Graphical Abstract

Highlights

- Study of global phosphoproteome response after activation of different RTKs
- Shp-2 is highly phosphorylated upon Pdgf, but not Fgf-2, stimulation
- Application of an allosteric Shp-2 inhibitor reveals phosphotyrosine targets of Shp-2
- Pdgf-receptor-dependent cell migration is controlled by Shp-2

In Brief

Batth et al. use mass spectrometry-based phosphoproteomics to analyze receptor tyrosine kinase signaling activated by different ligands, identifying hundreds of differentially regulated phosphotyrosine sites. Tyrosine phosphatase Shp-2 regulates global tyrosine phosphorylation in a Pdgf-receptor-dependent manner, affecting cellular outcomes.

Data and Software Availability

PXD005803
Large-Scale Phosphoproteomics Reveals Shp-2 Phosphatase-Dependent Regulators of Pdgf Receptor Signaling

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SUMMARY

Despite its low cellular abundance, phosphotyrosine (pTyr) regulates numerous cell signaling pathways in health and disease. We applied comprehensive phosphoproteomics to unravel differential regulators of receptor tyrosine kinase (RTK)-initiated signaling networks upon activation by Pdgf-β, Fgf-2, or Igf-1 and identified more than 40,000 phosphorylation sites, including many phosphotyrosine sites without additional enrichment. The analysis revealed RTK-specific regulation of hundreds of pTyr sites on key signaling molecules. We found the tyrosine phosphatase Shp-2 to be the master regulator of Pdgfr pTyr signaling. Application of a recently introduced allosteric Shp-2 inhibitor revealed global regulation of the Pdgf-dependent tyrosine phosphoproteome, which significantly impaired cell migration. In addition, we present a list of hundreds of Shp-2-dependent targets and putative substrates, including Rasa1 and Cortactin with increased pTyr and Gab1 and Erk1/2 with decreased pTyr. Our study demonstrates that large-scale quantitative phosphoproteomics can precisely dissect tightly regulated kinase-phosphatase signaling networks.

INTRODUCTION

With advances in proteomic technologies, it is possible to comprehensively analyze human cell proteomes on par with next-generation genomic sequencing (Bekker-Jensen et al., 2017; Kim et al., 2014). However, routine analysis of post-translational modifications (PTMs) remains challenging due to their generally low cellular abundance and dynamic nature. Above all, site-specific protein phosphorylation on serine, threonine, and tyrosine residues is of great importance in eukaryotic cells due to their crucial role in rapidly regulating essentially all intracellular signaling networks (Olsen et al., 2006). Protein phosphorylation is tightly regulated in healthy cells by the opposing action of protein kinases and phosphatases, which are the enzymes that write and erase this modification, respectively (Ubersax and Ferrell, 2007). Phosphotyrosine (pTyr) in particular remains elusive in its detection due to its highly transient and regulatory nature, which leads to overall lower phosphorylation stoichiometry and cellular abundance relative to its phosphoserine (pSer) and phosphothreonine (pThr) counterparts, which are readily observed in global phosphoproteomic experiments (Sharma et al., 2014). As a result, many large-scale cell signaling studies may inadvertently omit pTyr analysis and thereby overlook a vital layer of information regulating cell signaling and outcome. Obtaining greater molecular understanding of pTyr-driven signaling (alongside pSer and pThr) is the foundation for understanding signaling networks such as those initiated by receptor tyrosine kinases (RTKs), which are able to receive extracellular signals and relay them inside the cell plasma membrane, ultimately affecting cell fate, such as survival, differentiation, proliferation, and migration (Lemmon and Schlessinger, 2010). Consequently, RTKs and proteins involved in their downstream signaling tend to be highly oncogenic and thus the foremost drug targets in various cancers (Koytiger et al., 2013).

To address this challenge, we developed a streamlined phosphoproteomic framework based on high-pH reversed-phase chromatography (Wang et al., 2011) with automatic fraction concatenation followed by sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. This enabled global detection of pSer, pThr, and pTyr sites in a single analysis without the need for phosphotyrosine-specific antibody pull-downs. By primarily using phosphotyrosine site regulation as the proxy of early signaling events, the initial analysis allowed us to observe distinct activation of numerous signaling pathways with RTK ligand treatment. We found platelet-derived growth factor receptor (Pdgfr) (α and β subunits) to be a potent activator of most downstream signaling pathways. We observed that Shp-2 acts as a master regulator of the mitogen-activated protein kinase/extracellular-regulated kinase (Mapk/Erk) pathway for Pdgfr, and through the application of an allosteric Shp-2 phosphatase inhibitor, in combination with quantitative...
phosphoproteomics, our analysis revealed global rewiring of the tyrosine phosphoproteome in a Pdgf-β-dependent manner. This dataset also provides a list of putative Shp-2 substrates, which include proteins regulating downstream RTK signaling pathways important for cellular outcomes such as proliferation and migration. Collectively, our investigation may increase our mechanistic insight into RTK signal propagation and phosphatase action.

RESULTS

Antibody-Free Screen Enables Unbiased In-Depth Identification of Phosphorylation Sites

We employed an optimized phosphoproteomic workflow based on label-free quantitation to unravel regulators of downstream RTK signaling networks mediated by three activated RTKs. We investigated the global phosphosignaling response initiated by three RTKs in mice fibroblast NIH/3T3 cells after stimulation with their respective canonical ligands: platelet-derived growth factor beta receptor (Pdgf-β ligand), fibroblast growth factor receptor 1 (Fgf-2 ligand), and insulin-like growth factor 1 receptor (Igf-1 ligand), which were selected due to their abundance (Figure S1A). Their ligands induced a downstream phosphorylation response, evidenced by the activation and phosphorylation of Mapk/Erk (Figure S1B). We performed time-resolved quantitative phosphoproteomics to study the dynamic processes underlying the activation of RTK signal transduction. Samples were collected after 3 and 15 min of ligand stimulation, followed by efficient lysis and protein extraction, cysteine reduction, and alkylation in one step as previously reported (Jer sie-Christensen et al., 2016; Poulsen et al., 2013).

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We have previously demonstrated an efficient strategy for offline fractionation of phosphopeptides based on high-pH fractionation, which enabled us to identify a large number of tyrosine-phosphorylated peptides, similar to what is typically observed with the extensive use of pan-anti-pTyr antibodies (Batth et al., 2014). To establish a streamlined and comprehensive phosphoproteomic workflow, we automated the offline fractionation and concatenation (AUTOCON) steps before mass spectrometry (MS) analysis (Movie S1), which allowed us to process samples in batch mode and increase our throughput and reproducibility (Figure 1B). The phosphoproteomic workflow enabled the identification of 41,289 phosphorylation sites covering more than 7,000 phosphoproteins. We applied a strict quality filter. The resulting phosphosite intensities covered a large dynamic range, more than 6 orders of magnitude in each experiment, and 29,027 phosphorylation sites were quantifiable across conditions (Table S1). Analysis of the phosphorylated amino acid distribution showed that 84% of phosphorylation sites were on serine, whereas the 13% were threonine phosphorylation sites (Figure 1C). In addition, 851 (3%) of the total quantifiable phosphorylation sites belonged to tyrosine-containing peptides (Figure 1C), pinpointing a major strength of our workflow in achieving high pTyr coverage without pretreating cells with pervanadate.

Phosphotyrosine sites were roughly 2-fold lower in abundance compared to pSer and pThr (Figure S2A), which underscores the general challenges in detecting them by MS. Our workflow led to an efficient accumulation of phosphotyrosine-containing peptides over the 12 high-pH fractions (Figure S2B), resulting in the identification of pTyr sites on more than 500 proteins enriched for RTK signaling, kinase activity, and protein binding and inclined to be localized at the plasma membrane and cytoplasm (Figure S2C). These data demonstrate the streamlined and comprehensive phosphoproteomic workflow encompassing serine, threonine, and pTyr.

**Global Analysis of pTyr Sites**

We analyzed the ligand-induced distribution of the quantified pTyr sites to gain insights into pTyr signaling upon differential RTK stimulation. Specifically, the proportional response in the pTyr site intensities (ternary plot) (Figure 2) distinguished the stimulating condition under which the sites are distributed relative to one another. Activated Pdgfr was the most potent regulator of pTyr sites when compared to Fgfr1 and Igf1r, as shown by a large cluster of pTyr sites at the top of the ternary plot, indicating higher relative response. Density estimation of all phosphorylation sites under the three RTK stimulation conditions indicated that this phenomenon was not global and was limited only to pTyr, because similar distributions were not observed for pSer and pThr sites (Figure S3). We found 21 pTyr sites on the cytoplasmic tail near the C terminus of Pdgfr-α (11) and Pdgfr-β (10) subunits (Heldin and Lennartsson, 2013) to be exclusively detected upon Pdgf-ββ stimulation. In addition, we...
found six pTyr sites on Fgfr1 and fibroblast growth factor receptor 2 (Fgfr2) when stimulated with Fgf-2 (Figure 2; Table S1) (Furdui et al., 2006). Sites on canonical adaptor molecules such as Grb2 (pY209), Fns2 (pY306), and Irs2 (pY671) were also identified, indicating their preferential binding to activated Pdgfr, Fgfr1, and Igf1r, respectively (Figure 2). The analysis demonstrated the sensitivity of our workflow for screening pTyr sites without the need for pan-anti-pTyr antibody-based enrichment and comparable to state-of-the-art, antibody-based, phospho-tyrosine-focused studies (Abe et al., 2017). The number of tyrosine sites reported in this study were roughly 6-fold higher than those reported with the EasyPhos method, which was used to study insulin signaling (another RTK) in vivo over several time points (Humphrey et al., 2015).

**Phosphorylation Site Dynamics Reveal Preferentially Activated Signaling Pathways**

The distinct activation of pTyr sites upon ligand stimulation suggests that the response initiated by each ligand-RTK pair differentially regulate downstream signaling pathways (Francavilla et al., 2016). We visualized their temporal changes as quantitative dot blots (Figure 3A) and validated our results by western blot analysis and quantification (Figure 3B; Figure S4). We found Pdgf-ββ and Fgf-2 stimulation induced phosphorylation of the activation loop residues pT203 and pY205 on Mapk/Erk1 (Figure 3). However, we observed that Pdgf-ββ exclusively activated Pik3/Akt (i.e., Pik3ca pY508 and Akt1 pS473), as well as Jak/Stat (i.e., Stat3 pY705) and Plc-γ (i.e., Plcg1 pY783) pathways. Conversely, Fgf-2-treated cells pinpointed a preferential phosphorylation of Y614 and Y615 on focal adhesion kinase 1 (Ptk2 or Fak1) (Figure 3B). Surprisingly, Pdgf-ββ-stimulated cells displayed a reduction in the basal level of phosphorylation of Y614 and Y615 on Fak1 at both time points despite the relatively high pTyr response seen upon Pdgf-ββ stimulation. Because Igf-1 stimulation did not significantly induce activation of major signaling pathways, the remainder of this study was focused on the comparative analysis of the signaling responses mediated by Pdgfr and Fgfr1 activation. Collectively, these data support

Figure 3. Phosphoproteomic Analysis Reveals Differential Ligand-Induced Activation of Signaling Pathways

(A) Dynamic profiles of site phosphorylation on proteins involved in different signaling pathways upon Pdgf-ββ, Fgf-2, and Igf-1 stimulation are shown. The size of each dot is the mean of the site phosphorylation intensities among replicates, which were identified in each of the seven conditions. Bigger correlates with a strong induction of phosphorylation for the indicated site and condition. Relative maximum intensity values for each site were used to proportionally normalize the global site phosphorylation dynamic profile. Minimum values were set to 0.01 (on a scale to 1) for representative purposes.

(B) Lysates from NIH/3T3 cells stimulated with Pdgf-ββ, Fgf-2, and Igf-1 for the indicated time points were immunoblotted as indicated to validate the differential dynamic profile. Tubulin was used as loading control.

*Confirmed by western blot. **antibody cross-reaction for the activated form of Pdgfr-β as indicated by the manufacturer. See also Figure S4 and Table S1.
Shp-2 enzymatic activity (Figures 3A and 4A; Table S1) (Zhang et al., 2015). We reasoned that Pdgfr-mediated activation of Shp-2 might be responsible for Y614 and Y615 dephosphorylation on Fak1 (Mañes et al., 1999). We tested the impact of Shp-2 inhibition on Fak1 Y614 and Y615 dephosphorylation by a newly developed allosteric Shp-2 inhibitor SHP099 (Chen et al., 2016) and observed that Shp-2 inhibition in Pdgf-β-dependent cells led to increased Y614 and Y615 phosphorylation on Fak1 (Figure 4B). Although an increase of phosphorylation on Fak1 was also observed upon Fgf-2 treatment in the presence of the inhibitor, it was less pronounced relative to Pdgf-β stimulation (with SHP099), which inverted the phosphorylation dynamics on these two Fak1 pTyr sites. The results indicate that Shp-2 is an active player in Pdgf signaling and directly affects Fak1 phosphorylation.

**Figure 4. Pdgf-β-Induced Activation of Shp-2 Affects Y614 and Y615 Phosphorylation on Fak1**

(A) Lysates from cells stimulated with Pdgf-β, Fgf-2, and Igf-1 for 3 min were immunoblotted as indicated. Bands related to Fak1 phosphorylation are shown after a longer exposure to emphasize the differences across the conditions.

(B) Lysates from cells stimulated with Pdgf-β, Fgf-2, and Igf-1 for 3 min were analyzed by immunoblotting with the designated antibodies. When indicated, cells were treated with SHP099 for 30 min before ligand stimulation. Vinculin was used as loading control. SHP099, Shp-2 inhibitor. See also Figure S5.

The comprehensive phosphorylation workflow to investigate dynamic RTK signaling pathways.

**Fak1 De-phosphorylation Correlates with Pdgf-Dependent Shp-2 Activation**

We hypothesized the presence of a Pdgf-β-dependent regulatory mechanism leading to Y614 and Y615 dephosphorylation on Fak1. We speculated that activated tyrosine phosphatases might play a role in mediating this and confirmed the possibility with global inhibition of tyrosine phosphatase activity with pervanadate (Figure S5A). Thus, we examined phosphatases exclusively tyrosine phosphorylated upon Pdgfr activation. We found protein tyrosine phosphate non-receptor type 11 (Shp-2 encoded by PTPN11) to be a putative candidate, because it displayed pronounced (a more than 60-fold increase) pTyr at positions Y546 and Y584, sites known to positively regulate Shp-2 enzymatic activity (Figures 3A and 4A; Table S1) (Zhang et al., 2015). We reasoned that Pdgf-mediated activation of the comprehensive phosphorylation workflow to investigate dynamic RTK signaling pathways.

**Shp-2 Regulates the Global Pdgfr Phosphoproteome Landscape**

We characterized the overall impact of Shp-2 activation in Pdgfr-mediated phosphosignaling response with a stable isotope labeling by amino acids in cell culture (SILAC)-based approach, in combination with phosphotyrosine peptide pull-down (Figure 5; Figure S6A) (Ong et al., 2002). Our findings revealed global changes in phosphorylation upon Pdgfr activation in Shp-2-inhibited cells (Figure S6B). Approximately 20% (1,428) of 6,884 quantified phosphorylation sites were statistically regulated more than 2-fold in the presence of SHP099 with Pdgf-β stimulation (based on Student’s t test, p < 0.05) (Table S2). Relative to serine and threonine phosphorylation sites, pTyr sites were deemed Shp-2-putative substrates (Table S2).

Our results revealed the negative regulation of Fak signaling (pY614/615) by Shp-2/Pdgfr was reversed in the presence of SHP099. Furthermore, pY421 on cortactin (Cttn), a known important substrate of Fak signaling that is suggested to modulate cytoskeletal organization and cell motility, displayed dramatic increase in phosphorylation upon Pdgf-β stimulation when cotreated with SHP099 (Figure S7A; Table S2) (Tomar et al., 2012). We confirmed the dynamics of this key site on Cttn by using a site-specific antibody for pY421, which was abolished in the presence of Fak1 inhibitor PF-562271 (Figure 5B). In addition, Shp-2 and Fak1 were found to be constitutively associated and recruited to the Pdgfr signaling complex upon Pdgf-β stimulation (Figures S7B and S7C).

Moreover, the results confirmed the specificity of Shp-2 inhibitor (SHP099) in inhibiting the Mapk/Erk pathway activation via significant downregulation of phospho-Erk1/2 activation sites pY185/205 (Figure 5) (Chen et al., 2016). As a consequence, we identified many of the downregulated pSer sites that are predicted Erk1/2 substrates (Table S2). However, other signaling pathways were largely unaffected by Shp-2 inhibition, as determined by phosphorylation of prominent activation sites on Akt (pS473) and Stat (pY705), as well as pTyr sites on Ppck1 and Plik3c, which had unchanged or increased phosphorylation (Table S2). Our dataset demonstrates global upregulation of most phosphotyrosine signaling pathways by Shp-2 inhibition but accompanied by deactivation of the Mapk/Erk signaling cascade.
Shp-2 Regulates the Interaction of Pdgfr with Signaling Proteins

Some of the most significantly upregulated pTyr sites with Shp-2 inhibition, alongside Pdgf-ββ stimulation, were found on proteins harboring guanosine triphosphatase (GTPase)-activating protein (GAP) domains (Figure 5A). We found significantly upregulated pTyr sites on GAP proteins spanning several GTPase families, such as Rasa1, Arhgap12, and Tbc1d10a (Figure 5A; Table S2). Increased levels of Rasa1, a Ras-specific GTPase, were observed to coimmunoprecipitate with Pdgfr upon Shp-2 inhibition and...
Gfp-Gab1 DAPI Merge

A

[Images of fluorescence microscopy showing Gfp-Gab1 localization under different conditions: DMSO, Mock, SHP099, +Pdgf-ββ, and SHP099 + Pdgf-ββ.]

B

Gfp-Gab1 plasma membrane localization

% cells with P.M localized Gfp-Gab1

Legend:
- DMSO
- SHP099
- +Pdgf-ββ

C

Cell signaling pathway diagram: Inactive Erk1/2 → Grb2 → Gab1 → Shp2 → Rasa1 → Active Erk1/2

Legend (next page)

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Pdgf-ββ stimulation (Figure 5C). pTyr on the crucial adaptor protein Grb2-associated binder 1 (Gab1) at pY628 was significantly downregulated, alongside markers of Erk/Mapk pathway activation (via phospho-Erk1/2 sites pY185/205) in the presence of Shp-2 inhibitor. Gab1-induced Erk activation after Met receptor activation has previously been shown to depend on Shp-2 activity (Maroun et al., 2003). Therefore, we hypothesized that this could be equivalent in Pdgfr-activated cells. We found Gab1 to significantly decrease its interaction with Pdgfr in the presence of Shp-2 inhibition (Figure 5C). Because Gab1 is known to be targeted to the plasma membrane upon RTK stimulation (Chang et al., 2015), we observed an accumulation of Gab1 to the plasma membrane upon Pdgf-ββ stimulation (Figure 6). However, there was a significant reduction of Gab1 recruitment to the cellular plasma membrane after Pdgf-ββ stimulation in the presence of SHP099 (Figure 6B). The results indicate dynamic Shp-2-dependent regulation of key proteins, leading to changes in protein localization and pathway activation (Figure 6C).

**Shp-2 Affects Ligand-Dependent Cellular Outcomes**

We next sought to determine the impact of Pdgfr- and Fgfr1-mediated signaling on cellular outcomes. Pdgf-ββ-induced cell migration and proliferation were both affected by Shp-2 inhibition, whereas Fgf-2-induced cellular responses were not (Figure 7). Specifically, Shp-2 inhibition, alongside Pdgf-ββ costimulation, of cells significantly reduced the rate at which cells migrated, while this was not observed with Fgf-2 stimulation in the presence of inhibitor (Figures 7A an 7B). The opposite trend was observed for cell proliferation in cells coexposed to SHP099 and Pdgf-ββ, displaying slightly higher proliferation rates comparable to Fgf-2 treatment. Conversely, SHP099 treatment, in combination with Fgf-2 stimulation, failed to affect the proliferation rate significantly (Figure 7C). Collectively, the results indicate an active role for Shp-2 in dictating Pdgf-ββ-mediated cellular migration, but not Fgf-2-dependent signaling and cellular responses.

**DISCUSSION**

Tight regulation of the interplay among multiple cell signaling networks activated by RTKs is necessary to ensure normal cellular function. Identifying signaling proteins and quantifying their phosphorylation status directly by phosphoproteomics is emerging as an alternative to indirect approaches based on inferring cellular signaling network rewiring via genomic and transcriptomic studies (Yaffe, 2013). In this work, we demonstrate proof-of-principle of detecting and quantifying phosphotyrosine-containing peptides simultaneously with pSer and pThr without additional affinity purification via cocktails of pan-anti-pTyr antibodies. This foundation proved to be essential in the global investigation of endogenous RTK signaling and activation in murine fibroblast cells. The dataset will serve as a valuable resource for further follow-up of novel proteins and phosphorylation sites that may be involved in regulating downstream RTK response. Because Pdgfr is a crucial RTK implicated in numerous cancers and a prominent target for therapy, understanding its downstream signaling components could provide invaluable insight for treatment.

We conceptually demonstrated the potential of an antibody-free phosphoproteomic workflow by distinguishing the regulation of key downstream elements in Pdgfr signaling, such as Shp-2, Fak1, and Gab1, via pTyr. Although the relationships among these individual molecules have been well investigated as single- or two-component systems, our analysis pinpointed the interdependent regulation of each of these molecules simultaneously in an unbiased manner. The results highlight Shp-2 as a master regulator of downstream Pdgfr signaling while having modest to no influence on Fgfr signaling. Despite reduced cell migration upon Shp-2 inhibition, alongside Pdgfr activation, cell proliferation was surprisingly largely unaffected in our biological system. This could indicate different mechanisms of Mapk/Erk activation via activated Fgfr1 through distinct adaptor proteins such as Frs2. For instance, our results suggest that active Shp-2 is required for Gab1 recruitment to Pdgfr and the plasma membrane, which leads to full activation of the Erk/Mapk pathway. pTyr of Gab1 at pY628 has been suggested as a potential docking site for Shp-2 (Chan et al., 2010; Cunnick et al., 2001); however, it is possible that Shp-2 inhibition results in competitive Pdgfr interaction with an alternative protein superseding Gab1. It is conceivable that uncontrolled pTyr leads to the recruitment and localization of proteins and molecules that inhibit Erk1/2 activation. The significant increase in pTyr on several GAPs implies a crucial role in deactivating guanosine triphosphate (GTP)-bound Ras (active state), thus eliminating downstream activation of Erk via Ras/Raf/Mek. Unexpectedly, our results also indicate a potential role for GAPs, which have yet to be implicated with RTK signaling. Whether this results in reduced Gab1 pTyr and, as a consequence, reduced plasma membrane localization still needs to be determined. It is also plausible that additional molecular mechanisms and proteins could be involved in a synergistic or cumulative manner, leading to Mapk/Erk inhibition. Nonetheless, our dataset opens up the avenue for further examination of the role of several proteins that may regulate additional RTK signaling pathways in a similar manner and could serve as novel therapeutic targets.

The importance of phosphotyrosine signaling in health and disease is unquestionable, but global and unbiased tyrosine phosphoproteomic investigations have so far been limited due to its low cellular abundance. We demonstrated the benefits of

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**Figure 6. Immunofluorescence Imaging of Gfp-Gab1 Shows Treatment-Dependent Cellular Localization**

(A) Representative immunofluorescent cell images of Gfp-Gab1 cell nucleus with DAPI staining of NIH/3T3 cells under different treatment conditions. For imaging, cells were cooled to 4°C for 30 min before stimulation with Pdgf-ββ.

(B) Quantitation of Gab1 localization was determined by manually counting the number of cells, which displayed Gfp-Gab1 membrane localization. Four biological replicates (n = 4) performed on two separate days were used to determine statistical significance. Each biological replicate represents a different coverslip. Between 11 and 48 cells were counted for each coverslip. Error bars represent SD, and the p value was determined by unpaired two-tailed t test.

(C) Schematic model of the role of Shp-2 in Pdgfr signaling is illustrated. Inhibition of Shp-2 results in increased localization of Rasa1 to Pdgfr (as indicated by arrows) and the reverse for Gab1.
a global phosphoproteomic workflow for elucidating differential endogenous RTK signaling networks and expect similar workflows to be routinely applied directly toward analyzing signaling in tumor and cancer tissues. This provides opportunities to identify new biomarkers and drug candidates. As such, we hope that our datasets will serve as a beneficial foundation for further validation and mechanistic elucidation of several novel proteins that could be implicated in modulating downstream RTK signaling.

**EXPERIMENTAL PROCEDURES**

**Reagents**

The following commercial reagents were used: Fgf-2, Pdgf-ββ, and Igf-I (PeproTech); DMSO (Sigma-Aldrich); Shp-2 inhibitor SHP099 (Xcessbio); Fak1 inhibitor PF-562271 (Selleckchem); and Mek inhibitor U0126 (Cell Signaling Technology). Antibodies were as follows: rabbit anti-phospho-Fak (pY576/pY577); rabbit anti-Fak, rabbit anti-phospho-Akt (pS473); rabbit anti-Akt, mouse anti-phospho-Stat3 (pY705); rabbit anti-Akt, mouse anti-phospho-Stat3 (pY705); rabbit anti-Stat3, rabbit anti-Stat3.
anti-phospho-Pdgfr-β (pY751); rabbit anti-Pdgfr-β, mouse anti-phospho-
Fgr1 (pY653/654); rabbit anti-phospho-Plc1 (pY783); rabbit anti-phospho-
Ctn (pY421); rabbit anti-Ctn, rabbit anti-Plyc-1, rabbit anti-phospho-Shp-2
(pY542); rabbit anti-phospho-Shp-2 (pY580); rabbit anti-Shp-2, rabbit anti-
Gab1, mouse anti-phospho-Erk1/2 (pT202/pY204); and rabbit anti-Erk1/2
(Cell Signaling Technology); rabbit anti-Erk1/2, mouse anti-vinculin, and
mouse anti-tubulin (Sigma-Aldrich); rabbit anti-Fgr1, mouse anti-Ras GAP
(Santa Cruz Biotechnology); and mouse anti-Grb2 (BD Biosciences). Bead-
conjugated antibodies for phosphotyrosine enrichment were mouse anti-
phosphotyrosine (P-Tyr-100) and rabbit anti-phosphotyrosine (P-Tyr-1000)
(Cell Signaling Technology).

**Cell Culture and Ligand Stimulation**

NIH/3T3 Mus musculus fibroblast adherent cell cultures were purchased from
ATCC. Cells were cultured in DMEM (Gibco), 100 U/mL penicillin (Invitrogen),
and 100 μg/mL streptomycin (Invitrogen) and supplemented with 10%
newborn calf serum (NBCS) (Gibco) at 37°C in a humidified incubator with
5% CO2. For the ligand stimulation experiment, cells were serum-starved
overnight in serum-free medium. Cells were stimulated for the indicated time
points with 100 ng/mL of Pdgf-β, Fgf-2, or Igf-1. Ligands were replenished
every 24 hr for long-term stimulation. All experiments were performed at
80% confluence on Nunc Petri dishes (245 or 150 mm) except for the wound
healing scratch assay, for which 95% confluence was used. Experiments for
cell-based assays were performed in either 24-well plates or 6-well plates.
All cells were tested for mycoplasma with a PCR-based method every third
week.

**SILAC**

NIH/3T3 cells were cultured in high-glucose DMEM (Biowest) lacking lysine
and arginine supplemented with 10% dialyzed fetal bovine serum (Gibco),
2 mM L-glutamine (Gibco), 100 U/mL penicillin (Invitrogen), and 100 μg/mL
streptomycin (Invitrogen). Three cell populations were obtained: one labeled
L-[13C6]Arg (+6)} (Lys4, Arg6), and the third labeled with heavy variants of the
amino acids {L-[ 13C6, 15N2]Lys (+8) and L-[ 13C6, 15N4]Arg (+10)} (Lys 8,
Arg10). Medium and heavy variants of amino acids were purchased from
Cambridge Isotope Laboratories. According to determined protein concentra-
tion via Bradford assay (Bio-Rad), proteins for each SILAC condition were
mixed at 1:1:1 ratio.

**Cell Lysis and Western Blots**

For proteomic analysis, cells were quickly washed twice with cold 1× PBS
after stimulation. Bovine serum (6 M guanidinium hydrochloride and
25 mM Tris [pH 8]) containing 5 mM tris(2-carboxyethyl)phosphine (TECP)
and 5.5 mM chloroacetamide (CAA) was added to culture plates. Cells were
then scraped and collected. Following collection, the samples were boiled at
99°C for 10 min, followed by sonication using a tip. Bradford assay (Bio-
Rad, Hercules, CA, USA) was used to determine protein concentration. For
biochemical assays, cells were washed twice with cold 1× PBS after stimula-
tion. Cell extraction was performed in lysis buffer (50 mM Tris HCl [pH 7.6],
150 mM NaCl, and 1% Triton) supplemented with complete mini EDTA-free
protease inhibitor (Roche) and 1 mM of sodium fluoride (NaF), β-glycerol phos-
phate, and sodium orthovanadate (Na3PO4). Following centrifugation at
13,000 x g for a least 20 min at 4°C, the protein concentration was determined
using Bradford assay (Bio-Rad). When needed before stimulation, cells were
preincubated for 30 min with 10 μM U0126 and 10 μM PFF-52271 and for
1 hr with 10 μM SHP099 inhibitor. Control cells were preincubated with
DMSO alone. After sample collection, immunoblotting was performed as pre-
viously described (Francavilla et al., 2016). Quantification analysis was per-
formed with ImageJ software, and images were processed by Photoshop
and Illustrator software (CS6 version; Adobe). Each experiment was repeated
at least three times and produced similar results.

**Cell Proliferation Assay**

Cells were seeded in triplicate on 24-well plates at 4 x 104 cells/well, serum-
starved overnight, and treated for either 72 or 96 hr with 100 ng/mL of Fgf-2,
Pdgf-β, and Igf-1. When needed, cells were treated with 10 μM SHP099 inhib-
itor and 10 μM U0126 for the indicated time point, and control cells were
reated with DMSO alone. Medium with fresh ligands and inhibitor was reple-
ished every 24 hr. At each time point, viable cells were counted with trypan
blue exclusion method, and the ratio to unstimulated cells at time 0 was deter-
mined for each time point as previously described (Francavilla et al., 2016).
Values represent the means ± SEM from at least three independent experi-
ments performed in triplicate.

**Wound Healing Scratch Assay**

The migratory behavior of NIH/3T3 cells was evaluated using the scratch
(wound healing) assay. Equal numbers of cells were seeded in triplicate on
6-well plates and allowed to reach confluence. Cells were then serum-starved
overnight, and the wound was made by scratching a line across the bottom of
the dish on a confluent cells monolayer. One wound per well was made using a
sterile p-10 pipette tip spanning the well. Cells were rinsed gently with PBS
and then treated for 24 hr with 100 ng/mL of Pdgf-β, Fgf-2. When needed before
stimulation, cells were preincubated with 10 μM SHP099 inhibitor for 30 min.
Control cells were preincubated with DMSO alone. Migration of cells was
observed at premarked positions below the wells using a Leica DMi3000
inverted microscope at 5× magnification. Quantification was performed
manually by counting the number of migrating cells within the scratch cross-
sectional area. Values represent the means ± SEM from at least three indepen-
dent experiments performed in duplicate.

**Immunoprecipitation**

For pull-downs, stimulation was performed as described earlier and lysis was
performed in the same manner as for biochemical assays. NIH/3T3 cell lysates
(1 mg per condition) were pre cleared with anti-rabbit immunoglobulin G (IgG)
(Sigma-Aldrich) supplemented with Protein G-Sepharose beads (Invitrogen)
and inversion rotated for at least 2 hr at 4°C. After centrifugation at 1,000 rela-
tive centrifugal force (RCF) for 5 min, cleared samples were incubated with
anti-Pdgfr-β antibody overnight at 4°C (1:150 ratio). Protein G-Sepharose
beads were added for 1 hr at 4°C, and the immunoprecipitated proteins were
washed three times with ice-cold lysis buffer: once with 50 mM NaCl,
once with 150 mM NaCl, and once with Milli-Q H2O. Proteins were eluted
from beads by boiling in lithium dodecyl sulfate (LDS) sample buffer (Novex).

**Immunofluorescence Imaging**

For fluorescent microscopy, human Gab1 (transcript variant 1) was gateway
cloned into the destination vector pcDNA4/TO/GFP. Cells were grown on
coverslips and transfected with pcDNA4/TO/GFP-GAB1 using Fugene 6
(Promega) for 12 hr. After stimulation, cells were fixed in 4% formaldehyde
and permeabilized with PBS containing 0.2% Triton X-100 for 5 min. Cover-
slips were mounted with Vectashield mounting medium (Vector Laboratories)
containing the nuclear stain DAPI. Images were acquired with a Leica
DMi8000B wide-field microscope (Leica Microsystems) equipped with an
HC Plan-Apochromatic 63×/1.4 oil immersion objective. Image acquisition
and analysis were carried out with LAS X and ImageJ software.

**Sample Prep for MS**

Lys-C protease (Wako Chemicals, Richmond, VA, USA) was used to digest proteins to peptides for 2 hr at 37°C. Samples were then
desalted on a C18 sep-pak (Waters, Milford, MA, USA) and stored until further
processing. Following elution with 50% acetonitrile, samples were concen-
trated using a SpeedVac.

**Automated Concatenation and Fractionation of Tryptic Peptides**

Ultimate 3000 ultra-high performance liquid chromatography (UHPLC) (Dio-
nex, Sunnyvale, CA, USA), in conjunction with high-pH reversed-phase
chromatography, was used to separate and fractionate tryptic peptides.
Peptides were separated using a high-pH-compatible 250 x 4.6 mm C18 Waters
BEH X-Bridge peptide separation technology (PST) 3.6 μM Phenomenex
Kinetic H2O and buffer B (acetonitrile), and fractions were collected at 1 min intervals and AUTOICON into 10 fractions in a 24-well plate (Movie S1). Running at a constant 1 mL/min, the gradient was increased from 5% to 25% buffer B in 50 min and further increased to 70% buffer B in 5 min, where it was held for another 5 min. At this point, the fraction collection was stopped. Each fraction sampled 6 points across the gradient, resulting in a 6-mL volume per fraction. 100 µL were removed from each fraction for proteome analysis, 6 mL of 88% acetonitrile with 12% TFA was added to each fraction, and samples were stored.

**TiO2 Enrichment of Phosphopeptides**

Each fraction was enriched with metal oxide affinity chromatography (MOAC) enrichment in batch mode by addition of titanium dioxide (TiO2) beads (GL Sciences, Japan) preincubated with 2,3-dihydroxybenzoic acid (DHB) and mixed for 20 min at room temperature. Fractions 1–5 and 6–10 were combined, and double-TiO2 enrichment was performed to bring total number of fractions for phosphoproteome analysis per replicate to 12. TiO2 beads were collected using centrifugation and washed on C8 StageTips in a 96-well format. Phosphopeptides were eluted under basic conditions (5% ammonium hydroxide and 25% acetonitrile) and dried to remove residual acetonitrile. Phosphopeptides were then washed and loaded onto C18 StageTips in a 96-well format, where they were stored until further analysis by LC-MS/MS.

**Phosphotyrosine and pSer or pThr Peptide Enrichment**

Phosphotyrosine-containing peptides were enriched using a 1:1 mixture of bead-conjugated anti-phosphotyrosine antibodies (PTM scan pY100 and pY1000, Cell Signaling Technology). One vial of each antibody was used for two samples. Bead-conjugated antibodies were washed 4 x in 1 mL of PBS and resuspended in 3-(N-morpholino) propanesulfonic acid (MOPS) buffer. Peptides for phosphotyrosine enrichment were eluted from C18 Sep-Paks with 1.5 mL of 0.1% TFA twice and loaded on C18 StageTips, where they were stored until further analysis by LC-MS/MS.

For phosphorylated serine and threonine (pST) enrichment, supernatant from pTyr enrichment was acidified using TFA and washed on C18 Sep-paks. Sep-paks were eluted as described earlier, and ACN and TFA were added to final concentrations of 80% and 5%, respectively. TiO2 beads were added to the eluate, enriched for phosphopeptides, and prepared for LC-MS/MS analysis as described earlier. A second round of incubation was performed on the eluate, resulting in two samples for pST analysis.

**Nano-liquid Chromatography and MS Analysis**

Thermo Fisher Orbitrap HF (Bremen, Germany) mass spectrometer operating in positive mode coupled to Easy-nLC 1000 was used in the analysis of all samples. For phosphoproteomic analysis, all samples were analyzed with sensitive MS parameters. Spectra were acquired with survey MS scans at an Orbitrap resolution of 120,000 (full width at half maximum [FWHM]), from which the top 7 most abundant peaks were selected for higher-energy collisional dissociation (HCD) fragmentation and the resulting fragment spectra were collected at 60,000 MS/MS resolution. For proteome analysis, MS survey scan resolution of 60,000 was used and the HCD spectra were collected for the top 10 most abundant peaks at a resolution of 30,000. Peptides were separated in a 15-cm microbore column (75 µm inner diameter) packed in-house with 1.9 µM C18 beads (Dr. Maisch, Germany) at 250 nL/min. Identical fast gradient was used in the analysis of all samples. Buffer B (80% ACN and 0.1% formic acid) was increased from 5% to 35% in 55 min to 45% in 5 min. This was followed by a quick ramp up to 80% buffer B in 2 min. It was held there for additional 5 min before declining back down to 5% in 5 min, where it was held for an additional 5 min to re-equilibrate the column.

For single-shot analysis of SILAC pTyr and pST, sensitive scanning MS parameters were used as described earlier, except that the top 10 most abundant peaks were selected for HCD fragmentation. In addition, a longer gradient was used for single-shot analysis on a similar 15-cm column, consisting of an increase to 25% buffer B in 110 min followed by an increase to 40% buffer B in 25 min. After this, the column was increased to 80% buffer B in 5 min, where it was held for 5 min, followed by a ramp down to 5% buffer B for column re-equilibration.

**LC-MS/MS Database Search**

All files were processed together, and raw data were searched using MaxQuant v.1.5.3.33 with Andromeda search engine (http://www.maxquant.org) (Cox and Mann, 2008) against the Mus musculus uniprot database. The MS/MS spectra were searched with carbamidomethyl as a fixed modification on cysteine and variable modification oxidation on methionine, protein N-terminus acetylation, and phosphorylation on serine, threonine, and tyrosine, along with deamidation on asparagine and glutamine. A false discovery rate (FDR) of 1% was used at the peptide and protein level. Protein quantitation required minimum of two peptides without modifications.

**Phosphoproteomic Analysis**

Resulting identified phosphorylation sites (phospho STY(sites).txt table) from MaxQuant analysis were filtered only for those that were confidently localized (class I, localization probability ≥ 0.75). Replicates were retained only when >10,000 high-confident phosphorylation sites were present, resulting in a minimum of 5 replicates for each condition. Quantile normalization (Figure 1C) was used to normalize the phosphorylation data (Bolstad et al., 2003). A site was deemed quantifiable when it was observed a minimum of two times in at least one condition, resulting in >29,000 total quantifiable sites. The resulting quantile normalized sites were used for all analysis of phosphorylation data presented in this publication.

**SILAC Analysis**

SILAC phosphoproteome samples were analyzed with search parameters similar to those described earlier, with the addition of SILAC multiplicity indicating a triple SILAC setup. Resulting phosphorylation sites were analyzed in Perseus v.1.5.2. MaxQuant normalized ratios were used for quantitation. Phosphorylated serine, threonine, and tyrosine (pSTY) sites were expanded to differentiate multiple peptide phosphorylation states and filtered only for those that were confidently localized (class I, localization probability ≥ 0.75). Reverse and contaminant hits were removed from the analysis, and sites had to be observed for a minimum of 2 of 3 replicates in each of the 3 conditions to be considered for quantitation.

**Proteome Analysis**

Perseus v.1.5.2.6 was used for analysis of proteomics (Tyanova et al., 2016). For quantitative analysis of protein abundance changes, label-free quantitation (LFQ) values were employed (Cox et al., 2014), and intensity-based absolute quantification (iBAQ) analysis was used for relative expression analysis. Significant proteins were determined on log10-transformed LFQ values using a Student’s t test.

**DATA AND SOFTWARE AVAILABILITY**

The accession number for the MS proteomic data reported in this paper is PRIDE: PXD005803 (Vizcaino et al., 2014).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes seven figures, two tables, and one movie and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.02.038.
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AUTHOR CONTRIBUTIONS

J.V.O., C.F., and T.S.B. conceived and designed the project. T.S.B. developed the phosphoproteome workflow and carried out the proteomic experiments. T.S.B. and J.V.O. carried out the initial data processing and analysis. M.P. and A.P. provided assistance in proteomic experiments and performed biological, molecular, and cellular biological experiments and follow-ups and contributed to manuscript writing. M.A.X.T. performed the immunofluorescence microscopy experiments and analysis. T.S.B. wrote the initial draft of the paper. M.P., J.V.O., C.F., and A.P. edited and contributed to the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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