison of the spectra of the free Shp2NSH2 and the Shp2NSH2-Grb2SH2 complex showed that the average CSPs are relatively small. However, a limited number of residues show pronounced changes (Fig. 2f). The CSPs (>0.0075 ppm) were mapped onto a crystal structural representation of Shp2NSH2 (PDB code: 2SHP; Fig. 2g, h). From this, it is possible to interpret a mechanism for Grb2SH2-domain binding. The reported crystal structural data of Shp2 (PDB: 2SHP) reveal a non-phosphorylated auto-inhibited structure in which NSH2 directly interacts with the PTP domain, blocking the access of phosphorylated substrates. We see that a number of residues that are not within the Shp2NSH2-PTP interface are exposed to the isolated SH2, as well as NSH3 and CSH3 domains of Grb2 (Grb2SH2, Grb2NSH3 and Grb2CSH3, respectively). The data indicate that Grb2SH2 binds to Shp2NSH2 (Supplementary Fig. 2b), accompanied by a weaker interaction of Grb2CSH3. MST experiments on the same isolated domains of Grb2 binding to Shp2NSH2 confirmed that the interaction with Grb2SH2 was dominant (Kd = 33.8 ± 4.5 μM). Interactions with both the SH3 domains of Grb2 were substantially weaker (Grb2NSH3: Kd = 422 ± 21.5 μM and Grb2CSH3: Kd = 207 ± 16.8 μM (Fig. 2d, Table 1 and Supplementary Data 2).
releasing the ‘closed’ structure and hence activating the phosphatase.

**The CSH3 domain of Grb2 interacts with Shp2PTP.** Data shown in Figs. 1b and 1c revealed that, as well as binding to Shp2\textsubscript{SH2}, Grb2\textsubscript{Y160E} also formed an interaction with Shp2PTP. We explored this further using MST and found that the isolated Shp2PTP binds Grb2\textsubscript{Y160E} with a similar affinity to the interaction with Shp2\textsubscript{2SH2} ($K_d = 0.30 \pm 0.03 \mu M$; Fig. 3a, Table 1 and Supplementary Data 3). To identify which domain of Grb2 interacts with Shp2PTP we used GST-fused Grb2\textsubscript{SH2}, Grb2\textsubscript{NSH3} and Grb2\textsubscript{CSH3} constructs to precipitate recombinant Shp2PTP. The pull-down indicated that only Grb2\textsubscript{CSH3} can bind to the PTP domain (Fig. 3b, Supplementary Fig. 4d and Supplementary Data 3). SH3
Further details, see Table 1 and Methods. Data are presented as (mean ± SD) of technical triplicates.

Shp2NSH2 chemical shifts in the presence and absence of Grb2SH2. Overlay of a region of the 15N-1H HSQC (heteronuclear single-quantum coherence) interaction of Grb2SH2 to 15N-labelled Shp2NSH2. Minimal chemical shift perturbation upon Grb2SH2 mapped on Shp2NSH2 sequence.

Fluorescence resonance energy transfer between the two fluorophores are in close proximity (i.e., as distanced fluorophores through conformational change reverses this outcome. Steady-state FRET was measured whilst adding Grb2Y160E to Shp2ΔNSH (Fig. 4a) and Supplementary Data 4). The data clearly show that, prior to Grb2 addition, the BFP donor emission is low and GFP acceptor emission is high. Recovery of FRET donor BFP emission and a decrease in FRET acceptor GFP emission with increasing concentration of Grb2Y160E results in the growing population of Grb2Y160E–Shp2ΔNSH complex undergoing a conformational change from the auto-inhibited state to a state where the termini of Shp2ΔNSH are separated.

To assess whether the Grb2 binding-induced conformational change altered enzymatic activity, we conducted an in vitro phosphatase assay. A pY-containing peptide substrate (END-pYINASL) was incubated with non-phosphorylated Shp2ΔNSH and different concentrations of Grb2Y160E. Free phosphate generated from hydrolysis of the pY was measured by the absorbance of a malachite green molybdate phosphate complex (Fig. 4b and Supplementary Data 4). A significant increase in phosphatase activity was measured with the increasing presence of Grb2Y160E. Importantly, a similar level of turnover of the peptide was observed comparing the monomeric Grb2-induced phosphatase activity to that observed for phosphorylated Shp2 in the absence of the adaptor protein (pShp2; Fig. 4c and Supplementary Data 4). These in vitro experiments suggest that under basal conditions Shp2 activity can be upregulated through binding to monomeric Grb2 alone, and that this activity is at least as high as occurs when the phosphatase is phosphorylated as seen in growth factor-stimulated cells.

Monomeric Grb2Y160E is associated with the upregulation of Shp2 in cancer. Shp2 activity has been shown to play a key role in cancer progression. As Grb2Y160E can promote phosphatase activity in the absence of Shp2 phosphorylation, this could lead to a proliferative outcome in cells through upregulation of the Erk1/2 pathway without the need for the elevated kinase activity often associated with cancer phenotypes. The role of upregulation of non-phosphorylated Shp2 through binding to monomeric Grb2 in cancer has not previously been investigated. For example, in the triple-negative breast cancer cell line MDA-MB-468 in the absence of extracellular stimulation, we see negligible phosphatase activity to that observed for phosphorylated Shp2 in the absence of the adaptor protein (pShp2; Fig. 4c and Supplementary Data 4). These data are consistent with monomeric Grb2 being able to upregulate MAPK signalling (as shown by
Fig. 3 Characterisation of Grb2Y160E binding to Shp2PTP domain. 

**a** MST measurement of Shp2PTP binding to fluorescent labelled Grb2Y160E (0.1 µM). Both PTP domain and tandem 2SH2 domains show similar affinity to Grb2Y160E. Data are presented as (mean ± SD) of technical triplicates.

**b** GST pull-down experiment using GST-tagged individual Grb2 domain (NSH3, SH2 and CSH3) to precipitate Shp2 PTP domain. The result clearly indicates the interaction is mediated through Grb2 CSH3 domain. The blot represents three independent experiments. Inset: densitometric analysis of GST pull-down results. Results are represented as mean ± SD. Statistics were determined using an unpaired Student’s t test.

**c** Sequence analysis identifies six potential PxxP or R/KxxK (x is any amino residue) motifs for Grb2 CSH3 domain interaction. Synthesised peptides were used to test the interactions with Grb2 CSH3 domain (Atto 488 labelled, 0.1µM) using MST. The results show 322KPKK325, 366KCVK369 and 429PSDP432 interact with Shp2 PTP domain. Motif 429PSDP432 binds to Shp2 PTP domain with the highest affinity of 18 µM. See Table for detailed information and peptide sequences. Data are presented as (mean ± SD) of technical replicates (peptide 322–325 and peptide 429–432) or triplicates.

**d** ITC was used to corroborate the two binding events between Shp2 and Grb2Y160E. Grb2Y160E (200 µM) was titrated into Shp2Δ69 (25 µM). Twelve 3 µl injections of Grb2Y160E were titrated into Shp2Δ69. Top panel: baseline-corrected power versus time plot for the titration. Bottom panel: the integrated heats and the molar ratio of the Grb2Y160E to Shp2Δ69.
pErk1/2) through binding to Shp2 without the need for phosphorylation of the latter. Cell proliferation is a well-known marker for MAPK signalling upregulation so we investigated whether this was differentially affected by the presence of Grb2WT or Grb2Y160E expression using MDA468 cells. In the absence of extracellular stimulation, we observed that the expression of Grb2Y160E enhanced the level of cellular proliferation compared with wild type (Fig. 4e and Supplementary Data 4).

Discussion

Kinase and phosphatase activity have to be precisely controlled to limit aberrant signal transduction in cells. It has previously...
been demonstrated that Shp2 function is dependent on RTK activity37–41, and is accompanied by phosphorylation of tyrosine residues Y542 and Y580. This appears to facilitate a conformational change that abrogates an intramolecular interface between the NSH2 and PTP domains of Shp2 and relieves the auto-inhibited state26. Activated Shp2 can play a role in proliferative signalling through the Erk1/2 pathway3,4,37.

In this work, we show that the upregulation of Shp2 can also be accomplished in the absence of RTK activity, through the binding of the monomeric adaptor protein Grb2 (mGrb2). Monomeric Grb2 will be present in cells in which the adaptor protein is expressed at low concentrations (dimer-monomer equilibrium $K_d = 0.8 \mu M^{29,30}$), or as the result of phosphorylation on Y160 by constitutive, background RTK activity in the absence of ligand stimulation28. The SH2 and CSH3 domains of Grb2 form a bidentate interaction with the NSH2 and PTP domains of Shp2, respectively. Interaction with Shp2 in this way results in an mGrb2-dependent conformational change which opens the auto-inhibited state to facilitate phosphatase activity.

The relevance of a mechanism for Shp2 upregulation in non-stimulated cells is likely to be associated with the requirement of cells to maintain homoeostatic and metabolic ‘house-keeping’ function in the absence of extracellular stimulation. We have previously shown that similar functional activation of enzymes and adaptors associated with RTK-mediated signalling pathways occurs under basal conditions without the need for kinase upregulation (e.g., Pcly1,31 Shc32). This second tier of signalling (tier 2 signalling), below the profound and defined effects of full kinase activation resulting from extracellular stimulation, is likely to be important in maintaining cell viability and in responding to stress. Aberrancies in tier 2 signalling can also be associated with cancer pathology31. This appears to be reflected here where, through interaction with mGrb2, Shp2 can be engaged in signalling in cancer cells without the need for mutated, dysfunctional kinases. Therefore, in cells depleted for a given RTK, as is found in triple-negative breast cancer (e.g., in MDA-MB–468 cells, which do not express Her2; Fig. 4d), proliferative or metastatic signalling could be driven by the uncontrolled impact of monomeric Grb2-mediated Shp2 activation. The importance of the interaction with Grb2 may also be emphasised by the observation of two reported oncogenic Shp2 mutations in the NSH2, which are localised at the identified Grb2-binding site, namely T42A42 and N58K43. Change-of-function associated with these mutations would potentially release regulatory control of background tyrosine kinase-mediated signalling.

Non-stimulatory conditions portray many features of the early phases of tumour development and progression as well as prevailing in resistant cells treated with kinase inhibitors. Thus, the understanding of how signalling proteins behave and are controlled under these conditions is the necessary foundation to develop more efficient therapeutic strategies. It remains to be seen whether examples of mGrb2-mediated activation of Shp2 are common regulators of signalling in basal cells, and particularly whether aberrancies resulting from environmental change-related stress can drive tumorigenesis through perturbation of Grb2 concentration.

**Methods**

**Cell culture.** HEK293T cells were maintained in Dulbecco’s modified Eagle’s high glucose medium supplemented with 10% (v/v) fetal bovine serum and 1% antibiotic/antimycotic (Lonza) in a humidified incubator with 10% CO₂.

**Reagents.** Recombinant human epidermal growth factor (EGF) was purchased from R&D Systems. Anti-Grb2 (sc-8034) were purchased from Santa Cruz Biotechnology. Anti-EGFR (4267), anti-Grb2 (sc-8034), anti-pErk1/2 (9101), and anti-Shp2 (3754) were purchased from Cell Signalling Technology. Anti-RFP (A00682) was purchased from Santa Cruz Biotechnology. Anti-Grb2 antibodies were purchased from Cell Signalling Technology, Sigma or Abcam. Anti-Grb2 pY160 was synthesised from Genscript.

**Western blots.** Cells were grown in 10-cm dishes, serum-starved overnight and stimulated with 10 ng/ml EGF for 15 minutes. Cells were lysed with buffer containing 50 mM Hepes, pH 7.5, 1% (vol/vol) igepal-C630, 1 mM mg/ml bacitracin, 1 mM ethylenediaminetetraacetic acid, 10 mM NaF, 1 mM sodium orthovanadate, 10% (vol/vol) glycerol, 50 mM NaCl, 1 mM phenylmethylsulfonyl fluoride 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.

**Plasmids.** For the pull-down assay, gene fragments encoding Grb2 and Shp2 (28) were cloned into pGEX2T vector to construct GST-tagged Grb2 domains (GST-Grb2ΔNΔC, GST-Grb2ΔC and GST-Grb2ΔN) or Shp2 domains (GST-Shp2ΔC, GST-Shp2ΔΔC and GST-Shp2ΔΔN) using BamHI and NotI sites. For the cellular FLIM study, Grb2 genes (Grb2WT or Grb2Y160E) were cloned in pECFP vector, and Shp2 C-terminal tail truncation (Shp2ΔΔN) was cloned in pcDNA-RFP vector using HindIII and Pmel sites. For the in vitro FRET assay, Shp2ΔΔN was fused with an N-terminal RFP tag and a C-terminal GFP tag and was cloned in a pET28b vector. Mutagenesis (GST-Grb2ΔNΔC, GST-Grb2ΔC and GST-Grb2ΔN) was carried out using the Q5 site-directed mutagenesis kit (New England Biolabs) according to the manufacturer’s protocol. To express recombinant proteins in Escherichia coli for in vitro phosphatase assay, biophysical assay, pull-down assay and NMR studies, genes of interest were amplified using standard PCR methods. Following designated restriction enzyme digestions, fragments were ligated into pET28b (Shp2WT, Shp2ΔΔN, Shp2ΔΔC and Grb2ΔΔN) for expression in bacteria.

**Protein expression and purification.** Proteins were expressed in BL21 (DE3) cells. In all, 20 ml of cells grown overnight were used to inoculate 1 litre of LB media with antibiotic (50 μg/ml kanamycin for pET28b-backed plasmids or 50 μg/ml ampicillin for pGEX4T1-backed plasmids). The culture was grown at 37°C with
constant shaking (200 rpm) until the OD_{600} = 0.7. At this point, the culture was cooled down to 18°C and 0.5 mM of isopropyl β-D-thiogalactopyranoside was added at 1 h expression for 12 h before harvesting. Harvested cells were suspended in Talon buffer A (20 mM Tris, 150 mM NaCl and 1 mM β-ME, at pH 8.0) and lysed by sonication. Cell debris was removed by centrifugation (20,000 rpm at 4°C for 1 h). The soluble fraction was applied to an Akta Purifier System for protein purification. Elution was performed using Talon buffer B (20 mM Tris, 150 mM NaCl, 0.5 mM TCEP, at pH 7.5 and 10% (v/v) D2O at pH 8.0). Proteins were concentrated to 2 ml and applied to a Superdex 75 column using a HEPES buffer at pH 7.5 (20 mM HEPES, 150 mM NaCl and 1 mM TCEP, at pH 7.5).

**Pull-down/immunoprecipitation.** Purified protein or total transfected HEK293T cell lysate were prepared in 1 ml volume for pull-down or immunoprecipitation. In all, 50 µl of 50% glutathione beads (Sigma), Strept–Tactin beads, MERCK or RFP–Trap beads (Chromotek) were added and incubated overnight. The beads were spun down at 5000 rpm for 3 minutes, supernatant was removed and the beads were washed with 1 ml lysis buffer. This washing procedure was repeated five times in order to remove non-specific binding. After the last wash, 50 µl of 2× Laemmli sample buffer were added, the samples were boiled and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western blot analysis.

**Fluorescence lifetime imaging microscopy.** HEK293T cells were co-transfected with cyan fluorescent protein- and red fluorescent protein-tagged Grb2 and Shp2, respectively, (CFP-Grb2 and RFP-Shp2). After 24 h cells were seeded onto glass coverslips and allowed to grow for a further 24 h, fixed by the addition of 4% (w/vol) paraformaldehyde in PBS, pH 8.0. Following fixation and incubation in room temperature cells were washed 6–7 times with phosphate-buffered saline (PBS), pH 8.0. Coverslips were mounted on a slide with a mounting medium (0.1% Vybrant MTT Cell Proliferation Assay Kit (ThermoFisher) according to the manufacturer’s instructions. Labelling ef- ficiency was determined by measuring the absorbance at 280 nm and 488 nm. A solution of unlabeled protein was serially diluted in the presence of 100 mM labeled protein. The samples were loaded into capillaries (NanoTemper Technologies). Measurements were performed at 25°C in 20 mM HEPES buffer, pH 7.5, with 150 mM NaCl, 0.5 mM TCEP and 0.005% Tween 20. Data analyses were performed using a single exponential decay model.

**Isothermal titration calorimetry.** ITC experiments were carried out using a MicroCal iTC200 instrument (Malvern), and data were analysed using ORIGIN7 software. To avoid heats associated with protein dissociation, Grb2-Y10E was titrated into Shp2ΔSH2 at 25°C. The heat per injection was determined and subtracted from the control (buffer into Shp2ΔSH2) binding data. Data were analysed using a single independent site model using the Origin software.

**Microscale thermorheometry.** The Grb2 and Shp2 interactions were measured using the Monolith NT.115 MST instrument from NanoTemper Technologies. Proteins were fluorescently labelled with Atto 488 NHS ester (Sigma) according to the manufacturer’s protocol. Labelling efficiency was determined to be 1:1 (protein to dye) by measuring the absorbance at 280 nm and 488 nm. A solution of unlabeled protein was serially diluted in the presence of 100 mM labeled protein. The samples were loaded into capillaries (NanoTemper Technologies). Measurements were performed at 25°C in 20 mM HEPES buffer, pH 7.5, with 150 mM NaCl, 0.5 mM TCEP and 0.005% Tween 20. Data analyses were performed using NanoTemper Analysis software, v.1.5.41 and plotted using OriginPro 9.1.

**Biolayer interferometry.** BLI experiments were performed using a FortéBio Octet Red 384 using Anti-GST sensors. Assays were done in 384-well plates at 25°C. Association was measured by dipping sensors into solutions of analyte protein for 120 s and was followed by moving sensors to wash buffer for 120 s to monitor the dissociation process. Raw data show a rise in signal associated with binding followed by a diminished signal after application of wash buffer.

**In vitro phosphatase assay.** In vitro Shp2 activity assays were carried out using the Tyrosine Phosphatase Assay System (Promega) according to the manufacturer’s manual. In brief, reconstituted Shp2ΔSH2 was mixed with the phosphopeptide substrate in the presence or absence of different concentrations of Grb2 (Grb2_1× or Grb2_2×). The method is based on measuring the absorbance change after the formation of a reaction mixture of molybdate-malachite green phosphate complex of the free phosphate. The reaction time was 15 minutes for all experiments reported.

**MTT cell proliferation assay.** MDA-MB-231 or HeLa cells were seeded in 96-well plates. After overnight serum-starvation using phenol red-free medium, cells were labelled with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 4 hours at 37°C and dimethyl sulfoxide (DMSO) was used as the solubilizing agent to dissolve the formazan. Absorbance was measured at 470 nm. The percentage of viable cells was determined by normalising absorbance of culture medium treated with MTT and DMSO.

**Nuclear magnetic resonance spectroscopy.** Backbone resonances for the 'H, 15N, 13C labelled sample of Shp2ΔSH2 were assigned by recording a standard Bruker or BEST TROSY version48 of 3D backbone resonance assignment spectra (HNCAC, HNCOCA, HNACCB, CBCACNBH, HNCO and HNCACO) with NUS sampling technique (25% fraction). The titration experiments of Grb2ΔSH2 into Shp2ΔSH2 were recorded using 15N HSQC pulse sequence from Bruker standard library. For that, samples of 200 µM uniformly 15N labelled Shp2ΔSH2 in 20 mM HEPES, 150 mM NaCl, 0.5 mM TCEP, pH 7.5 and 100 µM spectrophotometer equipped with a Bruker AVANCE III 750 MHz and 950 MHz spectrometers equipped with a Bruker TCI triple resonance cryogenically cooled probes. Data were processed with XWINNMR Analysis software package49. CSPs for individual residues were calculated from the chemical shift for the backbone amide β (Δω37) and 15N (Δω15) using the following equation: CSP = [Δω37 + (0.154Δω15)]50. For the assignment of residues in the NSH2 domain of Shp2 coverage of non-proline residues was 81% complete. The first nine N-terminal residues remain unassigned and there is a gap in assignment of six and four residues at amino acid numbers 33–38 and 84–87, respectively.

**Statistics and reproducibility.** All data were expressed as mean and standard deviation. P values were determined by the Student’s unpaired t test (Figs. 1e and 2e were calculated using Student’s paired t test). The reproducibility was determined by biological replicates and repeating the experiment two or three times as described in the figure legends.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article. The authors declare that all data supporting the findings of this study are available within the article and its supplementary information files. All data needed to evaluate the conclusions in the paper are present in the paper or the Supplementary Materials. The plasmids used in this study are available and require a material transfer agreement with University of Leeds.

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Author contributions
C.-C.L. and J.E.L conceived of the project and designed experiments. C.-C.L., K.M.S. and Z.A. carried out experiments. L.W. and A.K. analysed NMR data. C.-C.L. and J.E.L. wrote the paper with input from all authors.

Competing interests
The authors declare no competing interests.

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