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Identification of L-lactate Dehydrogenase as a Protein Tyrosine Phosphatase 1B substrate using K-BIPS

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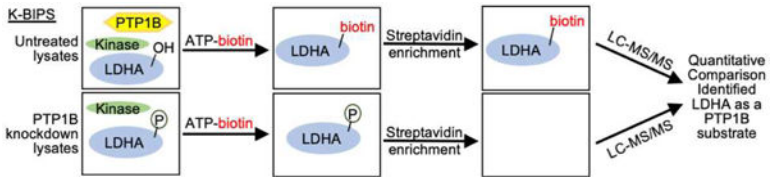
Abstract

Kinases and phosphatases are major players in a variety of cellular events, including cell signaling. Aberrant activity or mutations in kinases and phosphatases can lead to diseases, such as cancer, diabetes, and Alzheimer's. Compared to kinases, phosphatases are understudied, which is partly a result of the limited methods to identify substrates. As a solution, we developed a proteomics-based method called Kinase-catalyzed Biotinylation to Identify Phosphatase Substrates (K-BIPS) that previously identified substrates of Ser/Thr phosphatases using small molecule inhibitors. Here, for the first time, K-BIPS was applied to identify substrates of a tyrosine phosphatase, Protein Tyrosine Phosphatase 1B (PTP1B), using siRNA knockdown conditions. Eight possible substrates of PTP1B were discovered in HEK293 cells, including the known substrate Pyruvate Kinase. In addition, L-lactate Dehydrogenase (LDHA) was validated as a novel PTP1B substrate. With the ability to use knockdown conditions with Ser/Thr or Tyr phosphatases, K-BIPS represents a general discovery tool to explore phosphatases biology by identifying unanticipated substrates.

Graphical Abstract



For the first time, Kinase-catalyzed Biotinylation to Identify Phosphatase Substrates (K-BIPS) was used with tyrosine phosphatase, PTP1B, using siRNA knockdown. Biotinylation with ATP-biotin, streptavidin enrichment, and LC-MS/MS revealed possible PTP1B substrate, including L-lactate dehydrogenase (LDHA). LDHA was validated as a PTP1B substrate, confirming K-BIPS as a powerful tool to identify unanticipated phosphatase substrates.

Keywords

ATP analog; kinase; proteomics; Protein tyrosine phosphatase 1B; Lactate dehydrogenase A

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Supporting information for this article is given via a link at the end of the document.

Introduction

Phosphorylation is a crucial and reversible post-translational modification that can profoundly influence protein structure and function.[1] Phosphorylation is catalyzed by protein kinases, which transfer the γ-phosphoryl of ATP (adenosine 5'-triphosphate, Figure 1A) to the hydroxyl group of serine, threonine, or tyrosine.[2] Protein phosphatases catalyze the removal of the phosphoryl group (Figure 1A).[3] The tight regulation of kinases and phosphatases is vital for proper cell signaling,[4] and their abnormal activities can cause diseases, such as cancer, diabetes, and Alzheimer's.[5–10] Kinases and their substrates have been extensively studied in normal biology and disease, which has led to the development of many kinase inhibitors as clinical drugs.[11] Despite the known disease relevance, phosphatases and their substrates remain comparatively underexplored, with only recent progress towards clinically-viable phosphatase inhibitor drugs.[12]

Phosphatases are classified according to their amino acid substrate preference and structure, including Ser/Thr phosphatases, Tyr phosphatases, and dual-specificity phosphatases.[3] Prior work documented that isolated full-length protein tyrosine phosphatases (PTPs) maintain substrate specificity,[13] recognizing both the phosphotyrosine residue to be dephosphorylated and flanking amino acids.[14–15] Despite high substrate selectivity, the identification of cellular substrates of PTP proteins has relied on only a few strategies. One method involves substrate trapping with an inactive mutant of the phosphatase,[15] which requires recombinant mutant phosphatases. Several mass spectrometry approaches were used to identify substrates of PTPs, including SILAC labeling coupled with whole proteome analysis and affinity purification.[16–18] Combining the data from these methods, the DEPOD.org database of phosphatases and their substrates was created to promote further studies on phosphatases.[19] Despite the progress, the paucity of methods to identify substrates of phosphatases has limited the routine identification of cellular substrates under different cellular conditions.

As an additional method for phosphatase substrate identification, we previously developed K-BIPS (Kinase-catalyzed Biotinylation to Identify Phosphatase Substrates).[20] K-BIPS relies on the fact that cellular kinases accept γ-modified ATP analogs as cosubstrates. For example, kinases collaborate with ATP-biotin (Figure 1A) to attach a phosphoryl biotin tag to substrates, facilitating protein visualization and purification. Importantly for K-BIPS, the extent of protein biotinylation by ATP-biotin in lysates was reduced significantly in the presence of phosphatase inhibitors,[21–24] indicating that active phosphatases are required for full kinase-catalyzed biotinylation of cellular proteins. Based on this phosphatase dependence, K-BIPS compares the relative biotinylation of proteins in untreated and phosphatase-inactivated cell lysates after the ATP-biotin reaction. When phosphatases are active, phosphatase substrates will be dephosphorylated for subsequent biotinylation by ATP-biotin (Figure 1B, top). In contrast, substrates in the phosphatase-inactive sample are trapped in the phosphorylated state, preventing kinase-catalyzed biotinylation (Figure 1B, bottom). Possible substrate proteins can be identified by streptavidin enrichment, followed by a quantitative comparison of isolated proteins in the untreated and phosphatase-inactive samples using liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

In our initial disclosure of the K-BIPS method, phosphatases were inactivated by incubation with small molecule inhibitors, allowing identification of both known and new substrates of Ser/Thr phosphatases, including protein phosphatase 1 (PP1).[20] Unfortunately, few specific small molecule inhibitors of phosphatases are known,[12] limiting the general utility of K-BIPS. K-BIPS also was not established for PTPs in that initial study.

In this study, the K-BIPS method was used to discover substrates of PTPs using small interfering RNA (siRNA) knockdown conditions for phosphatase inactivation. As one of the best-studied PTPs, PTP1B (Protein Tyrosine Phosphatase 1B) was chosen for this proof-of-concept study. PTP1B is a negative regulator of insulin signaling and has emerged as a potential target for type 2 diabetes mellitus and obesity treatment.[25–26] PTP1B also acts as a tumor suppressor or tumor promoter through the dephosphorylation of specific substrates.[27] Known substrates of PTP1B include Janus Kinase 2 (JAK2), Insulin Receptor (IR), Insulin Receptor Substrate (IRS), and Pyruvate Kinase (PKM).[28–29] However, a full list of substrates linked to tumor formation, diabetes, or obesity is not yet identified due to the scarcity of methods. Using K-BIPS, eight high confidence protein hits were identified as putative PTP1B substrates, including PKM, a known substrate of PTP1B. In addition, L-lactate Dehydrogenase (LDHA) was validated as a new substrate of PTP1B. With the versatility to apply to any phosphatase using siRNA knockdown conditions, K-BIPS is a powerful tool to discover phosphatase substrates.

Results and Discussion

K-BIPS method validation

Before discovery of new substrates of PTP1B, the K-BIPS method was validated by testing the biotin labeling and enrichment of the known PTP1B substrate JAK2.[28] HEK293 cells were untreated, transfected with siRNA to knockdown PTP1B, or transfected with scrambled siRNA as a negative control. After cell lysis, the lysates were tested for successful knockdown by separating proteins by SDS-PAGE and visualizing PTP1B levels in each sample. As expected, reduced PTP1B was observed in input lysates of samples transfected with PTP1B siRNA (Figure 2A, lane 2) compared to untreated or scrambled siRNA control samples (Figure 2A, lanes 1 and 3). Quantification of three independent trials documented reproducible knockdown of 92 ± 4% (compared to untreated control, Figure S1B), indicating successful knockdown. For the K-BIPS method, each lysate was incubated with ATP-biotin for kinase-catalyzed biotinylation, and biotinylated proteins were enriched using streptavidin purification. After enrichment, proteins were separated by SDS-PAGE and JAK2 levels were visualized by Western blotting. As expected, a lower level of JAK2 was observed in the PTP1B siRNA knockdown sample (Figure 2A, lane 2) compared to the negative controls (Figure 2A, lanes 1 and 3). Quantification of three independent trials documented that siRNA knockdown reduced the amount of JAK2 enrichment by 45 ± 5% compared to the untreated control (Figure 2B, lane 2 vs 1). The reduced enrichment of JAK2 observed with PTP1B knockdown compared to controls confirms that differential biotinylation occurs under K-BIPS conditions. With conditions in place for effective K-BIPS substrate enrichment, the next step was to identify substrates using K-BIPS and LC-MS/MS analysis.

K-BIPS for PTP1B novel substrates identification

To identify new substrates of PTP1B, a full K-BIPS experiment was performed with LC-MS/MS analysis of enriched proteins. Lysates from untreated cells or cells transfected with PTP1B or scrambled control siRNA were incubated with ATP-biotin before streptavidin enrichment of biotinylated proteins. Enriched proteins were trypsin digested and analyzed by LC-MS/MS and label-free quantitation using MaxQuant.[30] In five independent trials, a total of 955 proteins were identified (Table S1). To identify putative substrates, fold enrichment values were calculated by dividing the MaxQuant intensity of each protein in the untreated and scramble control siRNA transfected sample by the intensity of that same protein in the PTP1B siRNA transfected sample. If both enrichment values were above 1.5, the protein was considered as a possible substrate hit. As an initial hit list, 53 proteins were enriched by at least 1.5-fold in 2 out of 5 trials (Table S2). Among the hits was pyruvate kinase (PKM). Previously, an isoform of PKM, PKM2, was identified as a substrate of PTP1B.[29] The presence of a known PTP1B substrate in the hit list validates that K-BIPS is useful for substrate identification.

Cellular compartment, biological process, and molecular function classifications of the 53 hit proteins (Table S2) were analyzed using DAVID 6.8 to compare to known PTP1B activities.[31–32] PTP1B localizes predominantly at the endoplasmic reticulum (ER) and accesses substrates through biosynthesis, endocytosis, and ER network movement.[33–34] Therefore, membrane-bound and cytoplasmic proteins are likely to be highly represented in the hit list. As expected, the majority of hit proteins are either cytoplasmic or membrane-bound (Figures 3A). In terms of biological processes, PTP1B and substrates play a role in cell-cell communication,[35–36] and the PTP1B substrate EPHA5 regulates cell-cell contacts in the pancreatic β-cell line.[35, 37] Consistent with this prior work, enriched substrates were involved in cell-cell adhesion (Figure 3B). Previous studies found that PTP1B induces translational initiation through enhanced activation of insulin-signaling pathway.[38] Similarly, the K-BIPS hit proteins were enriched in a variety of biological processes associated with protein expression, including RNA splicing, ribosomal large subunit assembly, the formation of the translation preinitiation complex, translational initiation, translation, and protein folding (Figure 3B). Related to molecular function, the hit proteins are also linked to protein expression, including poly(A) RNA binding, structural constituent of ribosome, and translation initiation factor and regulator activity (Figure S2B). In total, the analysis shows that many hit proteins are localized and maintain functions consistent with known PTP1B activities.

A possible advantage of K-BIPS is the use of biotinylation and enrichment to identify substrates, which is expected to isolate proteins independent of abundance, including low abundance proteins. To assess the quality of enrichment, the 53 hits were analyzed using the abundance values from Protein Abundance Database, PAXdb.[39] The abundance range of K-BIPS hit proteins was 1.01– 5619 ppm (Figure S3), which represents a similar range compared to the full proteome in HEK293 cells (0.01 to 10,000 ppm).[40] According to the analysis, K-BIPS enriched proteins without bias toward highly abundant proteins, which confirms the value of affinity purification in the method.

Secondary confirmation of LDHA as a K-BIPS hit

To select potential substrates for secondary validation, a higher confidence hit list was generated by identifying eight proteins that were enriched by more than 1.5-fold in at least 3 out of 5 trials (Tables 1 and S2). The known PTP1B substrate PKM was among this higher confidence list. From this high confident hit list, L-lactate dehydrogenase (LDHA) and DnaJ homolog subfamily A member 2 (DNAJA2) were selected for further confirmation as K-BIPS hit proteins.

As a secondary confirmation, the K-BIPS method was performed with Western blot analysis to confirm enrichment of LDHA and DNAJA2. The K-BIPS procedure was followed with biotinylated proteins in each sample enriched and separated by SDS-PAGE. As expected, a reduced level of biotinylated LDHA was observed in PTP1B knockdown compared to control samples (Figure 4A, compare lanes 2 to 1 and 3). Quantified data from four independent trials confirmed that LDHA enrichment was significantly lower in PTP1B knockdown cell lysate compared to untreated and scrambled siRNA controls (61 ± 8%, Figure 4B). We note that K-BIPS enrichment and Western blot visualization required knockdown of PTP1B by at least 85% (Figure S1B and S4B), providing a benchmark for the relative knockdown necessary for effective use of the K-BIPS method. The data confirmed that LDHA was a K-BIPS hit and a possible substrate of PTP1B.

In contrast to the results with LDHA, DNAJA2 did not show the expected K-BIPS enrichment pattern by Western blot analysis. All K-BIPS samples, including PTP1B knockdown samples, showed equal levels of DNAJA2 after enrichment (Figure S5, compare lanes 1 to 2, and 3). In this case with DNAJA2, Western blot analysis might not have been sensitive enough to monitor changes in enrichment. However, the fact that K-BIPS with gel analysis did not reproduce the LC-MS/MS data suggested that DNAJA2 might not be a substrate of PTP1B. This secondary K-BIPS data highlight the importance of confirmation assays to avoid possible false positive hits and focus additional validation studies on likely PTP1B substrates, such as LDHA.

Validation of the LDHA dephosphorylation by PTP1B

With successful confirmation of LDHA as a K-BIPS hit, LDHA was further validated as a PTP1B substrate using Phos-tag™ gel electrophoresis. Proteins in untreated, PTP1B siRNA transfected, or scrambled control siRNA transfected cell lysates were separated by SDS-PAGE containing the Phos-tag™ additive, which interacts with phosphate groups to alter protein migration as a function of phosphorylation state.[41] If LDHA is a substrate of PTP1B, the expectation was that PTP1B knockdown samples would contain a slower migrating phosphorylated form of LDHA not present in negative control samples. As expected, two slower migrating LDHA bands were observed in the PTP1B knockdown sample compared to untreated or scrambled control siRNA transfected samples (Figure 5A, compare lanes 2 to 1 and 3). Quantification of the band intensities from three independent trials confirmed that the levels of the two new phospho-LDHA bands were significantly increased in the PTP1B knockdown sample compared to controls (Figures 5B and 5C). The Phos-tag™ gel electrophoresis data indicated that the phosphorylation state of LDHA

increased as a function of PTP1B knockdown, suggesting that LDHA is a substrate of PTP1B.

According to the PhosphositePlus® database, LDHA has 21 phosphosites on Ser, Thr, and Tyr residues. The Phos-tag™ data is consistent with this prior work by documenting many phosphorylated LDHA bands (Figure 5A). With six known phosphotyrosine sites, the Phos-tag™ data where two bands showed elevated phosphorylation upon knockdown suggests that PTP1B influences the dephosphorylation of two sites. Further experiments are needed to identify the specific phosphotyrosines dephosphorylated by PTP1B.

LDHA is a subunit of LDH (lactate dehydrogenase) that catalyzes the conversion of pyruvate to lactate, which can lead to anaerobic glycolysis in tumor cells.[42] As a result, LDH is often used as a sensitive indicator of cellular metabolism in tumor cells.[42–43] LDHA is phosphorylated by oncogenic receptor tyrosine kinase FGFR1, which enhances the enzymatic activity of LDHA and leads to the Warburg effect and cancer growth.[44–45] Despite the role of dynamic phosphorylation in the activity of LDHA, the tyrosine phosphatases that dephosphorylate LDHA are not known. Here LDHA was validated as a substrate of PTP1B. Similar to the role of LDHA in cancer cells, PTP1B influences tumorigenesis through dephosphorylating substrates.[27] For example, PTP1B knockdown or small molecule inhibition affected the phosphorylation state and kinase activity of Src, resulting in reduced colony forming in colon cancer cells.[27] In a similar manner, PTP1B is known to negatively regulate IL-4/Jak/Stat6 signaling pathway that facilitate anti-tumor properties.[46] In addition to the role of PTP1B in Src-mediated functions and interleukin induced STAT6 signaling, the data presented here suggest that PTP1B might influence tumor cell growth by regulating LHDA activity and metabolism.

LDHA is also known to regulate glucose metabolism and insulin secretion.[47] As a result, LDHA is used as a biomarker to distinguish metabolic changes associated with obesity and diabetes.[48] Likewise, PTP1B is a negative regulator of insulin signaling, and also plays a role in diabetes and obesity.[25] Insulin signaling is initiated by activating insulin receptor (IR), recruiting of insulin receptor substrate (IRS), and promoting downstream kinase activities, which ultimately results in activation and translocation of glucose transporter proteins. PTP1B governs the phosphorylation state of both IR and IRS proteins to play a key role in regulating the insulin pathway.[26–27] Previous studies confirmed that PTP1B also regulates leptin signaling through dephosphorylation of JAK2, which induces resistance toward obesity.[49] In the case of insulin signaling, the data here suggests that PTP1B affects LDHA activity, in addition to IR and IRS activation, to control insulin signaling and glucose transport.

Conclusion

In summary, K-BIPS was successfully used to identify substrates of PTP1B using knockdown conditions. Combined with prior work, K-BIPS is compatible with both Ser/Thr and Tyr phosphatases using either phosphatase specific small molecule inhibitors or siRNA knockdown conditions, which shows the versatility of the method. K-BIPS can also flexibly involve any cell lysate or tissue homogenate with active kinases/phosphatases, including

lysates with activated cell signaling pathways to identify phosphatase substrates under various cellular conditions. Finally, with the availability of methods to permeabilize ATP-biotin into cells,[50–51] K-BIPS is compatible with cell-based analysis, which is currently ongoing in our lab.

Given its versatility, K-BIPS is complementary to other methods to discover phosphatase substrates, including substrate trapping or mass spectrometry-based approaches, resulting in an expanded toolbox of approaches for the biomedical community. Comparing the methods, although almost 50 substrates of PTP1B are known,[19] only one substrate, PKM, was identified by K-BIPS. Given the variety of cell lines and conditions used in prior PTP1B substrate identification studies,[15–17] the limited substrate overlap among the studies suggests that the substrate profile of PTP1B might be dependent on cell type and condition. Additional substrate identification studies are necessary to carefully dissect the substrates of PTP1B under additional cellular conditions. Moreover, with the recent progress towards clinically viable phosphatase inhibitor drugs, the substrate identification represents an enabling strategy to explore phosphatase functions, which can ultimately lead to new avenues of drug discovery.

Experimental Section

ATP-biotin synthesis

The synthesis of ATP-biotin was performed as previously described.[21]

PTP1B knockdown in HEK 293 cells

HEK293 cells (1×10^6) were grown in growth media (10 mL) comprised of DMEM media (Gibco) and 10% FBS (Fetal Bovine Serum, Gibco) in T75 flasks at 37 °C in a 5% CO_2 environment to 50% confluency. Cells were treated by adding growth media (8 mL), a pool of PTP1B-targeting siRNA (25 nM, Dharmacon, catalog number M-003529–04-0005) or a control non-targeting pool of siRNA (25 nm, Dharmacon, D-001206–14-05) dissolve in 950 μL of serum free DMEM media, and transfection reagent (40 μL in 960 μL of serum free media, Dharmacon, T-2001–02). As an untreated negative control, cells were treated with transfection reagent (40 μL in 1960 μL of serum free DMEM media) and growth media (8 mL) alone. After 72 hours of incubation at 37 °C in a 5% CO_2 environment, cells were harvested by removing the media, washing with DPBS (Dulbecco's Phosphate Buffered Saline, 5 mL, ThermoFisher), and incubating with a trypsin–EDTA solution (0.25% with phenol red, 3 mL, ThermoFisher) for 5 minutes at 37 °C. Cold DPBS (5 mL) was added to stop the trypsin reaction, and cells were collected by centrifugation at 1000 rpm, at 4 °C for 5 minutes. Cells were washed with cold DPBS (2 mL) once. The washed cell pellet was either stored at −80 °C or immediately lysed. For lysis, cells (23×10^6) were resuspended in lysis buffer (300 μL; 50 mM Tris pH 7.5, 150 mM NaCl, 0.5% Triton X-100, and 10% glycerol) containing Xpert protease inhibitor cocktail solution (1x, GenDEPOT) and rocked at 4 °C for 40 minutes. The soluble fraction was separated from cell debris by centrifugation at 13,200 rpm for 20 minutes. The protein concentration of the soluble fraction was determined by Bradford assay (BioRad) and stored as aliquots at −80 °C.

Assessment of PTP1B expression in knockdown cell lysates

Lysates from untreated, scrambled siRNA control transfected, or PTP1B siRNA-transfected cells (100 μg total protein) were heated at 95 °C for 1 min in Laemmli sample buffer (60 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 0.0005% bromophenol blue, and 2% beta-mercaptoethanol). Proteins in each sample were separated by 10% SDS-PAGE. Total proteins were visualized using a Typhoon imager (GE Healthcare Life Sciences) after staining with SYPRO® Ruby (Invitrogen™). For the PTP1B Western blot, proteins were transferred onto a PVDF membrane (Millipore, Immobilon-P) and probed with a PTP1B specific antibody (Cell Signaling Technology, 5311S).

K-BIPS protocol

Kinase-catalyzed biotinylation of proteins from untreated, scrambled siRNA control transfected, or PTP1B siRNA-transfected cell lysates (500 μg total protein) was initiated by adding ATP-biotin (2 mM) in a total volume of 120 μL. After a 2 hr reaction at 31 °C, excess ATP-biotin was removed by filtering the lysates from each sample separately through a 3 kDa centiprep spin columns (Millipore) twice, with water (200 μL) added in the second centrifugation, as described in the manufacturer's instructions. Streptavidin resin (200 μL of packed beads, 400 μL slurry, Genscript) was washed three times with phosphate-binding buffer (200 μL; 0.1 M phosphate pH 7.2, 0.15 M NaCl). The filtered samples were allowed to bind to the washed streptavidin resin (200 μL of packed beads for each sample) by rotating for 10 min at room temperature. The bound beads were washed with phosphate-binding buffer (200 μL) ten times, followed by washing three times with water (200 μL) and collecting by centrifugation at 0.5 rcf for 1 min at room temperature. The bound, biotinylated proteins were eluted by boiling the beads in 2% SDS in water (200 μL) for 8 minutes. The eluate was then concentrated using speed vac (Thermo Scientific). Proteins in the concentrated eluate were boiled at 95 °C for 1 min in Laemmli sample buffer and separated by 10% SDS-PAGE. As load and expression controls, input lysate before enrichment was also separated by 10% SDS-PAGE. Total proteins were visualized by SYPRO® Ruby stain (Invitrogen™) according to the manufacturer's guidelines. JAK2, LDHA, DNAJA2, and PTP1B levels were assessed by Western blotting after transfer onto a PVDF membrane (Millipore, Immobilon-P) and probing with JAK2 (Cell Signaling Technology, 3230S), LDHA (Lifespan Bioscience Inc. LS-C754739), DNAJA2 (Abcam-ab157216), and PTP1B (Cell Signaling Technology, 5311S) specific antibodies.

LC-MS/MS analysis for K-BIPS of PTP1B

The K-BIPS protocol was followed as described above. The full volume of each sample after streptavidin enrichment and concentration was desalted by running the samples 1 cm into a gel using 10% SDS-PAGE, followed by visualization using SYPRO® Ruby stain (Invitrogen™). Proteins were excised from the gel, and gel pieces were collected into Protein LoBind tube (Eppendorf™, Fisher Scientific). In-gel digestion was implemented as previously described.[52] The resulting dry peptides from the in-gel digestion were resuspended with 5% acetonitrile, 0.1% formic acid, and 0.005% trifluoroacetic acid. Peptides were separated using the Acclaim PepMap RSLC column and an Easy nLC 1000 UHPLC system (Thermo). A Q-Exactive mass spectrometer (Thermo) was used to analyzed

separate peptides using the following conditions; For MS1– 70,000 resolution with 375–1600 m/z, MS2– 17,500 resolution scans using 1.6 m/z window and 30% normalized collision energy for HCD. MaxQuant (ver 1.5.2.8)[30] was used to analyze the raw MS data combined with Uniprot human database (downloaded 04.07.2016). In the analysis, two missed tryptic cleavages were allowed, the iodoacetamide derivative of cysteine was set as a fixed modification, and acetylation of the N-termini was set as variable modifications. Mass tolerance for the parent ions was adjusted to 20 ppm for the first search, 4.5 ppm for the second, and 20 ppm for the fragment ions. The false discovery rate (FDR) was set to ≤1% for minimum protein. Peptide identification probability, as determined by reverse database search of the protein, required one unique peptide. The default settings were used for all other parameters.

Bioinformatics Analysis

The cellular compartment, biological process, and molecular function classifications of the proteins were analyzed using DAVID 6.8,[31–32] with the data plotted in Microsoft Excel. Abundance analysis was performed using the PAXdb database,[39] with the data plotted in Microsoft Excel.

LDHA validation by Phos-tag™ SDS-PAGE

Lysates from untreated, scrambled siRNA control transfected, or PTP1B siRNA-transfected cell lysates (75 μg total protein) were separated by 10% SDS-PAGE and 10% SDS-PAGE containing the Phos-tag™ additive (15 μM, FUJIFILM Wako Chemicals U.S.A. Corporation). SYPRO® Ruby stain was used to visualized total protein. To monitor LDHA and PTP1B levels, proteins in the two gels were transferred onto a PVDF membrane (Immobilon-P, Millipore sigma) and visualized with LDHA (Lifespan Bioscience Inc. LS-C754739) or PTP1B Cell Signaling Technology, 5311S) antibodies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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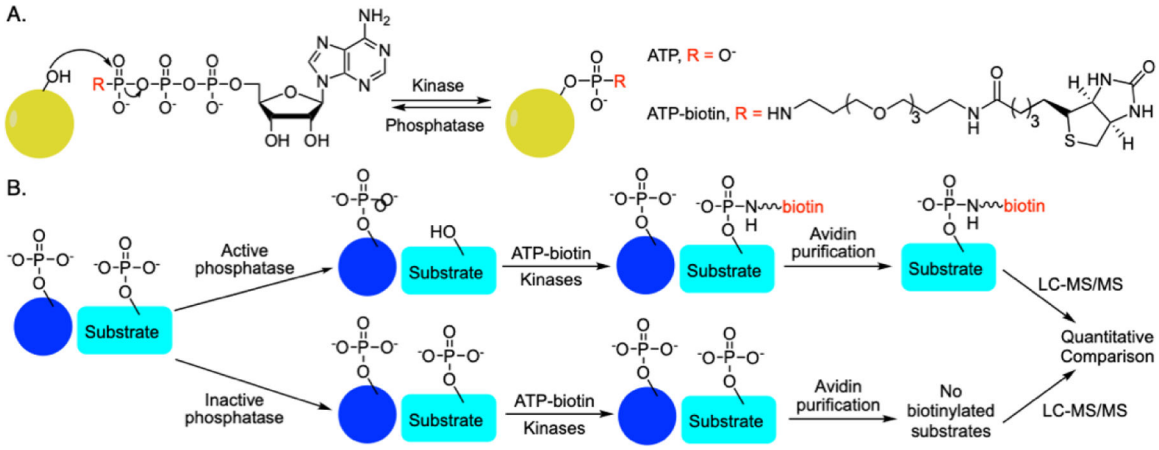

Figure 1.

Kinase-catalyzed phosphorylation and the K-BIPS method. A) Kinases transfer the γ-phosphoryl group of ATP or ATP analogs to protein substrates (yellow). In the case of ATP-biotin, a phosphoryl biotin tag is attached to kinase substrates. Phosphatases remove phosphoryl groups from phosphorylated proteins, although they are inefficient at removing phosphoryl biotin groups after kinase-catalyzed biotinylation. B) Active phosphatases in the cell lysate dephosphorylate substrates, which can subsequently undergo kinase-catalyzed biotinylation with ATP-biotin (top row). Substrates in lysates where phosphatases were inactivated or knockdown remain phosphorylated and unaffected by ATP-biotin (bottom row). After avidin enrichment of biotinylated proteins in both samples, quantitative comparison of proteins after LC-MS/MS analysis and label-free quantitation will identify potential substrates of the inactivated phosphatase, without observing non-substrates (blue).

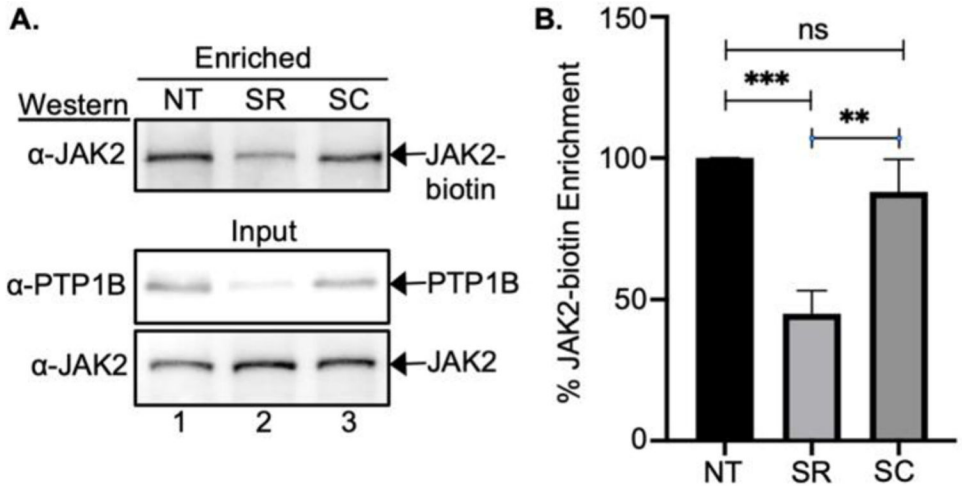

Figure 2.

Differential enrichment of known PTP1B substrate JAK2 by K-BIPS. A) HEK293 cells were untreated (NT) or transfected with PTP1B siRNA (SR) or scrambled control siRNA (SC). After lysis, the lysates were incubated with ATP-biotin. Biotinylated proteins were enriched with streptavidin resin and separated by 10% SDS-PAGE. Input lysates before enrichment were separated as load and expression controls. JAK2 and PTP1B levels were visualized by immunoblotting with specific antibodies. B) Enriched JAK2 levels were quantified using ImageJ 1.52a software and normalized as a percentage to NT samples. One-way ANOVA statistical analysis was performed with GraphPad Prism 8.2.1(***$p<0.001$, **$p<0.002$, ns-not significant). Full gel images, independent trials, and quantification are documented in Figure S1.

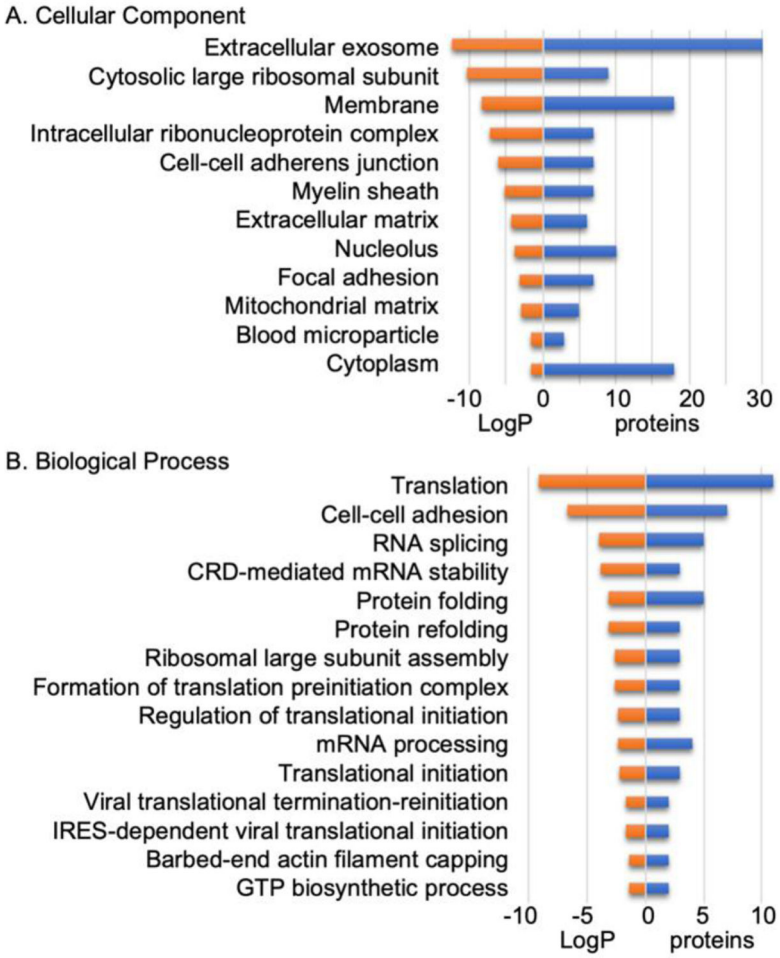

Figure 3. Functional classification of K-BIPS hits.

The 53 K-BIPS hit proteins were classified according to cellular component (A) and biological processes (B) using DAVID 6.8, with the log p value and number of proteins shown. Only the cellular component categories showing both a significant p value (>0.05) and at least 5 proteins are shown here, with the full data shown in Figure S2A.

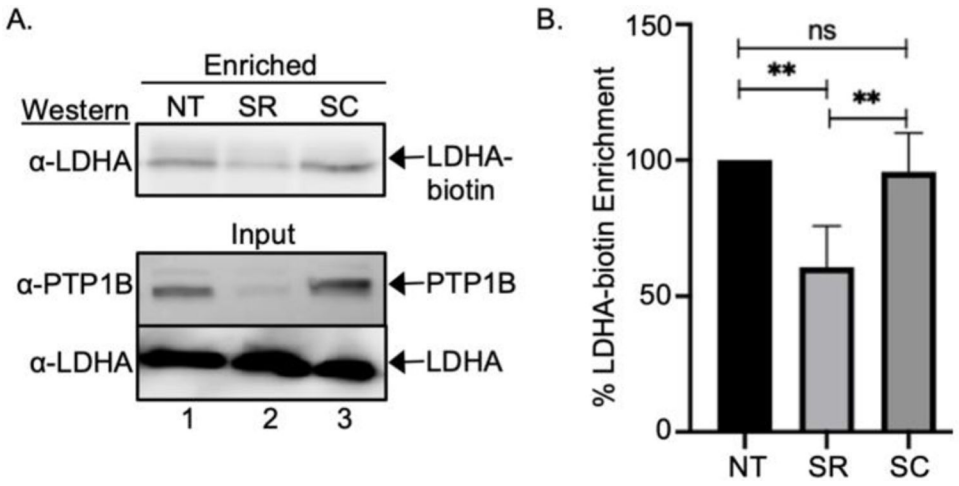

Figure 4. Secondary confirmation of LDHA as K-BIPS hit.

A) HEK293 cells were untreated (NT) or transfected with PTP1B siRNA (SR) or scrambled control siRNA (SC). After lysis, proteins in lysates were incubated with ATP-biotin. Biotinylated proteins were enriched with streptavidin resin and separated by 10% SDS-PAGE. Input lysates before enrichment were separated as load and expression control. LDHA and PTP1B levels were visualized by immunoblotting with specific antibodies. B) Enriched LDHA levels were quantified using ImageJ 1.52a software and normalized as a percentage to NT samples. One-way ANOVA statistical analysis was performed with GraphPad Prism 8.2.1(**$p<0.01$, ns- no significant). Full gel images, independent trials, and quantification are documented in Figure S4.

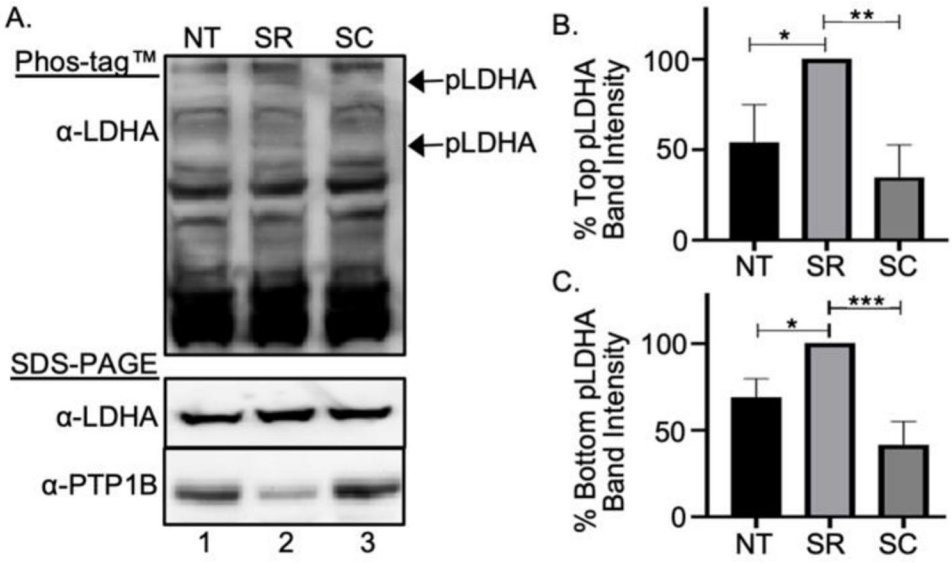

Figure 5. Validation of LDHA dephosphorylation by PTP1B.

A) HEK293 cells were untreated (NT) or transfected with PTP1B siRNA (SR) or scrambled control siRNA (SC). After cell lysis, proteins were separated by Phos-tag™ SDS-PAGE or traditional SDS-PAGE. Separated proteins were transferred onto PVDF membrane and LDHA and PTP1B were visualized with appropriate antibodies. Phosphorylated LDHA (pLDHA) bands present only with PTP1B knockdown (lane 2) are indicated with arrows. B-C) Intensities of the top pLDHA band (B) and bottom pLDHA band (C) in part A from three independent trials were quantified using ImageJ 1.52a software and normalized as a percentage to SR samples (set at 100%). One-way ANOVA statistical analysis was performed with GraphPad Prism 8.2.1 (*$p<0.03$, **$p<0.006$, ***$p<0.001$). Independent trials and quantification are documented in Figure S6.

Table 1.

High confidence PTP1B K-BIPS hit list*

Protein Name	Gene Name
L-lactate dehydrogenase A chain	LDHA
DnaJ homolog subfamily A member 2	DNAJA2
Pyruvate kinase PKM	PKM
Stress-70 protein, mitochondrial	HSPA9
40S ribosomal protein S25	RPS25
60S ribosomal protein L19	RPL19
Poly(rC)-binding protein 1	PCBP1
Protein LSM14 homolog A	LSM14A

*enriched by at least 1.5-fold in non-transfected and scrambled negative control siRNA transfected samples compared to PTP1B siRNA transfected samples in at least 3 out of 5 trials.