



Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: <http://www.elsevier.com/locate/ejmech>

Original article

Redox-based probes as tools to monitor oxidized protein tyrosine phosphatases in living cells

Francisco J. Garcia, Kate S. Carroll*

Department of Chemistry, The Scripps Research Institute, 130 Scripps Way, Jupiter, FL 33458, USA

ARTICLE INFO

Article history:

Received 20 February 2014

Received in revised form

15 June 2014

Accepted 18 June 2014

Available online xxx

Keywords:

Redox-based probes

Redox biology

Reactive oxygen species

Phosphatases

Bioconjugation

Nucleophile-based therapeutics

ABSTRACT

Reversible oxidation of protein tyrosine phosphatases (PTPs) has emerged as an important regulatory mechanism whereby reactive oxygen species (ROS) inactivates the PTP and promotes phosphorylation and induction of the signaling cascade. The lack of sensitive and robust methods to directly detect oxidized PTPs has made it difficult to understand the effects that PTP oxidative inactivation play in redox signaling. We report the use of redox-based probes to directly detect oxidized PTPs in a cellular context, which highlights the importance of direct approaches to assist in the study of physiological and pathophysiological PTP activity in redox regulation. We also demonstrate, as a proof-of-concept, that these redox-based probes serve as prototypes for the design and development of a new class of inhibitors for phosphatases. We envision a nucleophile reacting with the oxidized inactive catalytic cysteine to generate an irreversible thioether adduct which prevents the phosphatase from being reactivated and ultimately fortifies the signaling cascade. Our results reveal the potential of translation of our redox-based probes, which are used to understand redox cell circuitry and disease biology, to small-molecule nucleophile-based inhibitors, which may treat diseases associated with redox stress. This may have implications in the treatment of type 2 diabetes and cancer.

© 2014 Elsevier Masson SAS. All rights reserved.

1. Introduction

Reactive oxygen species (ROS) such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($\cdot OH$) are formed by the partial reduction of oxygen. Cellular ROS can be generated endogenously by univalent reduction of molecular oxygen to generate $O_2^{\cdot-}$, which can dismutate spontaneously or thorough the assistance of superoxide dismutase to give H_2O_2 . Superoxide formation typically results from the premature leakage of electrons from the electron transport chain, byproducts of xanthine and aldehyde oxidases, or induced through growth factor stimulation [1–3]. Substantial evidence indicates ROS functions as a signaling molecule during signal transduction in a diverse range of biological processes to mediate distinct physiological responses such as proliferation, differentiation, and apoptosis [4,5].

Protein tyrosine phosphatases (PTPs) are crucial regulators of signal transduction. This class of enzymes function as antagonists towards protein tyrosine kinases (PTKs) to control reversible tyrosine phosphorylation, which governs fundamental physiological

functions such as cellular growth, proliferation, differentiation, survival, metabolism, and motility [6]. PTPs are tightly regulated by several mechanisms ranging from differential expression, subcellular localization, limited proteolysis, post-translational modifications, ligand binding, and dimerization [7]. The PTP catalytic domain contains a unique microenvironment in which the catalytic cysteine exhibits a depressed pK_a and therefore exists as a thiolate anion at physiological pH. This facilitates the phosphatases to carry out their enzymatic function but also renders them sensitive to oxidation [8]. Upon exposure of the phosphatase to H_2O_2 , the catalytic cysteine residue is converted to a sulfenic acid (RSOH), which results in PTP inactivation. Reactions with cellular thiols can restore the oxidized PTPs to the catalytically active form [9]. Therefore, reversible PTP oxidation has emerged as an important cellular regulatory mechanism whereby ROS, such as H_2O_2 generated from physiological responses, promotes phosphorylation and induction of the signal cascade by oxidizing and inactivating the PTP at its catalytic site.

The lack of sensitive and robust methods for detecting oxidized phosphatases, as opposed to abundant redox-sensitive enzymes, has made understanding redox regulation of phosphatases in the cellular context challenging. The majority of approaches available to detect PTP oxidation are indirect and not apt for cell based

* Corresponding author.

E-mail address: KCarroll@scripps.edu (K.S. Carroll).

studies [10]. In-gel activity assays use radioactively labeled substrate to monitor reversible PTP oxidation [11]. The in-gel phosphatase assay is biased towards non-membrane bound phosphatases and is not quantifiable. Since cysteine thiols are known to react with electrophiles, a modified cysteinyl-labeling assay was developed that relies on biotinylated thiol-alkylating agents to expose reversibly oxidized phosphatases [12]. The modified cysteinyl-labeling assay is not a targeted approach and suffers from high background of non-PTP proteins (Fig. 1a). An alternative strategy to survey oxidized PTPs is to use an antibody generated against the conserved signature motif of phosphatases harboring the terminally oxidized (RSO₃H) active-site cysteine [13,14]. This immunochemical approach does not differentiate between phosphatases regulated by reversible oxidation (RSOH) from those that are inherently hyperoxidized (RSO₂H, RSO₃H).

Direct approaches to monitor protein oxidation currently rely on nucleophiles that exploit the selective reaction between RSOH (Fig. 1b). The most common example is 5,5-dimethyl-1,3-cyclohexanedione (dimedone) which generates a stable thioether adduct upon reacting with the RSOH [15]. The chemoselective reaction of several dimedone-based probes has been applied to detect oxidized proteins *in vitro* and *in situ* [16]. Based on the success of this critical reaction, several probes to exclusively monitor PTP oxidation have been developed. These PTP redox-based probes (RBPs) are composed of: 1) a dimedone-based warhead that forms a covalent adduct with the oxidized active-site cysteine; 2) a module that directs binding to the PTP catalytic site; and 3) a reporter tag used for the identification, purification, or direct visualization of the labeled protein [17]. Additionally, single-chain variable fragment (ScFv) antibodies directly detect unique

conformational changes associated with oxidized PTP1B [18]. Though the conformation sensing antibodies provides a direct approach to monitor PTP oxidation, they are specific for a single protein and may not be used to monitor oxidation of the entire classical PTP family.

The low cellular abundance of signaling proteins has made the detection of oxidized PTPs difficult. Herein, we report the use of the RBPs to detect oxidized phosphatases in cells and to investigate PTP regulation in redox signaling (Fig. 1c). Literature has reported that the bioorthogonal reaction is enhanced when the chemical reporter harbors an alkyne handle and is used in combination with an azide bearing detection tag [19]. In an effort to circumvent detection limitations of the low abundant phosphatases, we synthesized alkyne analogues of our previously reported RBPs to give the parent compound (DYn-0), biphenyl (BiPhYn-1), and naphthyl (NaphYn-1) probes (Fig. 1d). We've also adopted a more robust ligand for the Huisgen [3 + 2] cycloaddition reaction (click chemistry). We report the use of a more reactive tris(triazolylmethyl)amine-based ligand – BTTP as our ligand of choice for the bioorthogonal chemical reaction, as opposed to TBTA, to append reporter tags to the low abundant probe-modified proteins [20].

2. Results

The catalytic cysteine thiolate of PTP1B reacts with H₂O₂ to yield the RSOH, which rapidly condenses with the main-chain nitrogen of an adjacent serine residue to give the cyclic sulfenamide [21,22]. To determine whether dimedone could trap the PTP1B-SOH intermediate, we performed *in vitro* experiments with dimedone and the resulting protein S-dimedone adduct was detected using an

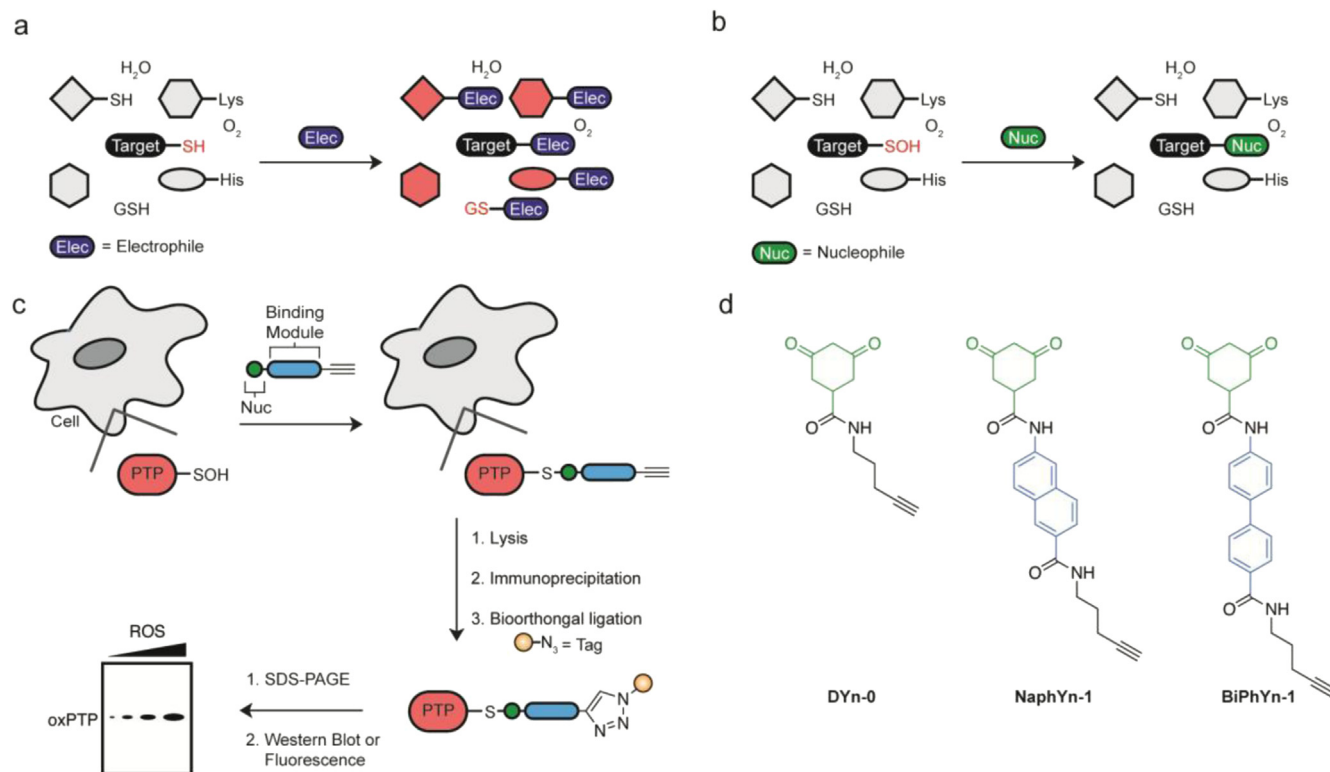


Fig. 1. (a) Electrophiles are promiscuous and may react with varying nucleophilic species found within a proteome. (b) Nucleophiles react selectively with the RSOH to generate a stable thioether bond. Figure adapted from Ref. [38]. (c) A direct approach to monitor the extent of PTP oxidation within a cell. Cells are treated with redox-based probes (RBPs) to selectively label the endogenously oxidized phosphatases. The cells are lysed and proteins of interest are immunoprecipitated. Bioorthogonal ligation is performed on the immunocomplex to append a reporter tag. Samples are resolved by SDS-PAGE and visualized by avidin blotting or fluorescence. (d) Structures of alkyne based RBPs used to directly monitor PTP oxidation.

immunochemical approach previously reported in our laboratory [23]. We treated recombinant PTP1B (aa 1–321) with increasing concentrations of dimedone in the presence of H_2O_2 . A stable adduct between dimedone and oxidized PTP1B was generated and detected by the antibody (Supplementary Fig. 1). In order to evaluate the capacity of RBPs to react towards the oxidized phosphatase, we treated PTP1B with increasing concentrations of the RBPs in the presence of H_2O_2 followed by the conjugation of a biotin tag via bioorthogonal ligation and visualization by avidin blotting. The data demonstrates that RBPs have increased sensitivity towards the oxidized phosphatase as opposed to the parent compound (Fig. 2a). Carbon acids, such as dimedone, can be oxidized by H_2O_2 to generate a trione species, which could act as an electrophile and form an adduct with the thiol form of PTP1B. It is important to note that the concentrations of H_2O_2 required to effect such a chemical reaction are significantly higher (mM) than those used in these experiments (μM) (unpublished data). Nonetheless, to further rule out this possibility we generated the sulfenic acid form of PTP1B, quenched this reaction with catalase, and then exposed the oxidized enzyme to the RBPs. Using this alternate workflow, no differences in PTP1B labeling by RBPs were observed, as expected

(Supplementary Fig. 2). A time dependent study with BiPhYn-1 and DYn-0 showed that BiPhYn-1 detects the oxidized PTP within 5 min as opposed to 30 min with the parent compound (Fig. 2b).

To determine whether the RBPs recognize and bind non-covalently to the phosphatase, we measured their potential to inhibit phosphatase activity using a fluorogenic substrate, 4-methylumbelliferyl phosphate (4-MUP). Aryl compounds can be prone to an aggregation-based mechanism of inhibition [24]. To test whether the RBPs inhibit PTP1B through such a mechanism, we performed activity assays in the presence of increasing concentrations of Triton-X 100 detergent. Notably, no differences were observed in the inhibition of PTP1B by BiPhYn-1 as the concentration of detergent was increased (Supplementary Fig. 3), indicating that this probe does not aggregate and subsequently inhibit PTP1B. BiPhYn-1 showed modest inhibition towards PTP1B ($IC_{50} = 0.49$ mM) as opposed to the parent compound DYn-0 ($IC_{50} = 4.7$ mM) and NaphYn-1 ($IC_{50} = 2.3$ mM) (Supplementary Fig. 4a). Since NaphYn-1 exhibited poor inhibition, we focused all subsequent efforts on further characterization of BiPhYn-1. First order reaction rates of the RBPs towards the RSOH were determined using a RSOH model system and revealed that the binding modules did not enhance the nucleophilicity of the dimedone warhead (Supplementary Fig. 4b) [25].

We used glutathione peroxidase 3 (Gpx3) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to evaluate the reactivity of the RBPs towards detection of RSOH in non-PTP proteins. Gpx3 and GAPDH were treated with BiPhYn-1 and DYn-0 in the presence of H_2O_2 followed by conjugation of the biotin tag and avidin blotting. Both compounds demonstrated equal labeling of non-PTPs suggesting that BiPhYn-1 maintains the ability to detect RSOH modifications in non-PTPs (Supplementary Fig. 5). The PTP super family is comprised of classical PTPs and dual specificity phosphatases (DUSPs), which dephosphorylate phospho-tyrosine and phosphoserine/threonine/tyrosine residues respectively. Since all PTPs contain a highly conserved catalytic site, we envisioned BiPhYn-1 would act as a global probe for all PTPs. We therefore tested the capacity of BiPhYn-1 in detecting a panel of oxidized PTPs in comparison to DYn-0. BiPhYn-1 was able to detect oxidized YopH, PTP1B, CDC25B, SHP-1, and VHR more robustly than DYn-0 (Supplementary Fig. 6). Taken together, these results designate the BiPhYn-1 RBP as a general tool for detecting oxidized PTPs.

Catalytically inactive PTP1B (C215S) was generated to ensure RBPs were targeting the catalytic cysteine. A loss of signal was observed when labeling C215S with BiPhYn-1 in the presence of H_2O_2 , as opposed to wild type (Supplementary Fig. 7a). Competition studies with phenyl vinyl sulfone (PVSF) [26], a mechanism based probe for PTPs, exhibited loss of detection of oxidized PTP1B with BiPhYn-1 in the presence of increasing concentrations of PVSF (Supplementary Fig. 7b) within cells. Finally, LC/MS/MS was used to map the site of modification by BiPhYn-1 (Supplementary Fig. 7c).

To ascertain whether BiPhYn-1 would detect oxidized phosphatases in a cellular context, COS1 cells were transfected with pJ3H-PTP1B to overexpress HA-tagged PTP1B (Supplementary Fig. 8). BiPhYn-1 was titrated into transfected cells for 1 h at 37 °C, 5% CO_2 . The cells were washed then lysed followed by immunoprecipitation of HA-tagged PTP1B using anti-HA agarose resin overnight. The probe-labeled phosphatase was visualized by appending a biotin tag via bioorthogonal ligation of the immuno-complex followed by avidin blot. BiPhYn-1 detects basal levels of oxidized PTP1B in a dose dependent manner (Fig. 2c).

Several works have reported that PTP1B is transiently inactivated by H_2O_2 produced by various cell stimuli and that phosphatase inactivation is important for induction of optimal tyrosine phosphorylation response [27–29]. One signaling pathway for which H_2O_2 production occurs is in response to the hormone insulin [30]. To verify that BiPhYn-1 can detect increased oxidized

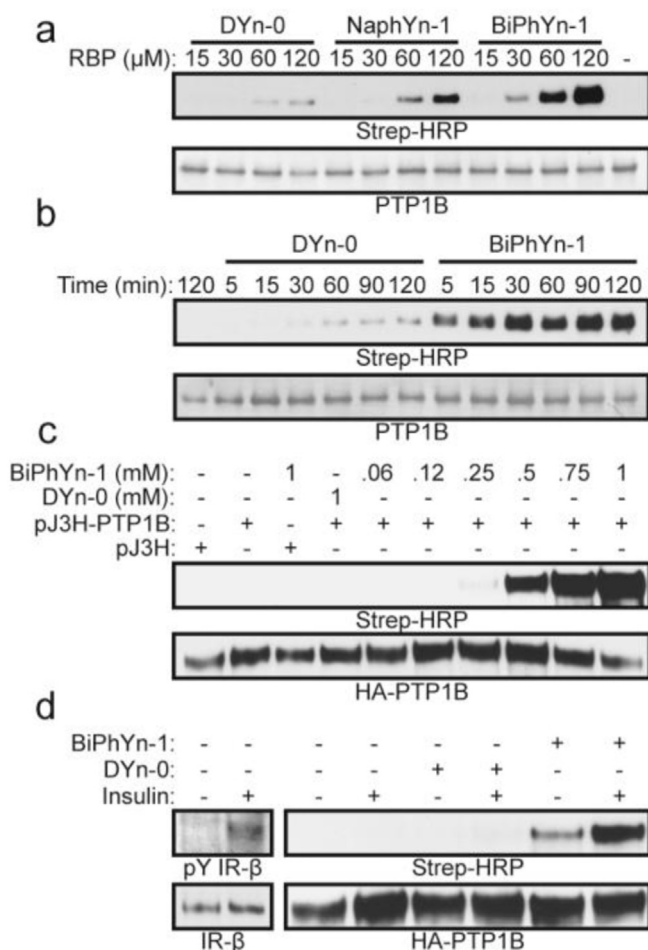


Fig. 2. RBPs exhibit enhanced selectivity towards oxidized PTP1B *in vitro* and *in situ*. Labeling of recombinant PTP1B (20 μM) with 10 equivalents H_2O_2 (200 μM) in the presence of 50 μM or increasing concentrations of RBP in (a) dose- and (b) time-dependent fashion displays RBPs selectivity towards oxidized PTP1B. (c) COS1 cells that had been transfected with pJ3H-PTP1B were treated with BiPhYn-1 and exhibit a dose-dependent detection of basal oxidized PTP1B. (d) 250 μM BiPhYn-1, but not DYn-0, detects increased levels of oxidized PTP1B initiated by insulin (100 nM) induced H_2O_2 production.

PTP1B upon ligand induced ROS formation, pJ3H-PTP1B transfected COS1 cells were treated with 100 nM insulin followed by the addition of BiPhYn-1. BiPhYn-1 was able to detect an increase in the levels of oxidized PTP1B upon insulin stimulation (Fig. 2d). This data suggests that the RBPs are robust tools to directly monitor levels of oxidized PTPs within a cell.

PTP1B has been shown to function as a negative regulator in the insulin signaling pathway. PTP1B influences the duration and amplitude of the insulin signaling response by catalyzing the removal of phosphoryl groups on tyrosine residues of the insulin receptor ($\text{IR}\beta$, pYpY 1162/1163) and insulin receptor substrates protein (IRS-1) [31,32]. Since BiPhYn-1 was able to detect increased levels of oxidized PTP1B in cells, the next step was to determine if the RBPs could modulate the extent of tyrosine phosphorylation. We hypothesized that the BiPhYn-1 RBP would react with the oxidized inactivated PTP in cells to enable prolonged signaling and downstream physiological effects for further investigations (Fig. 3a). To this end, CHO cells that express the human insulin receptor (CHO/hIRc) were incubated with a range of concentrations of BiPhYn-1 followed by treatment with or without insulin. BiPhYn-1 was able to increase the phosphorylation levels of $\text{IR}\beta$. The maximal effect of BiPhYn-1 on the levels of phosphorylation in CHO/hIRc cells peaked at 250 μM (Fig. 3b). At higher BiPhYn-1 concentrations the phosphorylation levels of the insulin receptor decreased potentially due to nonspecific effects. Due to the generic

scaffold associated with the RBPs, it is speculated that the probes may behave promiscuously and give rise to off-target effects via the inhibition of opposing but structurally related phosphatases in the PTP super family, ultimately leading to the reduction in the overall levels of phosphorylation at high RBP concentrations [33].

Insulin increases glucose uptake in cells by stimulating the translocation of the glucose transporter (GLUT4) from intracellular sites to the cell surface [34]. We next sought to determine whether the increased levels of phosphorylation of $\text{IR}\beta$ translated into an increase in glucose uptake. A fluorescent derivative of glucose, 2-NBD Glucose (2-NBDG), was used to ascertain whether the RBPs could trap the oxidized PTP1B to enhance glucose uptake in cells. COS1 cells were treated with increasing concentrations of the RBPs followed by simultaneous treatment with insulin and 2-NBDG. A dose-dependent increase in 2-NBDG uptake was observed in cells treated with BiPhYn-1 as opposed to cells treated with DYn-0 (Fig. 3b,c). Taken together, this data conveys that the RBPs can be used as a direct approach to facilitate cellular investigation of PTP redox regulation in cell signaling.

3. Conclusion

In summary, we report the use of redox-based probes to directly detect and monitor oxidized phosphatases in a cellular setting. These RBPs can facilitate the detection of ROS-inactivated PTPs associated

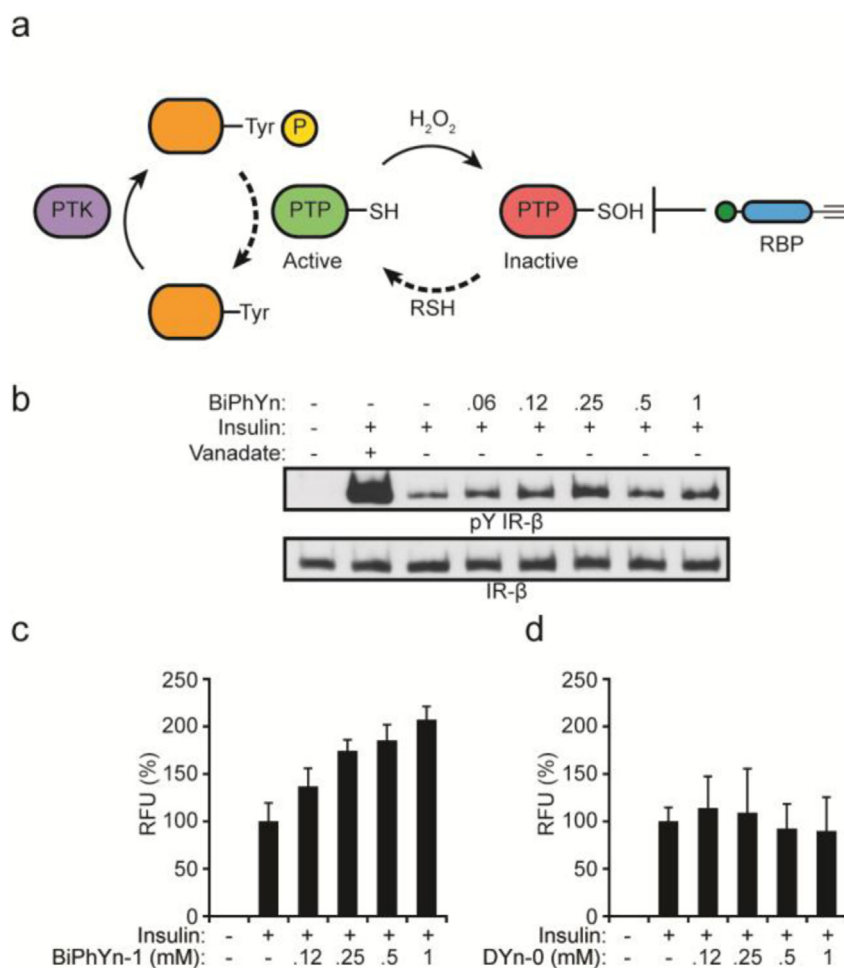


Fig. 3. (a) Reversible oxidative inactivation of protein tyrosine phosphatases (PTPs) is triggered by hydrogen peroxide (H_2O_2) reacting with the catalytic cysteine to generate a sulfenic acid (RSOH). Phosphatase activity is restored upon reaction with thiols. We hypothesize that trapping oxidized PTP1B with BiPhYn-1 can enhance tyrosyl phosphorylation of the insulin receptor β . (b) CHO/hIRc cells stimulated with 10 nM insulin in the presence of increasing concentrations of the RBP. (c, d) Enhanced glucose uptake observed in the presence of increasing concentrations of BiPhYn-1, but not DYn-0, when stimulating cells with 250 nM insulin in the presence of 50 μM 2-NBD glucose (2-NBDG).

with specific cell signaling pathways and help to outline the physiological roles of redox regulation. Our experiments supports and communicates the importance and ease of incorporating chemo-selective small molecule approaches to assist in the study of aberrant phosphatase activity brought about by increased ROS that is associated with pathophysiological states such as cancer. Additionally, PTP1B has been implicated as an important target for the treatment of type 2 diabetes and obesity. The development of cell permeable and bioavailable small molecule PTP inhibitors has been constrained by the highly conserved and highly polar phospho-tyrosine binding pocket [35]. As a result, many reported PTP inhibitors conform to highly charged anionic phosphatase mimetics that cannot cross the cell membrane [36]. Previously, aryl diketoacids were identified as pTyr surrogates that targeted and stabilized the “open” inactive conformation of PTP1B, thereby inhibiting the enzyme [37]. Recently it was demonstrated that stabilization of oxidized PTP1B, using single-chain variable fragment antibodies that target the unique conformation of oxidized PTP1B, enhanced phosphorylation and sustained insulin signaling [18]. We illustrate that our redox-based probes serve as a proof-of-concept for the development of a new class of small molecule inhibitors that target the oxidized inactive phosphatase via nucleophilic trapping of the oxidized catalytic cysteine. Our results revealed an increase in levels of phosphorylation of the insulin receptor as well as an increase in glucose uptake in the presence of the RPBs. This demonstrates that trapping of the oxidized phosphatase by way of small molecules bearing a nucleophilic site may be a practical means to inhibit further catalytic activity. The design of more selective and reactive “nucleophile-based inhibitors” may be used as a new approach for the treatment of diabetes and other disease states, which have been associated with aberrant phosphatase activity and amplified ROS production.

4. Materials and methods

More comprehensive experimental procedures, supplemental figures, and compound characterization can be found in the [Supporting information](#).

4.1. Recombinant protein labeling with RPBs

PTP1B and C215S PTP1B were buffer exchanged using a Nap-5 column (GE Healthcare Illustra) pre-equilibrated with 50 mM HEPES, 100 mM NaCl, 1 mM EDTA, pH 7.0. Simultaneous labeling of PTP1B or C215S PTP1B was performed by taking 20 μ M phosphatase and treating it with 10 equivalents (200 μ M) of hydrogen peroxide and indicated concentrations of sulfenic acid probes (DYN-0, NaPhYn-1, BiPhYn-1, or dimedone) for 1 h at room temperature while rocking. Excess RBP and EDTA were removed by passing the samples through a P30 column (Bio-Rad) pre-equilibrated with 50 mM HEPES, 100 mM NaCl, 0.1% SDS, pH 7.4. Time-dependent analyses were performed by taking an aliquot of the reaction mixture at various time points and passing through a pre-equilibrated P30 column to quench the reaction. RBP modified proteins were then treated with 100 μ M AzBiotin-PEG₄ (Invitrogen), a pre-mixed BTTP:CuSO₄ solution (200 μ M BTTP:100 μ M CuSO₄), and 2.5 mM sodium ascorbate for 1 h at room temperature while rocking. The click chemistry reaction was quenched with the addition of 1 mM EDTA. Protein samples were resolved by SDS-PAGE using Mini-Protean TGX 4–15% Tris-Glycine gels (BioRad) and transferred to a polyvinylidene difluoride (PVDF) membrane (BioRad). The PVDF membrane was blocked with 3–5% BSA in TBST for 1 h at room temperature, washed with TBST (3 \times), and immunoblotting was performed with HRP-streptavidin (GE Healthcare, 1:80,000). The PVDF membrane was washed with TBST (3 \times) and developed with ECL Plus chemiluminescence (Pierce) and imaged

by film. Equal loading of recombinant PTP1B was assessed by treating the membrane with a solution of 1:1 MeOH:R-250 Coomassie blue for 10 min, then allowing membrane to dry.

4.2. Cell culture

COS1 cells (ATCC) were maintained in a humidified atmosphere of 5% CO₂ at 37 °C and cultured in DMEM media (Invitrogen) supplemented with 10% FBS (Invitrogen), 1% penicillin–streptomycin (Invitrogen), 1% GlutaMax (Invitrogen), and 1% non-essential amino acids (Invitrogen). CHO cells overexpressing the human insulin receptor (CHO/hIRc) were a kind gift from Dr. Michael L. Tremblay. CHO/hIRc cells were maintained in a humidified atmosphere of 5% CO₂ at 37 °C and cultured in Ham's F-12 media (Corning) supplemented with 10% FBS, 1% penicillin–streptomycin, and 1% non-essential amino acids. For insulin treatment, cells were serum starved for 16 h prior to experimentation.

4.3. Labeling of oxidized PTP1B in COS1 cells

COS1 cells were plated on 100 mm dishes and transfected at 90% confluency according to manufactures instructions with pJ3H-PTP1B (Addgene plasmid 8601) for 48 h. COS1 cells were then washed with PBS, lifted with 0.25% trypsin–EDTA, harvested by centrifugation at 1500 g for 2 min, and then resuspended in serum-free DMEM at a density of 3–4 \times 10⁶ cells/mL. The resuspended cells were treated with DMSO or the indicated concentration of the redox-based probe for 1 h at 37 °C in a 5% CO₂ humidified atmosphere with periodic gentle agitation. Following treatment, cells were collected and washed with PBS (3 \times). For insulin treatment, cells were cultured in a similar fashion as above with the exception of being serum starved for 16 h prior to the experiment. After serum deprivation, cells were treated with 100 nM insulin (Calbiochem) for 2 min followed by the addition of the 250 μ M RPBs for 1 h at 37 °C in a 5% CO₂ humidified atmosphere with periodic gentle agitation.

4.4. Generation of cell lysate

COS1 and CHO/hIRc cells were harvested in a NP-40 lysis buffer [50 mM Tris–HCl pH 8.0, 137 mM NaCl, 10% glycerol, 1% NP-40, 50 mM NaF, 10 mM β -glycerolphosphate, 1 mM sodium vanadate, 1 \times EDTA-free protease cocktail inhibitors (Roche), and 200 U/mL catalase (Sigma)]. After 20 min incubation on ice with frequent mixing, cell debris was removed by centrifugation at 14,000 rpm at 4 °C for 15 min. Protein concentrations were determined by BCA assay (Pierce).

4.5. Immunoprecipitation

HA-PTP1B was immunoprecipitated from 500 μ g of cell lysate with 20 μ L anti-HA agarose (Pierce) as specified by manufacturer. The following day, the resin was pelleted with a 10 s burst at 12,000 \times g and the supernatant was saved. The resin was washed three times with TBST and twice with 50 mM HEPES, 100 mM NaCl, pH 7.4. The resin was then treated with 20 μ L of a pre-mixed click chemistry mix (100 μ M AzBiotin-PEG₄, 500 μ M BTTP, 250 μ M CuSO₄, and 2.5 mM sodium ascorbate in 50 mM HEPES, 100 mM NaCl, pH 7.4). The click reaction was allowed to mix for 1 h then terminated by the addition of 10 μ L Laemmli sample buffer without β -Me and boiling for 10 min.

4.6. Detection of phosphorylated IR β in CHO/hIRc cells

CHO/hIRc cells were plated onto 6-well plates and allowed to become adherent in complete media overnight at 37 °C in a humidified atmosphere of 5% CO₂. The cells were serum starved for

16 h prior to the experiment. CHO/hIRc cells were pre-treated with DMSO, 1 mM vanadate, and DYn-0 or BiPhYn-1 at various concentrations for 1 h at 37 °C, 5% CO₂. Media containing DMSO, vanadate, or RBPs was removed and cells were washed with PBS (3×) then stimulated with 10 nM insulin for 5 min. After stimulation, the media was removed and the cells were washed with ice cold PBS (3×). The cells were then lysed with the aid of a rubber policeman. 25 µg of clarified cell lysate was resolved by SDS-PAGE, transferred to PVDF membrane, and probed with appropriate antibodies.

4.7. 2-NBD glucose uptake assay

COS1 cells were plated in triplicate at a density of 3×10^4 cells/well in black clear bottom 96-well microplates (Corning) and allowed to become adherent in complete media overnight at 37 °C in a humidified atmosphere of 5% CO₂. The following day, the cells were gently washed with PBS (3×) and serum starved in glucose free DMEM (Corning) for 4 h at 37 °C, 5% CO₂. The cells were then treated with the RBPs for 1 h at 37 °C. Media containing RBPs was aspirated and glucose uptake was assessed with a Glucose Uptake Cell-Based Assay Kit (Cayman Chemicals) in which we treated cells with 50 µM 2-NBDG in the presence of 250 nM insulin (Calbiochem) for 15 min at 37 °C in a humidified atmosphere of 5% CO₂. Following stimulation, the cells were gently washed with PBS (2×) before the addition of 100 µL of the cell-based assay buffer. 2-NBDG uptake was measured using an EnVision plate reader (Perkin Elmer) with an excitation wavelength of 485 nm and emission wavelength of 535 nm.

Acknowledgments

This work was supported by the Camille Henry Dreyfus Teacher Scholar Award (K.S.C.) and the American Heart Association Scientist Development Award (0835419N, K.S.C.). We thank K.S. Gates (U. Missouri) for the PTP1B constructs, M.L. Tremblay (McGill University) for the CHO/hIRc cells, J. Yang (Vanderbilt) and D. Liebler (Vanderbilt) for mass spectrometry support. Finally, we would like to acknowledge T.H. Truong (Scripps) and M. LoConte (Scripps) for reviewing the manuscript.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.ejmech.2014.06.040>.

References

- [1] M. Giorgio, M. Trinei, E. Migliaccio, P.G. Pelicci, Hydrogen peroxide: a metabolic by-product or a common mediator of ageing signals? *Nat. Rev. Mol. Cell Biol.* 8 (2007) 722–728.
- [2] H. de Groot, A. Littauer, Hypoxia, reactive oxygen, and cell injury, *Free Radic. Biol. Med.* 6 (1989) 541–551.
- [3] M. Sundaresan, Z.X. Yu, V.J. Ferrans, K. Irani, T. Finkel, Requirement for generation of H₂O₂ for platelet-derived growth factor signal transduction, *Science* 270 (1995) 296–299.
- [4] E.A. Veal, A.M. Day, B.A. Morgan, Hydrogen peroxide sensing and signaling, *Mol. Cell.* 26 (2007) 1–14.
- [5] C.E. Paulsen, K.S. Carroll, Orchestrating redox signaling networks through regulatory cysteine switches, *ACS Chem. Biol.* 5 (2010) 47–62.
- [6] J.N. Andersen, P.G. Jansen, S.M. Echwald, O.H. Mortensen, T. Fukada, R. Del Vecchio, N.K. Tonks, N.P. Moller, A genomic perspective on protein tyrosine phosphatases: gene structure, pseudogenes, and genetic disease linkage, *Faseb J.* 18 (2004) 8–30.
- [7] J. den Hertog, A. Ostman, F.D. Bohmer, Protein tyrosine phosphatases: regulatory mechanisms, *Febs. J.* 275 (2008) 831–847.
- [8] J.J. Tanner, Z.D. Parsons, A.H. Cummings, H. Zhou, K.S. Gates, Redox regulation of protein tyrosine phosphatases: structural and chemical aspects, *Antioxid. Redox Signal.* 15 (2011) 77–97.
- [9] A. Ostman, J. Frijhoff, A. Sandin, F.D. Bohmer, Regulation of protein tyrosine phosphatases by reversible oxidation, *J. Biochem.* 150 (2011) 345–356.
- [10] R. Karisch, B.G. Neel, Methods to monitor classical protein-tyrosine phosphatase oxidation, *Febs. J.* 280 (2013) 459–475.
- [11] T.C. Meng, T. Fukada, N.K. Tonks, Reversible oxidation and inactivation of protein tyrosine phosphatases in vivo, *Mol. Cell.* 9 (2002) 387–399.
- [12] B. Boivin, S. Zhang, J.L. Arbiser, Z.Y. Zhang, N.K. Tonks, A modified cysteinyl-labeling assay reveals reversible oxidation of protein tyrosine phosphatases in angiomylipoma cells, *Proc. Natl. Acad. Sci. U. S. A.* 105 (2008) 9959–9964.
- [13] C. Persson, T. Sjoberg, A. Groen, K. Kappert, U. Engstrom, U. Hellman, C.H. Heldin, J. den Hertog, A. Ostman, Preferential oxidation of the second phosphatase domain of receptor-like PTP-alpha revealed by an antibody against oxidized protein tyrosine phosphatases, *Proc. Natl. Acad. Sci. U. S. A.* 101 (2004) 1886–1891.
- [14] R. Karisch, M. Fernandez, P. Taylor, C. Virtanen, J.R. St-Germain, L.L. Jin, I.S. Harris, J. Mori, T.W. Mak, Y.A. Senis, A. Ostman, M.F. Moran, B.G. Neel, Global proteomic assessment of the classical protein-tyrosine phosphatome and “Redoxome”, *Cell* 146 (2011) 826–840.
- [15] L.V. Benitez, W.S. Allison, The inactivation of the acyl phosphatase activity catalyzed by the sulfenic acid form of glyceraldehyde 3-phosphate dehydrogenase by dimedone and olefins, *J. Biol. Chem.* 249 (1974) 6234–6243.
- [16] C.E. Paulsen, K.S. Carroll, Cysteine-mediated redox signaling: chemistry, biology, and tools for discovery, *Chem. Rev.* 113 (2013) 4633–4679.
- [17] S.E. Leonard, F.J. Garcia, D.S. Goodsell, K.S. Carroll, Redox-based probes for protein tyrosine phosphatases, *Angew. Chem. Int. Ed. Engl.* 50 (2011) 4423–4427.
- [18] A. Haque, J.N. Andersen, A. Salmeen, D. Barford, N.K. Tonks, Conformation-sensing antibodies stabilize the oxidized form of PTP1B and inhibit its phosphatase activity, *Cell* 147 (2011) 185–198.
- [19] G. Charron, M.M. Zhang, J.S. Yount, J. Wilson, A.S. Raghavan, E. Shamir, H.C. Hang, Robust fluorescent detection of protein fatty-acylation with chemical reporters, *J. Am. Chem. Soc.* 131 (2009) 4967–4975.
- [20] W. Wang, S. Hong, A. Tran, H. Jiang, R. Triano, Y. Liu, X. Chen, P. Wu, Sulfated ligands for the copper(I)-catalyzed azide-alkyne cycloaddition, *Chem. Asian J.* 6 (2011) 2796–2802.
- [21] A. Salmeen, J.N. Andersen, M.P. Myers, T.C. Meng, J.A. Hinks, N.K. Tonks, D. Barford, Redox regulation of protein tyrosine phosphatase 1B involves a sulphenyl-amide intermediate, *Nature* 423 (2003) 769–773.
- [22] R.L. van Montfort, M. Congreve, D. Tisi, R. Carr, H. Jhoti, Oxidation state of the active-site cysteine in protein tyrosine phosphatase 1B, *Nature* 423 (2003) 773–777.
- [23] Y.H. Seo, K.S. Carroll, Profiling protein thiol oxidation in tumor cells using sulfenic acid-specific antibodies, *Proc. Natl. Acad. Sci. U. S. A.* 106 (2009) 16163–16168.
- [24] S.L. McGovern, E. Caselli, N. Grigorieff, B.K. Shoichet, A common mechanism underlying promiscuous inhibitors from virtual and high-throughput screening, *J. Med. Chem.* 45 (2002) 1712–1722.
- [25] T.P. Shiao, D.A. Erlanson, E.M. Gordon, Selective reduction of peptide isothiazolidin-3-ones, *Org. Lett.* 8 (2006) 5697–5699.
- [26] S. Liu, B. Zhou, H. Yang, Y. He, Z.X. Jiang, S. Kumar, L. Wu, Z.Y. Zhang, Aryl vinyl sulfonates and sulfones as active site-directed and mechanism-based probes for protein tyrosine phosphatases, *J. Am. Chem. Soc.* 130 (2008) 8251–8260.
- [27] S.R. Lee, K.S. Kwon, S.R. Kim, S.G. Rhee, Reversible inactivation of protein-tyrosine phosphatase 1B in A431 cells stimulated with epidermal growth factor, *J. Biol. Chem.* 273 (1998) 15366–15372.
- [28] T.C. Meng, D.A. Buckley, S. Galic, T. Tiganis, N.K. Tonks, Regulation of insulin signaling through reversible oxidation of the protein-tyrosine phosphatases TC45 and PTP1B, *J. Biol. Chem.* 279 (2004) 37716–37725.
- [29] K. Mahadev, H. Motoshima, X. Wu, J.M. Ruddy, R.S. Arnold, G. Cheng, J.D. Lambeth, B.J. Goldstein, The NAD(P)H oxidase homolog Nox4 modulates insulin-stimulated generation of H₂O₂ and plays an integral role in insulin signal transduction, *Mol. Cell. Biol.* 24 (2004) 1844–1854.
- [30] K. Mahadev, X. Wu, A. Zilbering, L. Zhu, J.T. Lawrence, B.J. Goldstein, Hydrogen peroxide generated during cellular insulin stimulation is integral to activation of the distal insulin signaling cascade in 3T3-L1 adipocytes, *J. Biol. Chem.* 276 (2001) 48662–48669.
- [31] B.J. Goldstein, A. Bittner-Kowalczyk, M.F. White, M. Harbeck, Tyrosine dephosphorylation and deactivation of insulin receptor substrate-1 by protein-tyrosine phosphatase 1B. Possible facilitation by the formation of a ternary complex with the Grb2 adaptor protein, *J. Biol. Chem.* 275 (2000) 4283–4289.
- [32] A. Salmeen, J.N. Andersen, M.P. Myers, N.K. Tonks, D. Barford, Molecular basis for the dephosphorylation of the activation segment of the insulin receptor by protein tyrosine phosphatase 1B, *Mol. Cell.* 6 (2000) 1401–1412.
- [33] L. Xie, S.Y. Lee, J.N. Andersen, S. Waters, K. Shen, X.L. Guo, N.P. Moller, L. Olefsky, D.S. Lawrence, Z.Y. Zhang, Cellular effects of small molecule PTP1B inhibitors on insulin signaling, *Biochemistry* 42 (2003) 12792–12804.
- [34] A.R. Saltiel, C.R. Kahn, Insulin signalling and the regulation of glucose and lipid metabolism, *Nature* 414 (2001) 799–806.
- [35] A.P. Combs, Recent advances in the discovery of competitive protein tyrosine phosphatase 1B inhibitors for the treatment of diabetes, obesity, and cancer, *J. Med. Chem.* 53 (2010) 2333–2344.
- [36] A.J. Barr, Protein tyrosine phosphatases as drug targets: strategies and challenges of inhibitor development, *Future Med. Chem.* 2 (2010) 1563–1576.
- [37] S. Liu, L.F. Zeng, L. Wu, X. Yu, T. Xue, A.M. Gunawan, Y.Q. Long, Z.Y. Zhang, Targeting inactive enzyme conformation: aryl diketocid derivatives as a new class of PTP1B inhibitors, *J. Am. Chem. Soc.* 130 (2008) 17075–17084.
- [38] T.H. Truong, K.S. Carroll, Redox regulation of epidermal growth factor receptor signaling through cysteine oxidation, *Biochemistry* 51 (2012) 9954–9965.

Redox-Based Probes as Tools to Monitor Oxidized Protein Tyrosine Phosphatases in Living Cells

Francisco J. Garcia and Kate S. Carroll

Department of Chemistry, The Scripps Research Institute, 130 Scripps Way, Jupiter, FL 33458, USA

Email: KCarroll@scripps.edu

Supporting Information

Table of Contents:	Page Number
I. Supplemental Methods – Biological	S2
II. Supplemental Methods – Chemical	S6
III. Supplemental Figures	S11

Biological Methods:

Materials:

Recombinant CDC25B, SHP-1, and VHR were purchased from Enzo Life Sciences. YopH was expressed and purified as previously reported [1]. Gpx3 was expressed and purified as described previously [2]. GAPDH was commercially purchased from Sigma.

Expression and purification of recombinant PTP1B:

The plasmid for PTP1B 321 was a kind gift from Dr. Kent S. Gates's laboratory. PTP1B 321 was expressed and purified as described previously [3]. The C215S mutant of PTP1B was generated using the QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene). The oligonucleotide primers designed to introduce the C215S mutation into PTP1B were: forward C215S, 5'-GAGCACGGGCCCCGTTGTGGTGAAGTCCAGTGCAGGCATCGGCAGGTCTGG-3'; reverse C215S, 3'-CCAGACCTGCCGATGCCTGCACTGGAGTGCACCACAACGGGCCCCGTGCTC-5'.

Labeling of recombinant proteins with RBPs:

PTP1B and C215S PTP1B were buffer exchanged using a Nap-5 column (GE Healthcare Illustra) pre-equilibrated with 50 mM HEPES, 100 mM NaCl, 1 mM EDTA, pH 7.0. Simultaneous labeling of PTP1B or C215S PTP1B was performed by taking 20 μ M phosphatase and treating it with 10 equivalences (200 μ M) of hydrogen peroxide and indicated concentrations of sulfenic acid probes (DYn-0, NaPhYn-1, BiPhYn-1, or dimedone) for 1 hour at room temperature while rocking. Excess RBP and EDTA were removed by passing the samples through a P30 column (Bio-Rad) pre-equilibrated with 50 mM HEPES, 100 mM NaCl, 0.1% SDS, pH 7.4. Time-dependent analyses were performed by taking an aliquot of the reaction mixture at various time points and passing through a pre-equilibrated P30 column to quench the reaction. Both Gpx3 and GAPDH were labeled as described previously [4]. Recombinant CDC25B, SHP-1, YopH, and VHR were buffer exchanged into aforementioned buffer using a pre-equilibrated P-6 column (Bio-Rad). 0.1 μ g/ μ L of recombinant phosphatases were treated with 100 μ M H₂O₂ and 50 μ M DYn-0 or BiPhYn-1 for 1 hour at 25 °C while rocking.

Click Chemistry (Recombinant Proteins):

RBP modified proteins were functionalized with a biotin reporter tag via the bioorthogonal Huisgen [3 + 2] cycloaddition (click chemistry). The samples were buffer exchanged into 50 mM HEPES, 100 mM NaCl, 0.1% SDS, pH 7.4. The samples were then treated with 100 μ M AzBiotin-PEG₄ (Invitrogen), a pre-mixed BTTP:CuSO₄ solution (200 μ M BTTP:100 μ M CuSO₄), and 2.5 mM sodium ascorbate for one hour at room temperature while rocking [5]. The click chemistry reaction was quenched with the addition of 1 mM EDTA.

Western Blot:

Protein samples were resolved by SDS-PAGE using Mini-Protean TGX 4-15% Tris-Glycine gels (BioRad) or NuPAGE Novex 4-12% Bis-Tris Midi gels and transferred to a polyvinylidene difluoride (PVDF) membrane (BioRad). After transfer, the PVDF membrane was blocked with 3-

5% BSA in TBST for 1 hour at room temperature. The membrane was washed with TBST (3X) and immunoblotting was performed with the following primary and secondary antibodies at the indicated dilutions: HRP-streptavidin (GE Healthcare, 1:8000 – 1:80000), IR β (Santa Cruz Biotechnology, 1:200), GAPDH (Santa Cruz Biotechnology, 1:1000), α -HA (Invitrogen, 1:1000), 4G10-phospho-tyrosine (Millipore, 1:1000), α -dimerone (1:1000), goat anti-rabbit IgG-HRP (Calbiochem, 1:5000 – 1:50000), and rabbit anti-mouse IgG-HRP (Invitrogen, 1:35000 – 1:50000) [6]. The PVDF membranes were washed with TBST (3X) and developed with ECL Plus chemiluminescence (Pierce) and imaged by film. Equal loading of recombinant PTP1B was assessed by treating the membrane with a solution of 1:1 MeOH:R-250 Coomassie blue for ten minutes, then allowing membrane to dry.

PTP1B activity assay:

PTP1B was buffer exchanged into 50 mM HEPES, 100 mM NaCl, 1mM EDTA, pH 7.0 using a pre-equilibrated Nap-5 column (GE Healthcare Illustra). The protein was further diluted to 100 nM in 32 mM HEPES pH 7.2, 5 mM NaCl, 2.5 mM EDTA, 0.83% glycerol, 0.02% Triton X-100, and 0.002% Brij-35. To a round-bottom black 96 well plate (Costar) was added PTP1B (50 nM, final) followed by the redox-based probes or DMSO. The reaction mixture was allowed to incubate for 15 minutes at 25 °C before initiating the assay with the addition of 4-MUP (50 μ M, final). The formation of fluorescent product was measured with a SpectraMax M5 plate reader with an excitation wavelength of 358 nm and emission wavelength of 449 nm over 15 minutes at room temperature.

Cell culture:

COS1 cells (ATCC) were maintained in a humidified atmosphere of 5% CO₂ at 37 °C and cultured in DMEM media (Invitrogen) supplemented with 10% FBS (Invitrogen), 1% penicillin-streptomycin (Invitrogen), 1% GlutaMax (Invitrogen), and 1% non-essential amino acids (Invitrogen).

CHO cells overexpressing the human insulin receptor (CHO/hIRc) were a kind gift from Dr. Michael L. Tremblay. CHO/hIRc cells were maintained in a humidified atmosphere of 5% CO₂ at 37 °C and cultured in Ham's F-12 media (Corning) supplemented with 10% FBS, 1% penicillin-streptomycin, 1% GlutaMax, and 1% non-essential amino acids. For insulin treatment, cells were serum starved for 16 hours prior to experimentation.

2-NBD Glucose Uptake Assay:

COS1 cells were plated in triplicate at a density of 3×10^4 cells/well in black clear bottom 96-well microplates (Corning) and allowed to become adherent in complete media overnight at 37 °C in a humidified atmosphere of 5% CO₂. The following day, the cells were gently washed with PBS (3X) and serum starved in glucose free DMEM (Corning) for 4 hours at 37 °C, 5% CO₂. The cells were then treated with the RBPs for one hour at 37 °C. Media containing RBPs was aspirated and glucose uptake was assessed with a Glucose Uptake Cell-Based Assay Kit (Cayman Chemicals) in which we treated cells with 50 μ M 2-NBDG in the presence of 250 nM insulin

(Calbiochem) for 15 minutes at 37 °C in a humidified atmosphere of 5% CO₂. Following stimulation, the cells were gently washed with PBS (2X) before the addition of 100 µL of the cell-based assay buffer. 2-NBDG uptake was measured using an EnVision plate reader (Perkin Elmer) with an excitation wavelength of 485 nm and emission wavelength of 535 nm.

Transfection of PTP1B into COS1 cells:

COS1 cells were plated on 100 mm dishes and transfected at 90% confluency according to manufactures instructions with pJ3H-PTP1B [7] (Addgene plasmid 8601) for 48 hours.

Labeling of oxidized PTP1B in COS1 cells:

Detection of oxidized proteins was performed in a similar fashion as described elsewhere [8]. 48 hours post-transfection, COS1 cells were washed with PBS, lifted with 0.25% trypsin-EDTA, harvested by centrifugation at 1500g for 2 minutes, and then resuspended in serum-free DMEM at a density of 3-4 x 10⁶ cells/mL. The resuspended cells were treated with DMSO or the indicated concentration of the redox-based probe for 1 hour at 37 °C in a 5% CO₂ humidified atmosphere with periodic gentle agitation. Following treatment for the indicated time, cells were collected and washed with PBS (3X). For insulin treatment, cells were cultured in a similar fashion as above with the exception of being serum starved for 16 hours prior to the experiment. After serum deprivation, cells were treated with 100 nM insulin (Calbiochem) for 2 minutes followed by the addition of the 250 µM RBPs for 1 hour at 37 °C in a 5% CO₂ humidified atmosphere with periodic gentle agitation. PVSF competition studies were performed in a similar fashion where cells were pre-treated with indicated concentrations of PVSF for 1 hour at 37 °C in a 5% CO₂ humidified atmosphere with periodic gentle agitation. The cells were washed and treated with 250 µM BiPhYn-1 for 1 hour at 37 °C in a 5% CO₂ humidified atmosphere with periodic gentle agitation.

Generation of cell lysate:

COS1 and CHO/hIRc cells were harvested in a NP-40 lysis buffer [50 mM Tris-HCl pH 8.0, 137 mM NaCl, 10% glycerol, 1% NP-40, 50 mM NaF, 10 mM β-glycerolphosphate, 1 mM sodium vanadate, 1x EDTA-free protease cocktail inhibitors (Roche), and 200 U/mL catalase (Sigma)]. After 20 minutes incubation on ice with frequent mixing, cell debris was removed by centrifugation at 14000rpm at 4 °C for 15 minutes. Protein concentrations were determined by BCA assay (Pierce).

Immunoprecipitation:

HA-PTP1B was immunoprecipitated from 500 µg of cell lysate with anti-HA agarose (Pierce) as specified by manufacturer. Briefly, 20 µL of anti-HA resin was pelleted with a 10 second pulse at 12000xg. Liquid was discarded and the resin was washed with 20 µL of TBS. The resin was pelleted and the liquid discarded. The lysate was added to the agarose and allowed to incubate overnight at 4 °C while rocking. The following day, the resin was pelleted with a 10 second burst at 12000xg and the supernatant was saved. The resin was washed three times with TBST and

twice with 50 mM HEPES, 100 mM NaCl, pH 7.4. The resin was then treated with 20 μ L of a pre-mixed click chemistry mix (100 μ M AzBiotin-PEG₄, 500 μ M BTTP, 250 μ M CuSO₄, and 2.5 mM sodium ascorbate in 50 mM HEPES, 100 mM NaCl, pH 7.4). The click reaction was allowed to mix for 1 hour. The reaction was terminated by the addition of 10 μ L Laemmli sample buffer without β -Me and boiling for 10 minutes.

Detection of phosphorylated IR β in CHO/hIRc cells:

CHO/hIRc cells were plated onto 6-well plates and allowed to become adherent in complete media overnight at 37 °C in a humidified atmosphere of 5% CO₂. The cells were serum starved for 16 hours prior to the experiment. CHO/hIRc cells were pre-treated with DMSO, 1 mM vanadate, and DYN-0 or BiPhYn-1 at various concentrations for 1 hour at 37 °C, 5% CO₂. Media containing DMSO, vanadate, or RBPs was removed and cells were washed with PBS (3X) then stimulated with 10 nM insulin for 5 minutes. After stimulation, the media was removed and the cells were washed with ice cold PBS (3X). The cells were then lysed with the aid of a rubber policeman as previously mentioned. 25 μ g of clarified cell lysate was resolved by SDS-PAGE, transferred to PVDF membrane, and probed with appropriate antibodies.

LC/MS/MS analysis:

Recombinant PTP1B was labeled as described above. After labeling, the protein was diluted with 50 mM ABC and reduced with 25 mM DTT for 20 min at 75 °C. Following reduction, the sample was alkylated with 100 mM IAM for 20 min at rt in the dark. The protein was then precipitated with MeOH:CHCl₃ (4:4:1 – Sample:MeOH:CHCl₃). The precipitated protein was washed twice with MeOH:CHCl₃ (1:1). The protein was re-suspended in 50 mM ABC and digested with sequencing grade trypsin at a 1:50 ratio for 16 hrs at 37 °C. The peptides were desalted using HLB SPE columns (Waters) and analyzed by LC/MS/MS using a Q Exactive (Thermo). Data was searched using Protein Prospector version 5.10.11 (MS-Product) and confirmed via manual inspection.

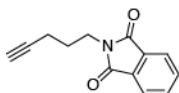
Chemical Methods:

Materials:

Reagents and solvents were purchased from Sigma or other commercial sources and were used without further purification.

Chemical Methods:

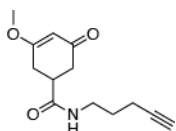
All reactions were performed under a nitrogen atmosphere in oven-dried glassware. Analytical thin layer chromatography (TLC) was carried out using Analtech Uniplat silica gel plates and visualized by UV. Flash chromatography was performed using silica gel (32-63 μM , 60 Å pore size) from Sorbent Technologies Incorporated. NMR spectra were obtained on a Bruker Avance 400 (400 MHz for ^1H ; 100 MHz for ^{13}C) in CDCl_3 , CD_3OD , or DMSO-d_6 (Cambridge Isotope Laboratories). ^1H and ^{13}C NMR chemical shifts are reported in parts per million (ppm) referenced to the residual solvent peak. Low-resolution electrospray ionization (ESI) mass spectra were obtained with an Agilent 6120 Single Quadrupole LC/MS.



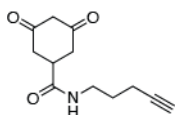
2-(pent-4-yn-1-yl)isoindoline-1,3-dione (1): To a dry RB flask under nitrogen was added 5-chloro-1-pentyne (3 g, 29.3 mmol), phthalimide (5.2 g, 35.2 mmol), K_2CO_3 (3.95 g, 29.3 mmol), and 58 mg of KI. The reagents were suspended in 30 mL of dry DMF and allowed to reflux at 70 $^\circ\text{C}$ for 16 hours. Reaction was cooled, quenched with addition of water, and extracted with ether (4 x 50 mL). The organic phases were combined, dried over MgSO_4 , filtered, concentrated, and purified by flash column chromatography in DCM. 4.9 g of yellow solid was obtained with a 78% yield. ^1H NMR (400 MHz, CDCl_3) δ 7.84 (q, J = 5.4 Hz, J = 3.0 Hz, 2H), 7.71 (q, J = 5.4 Hz, J = 3.0 Hz, 2H), 3.80 (t, J = 7.0 Hz, 2H), 2.27 (dt, J = 7.1 Hz, J = 2.6 Hz, 2H), 1.90-1.97 (m, 2H). ^{13}C NMR (100 MHz, CDCl_3) δ 168.3, 133.9, 132.1, 123.2, 82.9, 69.0, 37.1, 27.2, 16.2. ESI-MS m/z for $\text{C}_{13}\text{H}_{11}\text{NO}_2$ calculated 213.08; observed 214.1 $[\text{M}+\text{H}]^+$.



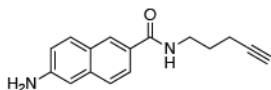
4-pentynamine (2): To a dry RB flask was added 4 g of (1) to 40 mL EtOH followed by the addition of hydrazine monohydrate until solution became homogenous (~20 mL). The reaction mixture was allowed to mix for 2 hours at 70 $^\circ\text{C}$ then allowed to cool to rt. The reaction was quenched with the addition of 30 mL water followed by acidification to pH 3.5 using 2 N HCl. The precipitate was filtered and the filtrate was concentrated. The concentrated residue was cooled to 0 $^\circ\text{C}$ followed by the addition of 10 N NaOH (~30 mL). The aqueous phase was extracted with DCM (4 x 50), combined, dried over MgSO_4 , filtered, and carefully concentrated at rt. 556 mg of product was obtained at a 36% yield. ^1H NMR (400 MHz, CDCl_3) δ 2.9133 (t, J = 7.0 Hz, 2H), 2.29 (dt, J = 7.0 Hz, J = 2.7 Hz, 2H), 1.98 (t, J = 2.7 Hz, 1H), 1.74-1.81 (m, 2H). ^{13}C NMR (100 MHz, CDCl_3) δ 83.4, 69.0, 40.3, 30.2, 22.8.



DYn-0_a (3): To a dry round bottom flask flushed with nitrogen was added 3-methoxy-5-oxocyclohex-3-enecarboxylic acid (300 mg, 1.76 mmol), EDC-HCl (505.9 mg, 2.65 mmol), and dry DMF (2 mL). The reaction was allowed to mix at rt for 30 min under nitrogen. 4-pentyneamine (293.1 mg, 3.53 mmol) was added and the reaction mixture was brought up to 75 °C and allowed to mix overnight under nitrogen. The reaction was quenched with addition of water and extracted with EtOAc. The organic layer was dried over MgSO₄, filtered, and concentrated. The crude material was not subjected to flash column chromatography and we obtained 329.2 mg of product (79% yield). ¹H NMR (400 MHz, CDCl₃) δ 5.36 (s, 1H), 3.73 (s, 3H), 3.36 (q, J = 12.7 Hz, J = 6.7 Hz, 2H), 2.90-2.95 (m, 1H), 2.79-2.86 (m, 1H), 2.54 (d, J = 11.8, 1H), 2.46-2.52 (m, 2H), 2.24 (dt, J = 9.7 Hz, J = 2.6 Hz, 2H), 1.75 (m, 2H). ¹³C NMR (100 MHz, CDCl₃) δ 197.7, 177.6, 172.4, 101.6, 83.4, 69.2, 56.0, 40.5, 39.7, 38.7, 31.4, 27.9, 16.0. ESI-MS *m/z* for C₁₃H₁₇NO₃ calculated 235.12; observed 236.2 [M+H]⁺.

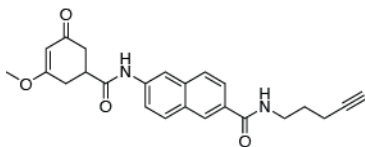


DYn-0 (4): In a round bottom flask, compound (3) (329.2 mg, 1.399 mmol) was suspended in a 1:1 Acetonitrile:Water solution (5 mL). 10% CAN (76.7 mg, .1399 mmol) was added and the reaction mixture was refluxed at 95 °C for 3 hrs. The reaction was then cooled and concentrated and directly purified by HPLC. ¹H NMR (400 MHz, DMSO-d₆) δ 7.97 (t, J = 5.4 Hz, 1H), 5.19, (s, 1H), 3.11 (q, J = 12.6 Hz, 6.8 Hz, 2H), 2.81-2.88 (m, 1H), 2.78 (t, 2.6 hz, 1H), 2.49-2.51 (m, 2H), 2.85 (dd, J = 17 Hz, J = 4.8 Hz, 2H), 2.15 (dt, J = 7.2 Hz, J = 2.6 Hz, 2H), 1.53-1.60 (m, 2H). ¹³C NMR (100 MHz, DMSO-d₆) δ 172.1, 103.2, 83.9, 71.3, 37.6, 27.9, 15.2. ESI-MS *m/z* for C₁₂H₁₅NO₃ 221.11; observed 222.1 [M+H]⁺.

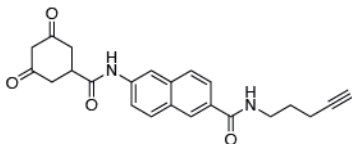


NaPhYn-1_a (5): To a dry round bottom flask flushed with nitrogen was added 6-amino-2-naphthoic acid (200 mg, 1.07 mmol), NHS (135.18 mg, 1.17 mmol), and dry DMF (2 mL). The reaction mixture was cooled in an ice-bath. EDC-HCl (224.71 mg, 1.17 mmol) was added and the reaction mixture was allowed to react at 0 °C for 30 min, then the reaction was brought up to rt and allowed to mix for an additional 3 hrs. The reaction was quenched with the addition of saturated sodium bicarbonate and extracted with EtOAc. The organic layer was dried over MgSO₄, filtered, and concentrated for the next step without further purification. To a dry round bottom flask flushed with nitrogen was added the NHS-ester (323 mg, 1.13 mmol) which was suspended in dry DMF (4 mL). 4-pentyneamine (292.5 mg, 3.52 mmol) and DIPEA (613 uL, 3.52

mmol) were subsequently added and the reaction mixture was allowed to mix at 75 °C overnight under nitrogen. The reaction was quenched with the addition of saturated sodium bicarbonate and extracted with EtOAc. The organic layer was dried over MgSO₄, filtered, and concentrated. Flash column chromatography was used for purification (1:1 EtOAc:Hexanes, 10% MeOH in EtOAc spiked with 1% TEA) to yield 188.2 mg of (5) (70% yield over 2 steps). ¹H NMR (400 MHz, CD₃OD) δ 8.17 (s, 1H), 7.72 (dd, J = 8.6 Hz, J = 1.9 Hz, 1H), 7.65 (d, J = 8.8 Hz, 1H), 7.52 (d, J = 8.8 Hz, 1H), 7.01 (dd, J = 8.7 Hz, J = 2.2 Hz, 1H), 6.94 (d, J = 2.1 Hz, 1H), 3.47-3.51 (m, 2H), 2.24-2.28 (m, 3H), 1.79-1.86 (m, 2H). ¹³C (100 MHz, CD₃OD) δ 170.7, 149.1, 138.4, 131.2, 128.4, 126.8, 125.1, 120.3, 108.4, 84.5, 70.2, 61.6, 40.3, 29.6, 21.0, 16.9. ESI-MS *m/z* for C₁₆H₁₆N₂O calculated 252.13; observed 253.2 [M+H]⁺.

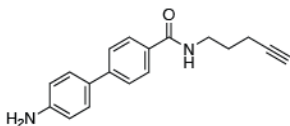


NaPhYn-1_b (6): To a dry round bottom flask flushed with nitrogen was added 3-methoxy-5-oxocyclohex-3-enecarboxylic acid (115.39 mg, .678 mmol), EDC-HCl (194.48 mg, 1.02 mmol), and DMAP (99.4 mg, .813 mmol). The flask was sealed under nitrogen. Compound (5) (188.2 mg, .746 mmol) was resuspended in dry DMF (2 mL) and added to the reaction mixture followed by the addition of TEA (95 uL, .678 mmol). The reaction mixture was brought up to 85 °C and allowed to mix overnight under nitrogen. The reaction was quenched with the addition of water and extracted with EtOAc. The organic phases were combined and dried over MgSO₄, filtered, and concentrated. Flash column chromatography was used for purification (1:1 Hexanes:EtOAc, 7:3 EtOAc:Hexanes, 5% MeOH in EtOAc) to yield 138.16 mg of product (46% yield). ¹H NMR (400 MHz, CD₃OD) δ 8.18 (d, J = 2.4 Hz, 2H), 7.80 (d, J = 8.9 Hz, 2H), 7.73 (q, J = 8.6 Hz, J = 5.4 Hz, 2H), 7.52 (dd, J = 8.8 Hz, J = 2.1 Hz, 1H), 5.35 (d, J = 1.2 Hz, 1H), 3.67 (s, 3H), 3.41 (t, J = 7.0 Hz, 2H), 3.19 – 3.21 (m, 2H), 3.10 – 3.16 (m, 1H), 2.56 – 2.60 (m, 1H), 2.51 – 2.53 (m, 2H), 3.15 – 2.21 (m, 2H). ¹³C NMR (100 MHz, CD₃OD) δ 200.3, 179.9, 173.5, 170.2, 139.0, 136.7, 130.8, 128.9, 128.5, 125.5, 121.9, 117.3, 102.2, 84.3, 70.0, 56.9, 42.3, 40.3, 40.2, 36.4, 32.5, 29.5, 16.8. ESI-MS *m/z* for C₂₄H₂₄N₂O₄ calculated 404.17; observed 405.2 [M+H]⁺.

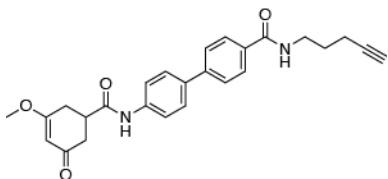


NaPhYn-1 (7): In a round bottom flask, Compound (6) (138.1 mg, .34 mmol) was suspended in a 1:1 Acetonitrile:Water solution (5 mL). 10% CAN (18.8 mg, .034 mmol) was added and the reaction mixture was refluxed at 95 °C for 3 hrs. The reaction was then cooled and concentrated and directly purified by HPLC. ¹H NMR (400 MHz, DMSO-d₆) δ 10.34 (s, 1H), 8.60 (t, J = 5.4 Hz, 1H), 8.37 (d, J = 7.4 Hz, 3H), 7.96 (d, J = 8.9 Hz, 1H), 7.87 (q, J = 8.7 Hz, J = 5.1 Hz, 3H), 7.65

(dd, $J = 4.4$ Hz, $J = 1.9$ Hz, 1H), 5.27 (s, 1H), 3.38 (q, $J = 12.7$ Hz, $J = 6.7$ Hz, 3H), 3.17-3.24 (m, 1H), 2.80 (t, $J = 2.6$ Hz, 1H), 2.49-2.51 (m, 2H), 2.25 (dt, $J = 7.2$ Hz, $J = 2.6$ Hz, 2H), 1.71-1.78 (m, 2H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 171.6, 166.3, 138.0, 134.6, 128.7, 127.0, 124.6, 120.4, 114.8, 103.3, 84.1, 71.3, 28.1, 15.5. ESI-MS m/z for $\text{C}_{23}\text{H}_{22}\text{N}_2\text{O}_4$ calculated 390.16; observed 391.2 $[\text{M}+\text{H}]^+$.

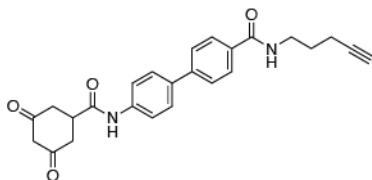


BiPhYn-1_a (8): To a dry round bottom flask flushed with nitrogen, was added 4-(4-aminophenyl)benzoic acid (400 mg, 1.88 mmol), NHS (237.4 mg, 1.93 mmol), and dry DMF (2 mL). The reaction mixture was cooled in a ice-bath. EDC-HCl (394.8 mg, 1.93 mmol) was added and the reaction mixture was allowed to react at 0 °C for 30 min, then the reaction was brought up to rt and allowed to mix for an additional 3 hrs. The reaction was quenched with the addition of saturated sodium bicarbonate and extracted with EtOAc. The organic layer was dried over MgSO_4 , filtered, and concentrated for the next step without further purification. To a dry round bottom flask flushed with nitrogen, was added the NHS-ester of (6) (506.7mg, 3.26 mmol) which was suspended in dry DMF (4 mL). 4-pentyneamine (271.4 mg, 3.26 mmol) and DIPEA (500 μL , 3.26 mmol) were subsequently added and the reaction mixture was allowed to mix at 75 °C overnight under nitrogen. The reaction was quenched with the addition of saturated sodium bicarbonate and extracted with EtOAc. The organic layer was dried over MgSO_4 , filtered, and concentrated. Flash column chromatography was used for purification (6:4 Hexanes:EtOAc; 10% MeOH in EtOAc spiked with 1% TEA) to yield 312.1 mg (61.2% yield over 2 steps) of (8) for future reactions. ^1H NMR (400 MHz, CD_3OD) δ 7.82 (d, $J = 8.4$, 2H), 7.60 (d, $J = 8.4$ Hz, 2H), 7.42 (d, $J = 8.6$ Hz, 2H), 6.78 (d, $J = 8.6$ Hz, 2H), 3.48 (t, $J = 6.9$ Hz, 2H), 3.31-3.33 (m, 1H), 2.25-2.28 (m, 2H), 1.80-1.87 (m, 2H). ^{13}C NMR (100 MHz, CD_3OD) δ 170.2, 163.8, 149.3, 145.8, 133.1, 132.8, 130.3, 130.0, 129.9, 128.8, 126.8, 116.6, 84.4, 70.2, 40.2, 38.0, 29.5, 16.9. ESI-MS m/z for $\text{C}_{18}\text{H}_{18}\text{N}_2\text{O}$ calculated 278.14; observed 279.1 $[\text{M}+\text{H}]^+$.

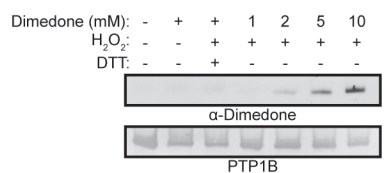


BiPhYn-1_b (9): To a dry round bottom flask flushed with nitrogen, was added 3-methoxy-5-oxocyclohex-3-enecarboxylic acid (190.8 mg, 1.12 mmol), EDC-HCl (320.5 mg, 1.68 mmol), and DMAP (205.3 mg, 1.68 mmol). The flask was sealed under nitrogen. Compound (8) (312.1 mg, 1.12 mmol) was resuspended in dry DMF (2 mL) and added to the reaction mixture followed by the addition of TEA (250 μL , 1.12 mmol). The reaction mixture was brought up to 85 °C and allowed to mix overnight under nitrogen. The reaction was quenched with the addition of water

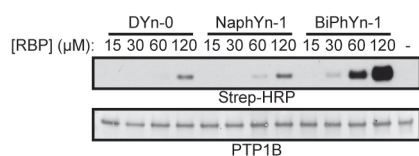
and extracted with EtOAc. The organic phases were combined and dried over MgSO_4 , filtered, and concentrated. Flash column chromatography was used for purification (6:4 EtOAc:Hexanes, 8:2 EtOAc:Hexanes, 10% MeOH in EtOAc) to yield 247 mg of product (51.7% yield). ^1H NMR (400 MHz, DMSO-d_6) δ 7.92 (d, J = 8.4 Hz, 2H), 7.74 (d, J = 8.4 Hz, 2H), 7.71 (d, J = 2.3 Hz, 2H), 7.55-7.65 (m, 2H), 5.38 (s, 1H), 3.71 (s, 3H), 3.35 (q, J = 12.4 Hz, J = 6.6, 3H), 2.23 (dt, J = 7.1 Hz, J = 2.6 Hz, 2H), 1.69-1.76 (m, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 196.1, 176.4, 171.0, 165.8, 142.0, 139.0, 133.8, 132.8, 127.8, 127.1, 125.8, 119.5, 101.4, 84.1, 71.3, 56.0, 30.8, 28.0, 15.5. ESI-MS m/z for $\text{C}_{26}\text{H}_{26}\text{N}_2\text{O}_4$ calculated 430.19; observed 431.2 $[\text{M}+\text{H}]^+$.



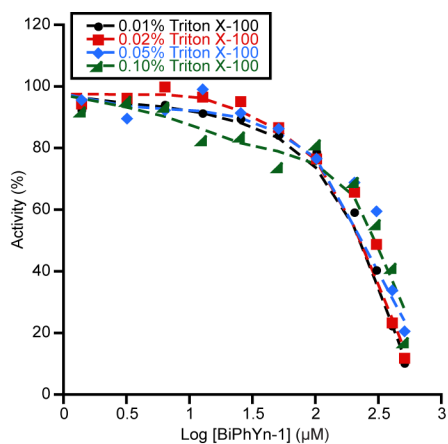
BiPhYn-1 (10): In a round bottom flask, Compound (9) (247 mg, .57 mmol) was suspended in a 1:1 Acetonitrile:Water solution (5 mL). 10% CAN (31.45 mg, .057 mmol) was added and the reaction mixture was refluxed at 95 °C for 3 hrs. The reaction was then cooled and concentrated and directly purified by HPLC. ^1H NMR (400 MHz, DMSO-d_6) δ 8.54 (d, J = 8.4 Hz, 2H), 7.91 (d, J = 8.4 Hz, 2H), 7.71 (d, J = 2.3 Hz, 2H), 7.55-7.65 (m, 2H), 5.38 (s, 1H), 3.34 (q, J = 12.4 Hz, J = 6.6, 3H), 2.23 (dt, J = 7.1 Hz, J = 2.6 Hz, 2H), 1.69-1.76 (m, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.3, 165.9, 142.0, 139.0, 133.7, 132.8, 131.3, 127.8, 127.08, 125.8, 119.5, 103.2, 84.1, 71.3, 45.4, 34.9, 28.0, 15.5, 8.4. ESI-MS m/z for $\text{C}_{25}\text{H}_{24}\text{N}_2\text{O}_4$ calculated 416.45; observed 415.1 $[\text{M}-\text{H}]^-$.



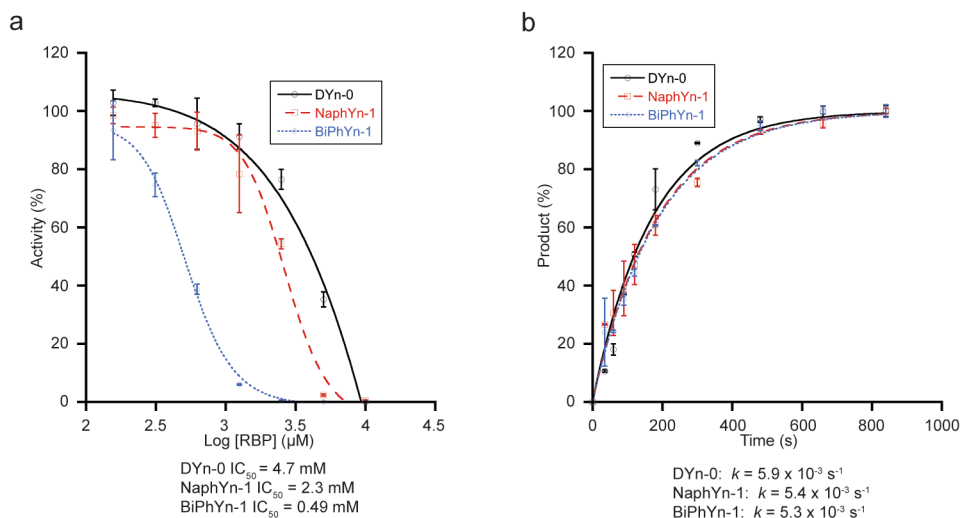
Supplementary Figure 1. Dimedone reacts with oxidized PTP1B. Recombinant PTP1B (20 μ M) was treated with dimedone in the presence of 10 equivalents H₂O₂ (200 μ M) for 1 h. The dimedone adduct was visualized with an α -dimedone antibody.



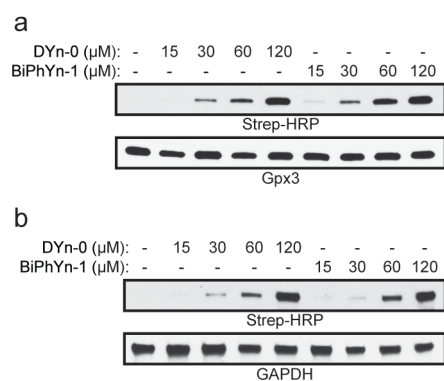
Supplementary Figure 2: RBPs solely react with oxidized cysteine of PTP1B. PTP1B (20 μM) was oxidized in the presence of 10 equivalences (200 μM) H₂O₂ for 15 minutes. Excess H₂O₂ was quenched in the presence of catalase. Following pre-oxidation of the phosphatase, the RBPs were added and allowed to react with oxidized PTP1B for 1 h.



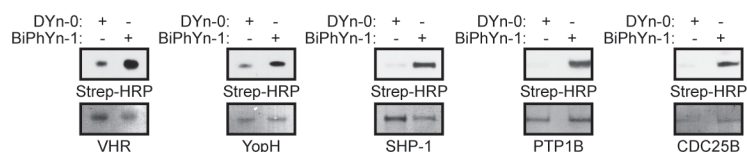
Supplementary Figure 3: RBPs do not act on PTP1B via aggregation-based inhibition. PTP1B activity was assessed by monitoring the dephosphorylation of 4-methylumbelliferyl phosphate (4-MUP) in the presence of increasing concentrations of Triton-X 100. No change in the inhibition profile of PTP1B with increasing concentrations of Triton-X 100 suggests that RBPs do not observe aggregation-based inhibition of target protein.



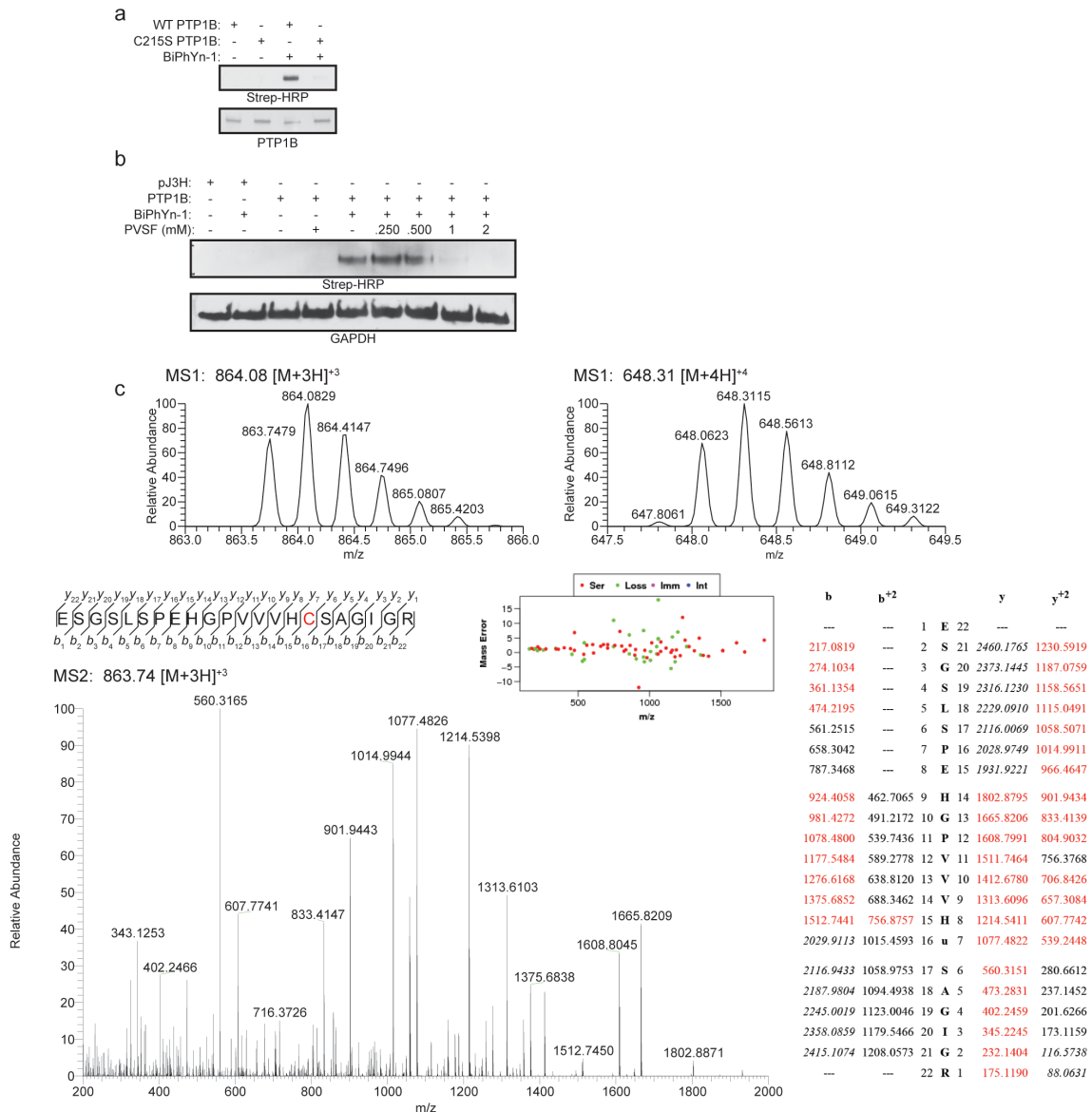
Supplementary Figure 4. Characterization of RBPs selectivity and nucleophilicity. (a) PTP1B activity was assessed by monitoring the dephosphorylation of 4-methylumbelliferyl phosphate (4-MUP) in the presence of increasing concentrations of RBPs. The calculated IC_{50} values are: DYn-0 = 4.7 mM; NaphYn-1 = 2.3 mM; and BiPhYn-1 = 0.49 mM. (b) The binding module does not enhance the nucleophile properties of the warhead. First order reaction rates of RBPs towards an RSOH model system yielded similar rates: DYn-0 = $5.9 \times 10^{-3} \text{ s}^{-1}$; NaphYn-1 = $5.4 \times 10^{-3} \text{ s}^{-1}$; BiPhYn-1 = $5.3 \times 10^{-3} \text{ s}^{-1}$.



Supplementary Figure 5. RBPs do not increase the general RSOH reactivity. (a, b) Gpx3 and GAPDH (25 μM) were treated with 1.5 equivalents H₂O₂ (37.5 μM) for 1 h in the presence of increasing concentrations of DYn-0 and BiPhYn-1.



Supplementary Figure 6. BiPhYn-1 detects global PTP oxidation. 0.1 $\mu\text{g}/\mu\text{L}$ PTP1B, YopH, SHP-1, VHR, and CDC25B were treated with 100 μM H_2O_2 and 50 μM RBP for 1 h followed by bioorthogonal ligation and visualization by avidin blotting.



Supplementary Figure 7. BiPhYn-1 targets the catalytic cysteine of PTP1B. (a) C215S PTP1B and WT PTP1B (20 μ M) were labeled with 50 μ M BiPhYn-1 in the presence of 10 equivalents (200 μ M) H_2O_2 for 1 h. Loss of signal was observed in C215S samples as opposed to WT. (b) PVSF, a mechanistic inhibitor of PTP1B, was titrated into pJ3H-PTP1B transfected COS1 cells for 1 h. The cells were then treated with 250 μ M of BiPhYn-1 for another hour and lysed. PTP1B was immunoprecipitated and subjected to bioorthogonal ligation to append a biotin reporter tag and visualization by avidin blotting. A loss of BiPhYn-1 labeled PTP1B was observed in the presence of increasing concentrations of PVSF. (c) LC/MS/MS analysis confirms catalytic Cys (C215) is modified by BiPhYn-1. Fragment ions in red indicate that they have been assigned to the spectra.

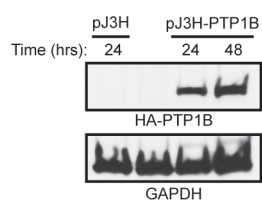


Figure S8. Transfection efficiency of pJ3H-PTP1B in COS1 cells over 48 h period.

- [1] Z.Y. Zhang, J.C. Clemens, H.L. Schubert, J.A. Stuckey, M.W. Fischer, D.M. Hume, M.A. Saper, J.E. Dixon, Expression, purification, and physicochemical characterization of a recombinant *Yersinia* protein tyrosine phosphatase, *J Biol Chem*, 267 (1992) 23759-23766.
- [2] C.E. Paulsen, K.S. Carroll, Chemical dissection of an essential redox switch in yeast, *Chem Biol*, 16 (2009) 217-225.
- [3] H. Zhou, H. Singh, Z.D. Parsons, S.M. Lewis, S. Bhattacharya, D.R. Seiner, J.N. LaButti, T.J. Reilly, J.J. Tanner, K.S. Gates, The biological buffer bicarbonate/CO₂ potentiates H₂O₂-mediated inactivation of protein tyrosine phosphatases, *J Am Chem Soc*, 133 (2011) 15803-15805.
- [4] T.H. Truong, F.J. Garcia, Y.H. Seo, K.S. Carroll, Isotope-coded chemical reporter and acid-cleavable affinity reagents for monitoring protein sulfenic acids, *Bioorg Med Chem Lett*, 21 (2011) 5015-5020.
- [5] W. Wang, S. Hong, A. Tran, H. Jiang, R. Triano, Y. Liu, X. Chen, P. Wu, Sulfated ligands for the copper(I)-catalyzed azide-alkyne cycloaddition, *Chem Asian J*, 6 (2011) 2796-2802.
- [6] Y.H. Seo, K.S. Carroll, Profiling protein thiol oxidation in tumor cells using sulfenic acid-specific antibodies, *Proc Natl Acad Sci U S A*, 106 (2009) 16163-16168.
- [7] C.L. Venable, E.U. Frevert, Y.B. Kim, B.M. Fischer, S. Kamatkar, B.G. Neel, B.B. Kahn, Overexpression of protein-tyrosine phosphatase-1B in adipocytes inhibits insulin-stimulated phosphoinositide 3-kinase activity without altering glucose transport or Akt/Protein kinase B activation, *J Biol Chem*, 275 (2000) 18318-18326.
- [8] C.E. Paulsen, T.H. Truong, F.J. Garcia, A. Homann, V. Gupta, S.E. Leonard, K.S. Carroll, Peroxide-dependent sulfonylation of the EGFR catalytic site enhances kinase activity, *Nat Chem Biol*, 8 (2012) 57-64.