Volume 1 | Number 1 | Jan 2013 | Pages 1–100

Molecular Biosystems

www.rsc.org/molecularbiosystems

THE BIOLOGY OF PLAGUE

ROYAL SOCIETY OF CHEMISTRY

This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the **Information for Authors**.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard **Terms & Conditions** and the **Ethical guidelines** still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/molecularbiosystems

Table of Contents (TOC):
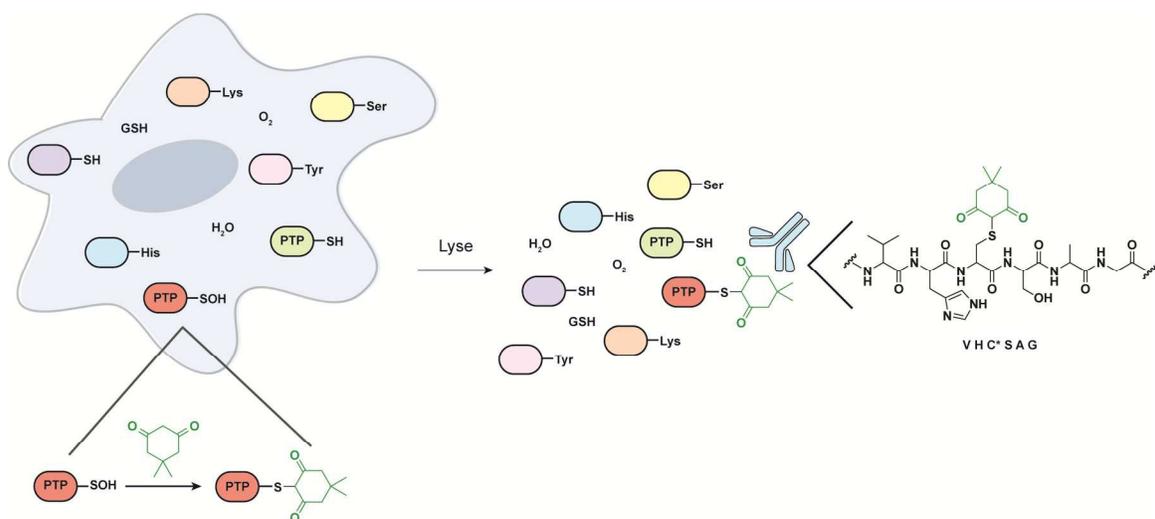
We report a simplified immunochemical approach to directly detect and quantify oxidized protein tyrosine phosphatases modified with dimedone.

Journal Name

ARTICLE

An Immunochemical Approach to Detect Oxidized Protein Tyrosine Phosphatases Using a Selective C-Nucleophile Tag

Francisco J. Garcia and Kate S. Carroll[*]

Protein tyrosine phosphatases are crucial regulators of signal transduction and function as antagonists towards protein tyrosine kinases to control reversible tyrosine phosphorylation, thereby regulating fundamental physiological processes. Growing evidence has supported the notion that reversible oxidative inactivation of the catalytic cysteine residue in protein tyrosine phosphatases serves as an oxidative post-translational modification that regulates its activity to influence downstream signaling by promoting phosphorylation and induction of the signaling cascade. The oxidation of cysteine to the sulfenic acid is often transient and difficult to detect, thus making it problematic in understanding the role that this oxidative post-translational modification plays in redox-biology and pathogenesis. Several methods to detect cysteine oxidation in biological systems have been developed, though targeted approaches to directly detect oxidized phosphatases are still lacking. Herein we describe the development of a novel immunochemical approach to directly profile oxidized phosphatases. This immunochemical approach consists of an antibody designed to recognize the conserved sequence of the PTP active site (VHC$_{DMD}$SAG) harboring the catalytic cysteine modified with dimedone (C$_{DMD}$), a nucleophile that chemoselectively reacts with cysteine sulfenic acids to form a stable thioether adduct. Additionally, we provide biochemical and mass spectrometry workflows to be used in conjugation with this newly developed immunochemical approach to assist in the identification and quantification of basal and oxidized phosphatases.

Introduction

Phosphorylation of tyrosine residues by protein tyrosine kinases (PTKs) is a key post-translational modification that orchestrates many aspects of cellular signaling ranging from proliferation, differentiation, cell survival, metabolism, cell migration, and cell-cycle control.[1] Tyrosine phosphorylation is tightly regulated by the opposing actions of protein tyrosine phosphatases (PTPs), which catalyze the hydrolytic dephosphorylation of phosphorylated tyrosine residues, thereby countering the activities of PTKs and maintaining a balance in cellular phosphorylation and signaling.

PTPs catalyze the dephosphorylation of phosphorylated tyrosine residues via a conserved catalytic cysteine residue, which exhibits a reduced pK$_a$ and exists as a stable thiolate as a consequence of being surrounded by basic amino acids that stabilize the anion. The unique architecture of the catalytic site renders the active site cysteine a potent nucleophile and facilitates PTPs to carry out their enzymatic function. PTPs have been shown to be tightly regulated by several mechanisms such as spatiotemporal expression, subcellular localization, proteolysis, dimerization, and post-translational modifications.[2] Recent studies have shown that the reversible oxidation of PTPs has emerged as an important general post-translational regulatory mechanism for members of this enzyme family under physiologic and pathologic conditions. Oxidation of PTPs converts the catalytic cysteine to a sulfenic acid (SOH) and, depending on the architecture of the catalytic microenvironment, can rapidly rearrange to form a cyclic sulfenamide with the nitrogen of a neighboring amide peptide backbone residue or a disulfide bond with a nearby cysteine residue.[3-7] The formation of either the intramolecular disulfides or cyclic sulfenamide within the phosphatase protects against overoxidation of the catalytic cysteine residue to the sulfinic (SO$_2$H) or sulfonic acid (SO$_3$H) oxoforms which are biologically irreversible.

A wide range of compounds have been shown to induce reversible oxidation of PTPs such as superoxide, hydroperoxides, peroxymonophosphate, hypothiocyanous acid, pyrroloquinoline, peroxymonocarbonate, peroxytetradecanoic acid, nitric oxide, hydroxyl radicals, and peroxidized lipids.[8-17] Additionally, PTP oxidation has been shown to be an intrinsic component of cell signaling whereby production of reactive oxygen species (ROS) is triggered by the activation of many classes of cell surface receptors including receptor tyrosine kinases (RTKs), integrins, cytokine receptors, G-protein-coupled receptors, and T- and B-cell receptors.[18-23] The most studied and biologically relevant oxidant is hydrogen peroxide (H$_2$O$_2$) due to its stability and selective reactivity towards cysteine thiolates.[24] The most relevant sources of ROS production are NADPH oxidase (Nox) enzymes following activation of various RTKs and the premature leakage of electrons from the respiratory chain of the mitochondria. Several studies have reported the oxidation of specific PTPs in response to different types of cell stimuli, including PTP1B in epidermal growth factor receptor signaling, SHP2 in platelet-derived growth factor signaling, both PTP1B and TCPTP in insulin signaling, and SHP1 in B-cell receptor signaling.[18, 19, 25, 26] Thus, these studies show that ROS acts as a second messenger leading to the transient reversible oxidation and inactivation of PTPs to sustain enhanced phosphorylation within signaling cascades.

Department of Chemistry, The Scripps Research Institute, 130 Scripps Way, Jupiter, FL 33458, USA. E-mail: kcarroll@scripps.edu; Tel.: +1 561 228 2471; Fax: +1 561 228 2919
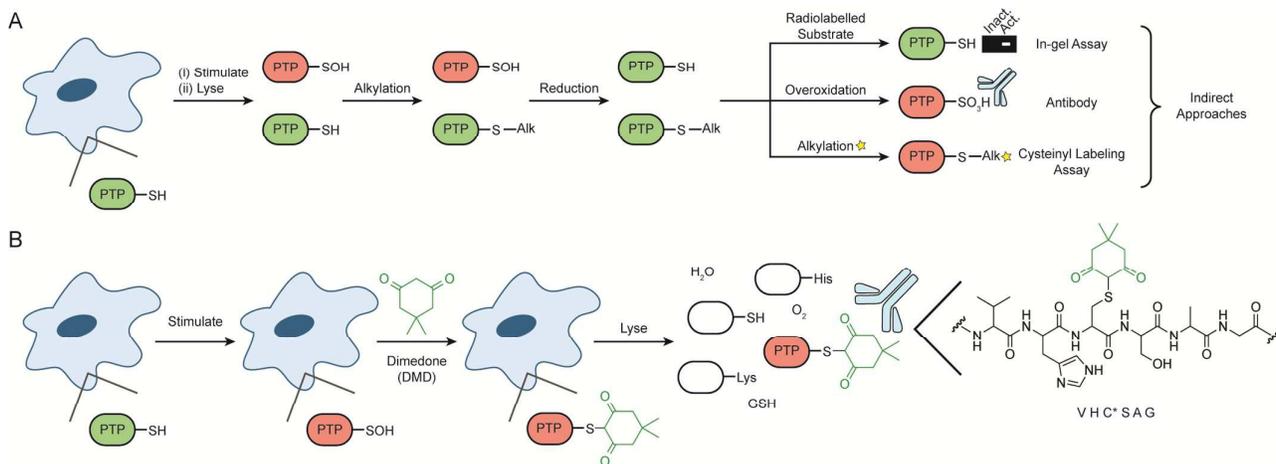
Figure 1: Approaches available to monitor PTP oxidation. (A) Indirect approaches include in-gel activity assays that monitor protein activity via radiolabeled substrate, antibodies that recognize a unique epitope consisting of the conserved signature motif within the active site of PTPs harboring the terminally oxidized catalytic cysteine residue, and tagged thiol-alkylating reagents. (B) A novel immunochemical approach, which utilizes nucleophiles such as DMD that chemoselectively react with protein sulfenic acids, to recognize the conserved catalytic motif of all PTPs bearing the DMD-thioether epitope.

Oxidative inactivation of protein tyrosine phosphatases constitutes a major regulatory mechanism of enzyme activity under physiologic and pathologic conditions. A lack of methods to monitor PTP oxidation has made it difficult to study their redox regulation within cells.[27] Current approaches to monitor PTP oxidation are mainly indirect and are dependent on electrophilic species that covalently modify the cysteine thiols (Figure 1A). Iodoacetate (IAA) bearing radiolabeled, biotinylated, or fluorescent tags have been used to monitor PTP oxidation whereby a decrease in the incorporation of the tagged-IAA is used to assess whether the phosphatase had been oxidized upon growth factor stimulation.[19, 21, 28] Since you are monitoring a loss of labeling as the PTP becomes oxidized, the major disadvantage of using tagged-IAA are sensitivity issues.   A modified cysteinyl-labeling assay was developed to remedy the limitations of the IAA-tagged approach and relied on a biotinylated thiol-alkylating (IAP-biotin) reagent to indirectly monitor PTP oxidation. The assay consists of three steps: (i) alkylation of active-site cysteine residues of PTPs that had not been oxidized; (ii) reduction and reactivation of reversibly oxidized PTPs; and (iii) alkylation of the aforementioned reduced PTPs with IAP-biotin.[29] The modified cysteinyl-labeling assay is not a targeted approach and suffers from high background of non-PTP proteins and low sensitivity.

Other indirect approaches, such as the in-gel phosphatase assay, have been developed to permit the identification of oxidized PTPs (Figure 1A). This assay relies on the alkylation of thiols that have not been oxidized within a cell lysate. The PTPs that have been oxidized are protected from alkylation and the lysate is subsequently resolved by SDS-PAGE containing a radioactively labeled substrate. The resolved oxidized PTPs are reactivated and refolded in the presence of reducing agents and their detection is based on reactivity with the radiolabeled substrate.[18, 30] The in-gel phosphatase assay has limitations and is biased towards non-membrane bound phosphatases and is not quantifiable.

Immunochemical strategies have also been developed to indirectly detect oxidation of PTPs. Antibodies, generated towards the conserved catalytic motif of PTPs ([I/V]HCSXGXGRS[S/T]G) bearing a sulfonic acid epitope on the catalytic cysteine, can be

used to detect oxidized PTPs. The detection strategy requires: (i) capping of free thiols with alkylating reagents, (ii) PTP immunoprecipitation, (iii) reduction of oxidized PTP, and (iv) treatment of PTP with pervanadate (PV) to generate the hyperoxidized SO_3H epitope (Figure 1A). Applications of this oxPTP antibody was used to show that UV-irradiation of cells could induce preferential oxidation of the second catalytic domain of RPTPα and monitor alterations in oxidation of SHP2 after treatment with platelet derived growth factor (PDGF).[31, 32]   Additionally, global quantification proteomic approaches have been combined with this immunochemical approach to assess PTP oxidation.[33]   The main limitations of this immunochemical approach is that it does not differentiate between phosphatases regulated by reversible oxidation from those that are inherently overoxidized. Moreover, the workflow for this immunochemical assay is cumbersome and may lead to false-positive results.

The detection of oxidized PTP via indirect methods require a workflow in which cells are lysed followed by subsequent manipulation of the samples. Cell lyses prior to capture of oxidized PTPs may lead to artificial oxidation due to the disruption of the various redox compartments within the cell. Direct approaches to monitor oxidized PTPs have been developed and circumvent issues with artificial oxidation brought about by cell lyses and egregious protein manipulation.   Nucleophiles such as 5,5-dimethyl-1,3-cyclohexanedione, also known as dimedone (DMD), have been shown to selectively react with protein sulfenic acids to generate a stable thioether adduct (Figure 1B).[34] Bifunctional chemical probes that incorporate reporter/affinity tags on the DMD nucleophile allows for the direct detection of oxidized proteins using a single reagent and has had success in the detection and quantification of various oxidized proteins, including PTPs.[35]   Several probes to exclusively monitor PTP oxidation have been developed. These PTP redox-based probes (RBPs) are composed of: (i) a DMD-based nucleophile which chemoselectively reacts with the oxidized catalytic cysteine; (ii) a module that directs binding to the PTP catalytic site; and (iii) a reporter tag used for the identification, purification, or direct visualization of the labeled protein.[36] These RBPs have been shown to detect basal and growth factor induced oxidized PTP1B in cells stimulated with insulin and have potential to

be used to study redox regulation of phosphatases via global proteomic assessment.[37]

DMD-specific antibodies, generated from a thiodimedone hapten consisting of the DMD thioether moiety on a cystamine backbone, are available to directly monitor protein oxidation via the protein-S-DMD adduct.[38] These antibodies have been applied to monitor PTP oxidation in vitro but may also be used to detect oxidized phosphatases in DMD-treated cells via immunoprecipitation of the desired PTP followed by immunoblotting.[37] Additionally, conformation sensing antibodies have been developed to directly detect unique conformational changes associated with oxidation of PTP1B to the cyclic sulfenamide.[39] Though the conformation sensing antibody provides a direct approach to monitor PTP oxidation, they are specific for a single phosphatase (PTP1B) and may not be used to monitor oxidation of the entire PTP family.

Oxidation of PTPs has emerged as an important mechanism for regulation of PTP activity and several approaches for oxPTP detection exist, improvements of the tools available are necessary and may aid in the investigation of PTP redox regulation. Herein we provide the development of an immunochemical approach that directly detects oxidized PTPs (Figure 1B). We also provide biochemical and mass spectrometry workflows to assist in the identification and quantification of basal and oxidized PTPs.

Materials and Methods

Synthesis of VHC$_{DMD}$SAG hapten and generation of antibodies

The heptapeptide was synthesized on solid phase using standard Fmoc main chain and Boc/Trt side chain protection chemistry. The VHCSAG peptide was treated with bromo-dimedone to generate the VHC$_{DMD}$SAG modified peptide. KLH (Keyhole Limpet Hemocyanin) protein was activated by adding sulfo-SMCC (sulfosuccinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylated), a hetero-bifunctional crosslinker containing an amine-reactive N-hydroxysuccininmide and a sulfhydryl-reactive malemide group. The hapten was conjugated to succinylated KLH overnight at pH 8 and the product was then purified over a PD10 gel filtration column (GE Healthcare). Two New Zealand White (specific pathogen-free grade) rabbits were immunized subcutaneiously with conjugate in a 50:50 emulsification with adjuvant [either Freunds complete (FCA) or Freunds incomplete (FIA) according to the following schedule: day 0 boost (FCA), day 14 boost (FIA), and day 28 boost (FIA). The rabbits were bled on days 35 and 40 after the primary boost, the red blood cells spun out by centrifugation, and the remaining antisera was IgG purified using a Nab Protein A Plus Spin Kit (Thermo Scientific).

Preparation of recombinant PTP1B and VHR

The construct of PTP1B was a kind gift from Kent S. Gates and was expressed and purified as previously described.[13] VHR was cloned into an expression vector containing a TEV cleavable 6x-His tag via ligation independent cloning (LIC) techniques. The cDNA for VHR (HsCD00000599) and the LIC vector, pMCSG28 (EvNO00340964) were purchased from DNAsu. The VHR DNA fragment was amplified with primers: 5 - GTCTCTCCCATGATGTCGGGCTCGTTCGAC - 3; and 5 - GGTTCTCCCCAGCCTAGGGTTTCAACTTCCCCTC - 3; using standard

PCR methods. The PCR product was purified using the Wizard SV gel and PCR cleanup system (Promega). The PCR products were treated with T4 DNA polymerase (New England BioLabs) and dTTP. The pMCSG28 vector was linearized by digestion with SmaI, purified using the Wizard SV gel and PCR clean-up system, and then treated with T4 DNA polymerase and dATP. The PCR product was annealed to vector DNA by mixing 3 µL T4-treated pMCSG28 with 2 µL T4-treated PCR product and incubated on ice for 30 minutes, followed by transformation into E. coli. After sequencing verified successful cloning, the vector was transformed into BL21(DE3) cells, grown in LB media, induced with IPTG, purified via Ni-NTA and size exclusion chromatography. Recombinant CDC25A, SHP2, PTEN, MKP1, and IF2 were purchased from Enzo Life Sciences.

Chemical modification of recombinant phosphatases

Stocks of the phosphatases were treated with 50 mM DTT for 20 minutes on ice, followed by removal of DTT via buffer exchange with Nap-5 columns (GE Healthcare) pre-equilibrated with labeling buffer (50 mM HEPES, 100 mM NaCl, 1 mM EDTA, pH 7.0). Protein concentrations were determined by A280 using a NanoDrop (Thermo). 10 µM PTP was treated with 10 (100 µM) or more equivalences of H_2O_2 for 1 – 2 hours in the presence of varying concentrations of nucleophile. Excess reagent was removed by passing the sample through a P-30 column (BioRad) pre-equilibrated with 25 mM ABC. The commercially purchased recombinant phosphatases were buffer exchanged into aforementioned labeling buffer using a pre-equilibrated P-6 column (Bio-Rad). 0.1 µg/µL of each recombinant phosphatase was treated with 100 µM H_2O_2 in the presence and absence of 10 mM DMD for 1 hour. The samples were then prepped for Western blot analysis.

Ratiometric analysis of basal and ROS-mediated oxidized VHR

Recombinant VHR was prepared and buffer exchanged as mentioned above. VHR was either treated with 10 mM light DMD in the absence of H_2O_2 or with 10 mM heavy DMD in the presence of varying concentrations of H_2O_2 for 2 hours at room temperature. Afterwards, the samples were passed through a pre-equilibrated P-30 column to remove excess reagent and then mixed at a 1:1 ratio. The samples were digested in solution using trypsin overnight, desalted using C-18 columns (The Nest Group), and immunoprecipitated with the PTP-S-DMD antibody. The samples were then subjected to MS/MS analysis.

Isotope-coded dimedone and iododimedone (ICDID) for quantification of protein sulfenic acid

Recombinant VHR was prepared and buffer exchanged as mentioned above. VHR was treated with 10 mM heavy dimedone in the presence of varying concentrations of H_2O_2 for 2 hours at room temperature. Afterwards, the protein mixture was treated with 10 mM iododimedone for 1 hour at room temperature. The protein mixture was then subjected to an in-solution digestion using trypsin overnight. The following day, the peptides were desalted using a C-18 column (The Nest Group) and immunoprecipitated with the PTP-S-DMD antibody. The samples were then subjected to MS/MS analysis.

Immunoprecipitation of PTP-S-DMD peptides

The tryptic digests were desalted using C-18 columns (The Nest Group) as described above. The desalted digest was concentrated

to dryness and resuspended in 1 mL of 1x PBS. PTP-S-DMD antibody (5 μL) was added to the peptide suspension and allowed to mix overnight at 4 °C on an end-over-end rocker. The following day, the peptide mixture was treated with 20 μL protein A/G PLUS-Agarose (Santa Cruz Biotechnology) and allowed to mix at 4 °C for 3 hours on an end-over-end rocker. The immunocomplex was collected by centrifugation at 1000 x g for 2 minutes, washed with 1x PBS (3 x's), washed with H_2O (3 x's), and eluted by incubation with 0.15% formic acid (80 μL) for 10 minutes (3 x's). The eluted peptides were concentrated to dryness, respusended in 50 μL 0.1% formic acid and analyzed by MS.

Mass spectrometry analysis

Samples were analyzed by liquid chromatography-tandem MS (LC-MS/MS) using an EASY-nLC II system coupled to a linear ion trap mass spectrometer model LTQ (Thermo Fisher Scientific). Peptides were concentrated and desalted on a RP precolumn (0.1 x 20 mm EASY-column, Thermo Fisher Scientific) and on-line eluted on an analytical RP column (0.075 x 100 mm EASY-column, Thermo Fisher Scientific), operating at 300 nL/min and using a gradient of 5% - 90% B over 45 minutes [solvent A: 0.1% formic acid (v/v); solvent B: 0.1% formic acid (v/v) in 80% acetonitrile].

Detection of oxSHP2 in A431 cells

A431 (ATCG) cells were maintained at 37 °C in a 5% CO_2 humidified atmosphere. Cells were cultured in high glucose DMEM (Corning) containing 10% FBS (Invitrogen), 1% GlutaMax (Invitrogen), 1% MEM nonessential amino acids (Invitrogen), and 1% penicillin-streptomycin (Invitrogen). For EGF treatment, cells were serum starved for 16 hours prior to experimentation. Cells were grown to 80% confluency prior to serum starvation. A431 cells were stimulated with 100 ng/mL EGF (or varying concentrations of EGF) for 5 minutes. Afterwards, cells were washed with PBS (3 x's) and treated with 10 mM DMD (or varying concentrations of DMD) for 1 hour at 37 °C, 5% CO_2. Cells were washed with PBS (3 x's) to remove excess nucleophile and the 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). SHP2 was immunoprecipiated from 1 mg of cell lysate.

Western blot analysis

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). After transfer, the PVDF membrane was blocked with 3% 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: PTP-S-DMD AB (1:200 – 1:1000), SHP2 (Santa Cruz Biotechnology; 1:200), goat anti-rabbit IgG-HRP (Calbiochem, 1:1000 – 1:50000), and rabbit anti-mouse IgG-HRP (Invitrogen, 1:35000) The PVDF membranes were washed with TBST (3X) and developed with ECL Plus chemiluminescence (Pierce) and imaged by film. Equal loading of recombinant phosphatases were assessed by treating the
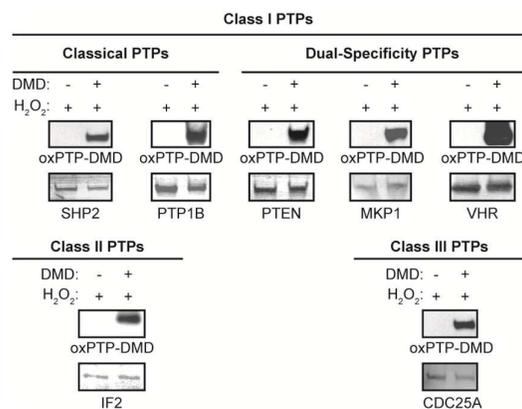
Figure 2: Profiling oxidized PTPs. Recombinant PTPs were treated with 10 equivalences of H_2O_2 in the presence of 10 mM DMD then analyzed by Western blot. The antibody is able to recognize the conserved PTP catalytic sequence harboring the DMD modification.

membrane with a solution of 1:1 MeOH:R-250 Coomassie blue for ten minutes, then allowing membrane to dry.

Results and Discussion

To detect H_2O_2 mediated oxidized PTPs, we envisioned an enhancement of both the oxPTP and the thiodimedone-specific antibody approach via complementation of the conserved active site peptide, representative of the classical PTPs, with the DMD moiety (Figure 1B).[31, 38] This enhanced antibody strategy will allow for improved detection of the oxidation-induced inactivation of PTPs. We designed and synthesized the PTP active site peptide (VHCSAG) via standard peptide coupling strategies. The peptide was then reacted with Bromo-DMD to give the desired conserved catalytic peptide harboring the stable DMD thioether adduct. The resulting peptide was modified to a five-carbon linker to incorporate the keyhole limpet hemocyanin (KLH). The resulting hapten-protein conjugate was used to elicit rabbit α-hapten-Ig gamma (IgG).

The human genome contains a set of 107 genes that encode for PTPs. This large enzyme family is subdivided into four classes based on their substrate specificity and amino acid sequence at the catalytic domain. Class I PTPs are comprised of classical and dual-specificity phosphatases, which dephosphorylate phospho-tyrosine and phospho- serine/threonine/tyrosine residues respectively. The class II phosphatases consist of low molecular weight PTPs (LMPTPs) and are encoded by the ACP1 gene. The class III phosphatases are made up of three genes encoding the Cdc25 family members. Finally, class IV are aspartic acid-based PTPs and are encoded by the four eyes absent homologue (EYA) gene. We expected that the antibody would be useful for the global detection of DMD-modified oxPTPs. To test this, we took various recombinant PTPs that were representative of the three cysteine-based PTP classes (I, II, and III) within the PTP superfamily and subjected them to oxidation in the presence or absence of DMD. Western blot analysis demonstrated a clear signal for all the PTPs that had been treated with H_2O_2 in the presence of DMD (Figure 2). These results suggest that the antibody may be suitable for global detection of DMD-modified oxPTPs. Future studies were performed on PTP1B, VHR, and SHP2 which all showed robust detection with the antibody.
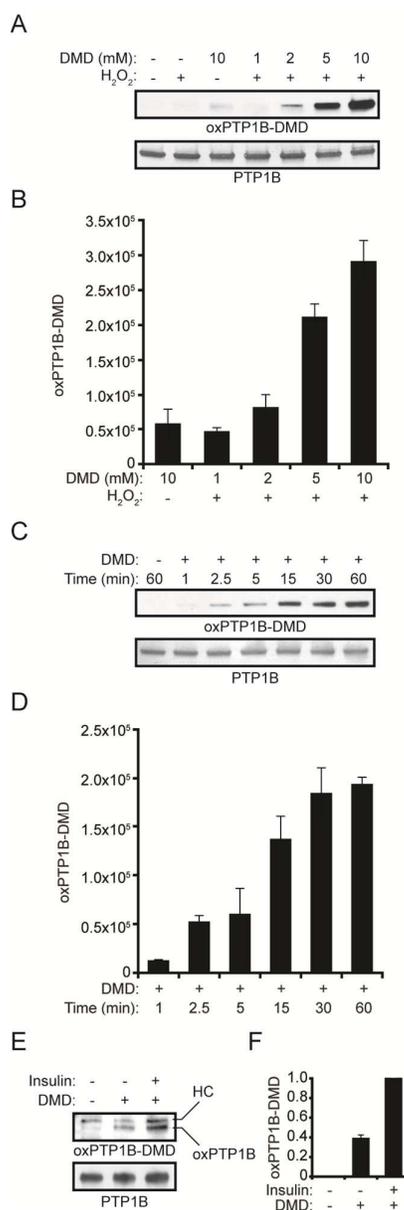
Figure 3: The PTP-S-DMD antibody recognizes oxidized phosphatases. Recombinant PTP1B (10 μM) was treated with 10 equivalences (100 μM) H_2O_2 in the presence of increasing concentrations of DMD and Western blot analysis revealed (A) does-dependent and (C) time-dependent enhancement of DMD-modified oxPTP1B in the presence of 5 mM DMD. Densitometric analysis of (B) dose-dependent and (D) time-dependent detection of recombinant oxPTP1B. (E) Increased levels of oxPTP1B were detected when HA-PTP1B transfected CHO/hIRc cells were stimulated with 100 nM insulin in the presence of 10 mM DMD. HC = heavy chain of IgG. (F) Densitometric analysis revealed a two-fold enhancement in levels of oxPTP1B after insulin stimulation.

Initial studies with recombinant PTP1B were performed to determine whether the antibody could detect DMD-modified oxPTPs in a dose- and time-dependent manner. 10 μM PTP1B was treated with varying concentrations of DMD in the presence of 10 equivalences (100 μM) H_2O_2. The protein was resolved by SDS-PAGE and subjected to Western blot analysis. The immunoblot

demonstrated that the antibody is able to detect DMD-modified oxPTP1B in a dose- (Figure 3A) and time-dependent fashion (Figure 3C). Densitometric analysis of the dose- (Figure 3B) and time-dependent (Figure 3D) Western blots reveal robust detection of this oxidative post-translational modification. We next evaluated the specificity of the antibody by determining whether it can fully discriminate between oxPTPs that have been modified with DMD from non-PTP redox-sensitive proteins, such as the antioxidizing enzyme – glutathione peroxidase 3 (Gpx3), that have also been shown to react with DMD. We treated Gpx3 with varying concentrations of DMD in the presence of H_2O_2 followed by SDS-PAGE and Western blot analysis and revealed that the antibody is solely selective for oxPTPs modified with DMD (Supplementary Figure 1A). Densitometric analysis confirmed only background (Supplementary Figure 1B). Finally, we tested the antibodies ability to differentiate between detection of DMD-modified oxPTPs from other nucleophiles that are structurally analogous to DMD. We treated recombinant PTP1B with H_2O_2 and varying concentrations of indandione (IND) and piperdinedione (PRD), both of which showed enhanced reactivity towards a small molecule SOH model as compared to DMD, and monitored their detection with the antibody.[40, 41] We observed detection of DMD-modified oxPTP1B only and no detection with the DMD nucleophilic isosteres (Supplementary Figure 1C). Taken together, these findings suggest that the antibody is a practical tool with high affinity and selectivity for detecting DMD-modified oxPTPs.

We next proposed to justify the usefulness of the antibody for facile detection of oxPTPs in cells. Previous antibody-based approaches that were developed to detect reversibly oxPTP used a monoclonal antibody that recognized the conserved sequence of PTPs bearing the catalytic cysteine terminally oxidized to the SO_3H. This approach required a cumbersome workflow in which cell lysates were treated with alkylating reagents to cap free thiols, followed by treatment with dithiothreitol (DTT) to regenerate the reversibly oxidized PTPs, and finally treatment with pervanadate to generate the SO_3H. We devised a scheme in which the antibody would allow for a simplistic workflow in the detection of oxPTPs (Supplementary Figure 2A). We envisioned that intact cells would be stimulated with growth factors to prompt the production of endogenous H_2O_2 associated with various cell signaling pathways. Following growth factor stimulation, the cells would be treated with DMD to trap the sulfenylated PTPs. Next, the cells would be lysed and the desired PTP would be immunoprecipiated and analyzed by Western blot analysis for DMD-modified oxPTPs using the PTP-S-DMD specific antibody. To evaluate this approach, we transfected CHO/hIRc cells with HA-tagged PTP1B (pJ3H-PTP1B) and subsequently stimulated the transfected cells with 100 nM insulin. Following insulin stimulation, the cells were treated with or without 10 mM DMD, lysed, and enriched for HA-PTP1B. Western blot analysis demonstrates the ease at which this antibody allows for the detection of oxPTP1B (Figure 3E). Densitometric quantification illustrated a two-fold increase of oxPTP1B after insulin stimulation (Figure 3F). Taken together, these results indicate that the PTP-S-DMD antibody offers a more rapid and simplistic approach for the direct detection of oxPTPs from a complex cellular milieu.

SHP2 directly interacts with the epidermal growth factor receptor (EGFR) and previous studies have shown that SHP2 is highly susceptible to reversible oxidative inactivation upon epidermal growth factor (EGF) stimulation.[42] We therefore wanted to determine whether the antibody would be able to detect SHP2
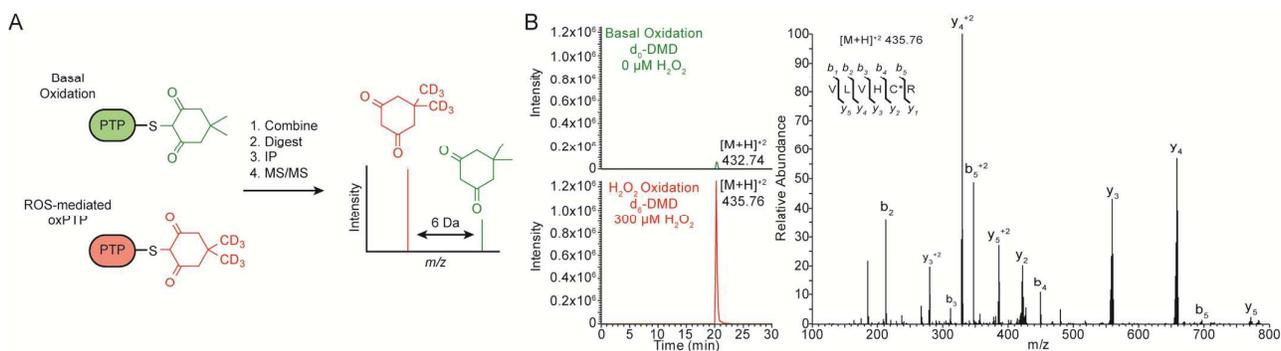
Figure 4: Immunoaffinity enrichment of DMD-modified oxPTP peptides for quantitative proteomic analysis. (A) The use of isotopic analogs of DMD, followed by immunoaffinnity enrichment of the DMD-modified PTP peptides, and subsequent mass spectrometry analysis provides a convenient method by which to identify and quantify oxPTPs. (B) Recombinant VHR was treated with 10 mM d_0-DMD in the absence of H_2O_2 and 10 mM d_6-DMD in the presence of 300 μM H_2O_2. After separate incubations, the samples were mixed, digested with trypsin, enriched, and subjected to MS/MS analysis. MS/MS analysis of 435.76 $[M+H]^{+2}$ corresponds to d_6-DMD modified oxVHR and displayed excellent coverage. Based on these results, we observed ~5.3% basal oxVHR using this technique.

oxidation upon stimulation with EGF in cells. A431 cells were stimulated with 100 ng/mL EFG followed by incubation with increasing concentrations of DMD. Western blot analysis of the immunoprecipitated SHP2 revealed a modest increase in DMD-modified SHP2 in the presence of 10 mM DMD (Supplementary Figure 2B). Densitometric quantification of the blot showed a two-fold increase in DMD-modified oxSHP2 after stimulation with EGF in the presence of 10 mM DMD (Supplementary Figure 2C). Time-dependent analyses were also performed with immunoprecipitated oxSHP2 and were in agreement with our *in vitro* studies (Supplementary Figure 2D). Stimulation of A431 cells with varying concentrations of EGF revealed a dose-dependent enhancement of DMD-modified oxSHP2 detection (Supplementary Figure 2E). Densitometric quantification of the blot showed a two- to three-fold increase in the levels of oxSHP2 in respects to increasing concentration of EGF (data not shown). In 1998, Lee *et al.* found that PTP1B is transiently inactivated after stiumulation of cells with EGF and determined that oxidative inactivation modulates the steady-state levels of phosphorylation necessary for growth stimulation.[19] To show the broad utility of our antibody, we performed a similar experiment in COS1 cells transfected with HA-PTP1B. The stimulation of COS1 cells with various concentrations of EGF displayed an expected dose-dependent increase in DMD-modified oxPTP1B (Supplemental Figure 2F). These results provide evidence for the usefulness of this immunochemical approach for the facile direct detection of oxidized phosphatases within a complex cellular setting.

Apart from immunochemical detection of oxidized phosphatases, we anticipated that the antibody would be useful for enriching DMD-modified PTP catalytic peptides for subsequent proteomic analysis. We initially tested whether the antibody could selectively pull down the DMD-modified catalytic peptide hapten. A solution of the hapten was prepared in PBS and treated with or without the antibody and allowed to mix overnight at 4 °C. The following day, the immunocomplex was pulled down with protein A/G agarose, washed extensively before eluting with 0.15% trifluoroacetic acid (TFA), and analyzed by LC/MS/MS. We observed a peak with a mass of 711.31 Da, corresponding to the $[M+H]^{+1}$ m/z of VHC*SAG (C* = DMD modified). MS/MS confirmed the sequence of the peptide (Supplemental Figure 3A). In the absence of the

antibody, no peaks were observed indicating that the hapten does not bind non-specifically to the IgG agarose beads. Thus the preliminary results suggest that the antibody is suitable for immunoprecipitating DMD-modified peptides.

We next sought to determine whether the antibody could enrich the DMD-modified catalytic peptide from a complex mixture. We choose VHR as a model system since previous studies indicated that oxidized VHR forms a stable SOH.[43] We therefore treated recombinant VHR with varying concentrations of H_2O_2 in the presence of 10 mM DMD, followed by in-solution digestion with trypsin, and enrichment of DMD-modified peptides with the antibody. We observed a H_2O_2 dose-dependent increase in the intensity of DMD-labeled VHR catalytic peptide (Supplemental Figure 3B). MS/MS analysis of the peak at 432.73 Da, which corresponds to the $[M+H]^{+2}$ m/z, confirmed the sequence of the catalytic peptide (Supplemental Figure 3B).

Isotopically labeled SOH probes have previously been developed to assist in quantifying the extent of protein SOH modification during growth factor mediated H_2O_2 production under normal and disease states.[44, 45] We envisioned that the antibody may be useful for differentiating proteins that are oxidized under basal conditions from those that are oxidized during growth factor induced H_2O_2 production (Figure 4A). We treated separate samples of VHR with either 10 mM of light-DMD or heavy-DMD in the absence or presence of H_2O_2, respectively. We then pooled the two samples together, generated a tryptic digest, immunoprecipitated the DMD-modified catalytic peptides, and subsequently analyzed the immunoprecipitated peptides by LC/MS/MS. We observed the light-DMD peak at m/z 432.74 $[M+H]^{+2}$ and heavy-DMD at m/z 435.76 $[M+H]^{+2}$. MS/MS of heavy-DMD modified VHR confirmed the sequence and area under the curve (AUC) calculations indicated ~5.3% basal oxidized VHR (Figure 4B).

Furthermore, we proposed that the antibody would be useful for distinguishing between SOH modified PTPs and PTPs that reside in the thiol state. We therefore utilized the isotope coded dimedone and iododimedone (ICDID) method for the quantitative analysis of protein SOH modification in conjugation with the PTP-S-

DMD approach to specifically monitor the extent of oxPTP formation in order to determine the extent or fraction of SOH modified PTPs (Figure 5A).[46] Here we treated VHR with varying concentrations of H_2O_2 in the presence of 10 mM heavy DMD to intercept any oxidized VHR. Afterwards, we treated the same sample with 10 mM iododimedone to trap any cysteine thiol that had not been oxidized. The samples were digested, DMD-modified catalytic peptides were immunoprecipitated with the antibody via immunoaffinity enrichment, and subsequently analyzed by LC/MS/MS. We observed a change in the ratio of heavy DMD modified VHR from light DMD modified VHR with increasing concentrations of H_2O_2 (Figure 5B). This suggested that with increasing concentrations of H_2O_2 more of the PTP exists as a SOH and not as a free thiol. Taken together, these studies indicate that the antibody may be a practical tool for identifying and quantifying oxidized phosphatases.

Conclusion

While approaches to identify specific oxidized PTPs are available, it has been difficult to develop general methods that detect and quantify any reversible oxidized PTPs in different physiological and pathological states. Therefore, tools that can directly detect SOH modified phosphatases through a simplistic workflow are necessary to help further our understanding of how this oxidative post-translational modification plays a role in disease states. The majority of current methods indirectly monitor phosphatase oxidation. Small molecules that chemoselectivley react with protein SOH have been developed to directly monitor PTP oxidation. These compounds are chemically selective for SOH, bear binding modules to direct them to the phosphatase super family, and are cell permeable.[36, 37] To this end, we have expanded the toolkit for the direct detection of this oxidative post-translational modification via the development, design, and implementation of an immunochemical approach. This immunochemical strategy was inspired from several known methods that detect oxidized phosphatases and improved on for facile implementations in workflows for the selective detection of oxidized phosphatases. Initial studies performed *in vitro* demonstrated that the antibody could detect DMD-modified phosphatases in a time- and dose-dependent fashion. Additionally, the antibody was shown to be selective for phosphatases modified with DMD as opposed to other proteins that are susceptible to oxidation and reaction with DMD. Furthermore, the antibody was shown to solely recognize the DMD epitope as opposed to other nucleophiles (IND or PRD) that share structural similarities but exhibit enhanced reactivity for SOH. Since all phosphatases contain a conserved catalytic domain, we confirmed that this immunochemical approach would be useful for global detection of oxidized PTPs. We then used the antibody to detect endogenous levels of oxidized phosphatases in cells. A431 cells, stimulated with varying concentrations of EGF, displayed a dose-dependent increase in the levels of oxidized SHP2. Finally, we proposed that this immunochemical approach would be useful for enrichment and subsequent proteomic analysis. A major strength of this approach is that it can be coupled to other previously developed technologies for quantification of protein sulfenic acids. Using heavy DMD, we were able to differentiate between basal oxidized PTPs from those that were oxidized in the presence of H_2O_2. Moreover, implementation of the ICDID method allowed for the quantification of protein SOH in the presence of various concentrations of H_2O_2. This new method for the direct detection of SOH modified
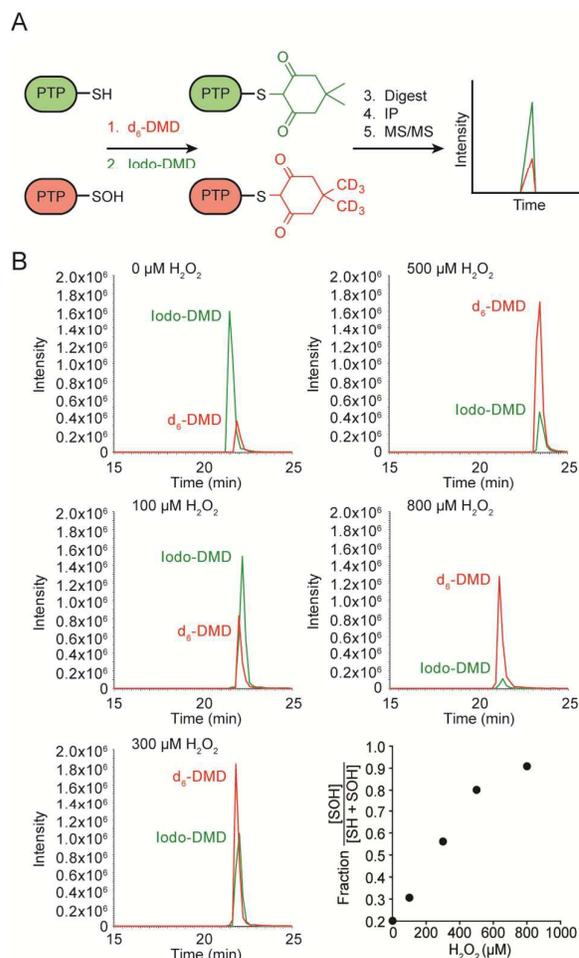
Figure 5: Quantification of oxPTPs using the Isotope-Coded Dimedone and Iododimedone (ICDID) strategy. (A) The ICDID strategy uses a set of probes that generate chemically identical proteins but differ in the specific mass of their labels depending on the original redox state of the cysteine residue. (B) The ICDID approach provides a means for quantitative profiling and comparison of thiol redox states in physiological and pathological conditions associated with oxidative stress. We observed iododimedone modified VHR at m/z 432.74 $[M+H]^{+2}$ and d_6-DMD modified oxVHR at m/z 435.76 $[M+H]^{+2}$ and the peaks were confirmed by MS/MS. The elution time of the light- and heavy-tagged peptide was approximately ~22 minutes. However, in some cases, small shifts in retention time were observed owing to slight variations in mobile phase, sample constituents, and/or column temperature.

phosphatases provides a powerful means to detect changes in cysteine oxidation in this protein family *in vivo* and should find a wide variety of applications for the study of biological processes revolving around redox signaling pathways that are central to human health and disease. Future applications of this approach can be used for detection, identification, and quantification of redox regulated phosphatases in cells during various cell signaling events and may be useful for a systems biology understanding of redox signaling. Recently its been shown that PTKs are susceptible to oxidative modification and our immunochemical approach can be extended for the development of PTK-S-DMD antibodies to assist in detecting redox-regulated kinases.[42] Additionally, with the discovery of highly reactive chemoselective nucleophiles towards SOH, we may be able to develop 2nd generation immunochemical

approaches which harbor these more reactive nucleophiles on conserved peptide sequences for key signaling proteins such as PTPs and PTKs.[40, 41]

Acknowledgements

References

1. M. A. Lemmon and J. Schlessinger, *Cell*, 2010, 141, 1117-1134.
2. J. den Hertog, A. Ostman and F. D. Bohmer, *The FEBS journal*, 2008, 275, 831-847.
3. A. Salmeen, J. N. Andersen, M. P. Myers, T. C. Meng, J. A. Hinks, N. K. Tonks and D. Barford, *Nature*, 2003, 423, 769-773.
4. R. L. van Montfort, M. Congreve, D. Tisi, R. Carr and H. Jhoti, *Nature*, 2003, 423, 773-777.
5. J. Yang, A. Groen, S. Lemeer, A. Jans, M. Slijper, S. M. Roe, J. den Hertog and D. Barford, *Biochemistry*, 2007, 46, 709-719.
6. C. Y. Chen, D. Willard and J. Rudolph, *Biochemistry*, 2009, 48, 1399-1409.
7. T. van der Wijk, J. Overvoorde and J. den Hertog, *The Journal of biological chemistry*, 2004, 279, 44355-44361.
8. W. C. Barrett, J. P. DeGnore, Y. F. Keng, Z. Y. Zhang, M. B. Yim and P. B. Chock, *The Journal of biological chemistry*, 1999, 274, 34543-34546.
9. S. Bhattacharya, J. N. Labutti, D. R. Seiner and K. S. Gates, *Bioorganic & medicinal chemistry letters*, 2008, 18, 5856-5859.
10. J. N. LaButti, G. Chowdhury, T. J. Reilly and K. S. Gates, *Journal of the American Chemical Society*, 2007, 129, 5320-5321.
11. A. E. Lane, J. T. Tan, C. L. Hawkins, A. K. Heather and M. J. Davies, *The Biochemical journal*, 2010, 430, 161-169.
12. K. Kimura, M. Takada, T. Ishii, K. Tsuji-Naito and M. Akagawa, *Free radical biology & medicine*, 2012, 53, 1239-1251.
13. H. Zhou, H. Singh, Z. D. Parsons, S. M. Lewis, S. Bhattacharya, D. R. Seiner, J. N. LaButti, T. J. Reilly, J. J. Tanner and K. S. Gates, *Journal of the American Chemical Society*, 2011, 133, 15803-15805.
14. A. Kuban-Jankowska, J. A. Tuszynski, P. Winter, M. Gorska, N. Knap and M. Wozniak, *PloS one*, 2012, 7, e52495.
15. M. F. Hsu and T. C. Meng, *The Journal of biological chemistry*, 2010, 285, 7919-7928.
16. F. G. Meng and Z. Y. Zhang, *Biochimica et biophysica acta*, 2013, 1834, 464-469.
17. M. Conrad, A. Sandin, H. Forster, A. Seiler, J. Frijhoff, M. Dagnell, G. W. Bornkamm, O. Radmark, R. Hooft van Huijsduijnen, P. Aspenstrom, F. Bohmer and A. Ostman, *Proceedings of the National Academy of Sciences of the United States of America*, 2010, 107, 15774-15779.
18. T. C. Meng, T. Fukada and N. K. Tonks, *Molecular cell*, 2002, 9, 387-399.
19. S. R. Lee, K. S. Kwon, S. R. Kim and S. G. Rhee, *The Journal of biological chemistry*, 1998, 273, 15366-15372.
20. K. Mahadev, A. Zilbering, L. Zhu and B. J. Goldstein, *The Journal of biological chemistry*, 2001, 276, 21938-21942.
21. J. Kwon, C. K. Qu, J. S. Maeng, R. Falahati, C. Lee and M. S. Williams, *The EMBO journal*, 2005, 24, 2331-2341.
22. D. K. Singh, D. Kumar, Z. Siddiqui, S. K. Basu, V. Kumar and K. V. Rao, *Cell*, 2005, 121, 281-293.
23. E. Giannoni, P. Chiarugi, G. Cozzi, L. Magnelli, M. L. Taddei, T. Fiaschi, F. Buricchi, G. Raugei and G. Ramponi, *The Journal of biological chemistry*, 2003, 278, 36763-36776.
24. C. E. Paulsen and K. S. Carroll, *Chemical reviews*, 2013, 113, 4633-4679.
25. T. C. Meng, D. A. Buckley, S. Galic, T. Tiganis and N. K. Tonks, *The Journal of biological chemistry*, 2004, 279, 37716-37725.
26. M. Capasso, M. K. Bhamrah, T. Henley, R. S. Boyd, C. Langlais, K. Cain, D. Dinsdale, K. Pulford, M. Khan, B. Musset, V. V. Cherny, D. Morgan, R. D. Gascoyne, E. Vigorito, T. E. DeCoursey, I. C. MacLennan and M. J. Dyer, *Nature immunology*, 2010, 11, 265-272.
27. R. Karisch and B. G. Neel, *The FEBS journal*, 2013, 280, 459-475.
28. R. F. Wu and L. S. Terada, *Science's STKE : signal transduction knowledge environment*, 2006, 2006, pl2.
29. B. Boivin, S. Zhang, J. L. Arbiser, Z. Y. Zhang and N. K. Tonks, *Proceedings of the National Academy of Sciences of the United States of America*, 2008, 105, 9959-9964.
30. T. C. Meng, S. F. Hsu and N. K. Tonks, *Methods*, 2005, 35, 28-36.
31. C. Persson, T. Sjoblom, A. Groen, K. Kappert, U. Engstrom, U. Hellman, C. H. Heldin, J. den Hertog and A. Ostman, *Proceedings of the National Academy of Sciences of the United States of America*, 2004, 101, 1886-1891.
32. C. Persson, K. Kappert, U. Engstrom, A. Ostman and T. Sjoblom, *Methods*, 2005, 35, 37-43.
33. 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 and B. G. Neel, *Cell*, 2011, 146, 826-840.
34. L. V. Benitez and W. S. Allison, *The Journal of biological chemistry*, 1974, 249, 6234-6243.
35. T. F. Brewer, F. J. Garcia, C. S. Onak, K. S. Carroll and C. J. Chang, *Annual review of biochemistry*, 2015, 84, 765-790.
36. S. E. Leonard, F. J. Garcia, D. S. Goodsell and K. S. Carroll, *Angewandte Chemie*, 2011, 50, 4423-4427.
37. F. J. Garcia and K. S. Carroll, *European journal of medicinal chemistry*, 2014, 88, 28-33.
38. Y. H. Seo and K. S. Carroll, *Proceedings of the National Academy of Sciences of the United States of America*, 2009, 106, 16163-16168.
39. A. Haque, J. N. Andersen, A. Salmeen, D. Barford and N. K. Tonks, *Cell*, 2011, 147, 185-198.
40. V. Gupta and K. S. Carroll, *Chemical Science*, 2016, DOI: 10.1039/C5SC02569A.
41. K. V. Ruddraraju, Z. D. Parsons, E. M. Llufrio, N. L. Frost and K. S. Gates, *J Org Chem*, 2015, DOI: 10.1021/acs.joc.5b01949.
42. C. E. Paulsen, T. H. Truong, F. J. Garcia, A. Homann, V. Gupta, S. E. Leonard and K. S. Carroll, *Nature chemical biology*, 2012, 8, 57-64.
43. J. M. Denu and K. G. Tanner, *Biochemistry*, 1998, 37, 5633-5642.
44. T. H. Truong, F. J. Garcia, Y. H. Seo and K. S. Carroll, *Bioorganic & medicinal chemistry letters*, 2011, 21, 5015-5020.
45. J. Yang, V. Gupta, K. S. Carroll and D. C. Liebler, *Nature communications*, 2014, 5, 4776.
46. Y. H. Seo and K. S. Carroll, *Angewandte Chemie*, 2011, 50, 1342-1345.