Functional Regulation of Dual Specificity Phosphatases: Modifications, Methods for Analysis, and Mass Spectrometry

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Functional Regulation of Dual Specificity Phosphatases: Modifications, Methods for Analysis, and Mass Spectrometry

Christopher A. Bonham

A Dissertation submitted to the Faculty of Graduate Studies through the Department of Chemistry and Biochemistry in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy at the University of Windsor

Windsor, Ontario, Canada.

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Functional Regulation of Dual Specificity Phosphatases: Modifications, Methods for Analysis, and Mass Spectrometry

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I. Co-Authorship Declaration

I hereby declare that this thesis incorporates material that is result of joint research, as follows:

Chapter 3 - Collaboration of Christopher A. Bonham with Adam Faccenda as co-first authors, and Dr. Xueji Zhang, under the supervision of Dr. Panayiotis O. Vacratsis and Dr. Bulent Mutus from the University of Windsor. A.F. synthesized the gold nanoparticles, performed nitric oxide analysis, SDS-PAGE and sample preparation. C.A.B. performed all MS experiments including sample preparation for mass spectrometry (MS) and differential labeling [Scheme 2] sample preparation and analysis. C.A.B., A.F., and B.M. wrote and prepared the manuscript. C.A.B. and A.F. performed revisions and edits. P.O.V. and X.Z. provided experimental insights.

Chapter 4 - Collaboration of Christopher A. Bonham with Dr. Aaron J. Steevensz as co-first authors, and Qudi Geng, under the supervision of Dr. Panayiotis O. Vacratsis from the University of Windsor. C.A.B. and P.O.V. were principal investigators of the research and co-corresponding authors. C.A.B. designed all experimental hypotheses. C.A.B. and A.J.S. performed all experiments, A.J.S. prepared mercury-immobilized affinity chromatography resin. C.A.B., A.J.S. and Q.G. performed mass spectrometry analysis. C.A.B. wrote and prepared the manuscript, A.J.S. and Q.G. prepared the figures. P.O.V. provided experimental insights and edited the manuscript.

Chapter 6 - Collaboration of Christopher A. Bonham with Norah E. Franklin as co-first authors, and Besa Xhabija, under the supervision of Dr. Panayiotis O. Vacratsis from the University of Windsor. C.A.B. designed, isolated, purified and characterized α-pSer58 MTMR2 antibody. C.A.B. and N.E.F. optimized and performed in vitro kinase assays respectively. C.A.B. optimized siRNA of ERK1/2 conditions, performed knock-down immunoblot (IB) experiments and endogenous serum starve immunoprecipitation (IP) and IBs. B.X. performed siRNA and serum starve immunofluorescence (IFA). N.E.F.
performed MAPK inhibition IPs, IBs and IFAs. C.A.B. performed hydrogen-deuterium exchange mass spectrometry experiments, epitope availability IPs and IBs, and PH-GRAM domain deletion IFAs. P.O.V., C.A.B. and N.E.F. wrote the manuscript, C.A.B. and B.X. performed revision experiments, writing and editing.

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ABSTRACT

Protein tyrosine phosphatases (PTPs) are a diverse family of signaling molecules capable of dynamic modes of post-translational regulation. Using a combination of chemical labeling and enrichment, targeted structural mass spectrometry (MS), and immunochemistry, we have discovered unique regulatory mechanisms among two members of the Class I PTP sub-group, VH1-like dual specificity phosphatases (DUSPs). Human YVH1 (hYVH1 or DUSP12) was found to be reversibly regulated by a variety of cellular oxidants, resulting in concomitant enzymatic inactivation and zinc ejection through the formation of disulfide bonds. This was one of the first accounts of PTP oxidation outside of the active site cleft, and of disulfide exchange reactions among PTPs to prevent irreversible oxidation of the active site. Furthermore, using gold nanoparticles and mercury-immobilized metal affinity chromatography, we developed novel methods for studying PTP thiol-oxidation, which can be readily applied to alternative redox-regulated biomolecules and systems biology applications. Similarly, we have optimized the development of a library of multifaceted, low pH thiol-labeling and enrichment reagents for application in quantitative analysis of biological thiols by MS.

Additionally, we have uncovered a mechanism which may participate in regulating sub-cellular localization of the lipid phosphatase myotubularin related-protein 2 (MTMR2). Specifically, we have evidence that dephosphorylation of MTMR2 Ser58 increases accessibility of key residues within a putative lipid-binding domain, which may lead to its stabilized localization to substrate-rich endosomes. Here, MTMR2 depletes phosphatidylinositol 3-phosphate (PI(3)P) substrate molecules, causing mis-localization of resident PI(3)P-binding proteins, and increased activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2). We have further identified ERK1/2 as kinases capable of phosphorylating MTMR2 Ser58. This resultant ERK1/2 activation may then lead to phosphorylation of MTMR2, destabilizing endosomal interactions through competitive shielding of key residues within the lipid-binding domain required for association, and thus completing a functional negative feedback mechanism to remove MTMR2 from PI(3)P-rich early endosomes. This may, in part, be critical for achieving
proper homeostatic endosomal signaling and maturation rates, acting to halt or slow maturation events to allow for increased receptor-signaling responses from these PI(3)P-depleted endosomes until an appropriate threshold is met, triggering ERK1/2-mediated attenuation of the stimulus through MTMR2 phosphorylation.
to those whose sacrifice made this possible, whom bore the brunt of mine
I would first like to thank Dr. Panayiotis O. Vacratsis for the opportunity and privilege to do research in his laboratory at the University of Windsor. His guidance, mentorship, friendship and patience was essential to my development as a research scientist. As he enters his 10th year at the University of Windsor (2013 - 2014), I am proud to of spent the last seven (one undergraduate and six postgraduate) of those years as a part of his research program. Always one to find the good in everything, especially living and enjoying both life and science, reminding us to keep perspective in our lifelong endeavours.

None of this would of been possible without the combined support of the Department of Chemistry and Biochemistry and the Department of Biological Sciences at the University of Windsor, and all the faculty and staff. This endeavour all began with an BSc. from the University of Windsor, and as such, is a testament to all those from these departments whom have taught me and I have acquired knowledge from over my undergraduate term. Moving onto MSc. and PhD. work, I would like to thank Dr. Sirinart Ananvoranich, Dr. Siryiam Pandey, Dr. Micheal B. Boffa, Dr. Lana Lee, Dr. Bulent Mutus, Dr. Marlys L. Kochinsky, Dr. Lisa A. Porter, Dr. Barbara Zielinski, Dr. Andrew Swan, Dr. John W. Hudson, Dr. Panayiotis O. Vacratsis, and members from their lab, past and present, for the use and training on equipment/instruments used for research within this dissertation. I would like to thank Marlene Bezaire, Elizabeth Kickham, Kimberly Lefebvre, Michelle Miglietta, Cathy Wilson for all of their office duties. A special thanks to the combined efforts of Marlene Bezaire and Elizabeth Kickham for all of their assistance over the years, with every and anything, literally. I would like to thank Tina Lepine, Una Lee, Nedhal Al-Nidawy, and Norah Franklin for the shared use of undergraduate lab equipment and reagents. I would like to thank Joe Lichaa for assistance with many computer-related ventures, Janeen Auld for mass spectrometry consultations, Erik Clausen, Louie Beaudry and Sinisa Jezdic for fixing and creating many pieces of lab equipment. I would like to thank Jerry Vriesacker, Candy Donaldson,
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**Figure 2.9 - Proposed model of sustainable activity under conditions of oxidative stress.**  
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**Supplementary Figure S2.1 - Mass fingerprint analysis of the zinc domain deletion variant hYVH1ΔCT1 tryptic peptides.**  
A) Reduced, carbamidomethylated hYVH1ΔCT1 mass fingerprint. Highlighted are the active site and N-terminal vicinal thiol-containing peptides at m/z 1305.74 and 1021.48 respectively.  
B) Oxidized, carbamidomethylated hYVH1ΔCT1 mass fingerprint. Highlighted are the reduced active site and N-terminal vicinal thiol-containing peptides at m/z 1248.65 and 964.27 respectively. Carbamidomethylated peaks are largely reduced or absent. Also highlighted is a putative intra-molecular disulfide peak at m/z 2211.28 of these target peptides. 67
**Supplementary Figure S2.2** - Differential thiol labeling mass fingerprints of hYVH1 tryptic peptides. A) Reduced, carbamidomethylated hYVH1 mass fingerprint. Highlighted is the extreme C-terminal vicinal thiol-containing peptide of the zinc-coordinating domain, m/z 1820.93. B) Reduced, N-ethylmaleimidyl hYVH1 mass fingerprint. Highlighted is the same vicinal thiol-containing peptide, m/z 1956.67. C) Oxidized, carbamidomethylated hYVH1 mass fingerprint. Highlighted is the absence of characteristic peak at m/z 1820.93. D) Differential thiol labeled hYVH1 mass fingerprint. Highlighted is the reappearance of peak m/z 1956.52.

**Scheme 3.1** - AuNP enrichment method for identifying S-nitrosylation sites in proteins. Free thiols of S-nitrosylated proteins are alkylated with iodoacetamide (IAM). Proteins are proteolyzed and AuNPs are introduced to the digest. Any S-NO peptides react with the AuNPs, releasing NO• to form AuNP-thiolate peptides complexes. The resultant AuNP-bound peptides are harvested by centrifugation and eluted off by thiol exchange using small molecular weight thiols. Released peptides are analyzed by mass spectrometry, and target peptide/protein assignment can be done by comparison to *in silico* digestion of known protein sequences or bioinformatic database mining.

**Scheme 3.2** - Simple modification of the AuNP-based enrichment technique discriminates between sites of S-nitrosylation, S-glutathionylation, and free thiols. Free thiols of S-nitrosylated and S-glutathionylated proteins are alkylated with iodoacetamide (IAM). Proteins are de-nitrosylated with ascorbate, differentially alkylated with N-ethylmaleimide (NEM) and proteolyzed. AuNPs are introduced to the digest where any S-NO peptides or free thiols, now S-NEM and S-carbamidomethylated (CAM) peptides, react with the AuNPs, and are enriched. S-glutathionylated peptides do not bind and thus remain in the supernatant. AuNPs are eluted by thiol exchange and released peptides are analyzed by mass spectrometry. Target peptide/protein assignment is confirmed by comparison to *in silico* digestion of known protein sequences or bioinformatic database mining.

**Figure 3.1** - AuNPs bind S-NO-PDI causing release of NO•. A) Increasing amounts of S-NO-PDI in phosphate buffered saline were added 1:1 to a constant amount (~5.3 × 10⁹) of AuNPs in argon-purged, septa-sealed 2 mL vials. A 500 μL aliquot of the headspace was then injected into the NOA to quantify NO•. Data on the graph represents the average of n = 3 independent experiments and *error bars represent S.D.* B) AuNPs from sealed vials were collected, harvested by centrifugation, washed, and incubated for the times indicated with SDS-PAGE sample buffer (+/- 100 mM DTT). The AuNPs were again collected by centrifugation, and the supernatants were resolved by SDS-PAGE. Protein was visualized by silver stain.

**Figure 3.2** - AuNPs successfully enrich thiol-containing peptides from S-NO-PDI Glu-C digests. A) Fully reduced PDI was alkylated with IAM, exchanged into 50 mM ammonium bicarbonate, and proteolyzed by a 10:1 ratio of PDI:Endoproteinase Glu-C. Peptide mass fingerprints were analyzed by MALDI-TOF MS and compared to *in silico* digests as described in 'Experimental Procedures'. B) Fully reduced PDI was S-
nitrosylated by S-nitrosoglutathione (GSNO), then alkylated with IAM, proteolyzed, and analyzed as above. C) Fully reduced PDI was S-nitrosylated, alkylated, and proteolyzed as above. The digest was added to $1.3 \times 10^{10}$ AuNPs and incubated for 30 min at 37 °C. The AuNPs were washed, and AuNP-bound peptides eluted by thiol exchange using 100 mM DTT. Peptide samples were desalted using Vivapure C-18 Microspin columns and analyzed by MALDI-TOF MS as above.

**Figure 3.3 - Tandem mass spectrometry of AuNP-enriched PDI peptides confirms thiol peptide association.** Tandem mass spectrum (MS/MS) of A) $b'$ domain thiol-containing peptide at $m/z = 951$, B) $a$ domain thiol-containing peptide at $m/z = 1692$, and C) $b'$ domain thiol-containing peptide at $m/z = 2352$ were analyzed by post-source decay as described in 'Experimental Procedures'.

**Figure 3.4 - AuNPs successfully enrich thiol-containing peptides from S-NO-hYVH1 tryptic digests.** A) Fully reduced hYVH1 was alkylated with IAM, exchanged into 50 mM ammonium bicarbonate, and proteolyzed by a 10:1 ratio of hYVH1:trypsin. Peptide mass fingerprints were analyzed by MALDI-TOF MS and compared to *in silico* digests as described in 'Experimental Procedures'. B) Fully reduced hYVH1 was S-nitrosylated by S-nitrosoglutathione (GSNO), then alkylated with IAM, proteolyzed, and analyzed as above. C) Fully reduced hYVH1 was S-nitrosylated, alkylated, and proteolyzed as above. The digest was added to $1.3 \times 10^{10}$ AuNPs and incubated for 30 min at 37 °C. The AuNPs were washed, and AuNP-bound peptides eluted by thiol exchange using 100 mM DTT. Peptide samples were desalted using Vivapure C-18 Microspin columns and analyzed by MALDI-TOF MS as above.

**Figure 3.5 - Tandem mass spectrometry of hYVH1 peptides shows presence of S-nitrosylation and S-glutathionylation.** Tandem mass spectrum (MS/MS) of A) active site Cys115-containing peptide at $m/z = 1248$, and B) S-glutathionylated active site Cys115-containing peptide at $m/z = 1553$, were analyzed by post-source decay as described in 'Experimental Procedures'.

**Figure 3.6 - AuNPs successfully enrich differentially labeled, thiol-containing peptides from S-nitrosylated and S-glutathionylated hYVH1 tryptic digests.** A) Fully reduced hYVH1 was S-glutathionylated then alkylated with IAM, and proteolyzed by a 10:1 ratio of hYVH1:trypsin. Peptide mass fingerprints were analyzed by MALDI-TOF MS and compared to *in silico* digests as described in 'Experimental Procedures'. B) Fully reduced hYVH1 was S-nitrosylated and alkylated with IAM. Sample was mixed with an undigested sample from A), then de-nitrosylated with ascorbate and alkylated with N-ethylmaleimide. Proteins were exchanged into 50 mM ammonium bicarbonate and processed as above. C) Digests from B) were added to $1.3 \times 10^{10}$ AuNPs and incubated for 30 min at 37 °C. The AuNPs were washed, and AuNP-bound peptides eluted by thiol exchange using 100 mM DTT. Peptide samples were desalted using Vivapure C-18 Microspin columns and analyzed by MALDI-TOF MS as above.

**Figure 3.7 - Tandem mass spectrometry of hYVH1 peptides shows presence of S-nitrosylation and S-glutathionylation using modified AuNP-enrichment scheme.**
Tandem mass spectrum (MS/MS) of A) active site Cys115-NEM peptide at \( m/z = 1373 \), B) \( S\)-glutathionylated Cys11-containing peptide at \( m/z = 2839 \), and C) \( S\)-glutathionylated Cys23-containing peptide at \( m/z = 3291 \) were analyzed by post-source decay as described in 'Experimental Procedures'.

**Figure 4.1 - Human YVH1 (DUSP12) is a thiol-rich dual specificity phosphatase.** Schematic displaying domain architecture of hYVH1. The N-terminal phosphatase domain contains the highly conserved \( HC_x R(S/T) \) motif along with three additional Cys residues. hYVH1 also possesses a unique C-terminal zinc-coordinating domain which coordinates two moles of zinc putatively through action of seven Cys residues.

**Figure 4.2 - Non-specific oxidation of hYVH1 thiols during proteolytic digestion at neutral pH.** Recombinant hYVH1 and CAM-labeled hYVH1 were subjected to an overnight trypsin digestion using a 1:25 protein:protease ratio in 50 mM ammonium bicarbonate pH 8, shaking at 37 °C. The resulting tryptic peptides were analyzed by MALDI-TOF MS with A) or without B) prior reduction with 25 mM TCEP.

**Figure 4.3 - Schematic workflows of Hg-IMAC-based methodology.** Left) Protein of interest is exposed to oxidative stress, sample is then acidified to quench the reaction and reduce the rate of oxidation “preserving” reversibly oxidized thiols (disulfides, \( S\)-glutathionylated, cyclic sulfenamides, \( S\)-nitrosylated thiols etc.). A rapid proteolytic digest using acid proteases (i.e. pepsin or Glu C) is then performed whereby peptides are then passed over Hg-IMAC resin to capture any reduced or \( S\)-nitrosylated thiols. Captured peptides are eluted using DTT and detected by MALDI-TOF MS. Resin flow-through is split into two fractions, with one fraction left untreated (oxidized thiols) and the other reduced with TCEP (reversibly oxidized thiols). Both fractions are then passed over fresh Hg-IMAC resin, resin is eluted, and eluant analyzed using MALDI-TOF MS. Peptides found in the reduced fraction not found in the non-reduced fraction are putative reversibly oxidized thiols. Right) Protein is treated as above, however, after acid quench, any reduced thiols are blocked with 4-DTP at low pH for 1 h. These reduced thiols have been labeled and can no longer participate in non-specific disulfide formation and can be differentiated from \( S\)-nitrosylated thiols. Moreover, the pH can be raised to 7 - 8 allowing for conventional protein digestion with trypsin and MALDI-TOF MS analysis.

**Figure 4.4 - Characterization of Hg-IMAC selectivity for wild type hYVH1 peptide thiols.** Both CAM-labeled and unlabeled wild type hYVH1 were trypsin digested, mixed, acidified, and incubated with Hg-IMAC resin for 1 hr using acetonitrile additive. Supernatant was collected and bound peptides were competitively eluted using DTT. All samples were analyzed by MALDI-TOF MS. A) Mixed peptides before Hg-IMAC enrichment. B) Flow-thru supernatant showing near depletion of thiol containing peptides while IAM labeled peptides remain. C) Peptide eluant from Hg-IMAC enrichment showing selective thiol-containing peptide enrichment as CAM-labeled peptides were not detected in the eluant.
Figure 4.5 - Characterization of Hg-IMAC selectivity for hYVH1 C115S peptide thiols. Both CAM-labeled and unlabeled hYVH1 C115S were trypsin digested, mixed, acidified, and incubated with Hg-IMAC resin for 1 hr using acetonitrile additive. Supernatant was collected and bound peptides were competitively eluted using DTT. All samples were analyzed by MALDI-TOF MS. A) Mixed peptides before Hg-IMAC enrichment. B) Flow-thru supernatant showing near depletion of thiol containing peptides while IAM labeled peptides remain. C) Peptide eluant from Hg-IMAC enrichment showing specific and selective thiol-containing peptide enrichment. Neither CAM-labeled peptides nor the C115S peptide were detected in the eluant. 114

Figure 4.6 - Characterization of reversibly oxidized thiols in the DUSP domain of hYVH1. hYVH1ΔCT1 was exposed to oxidative stress, acidified and rapidly proteolyzed with Glu C. Peptides were then passed over Hg-IMAC resin to capture any remaining reduced thiols. The resin flow-through was split into two fractions, with one fraction left untreated (oxidized thiols) and the other reduced with TCEP (reversibly oxidized thiols). Both fractions were then passed over fresh Hg-IMAC resin, the resin was eluted, and the eluants analyzed by MALDI-TOF MS. A) TCEP-reduced, Hg-IMAC eluant of reversibly oxidized thiol-containing peptides were observed, highlighting putative candidate(s). B) Non-TCEP-reduced, Hg-IMAC eluant of oxidized thiol-containing peptides were not observed, ensuring primary Hg-IMAC enrichment went to completion. C) TCEP-reduced, Hg-IMAC eluant of reversibly oxidized thiol-containing peptides from A) were CAM-modified with IAM to confirm enriched peptides contained free thiols as denoted by m/z shifts of 57. D) CAM-modified peptides from C) were digested by trypsin and re-analyzed to confirm candidate identification through both improvement of ionization efficiency and target mass identification by tandem MS. 116

Figure 4.7 - Identification of putative disulfide exchange within the active site of hYVH1 using 4-DTP labeling methodology. Both wild type hYVH1 and hYVH1 C115S were exposed to oxidative stress, acidified and rapidly labeled with 4-DTP. Samples were neutralized, digested with trypsin, equally partitioned, with one equivalent being reduced with TCEP, the other untreated. All samples were spotted and analyzed by MALDI-TOF MS. A) Oxidized, 4-DTP-labeled wild type hYVH1 displays putative disulfide linkages. B) Oxidized, 4-DTP-labeled, TCEP reduced wild type hYVH1 shows complete ablation of putative disulfides. C) Oxidized, 4-DTP-labeled hYVH1 C115S displays only a single putative disulfide linkage, suggesting disulfide exchange occurs involving this thiol. D) Oxidized, 4-DTP-labeled, TCEP reduced hYVH1 C115S also shows complete ablation of the lone putative disulfide, further suggesting evidence of exchange reactions. 118

Supplementary Figure S4.1 - Characterization of Hg-IMAC resin. A) Standard curve of Cys-HCl free thiol with DTNB showing linear relationship. B) Binding of Cys-HCl by Hg-IMAC resin as shown by reduction of free thiol with respect to increasing quantities of slurry, showing a linear relationship with slope denoting a binding capacity of 2.2 μM thiol/mL of slurry. C) Box plot for seven independently prepared batches of Hg-IMAC resin (data extrapolated to 1 mL of slurry) showing reproducibility. 120
Supplementary Figure S4.2 - Non-specific oxidation of hYVH1 thiols during proteolytic digestion at neutral pH. Recombinant hYVH1 C115S and CAM-labeled hYVH1 C115S were subjected to an overnight trypsin digestion using a 1:25 protein:protease ratio in 50 mM ammonium bicarbonate pH 8, shaking at 37 °C. The resulting tryptic peptides were analyzed by MALDI-TOF MS with A) or without B) prior reduction with 25 mM TCEP.

Supplementary Figure S4.3 - Characterization of Hg-IMAC selectivity for wild type hYVH1 peptide thiols. Both CAM-labeled and unlabeled wild type hYVH1 were trypsin digested, mixed, acidified, and incubated with Hg-IMAC resin for 1 hr using Tween-20 additive. Supernatant was collected and bound peptides were competitively eluted using DTT. All samples were analyzed by MALDI-TOF MS. A) Mixed peptides before Hg-IMAC enrichment. B) Flow-thru supernatant showing near depletion of thiol containing peptides while IAM labeled peptides remain. C) Peptide eluant from Hg-IMAC enrichment showing selective thiol-containing peptide enrichment as CAM-labeled peptides were not detected in the eluant.

Supplementary Figure S4.4 - Characterization of Hg-IMAC selectivity for hYVH1 C115S peptide thiols. Both CAM-labeled and unlabeled hYVH1 C115S were trypsin digested, mixed, acidified, and incubated with Hg-IMAC resin for 1 hr using Tween-20 additive. Supernatant was collected and bound peptides were competitively eluted using DTT. All samples were analyzed by MALDI-TOF MS. A) Mixed peptides before Hg-IMAC enrichment. B) Flow-thru supernatant showing near depletion of thiol containing peptides while IAM labeled peptides remain. C) Peptide eluant from Hg-IMAC enrichment showing specific and selective thiol-containing peptide enrichment. Neither CAM-labeled peptides nor the C115S peptide were detected in the eluant.

Supplementary Figure S4.5 - Schematic workflow of 4-DTP labeling efficiency and specificity. Protein sample is acidified, labeled with 4-DTP and partitioned equally. Samples are treated with or without TCEP to reduce 4-TP labeling, then neutralized and CAM-labeled with IAM. Samples are digested with trypsin and analyzed by MALDI-TOF MS to ensure specificity and completion of 4-DTP labeling under the given conditions.

Supplementary Figure S4.6 - Characterization of 4-DTP labeling efficiency and specificity for wild type hYVH1 peptide thiols. Wild type hYVH1 was acidified, labeled with 4-DTP and partitioned equally. Samples were treated with A) or without B) TCEP to reduce 4-TP labeling, then neutralized and CAM-labeled with IAM. Samples were digested with trypsin and analyzed by MALDI-TOF MS to ensure specificity and completion of 4-DTP labeling under the given conditions. A) Despite an extreme excess of IAM labeling agent, no CAM modified peptides are observed in the absence of TCEP reduction, whereas in B), multiple CAM modifications are observed.

Figure 5.1 - Synthetic scheme of isothiocyanate-cyclic activated disulfide reagents using oxidized glutathione. In the first step of synthesis, one amino group of excess GSSG reacts with an isothiocyanate derivative (ITC) to form ITC-GSSG. This product is
purified by RP-HPLC, followed by reduction of the disulfide bond by tris(2-carboxyethyl)phosphine (TCEP) generating a free thiol in the glutathione moiety (GSH) to form ITC-GSH. The free thiol is activated by 4,4'-dithiopyridine (4-DTP) forming ITC-GS-4-TP whereby the disulfide is subsequently attacked by the thiol of the thiourea to form the cyclic activated disulfide and release of 4-thiopyridone (4-TP). Adapted from Nielson et al., Anal. Biochem., (2012) 421:1, 115-120.

**Figure 5.2 - Separation of phenylisothiocyanate modified oxidized glutathione derivatives by anion exchange chromatography.** A) Reaction controls from synthetic schemes according to Nielsen et al. were injected and separated to use as comparative references. Reaction solvent (Blank), phenylisothiocyanate (PITC) without oxidized glutathione (GSSG), PITC with GSSG immediately quenched with ethanolamine, and GSSG without PITC were resolved by anion exchange chromatography. B) Synthetic reactions of PITC with GSSG were quenched at 10, 40 or 80 min and resolved by anion exchange chromatography. C) Structure and chemical characteristic of GSSG along with gradient conditions used in separations. In all cases, absorbance at λ = 254 nm and conductivity were monitored continuously. Conductivity of linear sodium chloride (NaCl) gradient shown in red.

**Figure 5.3 - Mass spectra of differentially resolved fractions by anion exchange chromatography.** Synthetic reactions quenched at A) t = 10 and B) 80 min were analyzed by liquid chromatography/mass spectrometry (LC/MS) as described in 'Experimental Procedures'.

**Figure 5.4 - Synthetic scheme of Edman's degradation.** Phenylisothiocyanate (PITC) first reacts with the amino terminus of a protein or peptide under mildly organic alkaline conditions to form a phenylthiocarbamoyl (PTC) adduct. This is then converted by both anhydrous and aqueous acids, and heat, to the phenylthiohydantoin (PTH)-amino acid derivative used in analysis, being accomplished by nucleophilic attack of the carbonyl carbon within the first peptide bond by a formed thiolate resulting from tautomerization of the PTC thiourea. Adapted from Edman, P., Acta Chem. Scand., 4 (1950) 34.

**Figure 5.5 - Suspected route and structure of synthetic by-product formation during phenylisothiocyanate reaction with oxidized glutathione.** A) The unique peptide bond formed in glutathione (GSH) leaves the free α-carboxyl group of glutamate proximally located to the thiourea thiolate for nucleophilic attack and loss of an oxygen, as opposed to attack of the carbonyl and hydrolysis of the amide bond. B) Proposed structure of the synthetic by-product cyclic PITC-GSSG-PITC at m/z = 849. C) Tandem mass spectrum (MS/MS) of synthetic by-product at m/z = 849 as analyzed by liquid chromatography/mass spectrometry (LC/MS) described in 'Experimental Procedures'.

**Figure 5.6 - Modified synthetic scheme of isothiocyanate-cyclic activated disulfide reagents using glutathione and 4,4'-dithiopyridine.** In the first step of this modified synthesis, glutathione (GSH) is reacted with 4,4'-dithiopyridine (4-DTP) to form GS-4-TP. Next, an isothiocyanate derivative (ITC) is added to react with the free amino group to form ITC-GS-4TP, with subsequent conversion to form either the desired cyclic
activated disulfide (ITC-CAD) and 4-thiopyridone (4-TP) or cyclic ITC-GS-4-TP and water ................................................................. 146

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Figure 5.10 - Timed ion chromatograms of phenylisothiocyanate-cyclic activated disulfide show sensitivity to reduction and alkylation. Unpurified reaction mixture containing phenylisothiocyanate cyclic activated disulfide (PITC-CAD) was reacted with A) water, B) N-ethylmaleimide (NEM), C) tris(2-carboxyethyl)phosphine (TCEP) and D) TCEP then NEM. Samples were analyzed by liquid chromatography/mass spectrometry (LC/MS) as described in 'Experimental Procedures', with data representing the timed ion chromatograms of m/z = 441 to detect the loss of PITC-CAD ................................................................. 152

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Figure 5.14 - Theoretical model of the minimal and optimal reaction characteristics involved in the formation of CAD-based reagent design. Reaction constraints would allow 'tuning' of CAD-based reagents through manipulation of the primary sequence of the peptide (R1, R2, R3 etc.), the ability to impose chemical reactivity past the minimal spatial limits of ITC thiolate (Y), and through functional derivatization of the ITC reagent (X). 157

Figure 6.1 - Characterization of a phospho-Ser58 MTMR2 antibody identifies ERK1/2 phosphorylation of recombinant MTMR2 in vitro. A) Phospho-Ser58 and nonphospho-Ser58 peptides were incubated with and without alkaline phosphatase, spotted on nitrocellulose membrane and probed with an in-house generated α-phospho-Ser58 MTMR2 (α-pSer58) antibody. B) HEK293 cells were transfected with empty vector (UT), FLAG-MTMR2, and FLAG-MTMR2 phospho-mimetic variants, FLAG-immunoprecipitated (IP: FLAG), treated with and without alkaline phosphatase, and probed for MTMR2 (IB: α-FLAG) and phospho-Ser58 MTMR2 (IB: α-pSer58). C) Purified bacterial recombinant MTMR2-His6 was incubated with various MAPKs in vitro as described in 'Experimental procedures' and phosphorylation of Ser58 MTMR2 was detected using the α-pSer58 antibody. Total MTMR2 and kinase levels were determined with their respective antibodies. 176

Figure 6.2 - siRNA-mediated depletion of ERK1/2 protein levels reduces MTMR2 Ser58 phosphorylation. A) HEK293 cells were transfected with control scramble siRNA or ERK1/2 siRNA, followed by FLAG-MTMR2 or FLAG-MTMR2 S58A and cultured as described in 'Experimental Procedures'. Cells were stimulated with 5 ng/ml EGF for 10 minutes, lysed and analyzed by immunoblot analysis. B) Graphs represent normalized quantitative immunoblot densitometry data of each indicated antibody to their respective α-actin loading control. Data represents mean and SEM values from at least n = 3 independent experiments, with ***P<0.001, **P<0.01 or *P<0.05. 178

Figure 6.3 - Inhibition of ERK1/2 activity decreases MTMR2 Ser58 phosphorylation. A) HEK293 cells were transiently transfected with empty vector (UT) or with FLAG-MTMR2, serum-starved (- serum) for 30 minutes, then treated with the MEK inhibitor U0126 to inhibit ERK1/2 activation (where indicated). Following stimulation with 5 ng/ml EGF for 5 or 30 minutes at 37°C, cells were lysed, FLAG-immunoprecipitated (IP: FLAG), then probed for phosphorylation of MTMR2 Ser58 by immunoblot (IB).
Immunoblots of all samples for FLAG was used to confirm equal transfections and loading IP levels. Total ERK1/2 and actin lysate immunoblots served as loading controls, while pERK1/2 immunoblots displayed U0126 efficacy and ERK1/2 activation by EGF. B) MTMR2 Ser58 phosphorylation was quantified by densitometry using ImageJ, normalized to FLAG IP levels and represented as fold change relative to serum-starved control without inhibitor. C) Changes in ERK1/2 phosphorylation (pERK1/2) thus activation is represented as the relative change in pERK1/2 levels compared to total ERK1/2 levels. All data represents mean and SD values from at least n = 3 independent experiments, with **P<0.01, *P<0.05.

Figure 6.4 - Endogenous MTMR2 Ser58 and ERK1/2 phosphorylation is influenced by altering serum conditions. A) HEK 293 cells were cultured and processed as described in 'Experimental Procedures', following either serum (+ 10% (v/v) serum; + serum) or low serum (+ 0.5% (v/v) serum; - serum) treatment for ~ 12 h. Both lysate and immunoprecipitates (IP) were subjected to immunoblot (IB) analysis using the indicated antibodies. B) Graphs represent normalized quantitative densitometry data to the indicated loading control (y-axis). Data represents mean and SD values from at least n = 3 independent experiments.

Figure 6.5 - Inhibition of the ERK1/2 pathway induces sub-cellular targeting of MTMR2 to Rab5-positive endosomes. A-C) HeLa cells were transiently transfected with FLAG-MTMR2 or FLAG-MTMR2 S58A and treated with the MEK inhibitor U0126 (ERK1/2) at 50 µM for 1 hour, SB203580 (p38) at 20 µM for 30 minutes and SP600125 (JNK) at 40 µM for 1 hour as indicated and described in 'Experimental Procedures'. Cells were analyzed by immunofluorescence for either FLAG-MTMR2 (grayscale or red) alone or with endogenous Rab5 (green). Merged images display regions of co-localization and are represented in expanded views. Images were collected using 40x oil objectives; Scale bars: 15 μm.

Figure 6.6 - Serum starvation induces subcellular targeting of MTMR2 to Rab5-positive endosomes. A) HeLa cells were transiently transfected with FLAG-MTMR2 and analyzed by immunofluorescence microscopy following either serum (+ 10% (v/v) serum; + serum) or low serum (+ 0.5% (v/v) serum; - serum) treatment as described in 'Experimental Procedures'. Cells were probed for FLAG-MTMR2 (red) and Rab5 (green). B) HeLa cells were treated as above and probed with endogenous MTMR2 (green) and Rab5 (red). Merged images display regions of co-localization and are represented in expanded views. Images were collected using 40x oil objectives; Scale bars: 15 μm.

Figure 6.7 - Depletion of ERK1/2 expression by siRNA induces sub-cellular targeting of MTMR2 to punctate regions. A) HeLa cells were transfected with control scramble siRNA or ERK1/2 siRNA, followed by FLAG-MTMR2 and cultured as described in 'Experimental Procedures'. Cells were stimulated with 5 ng/ml EGF for 10 minutes then analyzed by immunofluorescence analysis of FLAG-MTMR2 (green) and pERK1/2 (red). Images were collected using 40x oil objectives; Scale bars: 15 μm. B) Graphs represent the total number of puncta between control scramble siRNA and ERK1/2
siRNA using automated counting in ImageJ. Data represents mean and SD values from at least \( n = 3 \) independent experiments counting \( n = 10 \) cells per experiment, with ***\( P<0.001 \), **\( P<0.01 \) or *\( P<0.05 \).

**Figure 6.8 - Deuterium exchange of S58E MTMR2 suggests conformational changes may occur in a basic cleft within the PH-GRAM domain.** A,B) Purified bacterial recombinant MTMR2-His\(_6\) and MTMR2 S58E-His\(_6\) was analyzed by HDX-MS as described in 'Experimental Procedures'. Peptides of the PH-GRAM domain in MTMR2 S58E (red) showed significant alteration in their relative deuterium uptake compared to wild-type (black). Highlighted is the signature motif K\( X_n(K/R)XR \) characteristic of high affinity PIP-binding PH domains (increased font size and underlined) as well as neighbouring basic and hydrophobic amino acids (underlined). C) The mutated peptide containing residue S58E also shows decreased uptake (red). The peptide shown has Ser58 for clarity of the mutation site. Graphs represent calibrated average isotopic \( m/z \) values from each individual MALDI-TOF MS spectra and are represented as a collective average value. Primary sequence elucidation was determined by exact mass matching and/or tandem MS using post-source decay as previously described and as in 'Experimental Procedures'.

**Figure 6.9 - Crystal structure of the lipid phosphatase MTMR2 shows residues of the electropositive PH-GRAM domain regions undergoing changes in solvent accessibility upon Ser58 phosphorylation.** A) Crystal structure and B) surface electrostatic potential map of MTMR2 shows the position of target basic peptides found on the membrane proximal face (boxed) with respect to the locale of the active site cleft, both showing a net positive charge, presumably for polyphosphorylated lipid interactions. Inset highlights residues (Lys137, Arg172 and Arg173) responsible for the formation of this basic region within the PH-GRAM domain. Crystal structure and electrostatic potential map of MTMR2 from Begley et al., Proc. Nat. Acad. Sci., 103:4 (2006) 927-932.

**Figure 6.10 - Mutation of target basic residues in the PH-GRAM domain leads to increased accessibility of phospho-Ser58.** A) HEK293 cells were transiently transfected with the indicated FLAG-MTMR2 variants of target PH-GRAM domain residues and analyzed by co-immunoprecipitation (IP) using both \( \alpha \)-FLAG and \( \alpha \)-pSer58, and by immunoblotting (IB) with \( \alpha \)-FLAG, \( \alpha \)-pSer58 and \( \alpha \)-actin. B) FLAG-MTMR2 levels of \( \alpha \)-pSer58 IP samples were quantified by densitometry using ImageJ and normalized to FLAG lysate levels. C) FLAG-MTMR2 pSer58 levels of \( \alpha \)-FLAG IP samples were quantified by densitometry using ImageJ and normalized to FLAG-MTMR2 levels from \( \alpha \)-FLAG IP samples. D) FLAG-MTMR2 lysate levels were quantified by densitometry using ImageJ and normalized to actin lysate levels. All graphs represent normalized quantitative immunoblot densitometry, with data representing the mean and SEM values from at least \( n = 3 \) independent experiments.

**Figure 6.11 - Phospho-dependent endosomal localization of MTMR2 requires basic residues of the PH-GRAM domain.** A) HeLa cells were transiently transfected with the indicated FLAG-MTMR2 variants of target PH-GRAM domain residues along with the endosomal PI(3)P marker EGFP-2xFYVE, and analyzed by immunofluorescence...
microscopy as described in 'Experimental Procedures'. Cells were probed for FLAG-MTMR2 (red) and EGFP (green). Merged images display regions of co-localization, while images were collected using 40x oil objectives; Scale bars: 15 μm. ..................... 191
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACN</td>
<td>acetonitrile</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>APPL1</td>
<td>adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper containing 1</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>AuNPs</td>
<td>gold nanoparticles</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>CAD</td>
<td>cyclic activated disulfide</td>
</tr>
<tr>
<td>CAF</td>
<td>chemically assisted fragmentation</td>
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<tr>
<td>CAM</td>
<td>carbamidomethylated</td>
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<tr>
<td>CG</td>
<td>cysteine-glycine</td>
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<td>CHCA</td>
<td>α-cyano-4-hydroxycinnamic acid</td>
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<td>CIP</td>
<td>calf intestinal alkaline phosphatase</td>
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<tr>
<td>CMT</td>
<td>Charcot-Marie Tooth</td>
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<tr>
<td>Cys</td>
<td>cysteine</td>
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<td>Cys-HCl</td>
<td>cysteine hydrochloride</td>
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<tr>
<td>ΔCT1</td>
<td>C-terminal zinc-coordinating domain deletion mutant</td>
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<tr>
<td>DiFMUP</td>
<td>6,8-difluoro-4-methylumbelliferyl phosphate</td>
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<tr>
<td>DIGE</td>
<td>differential gel electrophoresis</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>D₂O</td>
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<td>DTNB</td>
<td>5,5'-dithio-bis(2-nitrobenzoic acid)</td>
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<td>4-DTP</td>
<td>4,4-dithiopyridine</td>
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<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>DUSP</td>
<td>dual specificity phosphatase</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetracetic acid</td>
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<tr>
<td>EEA1</td>
<td>early endosomal antigen 1</td>
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<td>EGF</td>
<td>epidermal growth factor</td>
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<td>EGFR</td>
<td>epidermal growth factor receptor</td>
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<tr>
<td>ERK1/2</td>
<td>extracellular signal-regulating kinases 1 and 2</td>
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<td>ESI MS</td>
<td>electrospray ionization mass spectrometry</td>
</tr>
<tr>
<td>FA</td>
<td>formic acid</td>
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<tr>
<td>FBS</td>
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<td>FITC</td>
<td>fluorescein-isothiocyanate</td>
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<td>FYVE</td>
<td>Fab1p, YOTB, Vac1p and EEA1</td>
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<td>Glu-C</td>
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<td>GRAM</td>
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<td>GSH</td>
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<td>GSNO</td>
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<td>oxidized glutathione</td>
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<td>H/DX MS</td>
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<td>HEK293</td>
<td>human embryonic kidney cells</td>
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<td>HeLa</td>
<td>Henrietta Lacks (cervical cancer cell line)</td>
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<td>Hg-IMAC</td>
<td>mercury-immobilized metal affinity chromatography</td>
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<td>HPLC</td>
<td>high performance liquid chromatography</td>
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<td>HRP</td>
<td>horseradish peroxidase</td>
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<td>Hsp70</td>
<td>heat shock protein 70</td>
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<tr>
<td>hYVH1</td>
<td>human orthologue of yeast vaccinia H1</td>
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<td>H₂O₂</td>
<td>hydrogen peroxide</td>
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<td>IAM</td>
<td>iodoacetamide</td>
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IB
imunoblot

IEC
ion exchange chromatography

IF
immunofluorescence

IgG
immunoglobulin G

IgY
immunoglobulin Y

IP
immunoprecipitation

ITC
isothiocyante

JNK
c-Jun NH2 terminal kinase

LC
liquid chromatography

MALDI-TOF MS
matrix assisted laser desorption ionization – time of flight mass spectrometry

MAPK
mitogen activated protein kinase

MEK1/2
MAPK/ERK kinases 1 and 2

Met
methionine

MS
mass spectrometry

MS/MS
tandem mass spectrometry

MTM
myotubularin

MTMR2
myotubularin related protein 2

m/z
mass-to-charge ratio

NBD-Cl
7-chloro-4-nitrobenzo-2-oxa-1,3-diazole

NEM
N-ethylmaleimide

NO
nitric oxide

PAR
4-(2-pyridylazo)-resorcinol

PBS
phosphate buffered saline

PCMB
parachloromercuribenzoic acid

PCR
polymerase chain reaction

PC12
Cell line derived from rat adrenal medulla pheochromocytoma

pD
potential of deuterium ion
<table>
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<tr>
<td>PDI</td>
<td>protein disulfide isomerase</td>
</tr>
<tr>
<td>pH</td>
<td>potential of hydrogen ion</td>
</tr>
<tr>
<td>PH</td>
<td>pleckstrin homology</td>
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<tr>
<td>PH-GRAM</td>
<td>pleckstrin homology-glucosyltransferases, Rab-like GTPase activators and myotubularins</td>
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<td>PI</td>
<td>phosphoinositide</td>
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<td>PVDF</td>
<td>polyvinylidene difluoride</td>
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<td>Rab</td>
<td>Ras superfamily of monomeric G proteins</td>
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<td>SDS-PAGE</td>
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<td>TBST</td>
<td>tris-buffered saline with tween-20</td>
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<td>VHR</td>
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<td>ZBD</td>
<td>zinc-binding domain</td>
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CHAPTER 1 - LITERATURE REVIEW

1.1 Regulation through cellular phosphorylation

Since its discovery in the late 1950's by Krebs and Fischer, phosphorylation-dependent regulation has grown to become known as a mechanism involved in nearly all aspects of cellular life [1-3]. This ubiquitous modification of biomolecules is thought to influence ~ one-third of the human proteome, being controlled by the catalytic functions of protein kinases and phosphatases [Fig. 1.1]. The diversity of functional consequences on targeted substrates is overwhelming; modification through phosphate addition or removal can result in the increase or decrease of enzymatic biological activity, stabilization or marking for destruction, facilitation or inhibition of movement between sub-cellular compartments, even in the formation or disruption of protein–protein interactions [3]. Coupled to the availability of phosphoryl donors (ie. ATP), reversible phosphorylation as a regulatory mechanism is a well suited choice as the most general mode of cellular maintenance utilized in eukaryotes.

Phosphorylation of cellular proteins typically occurs on serine, threonine and tyrosine (pSer, pThr and pTyr) residues, however, others residues have been reported as targets. These events are tightly controlled by two protein superfamilies, the protein kinases and the protein phosphatases, whereby the former catalyze the transfer of a phosphate moiety onto target substrates from the γ-phosphate of adenosine triphosphate (ATP), whereas the latter catalyze hydrolysis to regenerate native substrate and inorganic phosphate [Fig. 1.1]. This addition or removal of a phosphate molecule can invoke distinct chemical properties to a given substrate due to its negative charge distribution at physiological pH, thus its ability to form hydrogen and/or electrostatic bonds [4]. Since phosphorylation-dependent regulation is commonly observed among these families of enzymes themselves, this further exemplifies the layers of control afforded through such a mechanism over the orchestration of proper cellular physiology. Disruption of this homeostatic control leads to many disease states including cancer, diabetes and neurodegenerative disorders [5-8].
Figure 1.1 - Protein phosphorylation
1.2 The protein phosphatase superfamily

Although an abundance of cellular regulation is exerted through mechanisms mediated by reversible phosphorylation, the collectively small number of enzymes responsible for catalysis is astonishing. The protein kinase superfamily houses ~ 500 members, whereas the protein phosphatases consist of ~ 140 members, suggesting that among these enzymes, an even greater dynamic of regulation must act to control the fate of some ≥ 10000 protein substrates [9-12]. The protein phosphatase superfamily is partitioned into two main sub-families known as the protein Ser/Thr phosphatases (PS/TPs) and the protein tyrosine phosphatases (PTPs), not only on the basis of substrate specificity, but through large differences among three-dimensional structure, association with protein effectors, regulatory domains and catalytic mechanism [13-16]. The latter is partitioned even further into four sub-families, the largest of which is known as the class I cysteine-based PTPs [Fig 1.2] [6,10]. This sub-family can be further classified into either the classical PTPs or the vaccinia H1 (VH1)-like dual-specificity phosphatases (DUSPs) based on domain architecture and homology between catalytic domains. Dual specificity phosphatase classification originated from their inherent ability to dephosphorylate both pTyr and pSer/pThr containing substrates such as those of the mitogen-activated protein kinase (MAPK) family, however, this catalytic competency and substrate specificity varies greatly among members in this sub-family. For instance, the myotubularins dephosphorylate phosphoinositides (PIPs) [17,18] whereas the atypical DUSPs have been shown to target proteins, nucleics and molecules yet to be defined [10,19]. Interestingly, the first DUSP to be cloned was a protein encoded by a region of the open reading frame within the H fragment of a HindIII digestion of vaccinia virus DNA known as the H1 region (VH1) [20]. And while this enzyme has defined a major sub-family of PTPs, the distinct cellular functions of many of its related eukaryotic counterparts are yet to be defined, designating these enzymes as a members of the atypical sub-group of DUSPs [10,20].

1.3 Catalytic mechanism of protein tyrosine phosphatases

Despite the low sequence identity among the protein tyrosine phosphatase superfamily [14], these enzymes share both a highly conserved catalytic mechanism and domain architecture, in which their activities proceed through a thiol-phosphate intermediate
Figure 1.2 - The protein tyrosine phosphatase superfamily. From Julien et al., Nat. Rev. Cancer, 11:1 (2011) 35-49.
Figure 1.3 - A) Protein tyrosine phosphatase catalytic mechanism and B) deviations among active site clefts. Adapted from Tanner et al., Antioxid. Redox Signal., 15:1 (2011) 77-97 and from Begley et al., Proc. Nat. Acad. Sci., 103:4 (2006) 927-932.
Fig. 1.3A] [21-28]. The active site cleft of PTPs possess an invariant signature motif, HCX_{5}R(S/T), where it is this unique microenvironment that is responsible for reducing the pK_{a} of the catalytic cysteine thiol to enhance competency during the thiolate-based dephosphorylation of a diverse array of phosphorylated substrates [10,29-32]. Deviances in the depth and sequences within the active site cleft, along with the presence of additional regulatory domains, are all thought to assist in specific substrate targeting among the sub-groups within the PTP superfamily [Fig. 1.3B] [10,14,27,33,34].

Redundancy within the catalytic mechanism and domain architecture supports the notion that many of the PTPs exhibit a modular multi-domain arrangement [10]. Thus, most enzymes in this family, in addition to a catalytic domain, exist as multi-domain containing entities posing them for the capability of targeted regulation and diverse functionality [Fig. 1.4]. Although many of these inherent domains are involved in the regulation of protein-protein or protein-lipid interactions, several others are still poorly understood in terms of their functional regulation such as catalytically inactive PTP domains in which critical residues necessary for catalysis have been altered [10]. Some more unique examples include the novel C-terminal zinc coordinating domain of the atypical DUSP human orthologue of the yeast VH1-like phosphatase (hYVH1 or DUSP12) [35], and the N-terminal plekstrin homology-glucosyltransferases, Rab-like GTPase activators and myotubularins (PH-GRAM) domain of the lipid phosphatase myotubularin-related protein 2 (MTMR2) [36,37].

1.4 The dual specificity phosphatase hYVH1

One of the first eukaryotic DUSPs identified was a yeast VH1-like (YVH1) phosphatase cloned from *Saccharomyces cerevisiae* whose mRNA levels were dramatically induced by nitrogen starvation and low temperature [38]. It was later discovered to have a human orthologue (hYVH1), and has since been classified as an atypical DUSP based on its poor characterization and lack of MAPK targeting motifs [10,35]. The hyvh1 gene encodes a ubiquitously expressed, 340 amino acid protein, which in addition to an N-terminal phosphatase domain, possesses a novel C-terminal zinc coordinating domain that has been shown to coordinate 2 mols of zinc per mol of protein [Fig. 1.5] [35]. This cysteine-rich domain is highly conserved among YVH1
Figure 1.4 - Modular multi-domain arrangement of protein tyrosine phosphatases. From Alonso et al., Cell, 117:6 (2004) 699-711.
Figure 1.5 - Domain architecture of the atypical dual specificity phosphatase hYVH1
orthologues with this being the only phosphatase known to contain such a domain [35]. Moreover, orthologues are present as single gene copies and display an overall sequence conservation suggesting that there has been pressure against hyvhl gene duplication throughout evolution.

Deletion of the yvh1 gene in yeast disrupts normal growth processes, resulting in slow growth, a deficiency in spore maturation, and a decrease in glycogen accumulation, whereas insertion and expression of the hyvhl gene is capable of restoring a normal yeast growth phenotype [35,39-41]. Strikingly, although a catalytically defective point mutant is fully capable of complementing the slow growth phenotype, a hYVH1 variant lacking the non-catalytic C-terminal zinc-coordinating domain was unable to afford complementation [40]. It has also been demonstrated that the C-terminal zinc-coordinating domain of hYVH1 is not essential for intrinsic phosphatase activity \textit{in vitro}, however, it is required for interaction with the ATPase domain of heat shock protein 70 (Hsp70) [42]. Similarly, over-expression of wild type hYVH1, but not catalytically dead or zinc-coordinating domain deletion mutants, prevents cell death induced by Fas receptor activation, heat shock, and hydrogen peroxide (H2O2), the first known physiological role for hYVH1 catalytic function [42]. Support of hYVH1 as a pro-survival phosphatase was preceded by a high throughput siRNA screen of HeLa cells showing that knock-down of hyvhl was one of the top human phosphatases whose reduced protein expression led to the induction of apoptosis [43].

Discovering the ability of hYVH1 to protect cells from oxidative stress was rather paradoxical since most cysteine-based PTPs are inactivated under these conditions [28,44-48]. It was later determined, however, that cysteine residues within the zinc coordinating domain serve as a redox buffer, allowing hYVH1 to function under oxidative conditions known to inactivate PTPs [49]. Oxidation by H2O2 resulted in reversible and concomitant zinc ejection and enzymatic inactivation, whereby the zinc ejection correlated with loss of phosphatase activity suggesting a threshold component to the mechanism. As the primary oxidative modification of hYVH1 is formation of disulfide bonds, this mechanism prevents irreversible oxidation of the active site and zinc coordinating cysteines, allowing for restoration of both catalytic activity and zinc
coordination [49]. Since hYVH1 is the only member of the PTP superfamily shown to possess a zinc coordinating domain, this protective mechanism is likely a defining feature among YVH1 orthologues.

More recent findings have elegantly established YVH1 as a novel 60S ribosome biogenesis factor in yeast, and a necessary element for the formation of a fully mature 60S subunit [50-54]. Ribosome profiling revealed that YVH1 co-sediments with the late pre-60S subunit [52], whereas tandem affinity purification confirmed YVH1 association with members of this subunit [51,54]. In yvh1 deletion strains, defects in 60S subunit biogenesis are observed, resulting in increased levels of free 40S subunits, an accumulation of halfmer polysomes, and impaired nuclear export of late pre-60S particles [51,52]. Moreover, a distinct role of YVH1 in 60S subunit maturation has emerged as it has been shown to be an essential 'recycling factor' necessary for regulating the mutually exclusive association of Mrt4 and the stalk protein P0, with the 60S subunit. These events are thought to occur in a linear fashion, each being dependent on the former, whereby Mrt4 is dissociated by YVH1 during cytoplasmic translocation of the pre-60S subunit, followed by YVH1 dissociation by P0 en route to the formation of a mature 60S subunit [Fig. 1.6] [51-53]. This process of stalk protein P0 addition is a necessity for final maturation of 60S ribosomal subunits in the cytoplasm, exemplifying the critical importance of YVH1 in coordination of distinct trans-acting factors. In all cases, a YVH1 variant containing the zinc-coordinating domain alone was able to rescue YVH1 deletion strain phenotypes, suggesting that the catalytic domain is not required for the ribosome biogenesis role(s) of YVH1 [51-53].

Investigations that hYVH1 may possess a similar role in mammalian cells have discovered that it can also associate with the pre-60S ribosomal subunit endogenously. This association is not dependent on catalytic activity, however, regardless of catalytic competency, association is sensitive to treatment with oxidants, with dissociation occurring in a dose-dependent manner. Intriguingly, regions within both the catalytic and zinc-coordinating domains were found necessary for pre-60S association, as deletion of either ablated interaction with this ribosomal subunit [55]. Also, similar to yeast deletion studies, it was observed that knock-down of hvyh1 in HeLa cells promoted the mis-
Figure 1.6 - Linear mechanism of 60S ribosomal subunit maturation. From Lo et al., Mol. Cell, 39:2 (2010) 196-208.
localization of the ribosome trans-acting factor MRTO4, while knock-down of the ribosome stalk protein P0 resulted in failed dissociation of hYVH1 from the late-pre60S subunit and its nuclear exclusion [52,53]. Moreover, P0 knockdown resulted in MRTO4 mis-localization presumably through the inability to promote dissociation of hYVH1 from the pre-60S subunit, thus causing the inability of hYVH1 to promote MRTO4 dissociation for subsequent P0 docking and formation of mature 60S subunits [53]. It will be interesting to investigate if the redox-sensing function of hYVH1 regulates the rate of ribosome biogenesis during cellular stress through its direct associative actions with the late pre-60S ribosomal subunit.

Recent flow cytometry data has revealed that hYVH1 expression levels also regulate cell cycle progression suggesting that hYVH1 may represent a novel mediator of cell growth and proliferation during times of cellular stress. Transient overexpression of hYVH1 caused a significant increase in the G2/M cell population and in polyploidy, which was accompanied by a decrease in the G0/G1 population [56]. Consistent with most other findings, the zinc-coordinating domain is necessary and sufficient for hYVH1-mediated cell cycle changes, whereas phosphatase activity is dispensible. Moreover, siRNA-mediated knock-down of hYVH1 protein expression resulted in a dramatic increase in the G0/G1 population and in cellular senescence. Interestingly, phosphorylation studies of hYVH1 suggested a site of modification at Ser335 which regulates the sub-cellular targeting of hYVH1 and augments the hYVH1 G2/M phenotype [56]. As this site resides in close proximity to the zinc coordinating domain, it is thought the observed phenotypes are exerted through this essential domain, further establishing the crucial role of this novel domain in the physiological functions of hYVH1.

The importance of these functional roles of hYVH1 is highlighted by the fact that hyvh1 has been found up-regulated or amplified in a variety of cancers including neuroblastoma, retinoblastoma, intracranial ependymoma, and chronic myelogenous leukemia [57-60]. The hyvh1 gene is located on chromosome 1q21-23, a region often found amplified in a wide variety of neoplasms, and thus it is highly probable that the hyvh1 gene is overexpressed in other cancers as well [35,61]. It has become increasingly
clear that better characterizing the role of hYVH1 in regulating ribosome biogenesis, cell cycle progression, and cell survival, all processes that support tumour growth and survival, may uncover a direct function in human pathophysiology among these reported pleiotropic roles for human YVH1.

1.5 The myotubularin lipid phosphatase MTMR2

Myotubularins (MTMs) constitute one of the largest sub-groups among the PTP superfamily in eukaryotes [10,62,63], and although the majority of PTPs recognize phosphotyrosine containing proteins as substrates, MTM members dephosphorylate the lipid second messengers phosphatidylinositol 3-phosphates (PI3P) and PI(3,5)P2 [18,64-66]. Through the interaction and recruitment of effector proteins containing appropriate phosphoinositol (PIP)-binding modules, these phosphoinositol (PIPs) exert key contributions to membrane targeting, vesicular trafficking, and regulation of signal transduction pathways. Therefore, it is thought that PI kinases and phosphatases antagonize such effector molecules that utilize PI(3)P or PI(3,5)P2 as targeting ligands and/or allosteric activators [67]. Recent evidence in support of this hypothesis stems from the ability of MTMR2 to dephosphorylate endosomal PI(3)P resulting in mis-localization of the PI(3)P-binding Fab1p/YOTB/Vac1p/EEA1 (FYVE) domain and the novel PI(3)P-binding protein receptor-mediated endocytosis-8 (RME-8) [68,69].

It is not surprising that in addition to the catalytic domain, MTMs themselves possess several regulatory domains suspected to mediate both protein-protein and protein-lipid interactions [Fig. 1.7]. The three dimensional crystal structure of MTMR2 revealed that this enzyme possesses an N-terminal PH-like domain, a module known to associate with PIPs [70-72], within its suspected GRAM domain [37], which has since been coined a PH-GRAM domain [Fig. 1.8] [34,36]. Additionally, one of the more striking characteristics of human MTMs is the presence of at least six catalytically dead members, which have germline substitutions in catalytically essential residues within the PTP active site motif [Fig. 1.7] [63,73,74]. Even more surprising was the discovery that these inactive phosphatases form paired associations with active members, serving as adapters that regulate the localization and activity of their catalytically competent MTM counterparts [75-79]. It is becoming increasingly clear that along with PIP recognition,
**Figure 1.7 - Domain architecture of the myotubularin sub-group of dual specificity phosphatases.** From Begley et al., *Mol. Cell*, 12:6 (2003) 1391-1402.
multiple alternative interactions function in unison to regulate the targeted membrane association of PIP-binding proteins [80]. Combining PIP-association with such mechanisms as oligomerization, co-operative binding, and multiple domains or binding sites, not only acts to alter the stabilization of interactions, it also affords a far greater diversity of regulatory control over signal manipulation than that achieved through PIP levels alone [72,80]. The ability of MTMs to homo-dimerize, combined with the fact that inactive binding partners may function as naturally occurring substrate trapping mutants [81], further highlights the possibility that this multivalent, avidity-based, membrane association known as 'co-incidence detection' may exist among the MTMs [72,75,77,80].

Coming full circle, as first discovered by Krebs and Fischer, phosphorylation is often a central component in the overall regulation of protein function, including that of signaling enzymes. Moreover, their inaugural accounts were also the first evidence of phosphorylation-dependent allosteric regulation of enzymes [1,2]. In the case of lipid phosphatases and MTMs, upon discovery of its physiological substrate, this was all but too evident for the functionally similar lipid phosphatase, phosphatase and tensin homolog (PTEN) [82]. Numerous reports have been published describing the complex nature of PTEN phosphorylation and how this mechanism critically regulates its activities [83]. Although it is not yet fully clear, several lines of evidence suggest that phosphorylation of PTEN results in decreased activity, and the inability to interact with both PIPs and a binding partner that targets and stabilizes it at the membrane [84,85]. Collectively this has led to a model that phosphorylation of PTEN induces a 'closed conformation', whereby phosphorylation prevents interaction with PIPs and membrane stabilizing factors through allosteric blockage of key residues responsible for mediating these associations [85]. Dephosphorylation of these sites induces an 'open conformation' that allows PTEN access to membrane compartments, and thus, to act on its lipid substrate. In a similar, yet unrelated account, the PI(4)P phosphatase synaptojanin has also been found to be regulated by phosphorylation [86]. In resting neurons, synaptojanin is thought to be constitutively phosphorylated by cyclin-dependent kinase 5 (CDK5), inhibiting its interaction with the membrane targeting protein endophilin, resulting in mis-localization and inactivation [87]. In response to the appropriate stimuli, calcineurin dephosphorylates synaptojanin rendering it fully active and available to be
recruited to the proper sub-cellular compartment and perform its lipid phosphatase role [87].

As the lipid substrates for MTMs are also found present on membrane bilayers including endocytic vesicles, the mechanisms that control MTM sub-cellular targeting and/or phosphoinositol recognition are also predicted to be critically important for their function. Unlike PTEN and other well characterized lipid phosphatases, the physiological roles and cellular pathways affected by MTMs are still poorly understood. However, emerging evidence has implicated a role for phosphorylation-dependent allosteric regulation in the control of MTMR2 sub-cellular localization, thus access to its physiological substrates PI(3)P and PI(3,5)P2 [69]. It was observed that phosphorylation potently sequesters MTMR2 from substrate-rich endosomes, whereas a phosphorylation deficient variant strongly co-localized with PI(3)P/Rab5-positive endocytic vesicles. This resulted in depletion of PI(3)P, mis-localization of PI(3)P-binding proteins, and increased activation of growth factor receptor signaling pathways in response to ligand-mediated stimulation, notably in extracellular signal-regulated kinases 1 and 2 (ERK1/2) activity [68,69]. Since catalytic activity and hetero-dimerization with its inactive partner MTMR5 were found to be independent of phospho-status, regulation of MTMR2 sub-cellular localization is thought to occur through an allosteric mechanism [69]. Furthermore, it has been discovered that ERK1/2 are capable of phosphorylating MTMR2, strongly suggesting that the endosomal targeting of MTMR2 may be regulated through a phospho-dependent, ERK1/2 negative feedback mechanism [88]. Even more compelling was the observation that differential MAPK activities were shown to control the shuttling of MTMR2 between Rab5/PI(3)P-rich and adaptor protein containing a pleckstrin homology domain, phosphotyrosine binding domain (PTB) and a leucine zipper motif (APPL1)-containing endosomal subtypes in a phospho-dependent manner [88]. These results highlight that similar to PTEN and synaptojanin, phosphorylation is capable of potent allosteric regulation among the diverse cellular activities involving PIPs through direct influences on lipid phosphatases.

One of the more uncommon attributes of MTMs is that within this sub-group of PTPs, associations of three MTM family members have been well established among distinct
human pathological diseases [77]. Even more puzzling is that mutations in genes encoding highly homologous family members (MTM1 and MTMR2), with similar tissue expression patterns and a common substrate, results in different diseases. The mtm1 gene was discovered to be mutated in X-linked myotubular myopathy (XLMTM), a severe congenital muscular disorder characterized by hypotonia and generalized muscle weakness in newborn males [77,89]. In contrast, mutations in two other MTM genes, mtmr2 and mtmr13, have been shown to cause the neurodegenerative disorders Charcot-Marie-Tooth (CMT) disease 4B1 and 4B2 respectively [90,91]. Charcot-Marie-Tooth disease is one of the most prevalent hereditary neuromuscular diseases, afflicting approximately one in 2500 individuals. Charcot-Marie-Tooth disease 4B1 is an autosomal recessive disease comprised of a demyelinating neuropathy characterized by abnormal Schwann cell proliferation and folding of myelin sheaths in peripheral nerves [77]. The discovery that genetic mutations in the catalytically inactive MTMR13 can cause CMT4B2 was intriguing since it is a known associating partner of MTMR2 [92]. This indicates that mutations in genes whose products associate with active MTMs can result in similar neuromuscular disorders even when wild-type, catalytically active MTMs are present. Altogether, this suggests that MTMs are both differentially and highly regulated enzymes in which continued study is necessary to provide critical insight to links between pathophysiology and cellular activity.

1.6 Mass spectrometry

To draw attention to his recent advancements on Goldstein's discovery of 'kanalstrahlen' (canal rays) or 'positive rays' [93], Thomson published a monograph entitled "Rays of positive electricity and their application to chemical analyses" [94]. In it, he claimed "I feel sure that there are many problems in Chemistry which could be solved with far greater ease by this than by any other method". A bold statement to say the least, but since the publication of this monograph 100 years ago, analysis by means of 'positive rays' is arguably one of the most utilized modes of analysis among the industrial, experimental and natural sciences.

Although contributions by many throughout history have enabled the field of mass spectrometry, many consider Sir John Joseph Thomson (Nobel laureate for discovery of
the electron) as the founding father of mass spectrometry (MS). His discoveries were, however, only plausible due to the work of others in the field, a trait honoured in research science truly to this day. Although widely applicable to volatile and/or thermally stable chemicals, MS did not fully enter the biological sciences until some 75 years later upon the development of matrix-desorption and electrospray ionization by Hillenkamp et al. and Fenn et al. respectively [95,96], and the rest is, well, history.

As pertains to this document, the primary focus will be common MS-based technologies in the study of biological systems. However, to begin, a brief description of the nomenclature, pertinent equipment, and analytical read-out(s) will be outlined [97]. Mass spectrometry is simply the mass analysis of charged, gaseous ions (or pseudo-ions), which is enabled by manipulation of the position of these charged ions in space using electric and/or magnetic fields. To obtain free ions void of any other matter, analysis takes place under low pressure (ie. \textit{in vacuo}). As all elements have naturally occurring isotopes which vary by mass, mass spectrometry concerns itself with the mass of these individual isotopes of the elements, not the atomic mass of the elements, as the latter is the weighted average of the naturally occurring stable isotopes that comprise the element(s). Thus, the detected mass is not that of the molecular weight, but that of an isotope of a given charge (ie. $m/z$, dimensionless) or the mass of an ion's individual isotope(s) on the atomic scale divided by the number of charges it possesses. A molecular ion or precursor ion is formed within the ionization source (ie. a tryptic peptide) and it is the source of all fragment ions. A fragment ion results from the decomposition of precursor or molecular ions (ie. an ion formed by breakage of a peptide bond within the tryptic peptide precursor ion). A peak is a computerized recording of the mass spectrum, with the peaks representing the ions formed within the mass spectrometer (ie. ions are not found in mass spectra and peaks are not found in mass spectrometers). Moreover, peaks have intensities whereas ions have abundances, where in most instances, mass spectral peaks are plotted as relative intensities of the peak of highest intensity (ie. ion of most abundance). To note, mass spectral peaks and chromatographic peaks are different entities, however, common liquid chromatography/mass spectrometry (LC/MS) applications plot the sum of the ion current over time to produce a total ion chromatogram (TIC) (ie. the sum of all ion abundances at a given time over time). From
this, by selecting for a single \( m/z \), a plot of that specific ion current over time can be produced (either during or post analysis), which is referred to as an extracted or timed ion chromatogram (EIC or TIC).

The main features of a mass spectrometer are outlined, along with corresponding pressure requirements [Figure 1.9]; the sample inlet, the ion source, ion guides (arrows), mass analyzer, detector and computerized data system. First, it is evident that analyte must be introduced into the system, and this analyte must become ionized. Two of the most common procedures among biological MS are matrix-assisted laser desorption ionization (MALDI) [96,98] and electrospray ionization (ESI) [95], both being considered 'soft' ionization methods as they do not routinely fragment analytes upon ionization [Fig. 1.10]. As imagined, these processes are fundamentally different, however, achieve the same outcome; formation of charged, gaseous analytes. In the former, analyte is mixed with an organic, ultra-violet (UV) absorbing matrix, in a suitable solvent that has similar solubility characteristics to that of the analyte, to a final concentration of \( \sim 1:5000 \). This provides sufficient protection from subsequent radiation damage, prevents ionic interactions within the analyte upon desorption into the gas phase, and incorporates the analyte into a crystalline matrix critical for the success of MALDI experiments [98,99]. A pulsed \( N_2 \) laser at 337 nm then illuminates the spot, whereby the energy is absorbed by the matrix and transferred through to the analyte en route to gaseous ionization [Fig. 1.10A]. The exact mechanisms involved in the MALDI process of analytes remains under investigation, however, many have contributed to studying such phenomena [100].

In contrast, ESI, as its name implies, involves forcing an analyte, suspended in a suitable volatile solvent, through an electrically charged, fine tipped capillary resulting in the 'sprayed' dispersion of solution into fine droplets. To achieve the highest sensitivity, similar to the necessity for optimal co-crystalization in MALDI, ESI relies on formation of the 'Taylor cone'. This forms as a result of the electric field applied to the capillary, producing electrostatic forces which pull the liquid droplets out of the capillary toward the ground plate [100]. After an \( \sim 1 \) mm flight path, this jet of fluid spreads into a plume visible to the naked eye whereby the droplets rapidly diminish in size due to evaporation
Figure 1.10 - A) Matrix assisted laser desorption ionization and B) electrospray ionization sources
of the volatile solvent in the heated source. This electrostatic force alone is sufficient enough to promote evaporation of the small volumes analyzed during nano ESI (nESI) [101], however, the volumes processed by standard micro ESI sources require the aid of a desolvation gas which flows over the capillary to increase the concentration of analyte ions within the droplets [Fig. 1.10B]. The decreasing size in droplets increases repulsive forces between excess charges within the droplet, ultimately resulting in gaseous ionization of charged analytes [102,103].

Common mass analyzers include the time of flight (TOF) and the triple quadrupole. The operating principles of TOF instruments involve measuring the time required for an ion to travel from the ion source (typically MALDI for TOF instruments) to a detector ~1 - 2 m away [Fig. 1.11A] [104]. All of the charged, gaseous ions formed in the ion source receive the same kinetic energy (KE) during acceleration, and as such, those of different m/z will have correspondingly different velocities when drifting in a field free region (ie. the drift tube). During this time, ions separate into packets based on their velocity, which is directly proportional to their mass as shown in Equation 1.

\[ KE = \frac{1}{2}mv^2 \]  

where KE is the kinetic energy or accelerating voltage, m is the mass and v is the velocity.

Based on this, ions reach the detector in order of increasing m/z. One significant advantage of TOF mass analyzers is there is theoretically no upper limit of m/z values that can be measured, a limitation among most other mass analyzers which transmit and separate ions of different m/z using electric and/or magnetic fields such as triple quadrupoles. Linear TOFs do lack sufficient resolving power, however, refocusing and re-accelerating ions at the end of the drift tube using an ion mirror(s) (ie. reflectron) can circumvent this anomaly and allow for more structural details to be obtained (ie. tandem MS or MS/MS). As with most MS applications, this increased resolution comes at the expense of sensitivity. These two features are often traded off during any MS-based analysis, however persistent development of new hybrid instrumentation, ion optics and
Figure 1.11 - A) Time of flight and B) triple quadrupole mass analyzers
software packages continue to minimize the effects solicited by these compounding issues.

As the name implies, quadrupole instruments are constructed of four (quad) symmetrically arranged rods (or poles) to which an electric current is applied, and thus serve as electrodes. Operation of this mass analyzer is based on the motion of ions in modulating electric fields, whereby the pairs of rods opposite each other are connected to either the positive or negative terminal of a variable direct current (DC) source. In combination, variable radio frequency (RF) alternating current (AC) potentials are also applied to each pair of rods, collectively causing ions to oscillate dimensionally based on their rate of movement through the quadrupole (accelerating voltage), \(m/z\), and the frequency and magnitude of the AC signal [105]. Upon proper tuning of the fields, the ion-tragectory of certain \(m/z\) ratios is stabilized during transit through the quadrupole en route to the detector, while alternatively, any de-stabilized ions collide with the quadrupole electrodes, becoming neutralized and thus unable to reach the detector [Fig. 1.11B] [106]. Through modulating these fields, quadrupoles can reach nearly 4000 \(m/z\) mass ranges, not nearly that of the TOF, but their scanning speed and ease of tuning for targeted approaches makes them very powerful in biological MS applications.

Once charged, gaseous ions are formed and separated by mass, fragmentation of precursors, also known as tandem MS or MS/MS, can yield varying degrees of structural and chemical information. Although others exist, including metastable fragmentation (spontaneous decomposition of ions due to internal energy obtained from ionization in the source; known as post-source decay; PSD), a common approach to induce precursor ion fragmentation is through collision of ions with a neutral, inert gas. In this collisionally activated or induced dissociation (CAD or CID), ions convert some of their translational or kinetic energy into internal energy upon collision with the selected gaseous atoms or molecules, thus causing it to decompose [107,108]. Through the combined influences of proton mobility and relative bond energies within precursor ions, and the control of gaseous composition and collision energies, precursor decomposition can be finely tuned during CID to assist in breakage of targeted chemical bonds, making this a very useful tool in structural mass spectrometry. For instance, tuning the CID can
Figure 1.12 - Nomenclature of peptide precursor ion fragmentation
promote near exclusive peptide bond breakage into fragment ions, however, increasing the energy and/or altering the gaseous composition can result in more random breakage, providing either more or less information depending on that which is sought. Pertaining to the gas-phase fragmentation of peptides, the general model involves charge-directed bond cleavages via proton mobility [109]. Protonation among the peptide backbone initiates the charge-directed breakage of amide bonds, resulting in the formation of either y- or b-type ions, so designated by whether the charge remains on the C-terminal (y ion) or N-terminal (b ion) fragment after bond cleavage [Fig. 1.12].

Combination of CID 'tuning' with triple quadrupole mass analyzers, among others, provides the ability to develop targeted MS-based approaches. In its simplest form, a given precursor can be defined by the compilation of characteristic fragments it decomposes into at a given CID energy and gaseous composition. As such, a precursor can then be identified and defined by any fragment masses which are unique to that given precursor. Tandem MS (fragment ion scans/daughter ion scans) acquires fragment ion data of a given precursor [Fig. 1.13A], which allows one to tune the MS, and specifically focus target fragment ions of given precursors among the first and/or last quadrupoles. Collectively, these analytical approaches are termed precursor ion scans (PIS) and multiple reaction monitoring (MRM) [Fig. 1.13B,C]. As an analogy, these would be similar to fixing a fluorescence emission wavelength (λ) while scanning excitation λ's (in PIS) [Fig. 1.13B], or fixing both the emission and excitation λ's (in MRM) [Fig. 1.13C]. These techniques, referred to as 'targeted proteomics' among biological mass spectrometrists, are extremely powerful due to their accuracy, sensitivity and throughput. Moreover, this technology allows for 'finding needles in haystacks' such as cross-linked or modified peptides within complex mixtures. Detailed fragment ion characteristics allow for PIS to identify any precursor ion that is composed of, or contains, that specific fragment simply by fixing the 'emission λ' and scanning the 'excitation λ' [Fig. 1.13B]. Furthermore, combining targeted MS with targeted chemical labeling and enrichment of proteins, or chemical 'omics', has become a valuable tool in studying post-translational modifications both qualitatively and quantitatively. As these cellular modifications are routinely low in abundance, using such sensitive MS-based methods increases their analytical detection, boasting a clear advantage to their study via integrated MS
Figure 1.13 - Targeted mass spectrometry using A) daughter/fragment ion scanning, B) precursor ion scanning or C) multiple reaction monitoring
approaches. Moreover, coupled to MS, chemical labeling has become a *tour de force* in the field of structural biology in the form of hydrogen-deuterium exchange MS. In this technique, protein is diluted into deuterium oxide, and the base-catalyzed exchange of amide backbone protons with deuterons takes place. This exchange is acid-quenched, the protein rapidly digested by an acid protease, and subjected to MS analysis. The relative deuterium uptake of peptides can be calculated by their corresponding increase in \( m/z \), and thus, inferences on structural changes or binding interactions can be made based on alterations in solvent accessibility between control and stimulated states [*Fig. 1.8B*] [110].

Based on these criteria, it becomes clear to see that alterations in the mass of molecules can yield pertinent structural and potentially functional data. One common example of this among biological mass spectrometry is the addition of a phosphate group to a protein. Upon MS analysis, a mass shift will be observed for the modified residue containing-peptide precursor ion as compared to a dephosphorylated control. Moreover, based on the mass of the precursor, and the fragmentation pattern, not only can the intrinsic peptide sequence be identified, so can the site of modification and perhaps inferred function. This capability has been greatly bolstered by the sequencing of the human genome, which has since taken Thomson's 'positive rays', and mass spectrometry, into the complex world of systems biology.
The objectives of this thesis are to use targeted structural and biological mass spectrometry, immunochemistry, and chemical synthesis as discovery tools in studying the cellular biology of distinct signaling proteins of the protein tyrosine phosphatase superfamily. Of main focus is the redox- and phospho-dependent regulation of human YVH1 and myotubularin related protein-2 (MTMR2).

Specifically, we aim to:

1) Study the mechanism(s) of redox regulation of hYVH1 using spectrophotometry and differential thiol-labeling mass spectrometry

2) Develop novel labeling and enrichment strategies for studying post-translational modification of protein thiols and thiol-dependent biological processes both qualitatively and quantitatively using mass spectrometry

3) Design, isolate/purify, characterize, and employ phospho-specific antibodies and structural mass spectrometry-based methods to study the mechanism(s) of MTMR2 phosphorylation-dependent regulation
1.8 - REFERENCES


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CHAPTER 2 - REDOX REGULATION OF THE DUAL SPECIFICITY PHOSPHATASE HUMAN YVH1 THROUGH DISULFIDE BOND FORMATION

2.1 - INTRODUCTION

Human YVH1 (hYVH1; also known as DUSP12) is a member of the dual specificity phosphatase (DUSP) sub-family of protein-tyrosine phosphatases (PTPs) [1,2]. It is constructed of an N-terminal DUSP catalytic domain and a unique C-terminal zinc-coordinating domain [3]. Poor characterization and lack of mitogen-activated protein kinase targeting motifs further classify this enzyme as an atypical DUSP [1]. Human YVH1 exists as a lone isoform, with orthologues exhibiting high evolutionary conservation and similar domain organization, suggesting that this enzyme may participate in distinct processes fundamental to cellular physiology [3]. Deletion of the yvh1 gene in yeast disrupts normal growth processes [4], whereas insertion and expression of the hyvh1 gene is capable of restoring a normal yeast growth phenotype [3]. Moreover, amplification of the dusp12/hyvh1 gene has been reported in multiple sarcomas, implicating a role for hYVH1 in human pathological diseases [5-7].

Recently, deletion studies from our laboratory have shown that the C-terminal zinc-coordinating domain of hYVH1 is not essential for intrinsic phosphatase activity in vitro, however, it is required for interaction with the ATPase domain of heat shock protein 70 (Hsp70) [8]. Similarly, over-expression of wild type hYVH1, but not catalytically dead or zinc-coordinating domain deletion mutants, prevents cell death induced by Fas receptor activation, heat shock, and hydrogen peroxide (H2O2) [8]. Despite these findings, current information on hYVH1 biochemical, enzymatic and physiological functions remains limited.

PTPs and DUSPs share similar active site architecture and catalytic mechanism, characterized by a conserved HCX5R(S/T) motif [9,10]. The unique microenvironment formed within this catalytic cleft acts to reduce the pKa of an invariant cysteine residue, enhancing both its nucleophilicity and oxidation susceptibility [11,12]. Thus, the stimulated or constitutive generation of reactive oxygen species (ROS) can elicit
oxidative second messenger signaling responses capable of transient and reversible post-translationally inactivation of both PTPs and DUSPs through oxidation of the catalytic cysteine [13-15]. Oxidative susceptibility and modification varies among PTPs and DUSPs, a likely consequence of slight variations in active site conformations or mediated through unique regulatory domains [16-18]. Accumulating evidence suggests that redox-mediated oxidation of PTPs is a dynamic modification that can impose differential regulation among PTP family members [13,19]. Sulfenic acid, cyclic sulfenamide, and disulfide bond formation have all been shown to facilitate stable, reversible, active site modifications among various PTPs and DUSPs [14,20,21]. Furthermore, evidence suggests that oxidation predominantly and rapidly targets the active site cysteine, whereas other cysteinyl residues remain in the reduced state [15,21].

This study investigated the relationship between the C-terminal zinc-coordinating domain and the catalytic domain of hYVH1 during oxidative conditions. We provide data suggesting that the zinc-coordinating domain is targeted during oxidative stress to impede oxidation of the active site cysteine. Increased exposure to oxidative conditions readily induces disulfide bond formation within the zinc-coordinating and catalytic domains, resulting in concomitant zinc ejection and enzymatic inactivation. Zinc ejection is readily reversible and required for hYVH1 activity upon returning to reducing conditions. Thus, we propose a mechanism for protection of phosphatase activity through the intrinsic redox-buffering capacity of this unique zinc-coordinating domain.
2.2 - EXPERIMENTAL PROCEDURES

Cell culture, transfection, and Annexin V assay

HeLa cells (ATCC) were maintained as a monolayer in Dulbecco’s Modified Eagles Medium Nutrient Mixture F12 HAM (Sigma) supplemented with 10% (v/v) fetal bovine serum (Gibco by Life Technologies) at 37 °C and 5% CO₂. Transient transfection was carried out at 70% cellular confluency using FuGENE® 6 HD (Roche Applied Science) according to the manufacturer’s protocol. For H₂O₂ (ACP Chemicals Inc.) treatments, cells were exposed to the indicated concentrations for 1 h at 37 °C. Cell viability was determined using a fluoroscein isothiocyanate-conjugated Annexin V kit (Vybrant Apoptosis Assay Kit 3; Molecular Probes) according to manufacturer’s instructions and as described previously [8]. Cells were also treated with Hoechst 33342 dye (Molecular Probes) and viewed using fluorescence microscopy. Approximately 500 cells were counted per experiment using the Northern Eclipse software program, with apoptotic cells being detected by positive Annexin V staining. The percentage of apoptotic cells in the total sample population, as determined by Hoechst staining, was calculated. The data shown is as the average from \( n = 3 \) independent experiments.

Recombinant protein expression and purification

Bacterial expression of pGEX-4T1 GST-hYVH1 constructs and protein purification was as previously described with slight modification [3,8]. Production of the catalytically inactive variant GST-hYVH1 C115S and the C-terminal zinc-coordinating domain deletion mutant, GST-hYVH1ΔCT1, were as previously described [3,22]. Recombinant proteins were eluted with 50 mM Tris-HCl, 150 mM NaCl, 2.5 mM CaCl₂, 0.1% 2-mercaptoethanol, 80 units of thrombin (Sigma) pH 8, for 4 h at 4 °C from glutathione-agarose (Sigma) to remove the glutathione S-transferase tag. Purified protein samples were concentrated and exchanged into metal-free 50 mM Tris-HCl, 50 mM NaCl, 1 mM dithiothreitol (DTT; Sigma), 0.25 mM phenylmethylsulphonyl fluoride, pH 7.4, using Amicon Ultra-4 centrifugal filters (Millipore) at 4 °C. Metal-free buffers were prepared by passage through a Chelex-100 column (Sigma) to remove contaminating metals, with pH being checked and readjusted (if necessary) after each passage. All
metal-free buffers were tested for residual contaminants prior to experimental analyses via complexometric titrations to ensure trace levels were below the experimental detection limits. Protein concentration was determined against standard bovine serum albumin as per Bradford assay (Bio-Rad), and samples were aliquoted and stored at -80 °C until use. The purity of all proteins in this study was ~ 80–90%, as judged by SDS-PAGE analysis.

**Oxidation-induced enzymatic inactivation, thiol loss, and zinc ejection**

Phosphatase activity was monitored at 30 °C using the artificial substrate analog 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP; Invitrogen). Protein samples were exchanged into preheated (30 °C), metal-free 50 mM Tris-HCl, 50 mM NaCl, pH 7.4 using 0.5 mL Zeba™ Desalt Spin Columns (Pierce Biotechnology) followed by immediate treatment with H₂O₂. Concentrated stock solutions of H₂O₂ were prepared in non-chelex treated buffer to initiate production of ROS, and were subsequently diluted into metal-free buffer for use in experimental analyses. At defined time points, aliquots were mixed with 0.9 mM DiFMUP in 50 mM Tris-hydroxyaminomethane, 50 mM bis-Tris, 150 mM NaCl, pH 6, similar to previously described methods [20,22]. Samples were loaded into a 96-well FluroNunc plate (Sigma), and initial velocity data were recorded (DiFMUP; λ<sub>ex</sub> = 358 nm; λ<sub>em</sub> = 450 nm). Data were expressed as percentage activity of reduced hYVH1 samples in 5 mM DTT. Specific activity values were calculated and compared for both fully reduced hYVH1 and hYVH1ΔCT1. As a comparative control, phosphatase activity of the archetypical DUSP, vaccinia H1 related (VHR; Biomol), was monitored under similar conditions. Similarly, at defined time points of oxidation, samples were incubated with 20 mM DTT for 30 min to quench oxidation, followed by addition of DiFMUP as above to assess the reversibility of activity. Thiol quantitation was accomplished using a modified 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB; Sigma) assay similar to one described [23]. Samples, as those above, were treated for 10 min with defined concentrations of H₂O₂ and then incubated for 5 min with ~ 50 units of catalase. Reactions were mixed with 500 μM DTNB in 50 mM Tris-HCl, 50 mM NaCl, pH 7.4, for 30 min and absorbance was monitored (ε = 14
150 L*mol⁻¹*cm⁻¹ @ λ_{Abs} = 412 nm) [24]. Similarly, zinc release was monitored using a 4-(2-pyridylazo)-resorcinol/parachloromercuribenzoic acid (PAR/PCMB; Sigma)-based assay as described [25]. Aliquots were taken at defined time points (as above) and mixed with metal-free 100 μM PAR in 40 mM potassium phosphate buffer pH 7.5, and absorbance was monitored (PAR; ε = 66 000 L*mol⁻¹*cm⁻¹ @ λ_{Abs} = 500 nm). Addition of 50 μM PCMB in 100 μM PAR solution elicited complete zinc release, and the total absorbance was recorded. Data were expressed as percentage of total zinc (free zinc + protein bound zinc). All data represents the average result from at least n = 3 independent experimental analyses.

**Probing thiol redox-status using MALDI-TOF mass spectrometry**

A differential thiol labeling strategy similar to one previously described [25] was utilized to assess thiol redox-status in response to oxidation. Full length hYVH1 and hYVH1ΔCT1 were oxidized with 100-fold molar excess H₂O₂ for 1.5 h, precipitated with cold (-20 ℃) 10% trichloroacetic acid (wt/vol), and washed three times with cold (-20 ℃) 100% acetone. Pellets were resuspended in denaturing buffer (6 M Urea, 200 mM Tris-HCl, 10 mM EDTA, pH 8.5) supplemented with 100 mM iodoacetamide (IAM; Sigma) shaking for 1 h at 25 ℃. Each sample was divided and precipitated as above. Samples were resuspended in denaturing buffer in the presence or absence of 10 mM DTT shaking for 45 min at 25 ℃, followed by the addition of 100 mM N-ethylmaleimide (NEM; Sigma) shaking for 1 h at 25 ℃. Samples were again precipitated as above, resuspended in 50 mM ammonium bicarbonate, and digested with either sequencing-grade trypsin (Promega) or endoproteinase Glu-C (Roche Applied Science). Aliquots were collected at various time points, quenched with a final concentration 1% formic acid, and stored at -20 ℃ for future matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis. To further assist identification of inter-peptide disulfide bond formation, a tryptic digest of oxidized, iodoacetamide-labeled, wild-type hYVH1 was modified using the Ettan chemically assisted fragmentation (CAF) MALDI sequencing kit (Amersham Biosciences) as per the manufacturer’s protocol, without lysine modification. The resulting peptides have a
derivatized sulfonic acid group (136 Da) added to all free amines. In all cases, digests were spotted 1:1 with 10 mg/mL matrix solution (α-cyano-4-hydroxycinnamic acid in 60% acetonitrile, 1% formic acid; Sigma) on the target plate by the dried-droplet method. Peptide mass fingerprints and tandem mass spectrometry (MS/MS) using post source decay (PSD) was performed on selected parent ions as previously described [8] and compared to in silico fragmentation using the Protein Prospector software (http://prospector.ucsf.edu/).

**Metal-free enzymatic activity, reversibility of zinc binding and sulfenic acid analysis**

Protein samples were exchanged into metal-free 50 mM Tris-HCl, 50 mM NaCl, pH 7.4, as above, then treated for 10 min with or without a 2.5-fold molar excess of PCMB/total thiol content. Samples were exchanged again to remove excess PCMB and free zinc, incubated with or without 5 mM DTT for ~ 30 min, mixed with 0.9 mM DiFMUP as above, and analyzed for phosphatase activity. Similarly, full length hYVH1 was treated with PCMB, as above, or with 100-fold molar excess H₂O₂ for 1.5 h, to elicit zinc ejection. Samples treated with PCMB were incubated in the presence or absence of slight excess DTT for ~ 10 min without previous buffer exchange to facilitate reversible zinc binding. Samples oxidized with H₂O₂ were incubated in 50 units of catalase, and then in the presence or absence of slight excess DTT without previous buffer exchange. Slight excesses of DTT were necessary as it was observed that higher amounts of DTT effected zinc-PAR interactions, presumably through equilibrium based competitive binding. All samples were mixed with 100 μM PAR and the absorbance was monitored as described above. Thiol oxidation state was also monitored using the electrophilic reagent 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl; Sigma) as described [20,26]. Full length hYVH1 was exchanged, oxidized and quenched as above, then incubated with 1 mM NBD-Cl for 1 h. Samples were again exchanged as above and compared to a non-oxidized, NBD-Cl treated control by monitoring the absorbance range across λ₄₅₄ nm. All data represents the average result from at least n = 3 independent experimental analyses.
2.3 - RESULTS

*hYVH1 mediates protection against hydrogen peroxide induced cell death*

Our previous work demonstrated that hYVH1 can associate with Hsp70, and potently defend cells from insults such as heat shock, Fas receptor activation, and H$_2$O$_2$ [8]. Moreover, a catalytically inactive or a zinc domain deletion variant (hYVH1ΔCT1) failed to yield protection, indicating that both the phosphatase activity and the zinc coordinating domain are required for the cell survival function of hYVH1. To test the extent of hYVH1 cytoprotective ability, we subjected HeLa cells to increasing amounts of H$_2$O$_2$. Cells expressing hYVH1 were substantially more resistant to H$_2$O$_2$ cytotoxicity at 100 and 250 µM final concentrations as compared to control cells; ~ 27 fold and 7 fold less cell death respectively [Fig. 2.1]. However, at 500 µM H$_2$O$_2$, hYVH1 failed to protect cells to any significant extent. These results suggest that hYVH1 can protect cells up to a threshold level of oxidative stress, above which there may be a mechanism in place to down-regulate the cell survival ability of hYVH1.

*hYVH1 resists oxidative inactivation in vitro*

From our previous work it was also determined that hYVH1 catalytic activity exhibits substantial thermal stability *in vitro* when directly compared to the prototypical DUSP VHR. Moreover, we determined that full length hYVH1 had increased sustainability of catalytic activity under non-reducing and combined heat-shock/non-reducing conditions when compared to the catalytic domain variant hYVH1ΔCT1 [8]. It is believed that the nucleophilic cysteine residue within the active site of PTPs and DUSPs is rendered susceptible to oxidation under non-reducing conditions due to its reduced pK$_a$ value. However, hYVH1 possesses a unique zinc-coordinating domain containing seven Cys residues with suspected involvement in the coordination of 2 mols of zinc per mol of protein [3]. We hypothesized that by being an oxidative target itself, this domain may reduce the susceptibility of redox-induced inactivation at the active site Cys. Again, as a control, VHR was used, which shares 25% sequence identity with hYVH1 in the DUSP catalytic domain and has been shown
Figure 2.1 - hYVH1 protects cells from oxidative stress. HeLa cells were transiently transfected with empty vector (Control) or hYVH1 and treated with H$_2$O$_2$ (100 µM, 250 µM or 500 µM) for 1 hr at 37°C. A) Cell viability was detected using FITC-conjugated Annexin V as per manufacturer’s protocol and total cell number was determined using Hoechst 33342 dye. B) Graphical representation from $n = 3$ independent experiments expressing cell death as a percent of total cell number. Error bars represent the S.D.
sensitive to reversible H$_2$O$_2$-induced inactivation through formation of a stable sulfinic acid [20]. To assess oxidative resistance, wild type hYVH1, zinc domain deletion mutant (hYVH1ΔCT1), and VHR were subjected to H$_2$O$_2$ then assayed for phosphatase activity towards the exogenous substrate DifMUP. Under reducing conditions, the activity of VHR was significantly greater than that of both hYVH1 and hYVH1ΔCT1 [Fig. 2.2A]. In comparison, removal of the zinc-coordinating domain did not show significant deviation in activity when compared with wild type under reducing conditions [Fig. 2.2A]. In stark contrast, exposure to H$_2$O$_2$ at a molar ratio of 1:100 rapidly inactivates VHR (by ~ 45% after 2.5 min and ~ 60% after 10 min). Meanwhile, hYVH1 displayed greater resistance to the oxidative conditions, displaying activity loss of ~ 25% after 2.5 min and ~ 35% after 10 min [Fig. 2.2A,B]. The hYVH1ΔCT1 variant experienced activity loss similar to that of VHR, suggesting that the inherent resistance to inactivation by hYVH1 is mediated by the zinc-coordinating domain. The addition of excess DTT, post-oxidation, resulted in nearly complete regeneration (~ 80%) of phosphatase activity among all enzymes [Fig. 2.2B]. Furthermore, wild type hYVH1 demonstrated similar resistant behavior when titrated across an increasing molar ratio of H$_2$O$_2$ when compared with VHR and hYVH1ΔCT1 [Fig. 2.2C]. Collectively, these results demonstrate that the zinc-coordinating domain provides hYVH1 the capacity to impede redox-induced inactivation.

**Hydrogen peroxide-mediated thiol oxidation leads to zinc release**

The above findings suggest that the thiol-rich, C-terminal zinc-coordinating domain may be involved in resisting oxidative inactivation of hYVH1. To examine the effects of oxidative conditions on hYVH1 zinc coordination, we performed a PAR/PCMB assay to monitor zinc release in response to oxidation by H$_2$O$_2$. At ratios of H$_2$O$_2$ similar to those examined above, zinc was readily released from hYVH1. Moreover, the loss of phosphatase activity seemed to parallel the observed rate of zinc ejection at lower concentrations of H$_2$O$_2$ [Figs. 2.3A-C]. In all cases, spontaneous thiol oxidation and zinc release was determined to be negligible over the defined experimental times. These results suggest that zinc ejection and activity loss during increasing levels of oxidative
**Figure 2.2 - Analysis of hYVH1 inactivation by H\textsubscript{2}O\textsubscript{2}.**  
**A-B)** Inactivating effects of H\textsubscript{2}O\textsubscript{2} on DUSP catalytic activity.  
**A)** Initial velocity raw data comparing hYVH1, hYVH1\textDelta{}CT1 and VHR after incubation at 30 °C in a 1:100 molar ratio of H\textsubscript{2}O\textsubscript{2} or 5 mM DTT for 10 min.  
**B)** Designated enzymes (□ hYVH1, ■ hYVH1\textDelta{}CT1, □ VHR) were incubated as above for the defined time points then monitored for relative changes in activity. Inactivation and reactivation is expressed as percentage of fully reduced enzyme without H\textsubscript{2}O\textsubscript{2}.  
**C)** Similarly, designated enzymes were oxidized at the defined molar ratios for 20 min then assayed for remaining activity. Data was fit to a one phase exponential decay equation using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA, [www.graphpad.com](http://www.graphpad.com). All data analyses represent the mean of at least $n = 3$ independent determinations. Error bars represent the S.D.
stress is probably due to thiol oxidation within both the zinc-coordinating and catalytic domains. Since multiple Cys residues must become oxidized before zinc ejection is detected, the above trend also suggests that the zinc-coordinating cysteines may be preferentially oxidized over the active site Cys.

To examine thiol oxidation status more directly, a DTNB-based assay was performed. The catalytically dead mutant (hYVH1 C115S) was also analyzed to obtain some insight into the oxidation state of the active site cysteine residue. From primary sequence analysis, wild type hYVH1 and the hYVH1 C115S mutant contain 11 and 10 cysteine residues respectively, which were consistent with observed findings of total thiol content/mol of protein [Fig. 2.3D]. Upon treatment with increasing H$_2$O$_2$ concentrations, rapid loss of multiple free thiols was observed within 10 minutes as compared to untreated control. Since oxidation of the active site is reversible, conversion of the catalytic cysteine to a sulfenic or sulfonic acid is likely not occurring at appreciable levels, however, if a reversible cyclic sulfenamide or sulfenic acid is formed, we would expect an equal number of free thiols between wild type hYVH1 and the hYVH1 C115S mutant. Experimental evidence demonstrated that upon oxidation, wild type hYVH1 harboured approximately one less free thiol than the hYVH1 C115S mutant, which rather suggested that the active site cysteine may be participating in reversible intra-molecular disulfide bond formation during oxidative stress [Fig. 2.3D].

**Oxidation of hYVH1 results in intra-molecular disulfide bond formation**

The above data suggest that the active site Cys of hYVH1 may be capable of forming an intra-molecular disulfide bond. This ability would represent a mechanism to avoid irreversible oxidation during even extreme oxidative stress. Therefore, to further confirm the DTNB data, disulfide bonding status of both reduced and oxidized hYVH1 was analyzed using MALDI-TOF MS through the application of differential thiol labeling methodology [Fig. 2.4A]. Mass fingerprints of reduced and oxidized, carbamidomethylated (CAM) hYVH1 tryptic digests were obtained and compared for target peptide identification [Fig. 2.4B]. Reduced, CAM-hYVH1 displayed the
Figure 2.3 - hYVH1 zinc release, inactivation and thiol depletion by H₂O₂ exposure.  
**A-B)** Plots of hYVH1 time dependent enzymatic inactivation (●) and zinc release (□).  
Enzyme was incubated in increasing molar ratios of H₂O₂ (A, 1:50; B, 1:100) for the defined time points then analyzed for changes in activity or zinc release as described in “Experimental Procedures”.  
**C)** Similarly, hYVH1 was oxidized at the defined molar ratios for 20 min then assayed for remaining activity and bound zinc.  
**D)** Thiol oxidation of hYVH1.  hYVH1 (□) and hYVH1 C115S (■) were incubated with increasing molar ratios of H₂O₂ (1:0, 1:100, 1:500, and 1:1000) for 10 min then thiol content was analyzed using a DTNB-based assay as described in “Experimental Procedures”.  
Data was normalized to the concentration of enzyme.  All data analyses represent the mean of at least n = 3 independent determinations.  Error bars represent the S.D.
Figure 2.4 - MALDI-TOF MS approach to probe hYVH1 thiol redox status.  

**A)** Schematic diagram of the differential thiol labeling strategy utilized to identify Cys specific modifications of hYVH1 in response to H₂O₂-mediated oxidation.  

**B)** Table of target hYVH1 Cys containing tryptic and glutamic peptides.

<table>
<thead>
<tr>
<th>Residue</th>
<th>Protease</th>
<th>m/z</th>
<th>m/z</th>
<th>m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>97 - 105</td>
<td>CVAFIGQAR</td>
<td>964.50</td>
<td>1021.53</td>
<td>1089.55</td>
</tr>
<tr>
<td>110 - 121</td>
<td>AVLHGHAGVSR</td>
<td>1248.66</td>
<td>1305.68</td>
<td>1373.71</td>
</tr>
<tr>
<td>297 - 311</td>
<td>LGSFNWYGEQGSGGR</td>
<td>1706.71</td>
<td>1820.75</td>
<td>1956.80</td>
</tr>
</tbody>
</table>

**Start-End** | **Endoprotease** | **Glu-C (V8E)** |
<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>291 - 310</td>
<td>(D)GQLLCFKCSAKLGSFNWYGE</td>
<td>2201.05</td>
</tr>
<tr>
<td>311 - 331</td>
<td>(E)CSCGRWITPAQHIHKNRVDE</td>
<td>2488.20</td>
</tr>
<tr>
<td>222 - 244</td>
<td>(E)VLKCRKRRSLFRSSSLDHRE</td>
<td>2852.53</td>
</tr>
<tr>
<td>221 - 244</td>
<td>(D)EVLYKCRKRRSLFRSSSLDHRE</td>
<td>2981.57</td>
</tr>
</tbody>
</table>
characteristic active site peak at m/z 1305.74 along with that of an N-terminal proximal thiol at m/z 1021.48. No significant amounts of unmodified peptides were observed [Fig. 2.5A]. Oxidized CAM-hYVH1 displayed almost undetectable levels of both of these target peptides; however, both the active site and proximal thiol peptides appeared in their reduced form at m/z 1248.65 and 964.47, respectively. Interestingly, a peak at m/z of 2211.28 appeared, which is ~2 Da less than the resultant sum of these target peptides, suggesting formation of an intra-molecular disulfide bond [Fig. 2.5B]. The remaining tryptic digest was derivatized using CAF reagent labeling to facilitate unambiguous identification of this unknown peptide. Using this strategy, an intra-molecular disulfide-bonded peptide, upon sulfonation, would be doubly modified, once at each N-termini, causing a mass shift of 272 Da, which was detected for this peptide [m/z 2483.38; Fig. 2.6A]. Unambiguous identification was accomplished using MS/MS analysis by PSD of this parent ion, and the formation of an intra-molecular disulfide bond between the active site cysteine and the N-terminal proximal cysteine was confirmed [Fig. 2.6B].

Similarly, mass fingerprints of reduced CAM- or NEM-hYVH1 Glu-C digests were obtained and compared for target peptide identification [Fig. 2.4B]. Oxidized CAM-hYVH1 was subjected to differential thiol labeling, as described under “Experimental Procedures”, and mass fingerprints of all Glu-C digests were recorded. Reduced CAM or NEM digests displayed characteristic peaks, which were assigned to vicinal thiol-containing peptides of the C-terminal zinc-coordinating domain [Fig. 2.7A,B]. Upon oxidation, a loss of CAM m/z peaks 2315.26, 2602.66, and 2967.30 is observed, whereas formation of one NEM peptide at m/z 3101.84 is evident, suggesting that some reduced thiols exist during oxidation [Fig. 2.7C]. Oxidized, differentially labeled, CAM-hYVH1 resulted in the appearance of target NEM peaks at m/z 2738.61, 3231.65, and 3106.07, which correspond to the reduced NEM digest [Fig. 2.7B,D]. These observations suggest that in addition to the ability of the active site Cys to form an intra-molecular disulfide bond, the zinc-coordinating Cys residues in the C-terminal domain are putatively involved in a distinct disulfide bonding network. In both cases, higher oxidation states (sulfinic or sulfonic acids) of the thiolate moieties were not observed, further confirming predominant formation of disulfide bonds within both domains.
Figure 2.5 - Mass fingerprint analysis of hYVH1 tryptic peptides.  

A) Reduced, carbamidomethylated hYVH1 mass fingerprint. Highlighted are the active site and N-terminal proximal thiol-containing peptides at \( m/z \) 1305.74 and 1021.48 respectively.  

B) Oxidized, carbamidomethylated hYVH1 mass fingerprint. Highlighted are the reduced active site and N-terminal proximal thiol-containing peptides at \( m/z \) 1248.65 and 964.27 respectively. Carbamidomethylated peaks are largely reduced or absent. Also highlighted is a putative intramolecular disulfide peak at \( m/z \) 2211.28 of these target nentides.
Figure 2.6 - Mass fingerprint and MS/MS PSD analysis of CAF labeled hYVH1 tryptic peptides.  

A) Oxidized, carbamidomethylated, CAF labeled hYVH1 mass fingerprint. Highlighted is the doubly CAF labeled putative disulfide peak at m/z 2483.38.  

B) MS/MS analysis of the peak at m/z 2483.38. The y ion fragment series corresponds to the two target peptides AVLHVCHAGVSR and CVAFIGQAR denoting unambiguous identification of active site disulfide bond formation.
Figure 2.7 - Differential thiol labeling mass fingerprints of hYVH1 C-terminal glutamic peptides. A) Reduced, carbamidomethylated hYVH1 mass fingerprint. Highlighted are three vicinal thiol-containing peptides within the zinc-coordinating domain, m/z 2315.26, 2602.66, and 2967.30. B) Reduced, N-ethylmaleimidy l hYVH1 mass fingerprint. Highlighted are two of the vicinal thiol-containing peptides, m/z 2738.35, 3102.84, and 3231.43. C) Oxidized, carbamidomethylated hYVH1 mass fingerprint. Highlighted is the absence of characteristic peaks at m/z 2315.26, 2602.66, and 2967.30, and the presence of a peak at m/z 3101.84. D) Differential thiol labeled hYVH1 mass fingerprint. Highlighted is the reappearance of peaks at m/z 2738.61, 3231.65, and 3106.07.
Zinc coordination is reversible and required for optimal enzymatic activity

Disulfide bond formation within the zinc-coordinating domain would allow this domain to bind zinc under reducing conditions, and to form disulfide bonds under oxidative conditions, in a reversible, and thus, regulatory fashion. To assess if the C-terminal domain is capable of potentially governing regulatory events, reversibility of zinc coordination was tested. Treatment with either the thiol-reactive reagent PCMB or with H$_2$O$_2$ results in essentially complete zinc ejection in the absence of reducing reagents. This phenomenon is readily and rapidly reversible to near completion (~85%) upon the addition of reducing agents [Fig. 2.8A]. Interestingly, upon returning to reducing conditions, re-binding of zinc seems to be a necessity for complete recovery of catalytic activity. Complete zinc ejection and removal from the system leaves hYVH1 with ~40% activity in comparison to when hYVH1 zinc-binding is restored under reducing conditions [Fig. 2.8B]. Notably, the C-terminal zinc-coordinating domain deletion variant hYVH1ΔCT1 retains full activity under similar conditions [Fig. 2.8B]. This suggests that once the C-terminal cysteines are re-reduced, zinc coordination is required to prevent disturbing the phosphatase domain.

Collectively, the above results suggest that oxidation of hYVH1 is a reversible event. The non-enzymatic, reversible oxidation of thiols can only readily occur at lower thiol oxidation states, which include disulfide bonds, cyclic sulfenamides and sulfenic acids. To further confirm disulfide bond formation upon oxidation of hYVH1, NBD-Cl was used to probe for the presence of sulfenic acid modifications. Upon interaction with a free thiol, the formed adduct displays a $\lambda_{\text{max}}$ of 420 nm; however, interaction with a sulfenic acid results in formation of an adduct having a $\lambda_{\text{max}}$ of 347 nm, ultimately allowing for spectral identification between these species [20,26]. When NBD-Cl was used to probe hYVH1, no visible evidence of sulfenic acid formation was observed upon oxidation, confirming that disulfide bond formation is the predominant oxidative modification of hYVH1 cysteine thiols [Fig. 2.8C]. Taken together, hYVH1 is capable of reversible oxidation in both catalytic and zinc-coordinating domains, which is presumably mediated through disulfide bond formation.
Figure 2.8 - Effects of reversible zinc coordination on enzymatic activity and analysis of sulfenic acid formation.  

A) Reversible zinc coordination of hYVH1 under reducing conditions. Complete zinc ejection via H₂O₂ (■) or PCMB (●) is rapidly reversible upon subsequent treatment with slight excess of reducing agent.  

B) Effects of zinc coordination on enzymatic activity. Treatment of hYVH1 (□) or hYVH1ΔCT1 (■) with PCMB reversibly modifies all thiol residues, resulting in zinc ejection. Reduction with DTT after desalting restored hYVH1ΔCT1 with full activity, while hYVH1 remained partially inactive.  

C) Probing oxidized hYVH1 for sulfenic acid modification. Oxidation of hYVH1 results in loss of free thiols and preferential formation of reversible cystine bonds, not cysteinyl sulfenic acids. Data analyses (A-B) represent the mean of at least n = 3 independent determinations. Error bars represent the S.D. Data analysis (C) represents an individual trial of n = 3 independent experiments.
2.4 - DISCUSSION AND CONCLUSIONS

This current study has added insight to our initial findings that hYVH1 acts as a cell survival phosphatase in response to oxidative stress conditions. Moreover, we have revealed that the zinc-coordinating domain is critical for hYVH1 to resist oxidative inactivation of its phosphatase activity. Although other phosphatases have shown the ability to resist oxidation of their active sites by various mechanisms [16-18,21], this is the first report to demonstrate the involvement of a dynamic zinc-coordinating domain in facilitating oxidative resistance for a phosphatase.

The prototypical DUSP, VHR, was found to have higher activity than hYVH1 toward the artificial substrate DiFMUP under reducing conditions. However, in the presence of H$_2$O$_2$, VHR lost activity readily, whereas hYVH1 still maintained 60% of its original activity after 20 min of exposure. Inactivation was readily reversible upon treatment with excess reductant, which is consistent with multiple other studies of PTPs and DUSPs [11,20]. This result highlights the sensitivity of typical PTPs, such as VHR, to oxidative conditions and suggests that hYVH1 may be able to maintain activity during times of oxidative stress when other PTPs are rendered inactive. The cytoprotective nature of hYVH1-expressing cells also supports this claim [Fig. 2.1]. Why hYVH1 has lower intrinsic phosphatase activity \textit{in vitro} under reducing conditions as compared to VHR is puzzling given that it possesses all of the classical invariant residues known to be critical for catalytic activity of PTPs and DUSPs [27]. It may be that hYVH1 is highly specific for its physiological substrate or that it is functionally slow in terms of turnover rate to better suit being active under cellular stress conditions. We are currently pursuing the three dimensional structure of hYVH1 by x-ray crystallography and using hydrogen-deuterium exchange mass spectrometry to gain more insight into this issue and examine the structural relationship between the catalytic and the zinc-coordinating domains.

Another intriguing result was the observation that ejection of zinc in response to high levels of H$_2$O$_2$ correlated with a progressive decrease in phosphatase activity. The zinc-coordinating domain of hYVH1 is thought to contain two zinc-associated regions, with seven of the eight putative coordinating amino acids being Cys residues [3]. These
potential zinc-coordinating Cys residues may act as a redox buffer, becoming preferentially oxidized over the active site Cys. This hypothesis is supported by comparing the released zinc and remaining activity plots shown in Figure 3, where there seems to be a correlation between rate of zinc ejection and rate of inactivation. Ejection of zinc is not detected until the sequential oxidation of multiple Cys residues occurs resulting in release of free zinc ions, whereas enzymatic inactivation is strictly limited to the active site cysteine alone. Thus, this finding raises the possibility that the zinc-coordinating domain of hYVH1 may serve as a redox sink capable of protecting the active site Cys up to a certain threshold through preferential oxidation of zinc-coordinating cysteines.

To examine the extent of active site Cys oxidation, we measured free thiol content in response to increasing H₂O₂. The active site mutant (hYVH1 C115S) was used for a direct comparison. Control conditions produced the expected result, detecting the 11 Cys residues that are present in wild type hYVH1, while the hYVH1 C115S mutant was measured at the expected 10 Cys residues [Fig. 2.3D]. Under increasing oxidative conditions we were expecting that wild type and the C115S mutant would contain the same number of reactive free thiols due to oxidation of the active site Cys. Instead, we reproducibly detected approximately one less free thiol in wild type than in the C115S mutant. This result raised the possibility that the active site cysteine participated in the formation of an intra-molecular disulfide bond upon oxidation, resulting in one less reactive thiol for analysis via DTNB. Since the C115S mutant lacks this capability, an additional reactive thiol would remain available for detection by DTNB.

Efforts to definitively demonstrate the thiol redox status of multiple cysteine residues in hYVH1 were conducted using differential thiol labeling and MALDI-TOF MS [Fig. 2.4]. The targeted active site and an N-terminal cysteine-containing peptide were identified by mass fingerprint analysis of reduced, carbamidomethylated hYVH1 [Fig. 2.5A]. Upon oxidation, both characteristic peaks were lost; however, the presence of reduced forms of each peptide appeared along with an unknown peak at m/z 2211.28 [Fig. 2.5B]. No higher oxidation states of these Cys residues were observed, further suggesting disulfide bond formation was the mode of reversible oxidation, not sulfinic
acid. Notably, the putative disulfide mass of these peptides is 2211.17 Da, very close to the unknown peptide observed after oxidation. To decipher the identity of the unknown peptide, the tryptic digest was CAF-labeled to enhance and simplify MS/MS analysis by PSD. Initial evidence of an inter-peptidyl disulfide bond is denoted by a doubly labeled moiety, shown at \( m/z \) 2483.38 [Fig. 2.6A], with subsequent unambiguous identification by MS/MS PSD [Fig. 2.6B]. Mass spectrometry analysis of the C-terminal deletion variant hYVH1ΔCT1 also identified this intra-molecular disulfide bond formation upon oxidation [Suppl. Fig. S2.1]. These findings strongly support our initial studies that suggest sustainable activity under oxidative conditions as well as the formation of an intra-molecular disulfide bond within the active site. Moreover, presence of these disulfide-linked peptides in their respective reduced form in the mass fingerprint of oxidized, CAM-labeled samples further suggest disulfide bond formation due to the ability of MALDI to partially eradicate disulfide containing moieties. However, the formation of cyclic sulfe namides in small population can not be ruled out as these peptides are near identical mass to those of their fully reduced counterparts and thus have overlapping isotopic envelopes.

To probe the thiol status of the C-terminal zinc-coordinating cysteines, Glu-C peptides were identified by mass fingerprint analysis. Both CAM and NEM reduced peptides were identified as reference controls [Fig. 2.7A,B]. Only two characteristic peptides could be identified in the NEM mass fingerprint [Fig. 2.7B]. After oxidation, all characteristic peaks denoting zinc-coordinating vicinal thiol pairs were lost; however, a peak at \( m/z \) 3101.84 appeared, possibly denoting the presence of a reduced vicinal thiol pair after oxidation [Fig. 2.7C]. Differential labeling resulted in the appearance of all NEM peptides previously identified in the reference mass fingerprint, displaying the reversibility of the oxidative modification. Furthermore, higher oxidation states of these peptides were not readily observed, again suggesting that disulfide bonding is the likely form of modification. A peak broadening at \( m/z \) 3102.84 and 3106.07 appeared, which may be due to peptide isotopic overlap of a singly and doubly modified vicinal thiol pair at residues 221–244 and 222–244, respectively [Fig. 2.7D and Fig. 2.4B]. Additional supporting evidence was found when tryptic peptides were analyzed following the differential thiol labeling where we identified reversible thiol oxidation involving the
extreme C-terminal vicinal thiol pair [residues 302–316; Fig. 2.4B and Suppl. Fig. S2.2]. Overall, these data further support the working hypothesis that the C-terminal zinc-coordinating domain is capable of acting as a redox buffer via oxidation of vicinal thiol pairs, resulting in disulfide bond formation. Also, the presence of partially and fully reduced thiol pairs under oxidative conditions supports the idea that this region can potentially act as a reducing agent in promotion of a catalytically active phosphatase.

The capacity of hYVH1 to form disulfide bonds strongly supports the idea that enzymatic inactivation and zinc coordination are reversible phenomena. Upon treatment with micromolar concentrations of reducing agent, zinc-free hYVH1 was capable of rapid, re-coordination of ~ 85–90% total zinc [Fig. 2.8A]. Interestingly, examining the activity of zinc-free hYVH1 in the presence of reducing agents resulted in a marked decrease in activity of hYVH1 (~ 60%), whereas the C-terminal deletion variant hYVH1ΔCT1 (~ 10%) was unaffected [Fig. 2.8B]. These findings suggest that both reduced active site and proper zinc-coordination are necessary for full activity in vitro during re-reduction conditions, highlighting the importance of rapid re-binding of zinc following recovery from oxidative conditions. This reversible capacity of zinc coordination and disulfide bond formation suggests a dynamic regulatory mechanism under conditions of cellular oxidative stress [Fig. 2.9].

The precise biological role of hYVH1 remains unclear, and currently no substrate has been identified for this phosphatase. Since the phosphatase activity of hYVH1 is required for its cytoprotective function [8], identification of its phospho-target(s) during oxidative stress conditions is imperative for acquiring mechanistic understanding of how hYVH1 can protect cells from various insults. Other findings have recently suggested that YVH1 is a novel 60S ribosome biogenesis factor in yeast, and is a necessary element for the formation of a fully mature 60S subunit [28-31]. We have recently investigated if hYVH1 may possess a similar role in mammalian cells, and have discovered that it can associate with the pre-60S ribosomal subunit endogenously. This association is not dependent on catalytic activity, however, regardless of catalytic competency, the association is sensitive to treatment with oxidants, dissociating in a dose-dependent manner. Intriguingly, regions within both the catalytic and zinc-coordinating domains
Figure 2.9 - Proposed model of sustainable activity under conditions of oxidative stress. Under mild oxidative conditions, zinc is ejected due to thiols in the zinc-coordinating region serving as reducing agents, allowing the active site cysteine to remain reduced and catalytically competent. Severe oxidative conditions supersede the reducing power of the C-terminal thiols. However, the active site cysteine is protected from irreversible thiol oxidation (e.g. sulfonic acid) by disulfide bond formation with a proximal cysteine residue. Once reducing conditions are restored, the active site cysteine thiol is recovered while the C-terminal thiols rapidly re-coordinate zinc.
were found necessary for association, as deletion of either ablated interaction with the ribosomal subunit [22]. Thus, it will be interesting to investigate if the redox-sensing function of hYVH1 proposed in this study regulates the rate of ribosome biogenesis during cellular stress through its direct association with the pre-60S ribosomal subunit.

More recently, we have complemented these findings with various substrate trapping-mass spectrometry efforts having identified many of the suspected ribosomal subunit members as well as multiple novel interacting partners involved in RNA processing and regulation. Continued MS and hYVH1-mediated ribosome dynamics studies are currently ongoing to further understand the role of hYVH1 in regulating these processes of cellular physiology. Altogether, the discovery of this inherent redox defense mechanism has provided valuable insights into the protective value of the zinc-coordinating domain of hYVH1. This unique regulatory mechanism also sheds further light on the ability of certain cysteine-based phosphatases to cope with the destructive consequences of oxidative environments.
**Supplementary Figure S2.1 - Mass fingerprint analysis of the zinc domain deletion variant hYVH1ΔCT1 tryptic peptides.**  

**A)** Reduced, carbamidomethylated hYVH1ΔCT1 mass fingerprint. Highlighted are the active site and N-terminal vicinal thiol-containing peptides at m/z 1305.74 and 1021.48 respectively.  

**B)** Oxidized, carbamidomethylated hYVH1ΔCT1 mass fingerprint. Highlighted are the reduced active site and N-terminal vicinal thiol-containing peptides at m/z 1248.65 and 964.27 respectively. Carbamidomethylated peaks are largely reduced or absent. Also highlighted is a putative intra-molecular disulfide peak at m/z 2211.28 of these target peptides.
Supplementary Figure S2.2 - Differential thiol labeling mass fingerprints of hYVH1 tryptic peptides.  

A) Reduced, carbamidomethylated hYVH1 mass fingerprint. Highlighted is the extreme C-terminal vicinal thiol-containing peptide of the zinc-coordinating domain, m/z 1820.93.

B) Reduced, N-ethylmaleimidy l hYVH1 mass fingerprint. Highlighted is the same vicinal thiol-containing peptide, m/z 1956.67.

C) Oxidized, carbamidomethylated hYVH1 mass fingerprint. Highlighted is the absence of characteristic peak at m/z 1820.93.

D) Differential thiol labeled hYVH1 mass fingerprint. Highlighted is the reappearance of peak m/z 1956.52.
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CHAPTER 3 - GOLD NANOPARTICLE ENRICHMENT METHOD FOR IDENTIFYING S-NITROSYLATION AND S-GLUTATHIONYLATION SITES IN PROTEINS

3.1 - INTRODUCTION

The pioneering work of Stamler et al. [1] showed that NO• can modulate cellular signaling not only through the guanylate cyclase/cGMP pathway but also by S-nitrosylating proteins and small molecular weight thiols. A large volume of subsequent work has implicated S-nitrosylation (S-NO) of proteins in the regulation, sub-cellular compartmentalization, and degradation of proteins. Therefore, identification of the S-nitrosoproteome is an area of interest in beginning to elucidate reactive nitrogen species-(RNS)-mediated signaling pathways in health and disease [2-7].

To date, >1000 plant, animal, and prokaryotic thiol-containing proteins have been identified as potential S-nitrosylation targets [2]. However, the chemical characteristics of S-nitrosylated proteins, such as their low S-NO bond energy, photosensitivity, susceptibility to reduction by ascorbate, thiols, thiol reducing agents, and Cu (I), complicates their facile isolation and detection. Significant progress in identification of the S-nitrosoproteome was made by introduction of the “biotin-switch” assay by Jaffrey et al. [8]. Free protein-thiols are first blocked with an alkylation agent, followed by reduction of the protein S-nitrosothiols with ascorbate. In the final step, these newly formed thiols are reacted with thiol specific biotinylating agents, resolved by gel electrophoresis and detected by Western blot. Recent improvements to minimize ascorbate side reactions in the “biotin-switch” assay include the use of S-NO stabilizers [5] or the replacement of ascorbate with non-disulfide reducing, denitrosylating agents such as sinapinic acid [9]. Moreover, the “biotin-switch” method has also been used to directly isolate S-nitrosylated peptides from proteolyzed tissue lysates by capturing the biotinylated peptides on avidin beads for subsequent identification by liquid chromatography-tandem mass spectrometry (LC-MS/MS) techniques [10,11].

Saturation-labeling differential gel electrophoresis (DIGE) methods employing cysteine-specific fluorophores [12-15] have also been developed to identify both the thiol
proteome and S-nitrosoproteome. The DIGE approach employs a pair of fluorescent maleimide-conjugated cyanine dyes to specifically label free thiols in multiple protein samples. Mixtures of differentially labeled proteins are resolved on the same two-dimensional electrophoresis gel, and computer-aided analysis of the intensity differences between fluorophores compares the amount of S-nitrosylated (or thiolated) proteins.

Our aim was to use gold nanoparticles (AuNPs) to identify protein S-nitrosylation sites by taking advantage of the recently demonstrated property of AuNP reactions with S-NO proteins to yield NO• and AuNP-protein thiolates [16]. The experimental strategy of this method is outlined in Scheme 3.1, whereby residual free thiols of S-nitrosylated proteins are first alkylated with iodoacetamide (IAM). Proteins are next proteolyzed, and AuNPs are introduced to the digest. Any S-NO peptides are expected to react with the AuNPs, releasing NO•, and forming AuNP-thiolate peptides complexes. The resultant AuNP-bound peptides are then harvested by centrifugation and competitively eluted off of the AuNP surface by thiol exchange using an excess of small molecular weight thiols such as dithiothreitol (DTT). Released peptides are then analyzed by mass spectrometry, and target peptide/protein assignment can be done by comparison to in silico digestion of known protein sequences or bioinformatic database mining. Since alkylated peptides do retain some thiol character, they are likely to have a higher affinity than other amino acid residue side chains (i.e., groups with lower Au-affinity (GLA)-peptides) and to some extent, may be enriched under this scheme. We predict that the sterics of the bulky residues surrounding disulfide-containing peptides will preclude their interaction with AuNPs; however, less bulky glutathionylated peptides should interact with AuNPs yielding gold-thiolates.

Using this AuNP-based enrichment strategy, we have demonstrated that AuNPs are capable of reversibly binding protein thiols and thioethers causing de-nitrosylation of S-NO modified proteins and peptides. Furthermore, this property of AuNPs was found to provide a simple method for isolation, detection, and enrichment of S-modified peptides in a single step. Combination with differential thiol labeling and mass spectrometry, AuNP-based enrichment strategies are capable of unambiguous identification of protein S-modification sites.
Scheme 3.1 - AuNP enrichment method for identifying S-nitrosylation sites in proteins.
Free thiols of S-nitrosylated proteins are alkylated with iodoacetamide (IAM). Proteins are proteolyzed and AuNPs are introduced to the digest. Any S-NO peptides react with the AuNPs, releasing NO• to form AuNP-thiolate peptides complexes. The resultant AuNP-bound peptides are harvested by centrifugation and eluted off by thiol exchange using small molecular weight thiols. Released peptides are analyzed by mass spectrometry, and target peptide/protein assignment can be done by comparison to in silico digestion of known protein sequences or bioinformatic database mining.
3.2 - EXPERIMENTAL PROCEDURES

General information

Gold (III) chloride trihydrate, trisodium citrate dihydrate, reduced and oxidized glutathione, sodium nitrite, iodoacetamide, N-ethylmaleimide, dithiothreitol and formic acid were purchased from Sigma. All commercial reagents were used without further purification. Zeba™ Desalt Spin Columns, trifluoroacetic acid, recrystallized α-cyano-4-hydroxycinnamic acid, trifluoroacetic acid and ascorbic acid were purchased from Thermo Scientific. Siliconized micro-centrifuge tubes were purchased from Bio Plas Inc. and were used for all protein processing steps. Sequencing grade Trypsin was purchased from Promega, Endoproteinase Glu-C was purchased from Roche Applied Science. Vivapure C-18 Micro spin columns were purchased from Sartorius Stedim Biotech. High purity water and acetonitrile were purchased from Honeywell Burdick and Jackson.

Gold nanoparticle (AuNP) synthesis

Gold nanoparticles were synthesized according to the method of Grabar et al [17]. All glassware was washed and rinsed thoroughly with Milli-Q (18.2 MΩ) high purity water. In a 1 L double-neck round bottom flask, 197 mg gold (III) chloride trihydrate (HAuCl₄•3H₂O) was dissolved in 500 mL of Milli-Q water and refluxed for 1 h in a hot-oil bath (~ 100 °C) with vigorous stirring. Following reflux, 571 mg trisodium citrate dihydrate (Na₃C₅H₅O₇•2H₂O) was dissolved in 50 mL Milli-Q water and injected rapidly into the gold chloride solution. A distinct colour change can be seen from yellow to clear, then to dark burgundy (almost black) signifying the formation of a colloidal solution. After 10 min, the heat was removed and the solution was allowed to cool to room temperature. The resulting nanoparticles are 11 ± 1 nm in diameter, exhibit a maximum absorbance at 521 nm, and are stable for months at 4 °C. The resulting concentration is ~ 1.3 x 10¹⁰ AuNPs/μL.
**S-nitrosoglutathione (GSNO) synthesis**

All steps were performed in the dark due to the photosensitivity of the S-NO bond. To begin, 10.3 mg sodium nitrite (NaNO₂) was dissolved in 4 mL of ice-cold 0.5 M HCl, while 46.1 mg of reduced glutathione (GSH) was dissolved in 1 mL of ice-cold 0.5 M HCl, then added to the NaNO₂ solution. The mixture was incubated in the dark at 4 °C for 30 min and the pH was adjusted to 7.4 by the addition of dilute NaOH. GSNO concentration was determined from the absorption maximum of the S-NO bond at 335 nm (ε<sub>335nm</sub> = 980 L*mol⁻¹*cm⁻¹). The resulting solution was stored as 1 mL aliquots at -80 °C.

**Protein nitrosylation and alkylation**

Recombinant PDI and hYVH1 were purified as previously described [18,19]. Fully reduced proteins (2 μM) were nitrosylated by incubation with 1 mM GSNO in 100 mM sodium phosphate buffer pH 7.4 for 1 h at room temperature. Iodoacetamide (IAM) was then added to a final concentration of 10 mM and incubated for 2 h at room temperature. Excess GSNO and IAM were removed by two passes through Zeba™ Desalt Spin Columns as per the manufacturer’s specifications. At this step, the buffer was exchanged to 50 mM ammonium bicarbonate (pH ~ 8, unadjusted).

**Protein nitrosylation, glutathionylation and differential alkylation**

Fully reduced recombinant hYVH1 (30 μM) was nitrosylated or glutathionylated by incubation with 1 mM GSNO or oxidized glutathione (GSSG) in 25 mM Tris-HCl pH 7.5, 50 mM NaCl in the dark, shaking for 1 h at room temperature. Iodoacetamide was then added to a final concentration of 20 mM and incubated in the dark, shaking for 1 h at room temperature. Both samples were desalted as above, except nitrosylated hYVH1 was exchanged into 25 mM Tris-HCl pH 7.5, 50 mM NaCl. Ascorbate, N-ethylmaleimide, and copper (II) sulfate were then added to final concentrations of 30 mM, 20 mM, and 300 nM respectively. The sample was incubated in the dark, shaking for 3 h at room temperature, then desalted as above for subsequent proteolysis.
**Proteolytic digestion**

A 10:1 protein:protease (by mass) ratio was maintained for all digests. PDI was digested for 18 h with Endoproteinase Glu-C shaking at room temperature. hYVH1 was digested for 12 h with Trypsin shaking at 37 °C. For solution digest spectra, the samples were quenched by a final concentration of 1% formic acid.

**AuNP-peptide binding and elution**

AuNPs (500 μL) were pelleted by centrifugation (16 000 x g for 15 min). The supernatant was discarded and 40 μL of unquenched protein digests were added to the AuNP pellet and incubated 30 min at 37 °C. The AuNP-peptide complex was centrifuged, the supernatant was aspirated and the AuNP-peptide pellet was washed in 200 μL of 10 mM ammonium bicarbonate (pH ~ 7.6, unadjusted) three times. After the wash-spin steps, the AuNP-bound peptides were then eluted in 20 μL of 100 mM DTT. Following addition of DTT, the AuNPs were sonicated for 5 min in a water bath to disperse any AuNP aggregates. This was followed by a 2 h incubation after which the AuNPs were again pelleted and the supernatant harvested. To ensure the complete elution of peptides from the AuNP surface, a second addition of 20 μL 100 mM DTT was incubated with the AuNPs for 8 h with gentle agitation. The AuNPs were centrifuged and the resulting supernatant was combined with that from the first DTT elution step. Samples were acidified by the addition of 100 μL 0.1% trifluoroacetic acid, then desalted and concentrated using Vivapure C-18 Micro spin columns as per the manufacturer’s protocol. All digests were spotted 1:1 with 10 mg/mL matrix solution (recrystallized α-cyano-4-hydroxycinnamic acid in 60% acetonitrile, 0.1% trifluoroacetic acid) on the target plate by the dried droplet method and analyzed by matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) using an Applied Biosystems Voyager DE-Pro Mass Spectrometer. Peptide mass fingerprints and tandem mass spectrometry (MS/MS) using post source decay (PSD) was performed on selected parent ions as previously described [19] and compared to *in silico* fragmentation using the Protein Prospector software (http://prospector.ucsf.edu/).
3.3 - RESULTS AND DISCUSSION

**AuNPs demonstrate a saturable capacity to release NO• from S-NO proteins**

Protein disulfide isomerase (PDI) is an ∼ 57 kDa endoplasmic reticulum resident thiol oxidoreductase which catalyzes isomerization and rearrangement of disulfide bonds to ensure the proper folding of nascent proteins [20]. PDI is also secreted from cells where it associates with the cell surface [21,22] and has previously been shown susceptible to S-nitrosylation (S-NO) both *in vitro* and *in vivo* [18,23]. In order to confirm the ability of gold nanoparticles (AuNPs) to denitrosylate S-NO proteins, we mixed increasing amounts of S-NO protein disulfide isomerase (S-NO-PDI) [18] with citrate-capped AuNPs (10-12 nm) [24] in septa-sealed vials. After 1 min, the headspace was removed and injected into a Sievers chemiluminescent nitric oxide (NO•) analyzer (NOA) for quantification of NO• release. These experiments clearly indicate that NO• is released upon interaction of AuNPs with S-NO-PDI. Nitric oxide release reaches a plateau indicating that the AuNP surface becomes saturated with protein above ∼ 50 pmol of PDI [*Fig. 3.1A*].

*Exchange of AuNP-bound proteins by small molecular weight thiols*

To further examine both the mode of denitrosylation and the ability of AuNPs to bind protein thiols, AuNPs from the vials above were isolated, washed, incubated with either non-reducing (-DTT) or reducing (+DTT) SDS-PAGE sample loading buffer, and resolved by 10% SDS-PAGE. Resolved proteins were visualized by silver staining, and comparison of the sample treatments indicated that PDI was only detectable in the DTT containing samples, thus thiol-bound PDI can be released from AuNPs by thiol exchange. This further suggests that S-NO-PDI denitrosylation is mediated through the formation of AuNP-thiolate interactions [*Fig. 3.1B*].
Figure 3.1 - AuNPs bind S-NO-PDI causing release of NO•. **A)** Increasing amounts of S-NO-PDI in phosphate buffered saline were added 1:1 to a constant amount (~$5.3 \times 10^9$) of AuNPs in argon-purged, septa-sealed 2 mL vials. A 500 μL aliquot of the headspace was then injected into the NOA to quantify NO•. Data on the graph represents the average of $n = 3$ independent experiments and error bars represent S.D. **B)** AuNPs from sealed vials were collected, harvested by centrifugation, washed, and incubated for the times indicated with SDS-PAGE sample buffer (+/- 100 mM DTT). The AuNPs were again collected by centrifugation, and the supernatants were resolved by SDS-PAGE. Protein was visualized by silver stain.
**AuNPs selectively enrich thiol-peptides from S-nitrosylated proteins enabling the identification of S-nitrosylation sites**

PDI structural domains are arranged in an \(a-b-b'-a'\) topology, where the active site domains, \(a\) and \(a'\), contain the vicinal thiols Cys36XXCys39 and Cys379XXCys382 respectively. In addition, the \(b\) and \(b'\) domains each contain an additional free thiol at positions Cys295 and Cys326 respectively. Previous studies have shown that \(a'\) active site thiols are susceptible to oxidation such that the native enzyme performs its catalytic function in a half-oxidized state (i.e., \(a\) thiols reduced; \(a'\) thiols oxidized) [18,25].

hYVH1 (or dual specificity phosphatase 12; DUSP12) is a member of the cysteine-based protein tyrosine phosphatase (PTP) superfamily and has been shown to be a cell survival phosphatase in response to various cellular insults [26,27]. This 37.5 kDa protein is constructed of an amino terminal catalytic domain containing four free thiols, Cys11, Cys23, Cys97, and the active site nucleophile Cys115. Unique to this protein is a carboxyl-terminal domain containing seven Cys residues which participate in the coordination of 2 mols of zinc [28]. Recent findings show both domains undergo redox regulation [19], however their susceptibility to \(S\)-nitrosylation has not been demonstrated. Of these, only thiols within the catalytic domain and the vicinal Cys312XXCys314 pair are detectable in tryptic mass fingerprints. Remaining thiols reside in small, un-resolvable peptide fragments owing to the rich lysine and arginine content within the zinc-coordinating domain.

In fully reduced PDI and hYVH1, all detected thiols could be carbamidomethyl (CAM)-labeled with iodoacetamide (IAM) [**Fig. 3.2A** and **Fig. 3.4A**]. When fully reduced PDI or hYVH1 were \(S\)-nitrosylated prior to alkylation, all detected thiols were partially protected from CAM labeling [**Fig. 3.2B** and **Fig. 3.4B**]. Consistent with the NOA analyses, this strongly suggested the formation of pools of \(S\)-nitrosylated thiols in both PDI and hYVH1, as they resisted alkylation by IAM. The \(m/z\) values for these peptides correspond to those of free thiol peptide masses due to \(S\)-NO bond lability during nitrogen laser desorption ionization at 337 nm in MALDI-MS analyses [**Fig. 3.2B** and **Fig. 3.4B**] [29].
Figure 3.2 - AuNPs successfully enrich thiol-containing peptides from S-NO-PDI Glu-C digests.  A) Fully reduced PDI was alkylated with IAM, exchanged into 50 mM ammonium bicarbonate, and proteolyzed by a 10:1 ratio of PDI:Endoproteinase Glu-C. Peptide mass fingerprints were analyzed by MALDI-TOF MS and compared to \textit{in silico} digests as described in 'Experimental Procedures'.  B) Fully reduced PDI was S-nitrosylated by S-nitroso glutathione (GSNO), then alkylated with IAM, proteolyzed, and analyzed as above.  C) Fully reduced PDI was S-nitrosylated, alkylated, and proteolyzed as above. The digest was added to $1.3 \times 10^{10}$ AuNPs and incubated for 30 min at 37 °C. The AuNPs were washed, and AuNP-bound peptides eluted by thiol exchange using 100 mM DTT. Peptide samples were desalted using Vivapure C-18 Microspin columns and analyzed by MALDI-TOF MS as above.
Figure 3.3 - Tandem mass spectrometry of AuNP-enriched PDI peptides confirms thiol peptide association. Tandem mass spectrum (MS/MS) of A) $b'$ domain thiol-containing peptide at $m/z = 951$, B) $a$ domain thiol-containing peptide at $m/z = 1692$, and C) $b'$ domain thiol-containing peptide at $m/z = 2352$ were analyzed by post-source decay as described in 'Experimental Procedures'.
Figure 3.4 - AuNPs successfully enrich thiol-containing peptides from S-NO-hYVH1 tryptic digests.  

A) Fully reduced hYVH1 was alkylated with IAM, exchanged into 50 mM ammonium bicarbonate, and proteolyzed by a 10:1 ratio of hYVH1:trypsin. Peptide mass fingerprints were analyzed by MALDI-TOF MS and compared to *in silico* digests as described in ‘Experimental Procedures’.  

B) Fully reduced hYVH1 was S-nitrosylated by S-nitrosothioglutathione (GSNO), then alkylated with IAM, proteolyzed, and analyzed as above.  

C) Fully reduced hYVH1 was S-nitrosylated, alkylated, and proteolyzed as above. The digest was added to 1.3 × 10¹⁰ AuNPs and incubated for 30 min at 37 °C. The AuNPs were washed, and AuNP-bound peptides eluted by thiol exchange using 100 mM DTT. Peptide samples were desalted using Vivapure C-18 Microspin columns and analyzed by MALDI-TOF MS as above.
Figure 3.5 - Tandem mass spectrometry of hYYH1 peptides shows presence of S-nitrosylation and S-glutathionylation. Tandem mass spectrum (MS/MS) of A) active site Cys115-containing peptide at m/z = 1248, and B) S-glutathionylated active site Cys115-containing peptide at m/z = 1553, were analyzed by post-source decay as described in 'Experimental Procedures'.
AuNP-pulldowns of the peptide digests effectively enriched for the target thiol-containing peptides, resulting in overall simplification of the mass fingerprints and subsequent annotation. Both free thiol and CAM-labeled peptides were observed in PDI and hYVH1 digests, allowing for detection of both S-nitrosylated and free thiol peptide pools [Fig. 3.2C and Fig. 3.4C]. Mass fingerprints of PDI revealed that the vicinal thiols in the α domain (Cys36 and Cys39) and those in the β (Cys295) and β′ (Cys326) domains are susceptible to S-nitrosylation. The absence of the α′ domain vicinal thiols in the AuNP-pulldowns [Fig. 3.2C] and their presence in the solution digests [Fig. 3.2B] is in agreement with previous studies indicating that the vicinal thiols in the α′ active site domain are quite susceptible to oxidation in comparison to their α domain counterparts [18,25]. Furthermore, the absence of α′ domain thiols in the AuNP-pulldowns suggests that, as predicted, AuNPs have limited interaction with disulfide-linked peptides [Scheme 1]. As for hYVH1, most thiol-containing peptides appeared in the reduced state being indicative of S-nitrosylation, however, CAM-labeled peptides were also observed suggesting that these too could be differentiated among free and S-NO thiols [Fig. 3.4C]. Surprisingly, GSNO seemed to promote formation of small amounts of S-glutathionylation for the active Cys115 peptide of m/z = 1553 which was confirmed by tandem MS (MS/MS) [Fig. 3.5B]. Target thiol-containing peptide identities from both experiments were confirmed by MS/MS [Fig. 3.3 and Fig. 3.5].

**AuNPs interact with multiple peptide-thiol species allowing for combined identification of differentially S-modified residues**

The fact that CAM-labeled peptides were pulled down by AuNPs raised the possibility that thiols and thioethers (i.e. Methionine; Met) retain a higher affinity for gold in comparison to other functional groups in proteins, even upon chemical modification by alkylation or thiolation. Therefore, a simple modification of the AuNP-based enrichment technique should permit discrimination between sites of S-nitrosylation, S-glutathionylation, and free thiols [Scheme 3.2]. To test this, separate batches of hYVH1 were either partially S-nitrosylated or partially S-glutathionylated. The remaining free thiols were alkylated with IAM, while the S-NO-thiols were denitrosylated with ascorbate
Scheme 3.2 - Simple modification of the AuNP-based enrichment technique discriminates between sites of S-nitrosylation, S-glutathionylation, and free thiols.

Free thiols of S-nitrosylated and S-glutathionylated proteins are alkylated with iodoacetamide (IAM). Proteins are de-nitrosylated with ascorbate, differentially alkylated with N-ethylmaleimide (NEM) and proteolyzed. AuNPs are introduced to the digest where any S-NO peptides or free thiols, now S-NEM and S-carbamidomethylated (CAM) peptides, react with the AuNPs, and are enriched. S-glutathionylated peptides do not bind and thus remain in the supernatant. AuNPs are eluted by thiol exchange and released peptides are analyzed by mass spectrometry. Target peptide/protein assignment is confirmed by comparison to in silico digestion of known protein sequences or bioinformatic database mining.
and differentially alkylated with a second thiol reactive agent, N-ethylmaleimide (NEM). The samples were mixed, proteolyzed, and subjected to AuNP-pulldowns. The peptides were released by thiol exchange and identified by MS [Fig. 3.6].

The mixed peptide solution indicated the presence of S-glutathionylated, S-CAM, and S-NEM peptides [Fig. 3.6B]. The reduced peak intensities of the NEM-modified peptides are indicative of ion suppression in mixed digests. As predicted, the AuNP-pulldown significantly enriched for free thiol, alkylated thiol, and Met-containing peptides [Fig. 3.6C]. This AuNP-enrichment for S-modified peptide thiols indicates that upon partial S-nitrosylation of hYVH1, Cys11 and Cys115 are S-nitrosylated. On the other hand, Cys11, Cys23, Cys97, Cys312, Cys314, and the active site Cys115 are susceptible to S-glutathionylation [Fig. 3.6C, Fig. 3.7 and Fig. 3.5B]. In addition, this technique was able to enrich for Met-containing peptides, along with decreasing sample complexity and thus increasing signal to noise ratios. Future adaption to liquid chromatography/mass spectrometry will act to further the analytical capabilities of this AuNP-based method, potentially providing a system to study the S-nitrosoproteome.

In summary, we have for the first time demonstrated that thiols and thioethers retain a higher affinity for gold in comparison to other functional groups in proteins, even upon chemical modification by alkylation or thiolation. We have adopted this property of AuNPs to devise a simple, novel method by which AuNPs can be used to isolate, detect, and enrich for S-modified peptides in a single step. When combined with thiol modification and mass spectrometry, our AuNP-based method can unambiguously identify sites of protein S-modification, setting the stage for future AuNP-based applications in studying protein S-nitrosylation and beyond.
Figure 3.6 - AuNPs successfully enrich differentially labeled, thiol-containing peptides from S-nitrosylated and S-glutathionylated hYVH1 tryptic digests. A) Fully reduced hYVH1 was S-glutathionylated then alkylated with IAM, and proteolized by a 10:1 ratio of hYVH1:trypsin. Peptide mass fingerprints were analyzed by MALDI-TOF MS and compared to in silico digests as described in 'Experimental Procedures'. B) Fully reduced hYVH1 was S-nitrosylated and alkylated with IAM. Sample was mixed with an undigested sample from A), then de-nitrosylated with ascorbate and alkylated with N-ethylmaleimide. Proteins were exchanged into 50 mM ammonium bicarbonate and processed as above. C) Digests from B) were added to $1.3 \times 10^{10}$ AuNPs and incubated for 30 min at 37 °C. The AuNPs were washed, and AuNP-bound peptides eluted by thiol exchange using 100 mM DTT. Peptide samples were desalted using Vivapure C-18 Microspin columns and analyzed by MALDI-TOF MS as above.
Figure 3.7 - Tandem mass spectrometry of hYVH1 peptides shows presence of $S$-nitrosylation and $S$-glutathionylation using modified AuNP-enrichment scheme. Tandem mass spectrum (MS/MS) of A) active site Cys115-NEM peptide at $m/z = 1373$, B) $S$-glutathionylated Cys11-containing peptide at $m/z = 2839$, and C) $S$-glutathionylated Cys23-containing peptide at $m/z = 3291$ were analyzed by post-source decay as described in 'Experimental Procedures'. 
3.4 - REFERENCES


CHAPTER 4 - INVESTIGATING REDOX REGULATION OF PROTEIN TYROSINE PHOSPHATASES USING LOW PH THIOL LABELING AND ENRICHMENT STRATEGIES COUPLED TO MALDI-TOF MASS SPECTROMETRY

4.1 - INTRODUCTION

Cells utilize a vast array of strategic phosphorylation events to properly respond to extracellular stimuli. To achieve this homeostatic phosphorylation, kinases and phosphatases must be tightly controlled using a variety of temporal and spatial regulatory mechanisms. Although often considered the unfavourable by-products of aerobic respiration, increasing evidence has highlighted the role of cellular oxidants as second messenger molecules that participate in maintenance of this balanced phosphorylation [1]. Disruption of this delicate equilibrium can cause or promote numerous human disease states including cancer, diabetes, and neurodegenerative disorders [2-4].

Reactive oxygen and nitrogen species (ROS/RNS) have been shown to target multiple members of the protein tyrosine phosphatase (PTP) superfamily, providing a tier of transient and reversible post-translational regulatory control [5]. PTPs share a highly conserved active site architecture and catalytic mechanism, in which their catalytic activities depend on nucleophilic cysteine thiolates [6,7]. Moreover, PTPs possess an invariant signature motif, H CX$_3$R(S/T), responsible for the thiolate-based dephosphorylation of an ever increasing diversity of phosphorylated substrates [8-11]. This tertiary architecture and microenvironment reduces the $pK_a$ of the catalytic thiol to enhance catalytic competency, but also renders these enzymes susceptible to reversible oxidative inactivation [12-15]. As such, these enzymes pose as molecular ‘redox switches or sensors’ and can be regulated by discrete, subtle and/or sudden changes in cellular redox homeostasis [16].

The prevalence, type(s) and effects of oxidative modifications among PTP family members continues to diversify; a likely consequence of slight variations in active site conformations or mediation through unique regulatory domains [14,17]. Sulfenic acid, cyclic sulfenamide, nitrosylation, glutathionylation, sulfhydration and disulfide bond
formation have all been shown to facilitate stable, reversible, active site modifications among various PTP family members [13,18-22]. Thus, it was first suggested that oxidation predominantly and rapidly targets the active site cysteine resulting in inactivation, whereas other cysteinyl residues remain in the reduced state. More recently, however, accumulating evidence is beginning to re-define redox regulation of PTPs as a far more dynamic modification capable of differential regulation. In some cases, redox sensitive cysteine residues outside of the active site contribute to preserving, or are even a necessity for, catalytic activity under oxidative conditions [23-25].

Tools for studying PTP redox modifications are continually being developed, allowing for extraction of pertinent information regarding these regulatory post-translational modifications [26]. Recently, oxidative modification and conformation sensing antibodies have been developed as unique tools for studying individual PTP redox events at both the protein and/or proteomic level [27-30]. However, the economics and feasibility of such applications requires targeted and previously determined phenotypes which limits their use in more practical applications such as preliminary screening. Some of the more routine methods for examining protein thiol redox status rely heavily on differential labeling, whereby reversibly oxidized protein or peptide thiols are labeled with some 'reporter tag(s)' for subsequent identification using a variety of analytical techniques [31]. Efforts from our laboratory have focused mainly on development of novel labeling and enrichment strategies allowing for quantitative and/or qualitative identification of protein thiol redox status using mass spectrometry (MS) [23,32,33]. However, as in these and nearly all labeling methods, labeling steps must proceed for several hours at near neutral pH, in large molar excesses, to ensure optimal and complete reactivity of thiolates for the specific labeling agents. It is under these conditions that targeted thiolates are also prone to non-specific oxidation events including those catalyzed by common buffer contaminants such as molecular oxygen and trace metals (ie. disulfide bonds, sulphonates etc.) which hampers their accurate analysis and interpretation [31]. This issue can be partly circumvented by purging or degassing solutions used in sample handling, and performing cellular lysis under anaerobic conditions as those described by Meng et al. [34,35]. These precautions are effective in limiting oxidation by ROS/RNS, but have decreased abilities in preventing disulfide exchange reactions as
those commonly observed among members of the dual specificity phosphatase (DUSP) sub-families [18]. Thus, accurate analysis of thiol oxidation, both in vivo and in vitro, balances between the ability to suppress non-specific thiol exchange/oxidation and the specificity and reaction rate of labeling reagents used for modification, enrichment and detection.

Recently, our laboratory has been characterizing the activities of human YVH1 (hYVH1, also known as DUSP12), an atypical dual specificity phosphatase member of the PTP superfamily [10]. This unique enzyme possesses an N-terminal DUSP catalytic domain along with a C-terminal, thiol-rich, zinc-coordinating domain [Fig. 4.1] which has been shown to be indispensible for its biological activities below. YVH1 orthologues exhibit high evolutionary conservation, similar domain organization, and have recently been implicated in processes such as ribosome biogenesis, cell survival and cell cycle regulation [36-42]. Moreover, we previously revealed that oxidation of hYVH1 results in reversible, concomitant, enzymatic inactivation and zinc release in vitro through formation of intra-molecular disulfide bonds. This inactivation is more pronounced in the absence of the zinc-coordinating domain, however, reversibility is independent of this domains presence suggesting that oxidation preferentially targets this intrinsic redox buffering region, slowing the rate of oxidative inactivation [23]. We have since observed that similar to the yeast orthologue, hYVH1 interacts with the mammalian pre-60S ribosomal subunit, but interestingly, oxidation ablates this association in a dose dependent manner [42]. Although no bona fide physiological substrate or function have been elucidated, amplification of the dusp12/hyvh1 gene has been reported in multiple sarcomas, implicating roles of hYVH1 among human pathophysiology, highlighting the need for continued study [43-45].

We have begun to develop rapid, specific, sensitive, and reversible low pH thiol labeling schemes for screening PTP oxidative modification, specifically those involving, but not limited to, disulfide exchange reactions as those observed by Chen et al. for SHP-1 and SHP-2 [18]. From our previous studies on the redox regulation of hYVH1, we have employed novel labeling and enrichment strategies in combination with mass spectrometry to develop a platform of simple, rapid, and diverse labeling approaches for
**Figure 4.1 - Human YVH1 (DUSP12) is a thiol-rich dual specificity phosphatase.**
Schematic displaying domain architecture of hYVH1. The N-terminal phosphatase domain contains the highly conserved HCX_3R(S/T) motif along with three additional Cys residues. hYVH1 also possesses a unique C-terminal zinc-coordinating domain which coordinates two moles of zinc putatively through action of seven Cys residues.
studying disulfide exchange reactions. Our data suggests that exchange reactions occur quite readily among vicinal thiols within the catalytic domain of this DUSP similar to results shown by Chen et al. [18]. We find that the active site cysteine is capable of participating in multiple disulfide bond reactions in addition to those previously described, to further provide protection against irreversible oxidation events. Moreover, we have found evidence of other disulfide pairs, exhibiting the applicability of the methodology to identifying reversible thiol oxidation. Ultimately, these methods serve to provide an economical means for preliminary screening of targeted redox-active thiols within PTP superfamily members in vitro, to in turn, warrant an in depth analysis under more detailed physiological settings.
4.2 - EXPERIMENTAL PROCEDURES

Site-directed mutagenesis, recombinant protein purification and characterization of mutant variants by MS

Target cysteine residues are identified through structural bioinformatics and/or redox-active thiol enrichment (below), and mutated to serine residues using PCR-based site-directed mutagenesis. Mutated constructs are purified by conventional means in a suitable host organism and characterized by mass spectrometry. Synthesis of pGEX-4T1 GST-hYVH1 mutant constructs was as previously described for hYVH1 C115S and hYVH1ΔCT1 [23,36,38]. Briefly, PCR-based site-directed mutagenesis was performed following manufacturers protocol (Stratagene). All mutated plasmid DNA constructs were confirmed by DNA sequencing (AGCT Corp).

Recombinant proteins were purified as previously described, being eluted with 50 mM Tris-HCl, 150 mM NaCl, 2.5 mM CaCl₂, 0.1% 2-mercaptoethanol, 80 units of thrombin (Sigma) pH 8, at 4 °C from glutathione-agarose (Sigma) to remove the glutathione S-transferase tag [23]. Target constructs were brought to 1 mM dithiothreitol (DTT; Sigma), 5% glycerol (ACP Chemicals Inc.) and 0.5 mM phenylmethylsulfonylfluoride (PMSF; MP Biomedicals), 0.2 µm sterile filtered, aliquoted at 130 µL and stored at -80 °C until use. All protein concentrations were determined against standard bovine serum albumin (BSA; Sigma) as per the Bradford assay (Bio-Rad Laboratories Inc.), while purity of all proteins in this study was approximately 80–90% as judged by SDS-PAGE analysis.

To characterize individual constructs along with digestion conditions at the protein level, each construct (~ 20 µg) was carbamidomethylated (CAM) by incubation with 25 mM iodoacetamide (IAM; Sigma) for 60 min, shaking, at room temperature in the dark. Both CAM-labeled and unlabeled samples of each construct were next exchanged out of reducing/labeling conditions by precipitation, on ice, with cold (-20 °C) trichloroacetic acid (TCA; Sigma) at a final concentration of 15%. Pellets were collected after 30 - 60 min by centrifugation at 23 500 x g at 0 - 4 °C, washed 3 times with cold (-20 °C) acetone, then resuspended in 50 mM ammonium bicarbonate (Fisher Scientific) for subsequent proteolytic digestion. To each sample, trypsin (Promega) was added to a final
protease:protein ratio of 1:25, with digestion proceeding for 16 h, shaking at 37 °C. Digestions were quenched by addition of formic acid (FA; Sigma) to a final concentration of 0.1%. Labeled and unlabeled samples were combined, mixed, then split into equal volumes, where one was reduced with tris(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl; Pierce Biotechnology) shaking at room temperature for 15 min at a final concentration of 25 mM. Samples were spotted 1:1 with α-cyano-4-hydroxycinnamic acid (CHCA; Pierce Biotechnology) matrix constituted in 65% acetonitrile/water (Fisher Scientific) supplemented with 0.1% trifluoroacetic acid (TFA; Pierce Biotechnology) by the dried-droplet method and analyzed by matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS). Remaining peptide samples were diluted to approximately 5 ng/µL in binding buffer (200 mM phosphate buffer pH 4 with additive; see below) for subsequent loading onto resin as described below.

*Preparation and characterization of Mercury-Immobilized Metal Affinity Chromatography (Hg-IMAC) resin*

The outlined procedures are primarily based on those for BioRad’s discontinued Affi-Gel 501, an organomercurial derivative of Affi-Gel 10 N-hydroxysuccinimide activated agarose gel recently adapted by others [46-48]. The following protocol is for preparation of approximately 4 mL of Hg-IMAC resin. Before use, the chemical compatibility of all plastics/vessels with the chemicals used throughout this synthesis should be determined. Also, we strongly advise strict adherence to Good Laboratory Practice (GLP) procedures and consultation of appropriate Material Safety Data Sheets (MSDS) during and before synthesizing Hg-IMAC resin.

Affi-Gel 10 resin (Bio-Rad) is warmed to room temperature for ~ 30 min in the dark to limit hygroscopy, which promotes hydrolysis of the activated succinimidyl ester. Dissolve 83.76 mg of para-amino-phenylmercuric acetate (Sigma) in 1.2 mL of anhydrous dimethylformamide (Sigma). Mix via vortex and/or nutation for 20 - 30 min at room temperature to ensure full dissolution of all organomercurial compound. Agitate Affi-Gel 10 resin to form a homogeneous suspension, then quickly transfer 4 mL into a
clean 10 mL glass column with granulated frit, pre-rinsed with anhydrous dimethylformamide/2-propanol (Fisher Scientific). It is imperative to eliminate residual water, as water promotes rapid hydrolysis of succinimidyl esters, decreasing labeling efficiency of subsequent steps, and thus, binding capacity of Hg-IMAC resin. Wash resin with a minimum of 3 column volumes (12 mL) of anhydrous 2-propanol, then resuspend resin in 1 column volume of anhydrous 2-propanol, ensuring not to let resin dry. If resin dries and cracks, wash extensively with 2-propanol with periodic aspiration to remove trapped air. If the resin has begun to aggregate, restart the synthesis. Add the para-amino-phenylmercuric acetate solution to the resin (60 μmol/mL of gel) and aspirate carefully as to not introduce air bubbles into the resin. Cover the column with tin foil and incubate for 4 h with periodic aspiration to mix the resin. This constitutes a four-fold excess of organomercurial amine to available succinimide esters of the resin. At time, add 40 μL of ethanolamine (Sigma) to block any unreacted succinimide esters (164 μmol/mL of gel), aspirate and incubate as above for 1 h. Control resin is prepared by excluding para-amino-phenylmercuric acetate and blocking with excess ethanolamine.

Wash resin with 3 column volumes (12 mL) of dimethylformamide, followed by 12 column volumes (48 mL) of 2-propanol. Store the resin in 2 column volumes (8 mL, approx. 30% slurry) of 2-propanol in the dark at 4 °C. If require or prefer higher percentage slurry, centrifuge resin at 2500 x g for 5 - 10 min or allow to settle at 4 °C and remove the desired volume of 2-propanol. The resin should be used within one month, however, can be continually tested for efficacy by analysis of binding capacity prior to use. In our experience, resin maintained binding capabilities over 2+ months, however it is recommended that binding capacity is checked periodically to ensure reproducibility and efficacy.

To determine the binding capacity of Hg-IMAC, prepare 1 mg/mL (5.7 mM) stock standard solution of cysteine hydrochloride (Cys-HCl; Sigma) in water. Dilute ten-fold in water (570 μM) for use in binding capacity and standard curve analysis. Prepare stock 2 mM solution of 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB; Sigma) in 50 mM sodium acetate pH 4.5. A 12 point linear standard curve is prepared by mixing Cys-HCl to final concentrations ranging from 5.7 μM to 114 μM with DTNB at a final concentration of
200 µM in 100 mM phosphate buffer (ACP Chemicals Inc.) pH 7.4 with a 1 mL final reaction volume and recording the Abs$_{412\text{nm}}$ as per Riddles et al. [49].

Equilibrate and activate various amounts of Hg-IMAC resin slurry (0, 10, 20, 30, 40, 60, 80 µL; see below for equilibration/activation steps) and incubate with 570 µM of Cys-HCl, mixing by rotation for 45 min. For comparison, perform similar steps using the control resin. Collect resin by centrifugation at 4000 x g for 6 min, then mix 100 µL of each supernatant with DTNB at a final concentration of 200 µM in 100 mM phosphate buffer pH 7.4 with a 1 mL final reaction volume and record the Abs$_{412\text{nm}}$ as per Riddles et al. [49]. Calculate the remaining thiol content and convert to µmol/mL of slurry. In our experience, the binding capacity of Hg-IMAC resin is reproducibly around 2.3 +/- 0.2 µmol/mL of slurry [Suppl. Fig. S4.1] when performed in a 1:1 phosphate buffered saline (PBS; Thermo Scientific):water buffer pH 3.5 (adjusted with 85% phosphoric acid; ACP Chemicals Inc.) supplemented with a final concentration of 10% acetonitrile, and is consistent with other values in the literature [47]. Also, binding capacity studies have been performed over a range of pH's, buffers, and solvents, showing no significant deviations from the reported value above.

**Selective enrichment of cysteine containing peptides using Hg-IMAC resin**

Hg-IMAC resin slurry (at 100 µL/reaction) was activated by transferring to a 1.5 mL conical microcentrifuge tube, harvesting by centrifugation at 4000 x g for 1 min at room temperature, washing 2 x with equilibration buffer (PBS), 2 x with activation buffer (100 mM ammonium chloride), then 2 - 3 x with binding buffer (200 - 400 mM phosphate buffer pH 4 with additive; see below). Protein digests as described above (500 µL at 5 ng/µL) were loaded onto the resin and incubated at room temperature, nutating in the dark for 60 min. Resin was collected by centrifugation at 4000 x g for 1 min, and supernatant removed for storage. Resin was washed 3 x with wash buffer (10 mM phosphate buffer pH 4), with bound peptides eluted 2 x with 50 µL 100 - 200 mM DTT shaking 30 min at room temperature. Both samples were concentrated by rotary evaporation at 30 °C to approximately 20 - 40 µL, spotted 1:1 with CHCA matrix as above, and analyzed by MALDI-TOF MS. If necessary, samples were diluted 1:1 with
0.1% TFA/water and desalted/concentrated using ZipTip® C18 pipette tips (Millipore Corp.) as per manufacturers protocol. Samples were again spotted 1:1 with CHCA matrix and analyzed by MALDI-TOF MS as above.

To note, reduction of surface tension in aqueous buffers during Hg-IMAC activation and peptide enrichment can be accomplished by adding final concentration of 10% acetonitrile or 0.001% Tween-20 as additives to all buffers. Tween-20 reproducibly provided slightly better enrichment, however, must be removed prior to any MS analysis (either MALDI or liquid chromatography; LC/MS). Failure to remove residual Tween-20 results in potent suppression of peptide ions and can be a detrimental contaminate in LC/MS instrumentation, resulting in tedious decontamination procedures. Thus, we suggest to use Tween-20 only when interested in the Hg-IMAC eluant sample, as excess can be rinsed away during resin washing. If the supernatant is of interest analytically and/or LC/MS is being employed, we advise to substitute Tween-20 (Bio-Rad Laboratories Inc.) for acetonitrile.

**Screening reversible thiol oxidation with Hg-IMAC enrichment and 4,4-dithiopyridine labeling coupled to MS**

Molar ratio(s) of protein:H₂O₂ and incubation time(s) are empirically determined based on a variety of characteristics such as number of suspected redox active thiols, physiological concentration ratios, and detection limits. A simple activity, DTNB or 4,4-dithiopyridine (4-DTP) assay which titrates the redox reversibility will strongly aid in defining the starting molar ratios to be explored, however, a simple titration may also be applied directly to the Hg-IMAC scheme instead [13,49,50]. Take care not to dilute working protein reaction volume too drastically by the addition of H₂O₂, routinely calculate the addition of 20 µL concentrated H₂O₂ to achieve the desired final molar ratio. Since most reaction volumes are quite small, all pH values are checked on equivalent reaction mixes containing appropriate scaled up volumes (not concentrations) of each pertinent component. For small volumes of true reaction mixes, pH was occasionally monitored by spotting ~ 2 µL on pH paper to ensure reaction conditions are as expected. All pH values for buffers were obtained using an Orion 410Aplus pH meter equipped
with an Orion glass Ag/AgCl Sure-Flow Electrode, under 3-point calibration using standard buffer solutions (Fisher Scientific Buffer solutions pH 1.00 and 4.00; ACP Chemicals Inc. Buffer solutions pH 7.00 and 10.00).

Reduced protein was rapidly thawed to 0 °C and placed on ice. Samples were exchanged into reaction buffer (20 mM Tris-HCl pH 7.3, 25 mM NaCl or equivalent) using Zeba™ Desalt Spin Columns (Pierce Biotechnology) as per manufacturers protocol. During column preparation for exchange, stock solutions of H₂O₂ (ACP Chemicals Inc.) were prepared in reaction buffer. Once exchanged, the desired volume of protein was quickly transferred to a suitable microcentrifuge tube, followed by the addition of the appropriate volume of stock H₂O₂ to achieve the necessary molar ratio. The sample was gently mixed and incubated shaking at room (or desired) temperature for defined time(s). Following incubation, the reaction was quenched by addition of 1 M phosphate buffer (phosphate:reaction, 1:4, v:v) to 200 mM final concentration pH ~4.

Here, samples were divided into one of two schemes [Fig. 4.3], where initially a candidate list of redox-active and inactive protein thiols is formed for the subsequent targeted site-directed mutagenesis scheme. The devised schemes to identify candidate redox-regulated protein thiols are based on the merging of previously described methods with key modifications [23,47,50]. First, an acid protease (GluC; Roche Applied Science, immobilized pepsin; Pierce Biotechnology, or equivalent) at 1:10 protease:protein ratio was added to initiate digestion, and sample was incubated at 25 °C shaking for ≤ 1 h. During digestion, the appropriate amount Hg-IMAC resin was equilibrated and activated as described above. After digestion, digests were added to activated resin, and incubated as described. During the 1 h incubation, a second batch of Hg-IMAC resin was equilibrated and activated as described above. After 1 h, resin was collected by centrifugation, supernatant removed and partitioned equally into separate microcentrifuge tubes. One equivalent was reduced with 4 mM TCEP for approximately 10 - 20 min at 25 °C, while the second equivalent was diluted with an equal volume of water. After reduction, the peptide samples were diluted 1:1 with 200 mM phosphate binding buffer pH 4 containing the desired additive (10% acetonitrile or 0.001% Tween-20). Each of the peptide pools were loaded onto the second activated Hg-IMAC resin.
batches and incubated as described. During incubation, the primary Hg-IMAC resin containing redox-inactive and reduced thiols was washed and eluted as described. Once time was met for secondary Hg-IMAC incubations, resins were washed and eluted as described, with all samples analyzed as previously described above.

Based on the targeted thiols identified, we made an appropriate Cys to Ser point mutant to solidify our data sets. We analyzed wild-type and C115S hYVH1 for presence of disulfide pairs among the active site cysteine and N-terminal thiols within the catalytic domain. After quenching by addition of 1 M phosphate buffer (phosphate:reaction, 1:4, v:v) to 200 mM final concentration pH ~ 4, 4-DTP (Acros Organics) was added at a 1:10 reagent:protein ratio to initiate labeling, and incubated at 25 °C shaking for 20 - 40 min. Labeling efficacy of 4-DTP was confirmed by subsequent addition of IAM in the absence and presence of TCEP [Suppl. Fig. S4.5 and Suppl. Fig. S4.6]. At time, the pH was neutralized through buffer exchange of protein sample into 50 mM ammonium bicarbonate using Zeba™ Desalt Spin Columns as per manufacturers protocol. Trypsin was added at approximately 1:20 protease:protein ratio to initiate digestion, and samples were incubated at 37 °C shaking. Digests were spotted, as above, every 30 min to maximize signal:noise ratio and avoid ion suppression effects. Similarly, aliquots were taken at each time point and reduced with TCEP to further locate disulfide bonding pairs prior to spotting. All samples were analyzed by MALDI-TOF MS as described above.
Examination of protein thiol redox status continually battles between suppression of non-specific thiol exchange/oxidation and efficacy of labeling conditions used in the modification, enrichment and detection steps. The wide variety of potential redox modifications, most of which are labile and reversible, greatly confounds all aspects of their accurate analysis [31]. Members of the PTP superfamily show great dynamic range among the redox reactions in which they can undergo, with examples representing most all of the common cellular redox modifications [14]. This phenomenon acts to complicate studies regarding PTP redox regulation, but also to highlight the necessity for continual development of methods for expanding current knowledge regarding these extremely important contributors to signal transduction. However, an economical means for preliminary screening of targeted redox-active thiols within PTP superfamily members in vitro would enable more functional studies regarding the specific regulation afforded through Cys-based oxidation and of those cellular pathways potentially involved in their reversible regulation.

Coupling thiol labeling/enrichment with 'bottom up' mass spectrometric techniques has expanded the analytic abilities for studying the vast array of regulatory redox protein modifications at the proteomic level [28,48,51,52]. Moreover, recent reviews have suggested that the levels of protein Cys oxidation are ~ 10% under normal cellular conditions, while in response to oxidants, those levels are capable of reaching > 40% of total cellular protein Cys residues, illustrating the redox proteome as a prevalent aspect of cellular physiology [53]. But as previously mentioned, probing redox status of peptide thiols readily falls victim to formation of non-specific oxidation artifacts during both labeling and digestion steps, mainly due to the inherent reaction conditions (large molar excesses, pH ~ 8, extended times) [31]. Common labeling agents (IAM etc.) offer very little affordability in monitoring reaction efficiency, and as such, put potential limitations on data acquisition post digestion. These limitations are exemplified upon observing data from conventional tryptic protein digests obtained using classical conditions without previously labeling protein thiols [Fig. 4.2 and Suppl. Fig. S4.2]. Here, hYVH1 digests,
Figure 4.2 - Non-specific oxidation of hYVH1 thiols during proteolytic digestion at neutral pH. Recombinant hYVH1 and CAM-labeled hYVH1 were subjected to an overnight trypsin digestion using a 1:25 protein:protease ratio in 50 mM ammonium bicarbonate pH 8, shaking at 37 °C. The resulting tryptic peptides were analyzed by MALDI-TOF MS with A) or without B) prior reduction with 25 mM TCEP.
spiked with carboxymethylated (CAM) digests for relative comparison, were analyzed in the presence and absence of TCEP after overnight digestion. One can clearly note that addition of a reductant post digest results in a drastic increase in reduced thiol peptide peaks relative to their CAM-labeled counterpart, highlighting the propensity of non-specific oxidation occurring during typical tryptic digestion. Thus, if labeling efficiency is limited/unknown, or a label-free analysis is sought, potential limitations are apparent between most existing thiol-labeling MS-based approaches.

To circumvent these potential limitations among current MS-based approaches used in studying protein thiol redox regulation, we have devised a scheme using commercially available and well characterized thiol labeling reagents [Fig. 4.3]. Our current platform assesses three main aspects of the traditional work-flow; time, pH and label characteristics. First, decreasing time during all steps will act to limit potential non-specific oxidation as well as increase throughput, thus posing this as a method for rapid screening of candidate, reversibly oxidized/regulated protein thiols. Next, decreasing the pH (≤ 4) will further limit oxidative artifacts as thiols (-SH) are far less reactive than their deprotonated counterparts, thiolates (-S-) [54]. Moreover, in combination with digestion, these moieties will become even less prone to artifactual oxidation, as protein thiol pKa values are largely governed by tertiary microenvironments, which are lost during proteolytic digestion. This would be the case for the highly conserved active site cysteine residues characteristic of PTP superfamily members, making this method directly applicable to the study of such events. Also, in examples such as PTP1B where a stable cyclic sulfenamide is formed, acidification may also disfavour non-specific reactions of these spatially proximal reactants (ie. active site cysteine with neighbouring amide nitrogen). Acidification promotes formation of the less-reactive catalytic thiol (-SH), while also hovering the hydrogen exchange minima of amide hydrogens among the peptide backbone (pH ~ 2.5) [55]. These events suggest that reaction kinetics of sulfenamide formation would be greatly reduced as both reactants would predominantly exist as stably protonated species, thus disfavouring reactivity based on their chemical state. Lastly, coupling these phenomena to labeling and/or enrichment strategies has further strengthened the proposed scheme for analysis of redox regulated thiols.
Figure 4.3 - Schematic workflows of Hg-IMAC-based methodology. Left) Protein of interest is exposed to oxidative stress, sample is then acidified to quench the reaction and reduce the rate of oxidation “preserving” reversibly oxidized thiols (disulfides, S-glutathionylated, cyclic sulfenamides, S-nitrosylated thiols etc.). A rapid proteolytic digest using acid proteases (i.e. pepsin or Glu C) is then performed whereby peptides are then passed over Hg-IMAC resin to capture any reduced or S-nitrosylated thiols. Captured peptides are eluted using DTT and detected by MALDI-TOF MS. Resin flow-through is split into two fractions, with one fraction left untreated (oxidized thiols) and the other reduced with TCEP (reversibly oxidized thiols). Both fractions are then passed over fresh Hg-IMAC resin, resin is eluted, and eluant analyzed using MALDI-TOF MS. Peptides found in the reduced fraction not found in the non-reduced fraction are putative reversibly oxidized thiols. Right) Protein is treated as above, however, after acid quench, any reduced thiols are blocked with 4-DTP at low pH for 1 h. These reduced thiols have been labeled and can no longer participate in non-specific disulfide formation and can be differentiated from S-nitrosylated thiols. Moreover, the pH can be raised to 7 - 8 allowing for conventional protein digestion with trypsin and MALDI-TOF MS analysis.
Organomercurials have long since been known to have an extremely high affinity for thiols, reacting both rapidly and stoichiometrically, even displacing thiol-bonded nitric oxide through preferred formation of a stable mercaptide. This reactivity has a pH maximum of approximately pH 5, exhibiting the practical applicability of these chemicals towards both low pH labeling and/or enrichment [56]. Immobilization of such compounds has also allowed for the selective enrichment of target thiol-containing peptides, such as the recent application used in study of the S-nitroso-proteome [46,48]. This ability to enrich for targets acts to decrease spectral complexity, while increasing signal:noise ratio, thus sensitivity and accuracy, to ease subsequent analysis. Since immobilized organomercurials are capable of displacing nitric oxide from S-nitrosylated biomolecules, a second reagent capable of rapid, stoichiometric and low pH labeling of thiols can be exploited to differentiate this sub-population from alternative redox-based thiol modifications. Such a candidate is 4,4-dithiopyridine (4-DTP), and although the above features are highly uncommon for most every thiol labeling agent, they are even further complimented by the ability of 4-DTP to react with free thiols quantitatively, forming 4-thiopyridone (4-TP), a strongly absorbing chromophore ($\varepsilon = 21,400 \text{ L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ @ $\lambda = 324 \text{ nm}$) which allows for labeling efficiency to be monitored spectrophotometrically [50]. Furthermore, unlike other thiol-based labeling agents such as DTNB, as the name implies, the resultant product 4-TP contains a redox-inert thione (thio-ketone) moiety. This reaction product is thus unable to participate in additional disulfide exchange reactions, further exemplifying this reagent as a useful primary thiol blocking agent in studying redox regulation of protein thiols.

Although multiple work-flows can be built on this platform, we have chosen to highlight one such scheme, which enables analysis of both redox-inactive and redox-active protein thiols in a single experimental run, and infer relative kinetics/quantities of each. As a proof of concept, both wild-type hYVH1 and hYVH1 C115S (catalytic Cys to Ser mutation) digests were spiked with CAM-labeled counterparts, reduced with TCEP, and loaded onto Mercury-Immobilized Metal Affinity Chromatography (Hg-IMAC) resin at pH ~ 3.5 [Fig. 4.4 and Fig. 4.5]. Selective enrichment of thiol-containing peptides was observed; alkylated, CAM-labeled peptides, as well as non-thiol, methionine containing peptides, remained in the flow through supernatant [Fig. 4.4B and Fig. 4.5B].
Moreover, mutation of the active site cysteine resulted in loss of this corresponding peptide in the eluant, suggesting that intrinsic peptide composition has no effect on Hg-IMAC selectivity and peptide enrichment [Fig. 4.4C and Fig. 4.5C]. Furthermore, since it is possible to elute thiol-containing peptides using performic acid through formation of sulfoxides (SO\textsubscript{x}H) [47], taken together, this data suggests that Hg-IMAC is capable of selective enrichment of strictly thiol-containing peptides at low pH in the presence of the reducing agent TCEP. To note, this enrichment method is also highly diverse, allowing for individual tailoring of experimental procedures. For example, binding is quite rapid, approaching completion in approx. 30 min, however this rate can be 'tuned' by altering the amount of resin and/or the amount of protein/peptide loaded. Moreover, efficient binding is observed over a large dynamic buffer and pH range. For example, 10% acetonitrile, Tween-20 and guanidium hydrochloride additives displayed no substantial effects on peptide binding or enrichment over pH values ranging from approx. pH 3 - 7, highlighting the wide practical applicability of this methodology to a variety of experimental conditions and hypotheses [Suppl. Fig. S4.3 and Suppl. Fig. S4.4].

Based on the above findings, we have been successful in extending this workflow to studying redox-regulated thiols within the catalytic domain of hYVH1 [Fig. 4.3; Left]. Briefly, reduced hYVH1\textsubscript{ΔCT1} was rapidly exchanged into neutral buffer and immediately stimulated with H\textsubscript{2}O\textsubscript{2}. Oxidation was next quenched by the addition of a large molar excess of phosphate buffer pH 3.5 to effectively drop the reaction pH to approx. pH 4. This step serves a dual purpose as it is widely known that phosphate buffers act to competitively inhibit members of the PTP superfamily, as phosphate ions can readily occupy the active site [13]. This provides added analytic advantages, preventing non-specific oxidation of the catalytic cysteine residue during subsequent sample processing through a direct competition with H\textsubscript{2}O\textsubscript{2}. Concomitant addition of an acid protease (GluC, pepsin or equivalent) initiates digestion, which is followed by an initial Hg-IMAC enrichment step. The flow through supernatant of this first enrichment is equally partitioned and treated with or without TCEP, then subjected to a second Hg-IMAC enrichment, while the initial resin is washed and eluted for subsequent determination of reduced, redox-inactive thiols. Secondary Hg-IMAC enrichments are next washed and eluted for analysis of reversibly oxidized, redox-active thiols.
Figure 4.4 - Characterization of Hg-IMAC selectivity for wild type hYVH1 peptide thiols. Both CAM-labeled and unlabeled wild type hYVH1 were trypsin digested, mixed, acidified, and incubated with Hg-IMAC resin for 1 hr using acetonitrile additive. Supernatant was collected and bound peptides were competitively eluted using DTT. All samples were analyzed by MALDI-TOF MS. A) Mixed peptides before Hg-IMAC enrichment. B) Flow-thru supernatant showing near depletion of thiol containing peptides while IAM labeled peptides remain. C) Peptide eluant from Hg-IMAC enrichment showing selective thiol-containing peptide enrichment as CAM-labeled peptides were not detected in the eluant.
Figure 4.5 - Characterization of Hg-IMAC selectivity for hYVH1 C115S peptide thiols. Both CAM-labeled and unlabeled hYVH1 C115S were trypsin digested, mixed, acidified, and incubated with Hg-IMAC resin for 1 hr using acetonitrile additive. Supernatant was collected and bound peptides were competitively eluted using DTT. All samples were analyzed by MALDI-TOF MS. A) Mixed peptides before Hg-IMAC enrichment. B) Flow-thru supernatant showing near depletion of thiol containing peptides while IAM labeled peptides remain. C) Peptide eluant from Hg-IMAC enrichment showing specific and selective thiol-containing peptide enrichment. Neither CAM-labeled peptides nor the C115S peptide were detected in the eluant.
Of interest to our study were these redox-active thiols or reversibly oxidized thiols of hYVH1's catalytic domain. It was observed that indeed, hYVH1 possesses other thiols within the catalytic domain that undergo reversible oxidation upon exposure to H$_2$O$_2$, which were attributed to the two most N-terminal thiols [Fig. 4.6A]. Moreover, in the absence of TCEP, no peptides were observed, confirming that the initial Hg-IMAC enrichment went to completion, removing all redox-inactive, reduced thiols [Fig. 4.6B].

To further confirm certainty of peptide/thiol candidates, reversibly oxidized peptides from the TCEP reduced Hg-IMAC enrichment experiment were carbamidomethylated using IAM. This modification caused all peptides to shift by multiples of m/z 57 characteristic of CAM modification, confirming that all enriched peptides did indeed possess intrinsic cysteine thiols [Fig. 4.6C]. This also displays that Hg-IMAC does not interact with disulfide-linked peptides, to again, show thiol-dependent specificity.

As glutamic acid terminated peptides do not ionize as efficiently as those of tryptic descent, a secondary digest of these CAM-modified, glutamic peptides was performed to increase ionization efficiency as well as ease target peptide identification [Fig. 4.6D]. Upon tryptic digestion, it clearly identifies that indeed, other catalytic domain thiols of hYVH1 participate in reversible thiol modifications, suggesting that the redox regulation of this DUSP may not be limited to the previously described regulation, but instead adapt a disulfide exchange mechanism similar to that observed for SHP-1 and SHP-2 [18]. To note, as with any MS-based approach, a potentially strong limitation exists within the described method of analysis with respect to ion suppressive effects. One can partially alleviate these influences by further separation of eluted peptides prior to MS analysis by reversed-phase high performance liquid chromatography or equivalent separations chemistry to enhance individual peptide resolution. In foresight, this entire workflow has been constructed for adaption to high-throughput LC/MS-based platforms which would further extend the analytic capabilities instrumentally, while also combating the potential limitations described above.

To begin elucidation of this potential mechanism governing the redox regulation of hYVH1, we have adapted a novel labeling strategy involving 4-DTP as a primary thiol blocking agent as described above. The efficacy of this reagent as a primary thiol
Figure 4.6 - Characterization of reversibly oxidized thiols in the DUSP domain of hYVH1. hYVH1ΔCT1 was exposed to oxidative stress, acidified and rapidly proteolyzed with Glu C. Peptides were then passed over Hg-IMAC resin to capture any remaining reduced thiols. The resin flow-through was split into two fractions, with one fraction left untreated (oxidized thiols) and the other reduced with TCEP (reversibly oxidized thiols). Both fractions were then passed over fresh Hg-IMAC resin, the resin was eluted, and the eluents analyzed by MALDI-TOF MS. 

A) TCEP-reduced, Hg-IMAC eluant of reversibly oxidized thiol-containing peptides were observed, highlighting putative candidate(s).

B) Non-TCEP-reduced, Hg-IMAC eluant of oxidized thiol-containing peptides were not observed, ensuring primary Hg-IMAC enrichment went to completion.

C) TCEP-reduced, Hg-IMAC eluant of reversibly oxidized thiol-containing peptides from A) were CAM-modified with IAM to confirm enriched peptides contained free thiols as denoted by m/z shifts of 57.

D) CAM-modified peptides from C) were digested by trypsin and reanalyzed to confirm candidate identification through both improvement of ionization efficiency and target mass identification by tandem MS.
A blocking agent is highlighted by its ability to block subsequent CAM labeling, a condition that can be, as expected, ablated by TCEP reduction prior to CAM labeling [Suppl. Fig. S4.5 and Suppl. Fig. S4.6]. Although a variety of reversible modifications can be stimulated by H₂O₂, we suspected that the likely candidate was disulfide bonding based on our, and Chen et al., previous findings [18,23]. Following our alternative scheme [Fig. 4.3; Right] we were able to calculate putative disulfide masses for potential tryptic dipeptides between the active site cysteine (Cys¹¹⁵) and N-terminal cysteine residues (Cys¹¹ and Cys²³). Upon analysis, we observe sound evidence for the formation of a disulfide bond between the active site and an N-terminal thiol (Cys¹¹⁵-S-S-Cys²³ disulfide linkage) [Fig. 4.7A]. This species was ablated by addition of TCEP, and moreover, mutation of the catalytic cysteine to serine (C115S) also resulted in loss of this species, suggesting that a disulfide linkage is forming between these residues [Fig. 4.7B,C]. Surprisingly, we also observed a suspected linkage between Cys²³ and the vicinal thiol Cys¹¹ (Cys¹¹-S-S-Cys²³ disulfide linkage) which was also ablated by treatment with TCEP [Fig. 4.7A-D]. Overall, these findings have begun to provide evidence for a disulfide exchange mechanism which prevents irreversible thiol oxidation in a manner similar to that of SHP-1 and SHP-2 for the atypical DUSP, hYVH1. Current site-directed mutagenesis efforts creating serine point mutants for individual cysteines within the catalytic domain of hYVH1 are underway to begin detailed characterization of the ordered steps within this disulfide exchange mechanism.
Figure 4.7 - Identification of putative disulfide exchange within the active site of hYVH1 using 4-DTP labeling methodology. Both wild type hYVH1 and hYVH1 C115S were exposed to oxidative stress, acidified and rapidly labeled with 4-DTP. Samples were neutralized, digested with trypsin, equally partitioned, with one equivalent being reduced with TCEP, the other untreated. All samples were spotted and analyzed by MALDI-TOF MS.  

A) Oxidized, 4-DTP-labeled wild type hYVH1 displays putative disulfide linkages.  

B) Oxidized, 4-DTP-labeled, TCEP reduced wild type hYVH1 shows complete ablation of putative disulfides.  

C) Oxidized, 4-DTP-labeled hYVH1 C115S displays only a single putative disulfide linkage, suggesting disulfide exchange occurs involving this thiol.  

D) Oxidized, 4-DTP-labeled, TCEP reduced hYVH1 C115S also shows complete ablation of the lone putative disulfide, further suggesting evidence of
Using a combination of low pH thiol labeling and enrichment strategies, we have simplified the experimental workflow during sample preparation of redox-regulated protein thiols. As a proof-of-concept, we have examined the redox-regulation of hYVH1 and elucidated a potential disulfide exchange mechanism. Such an added layer of regulation may contribute to the ability of hYVH1 to cope with cellular insults such as oxidative stress [23,38]. Moreover, we are currently applying this strategy to large-scale redox proteomic experiments of both mammalian and *Daphnia pulex* origins for investigation of global cellular redox regulation of PTP family members. Through coupling to mass spectrometry, we have integrated new additions to current thiol labeling/enrichment schemes used in detection of redox sensitive protein thiols including those which regulate the activities of PTPs.
Supplementary Figure S4.1 - Characterization of Hg-IMAC resin. A) Standard curve of Cys-HCl free thiol with DTNB showing linear relationship. B) Binding of Cys-HCl by Hg-IMAC resin as shown by reduction of free thiol with respect to increasing quantities of slurry, showing a linear relationship with slope denoting a binding capacity of 2.2 μM thiol/mL of slurry. C) Box plot for seven independently prepared batches of Hg-IMAC resin (data extrapolated to 1 mL of slurry) showing reproducibility.
Supplementary Figure S4.2 - Non-specific oxidation of hYVH1 thiols during proteolytic digestion at neutral pH. Recombinant hYVH1 C115S and CAM-labeled hYVH1 C115S were subjected to an overnight trypsin digestion using a 1:25 protein:protease ratio in 50 mM ammonium bicarbonate pH 8, shaking at 37 °C. The resulting tryptic peptides were analyzed by MALDI-TOF MS with A) or without B) prior reduction with 25 mM TCEP.
Supplementary Figure S4.3 - Characterization of Hg-IMAC selectivity for wild type hYVH1 peptide thiols. Both CAM-labeled and unlabeled wild type hYVH1 were trypsin digested, mixed, acidified, and incubated with Hg-IMAC resin for 1 hr using Tween-20 additive. Supernatant was collected and bound peptides were competitively eluted using DTT. All samples were analyzed by MALDI-TOF MS. A) Mixed peptides before Hg-IMAC enrichment. B) Flow-thru supernatant showing near depletion of thiol containing peptides while IAM labeled peptides remain. C) Peptide eluant from Hg-IMAC enrichment showing selective thiol-containing peptide enrichment as CAM-labeled peptides were not detected in the eluant.
Supplementary Figure S4.4 - Characterization of Hg-IMAC selectivity for hYVH1 C115S peptide thiols. Both CAM-labeled and unlabeled hYVH1 C115S were trypsin digested, mixed, acidified, and incubated with Hg-IMAC resin for 1 hr using Tween-20 additive. Supernatant was collected and bound peptides were competitively eluted using DTT. All samples were analyzed by MALDI-TOF MS. 

A) Mixed peptides before Hg-IMAC enrichment. 

B) Flow-thru supernatant showing near depletion of thiol containing peptides while IAM labeled peptides remain. 

C) Peptide eluant from Hg-IMAC enrichment showing specific and selective thiol-containing peptide enrichment. Neither CAM-labeled peptides nor the C115S peptide were detected in the eluant.
Supplementary Figure S4.5 - Schematic workflow of 4-DTP labeling efficiency and specificity. Protein sample is acidified, labeled with 4-DTP and partitioned equally. Samples are treated with or without TCEP to reduce 4-TP labeling, then neutralized and CAM-labeled with IAM. Samples are digested with trypsin and analyzed by MALDI-TOF MS to ensure specificity and completion of 4-DTP labeling under the given conditions.
Supplementary Figure S4.6 - Characterization of 4-DTP labeling efficiency and specificity for wild type hYVH1 peptide thiols. Wild type hYVH1 was acidified, labeled with 4-DTP and partitioned equally. Samples were treated with A) or without B) TCEP to reduce 4-TP labeling, then neutralized and CAM-labeled with IAM. Samples were digested with trypsin and analyzed by MALDI-TOF MS to ensure specificity and completion of 4-DTP labeling under the given conditions. A) Despite an extreme excess of IAM labeling agent, no CAM modified peptides are observed in the absence of TCEP reduction, whereas in B), multiple CAM modifications are observed.
4.6 - REFERENCES


[42] C.M. Mailloux, Structure-function characterization of the human dual specificity phosphatase hYVH1, Department of Chemistry and Biochemistry, University of Windsor, Windsor, 2010, pp. 132.


CHAPTER 5 - MIXED DISULFIDE-BASED LABELING AGENTS FOR THE DETECTION, ENRICHMENT AND QUANTIFICATION OF BIOLOGICAL THIOLS

5.1 - INTRODUCTION

Biological thiols play a critical role in normal cell physiology, not only in maintenance of redox homeostasis of the cellular milieu, but in protein structure, modification/regulation and catalysis. Increasing evidence is beginning to uncover that the protein families affected through thiol-mediated regulation are becoming as diverse as the wide variety of potential thiol modifications themselves [1-6]. To this end, continued method development is necessary to refine those current studies, but also to drive the discovery of new ones.

Most studies aimed at studying thiol modification use indirect, reversible or differential thiol labeling [7]. These methods have been quite powerful in defining the redox proteome, however, as a stand-alone method of analysis, these approaches tend to be limited in their abilities to decipher between the many types of thiol oxidation, reporting only reversibly oxidized thiols. These applications further suffer from sample handling issues that often leave target thiols prone to non-specific reactions limiting both their sensitivity and accuracy [7]. Efforts have been made to circumvent formation of these artifacts, but complete removal has been challenging to achieve [8,9].

Recent efforts to study thiol modifications have employed chemical 'omics' approaches using a variety of reagents with diverse properties such as cell permeability, rapid low pH labeling, and/or tailored reactivity [10]. Recent examples have been successfully applied to the study of S-glutathionylation, S-nitrosylation and sulfenylation, whereby synthetic labeling agents impose unique reactive properties or functional groups on thiol-containing targets for subsequent enrichment and/or analysis [1,11]. In the case of the protein tyrosine phosphatase (PTP) superfamily, which have been shown to be susceptible to oxidation through their active site cysteine, use of substrate mimicking labeling agents can isolate non-oxidized, active enzymes, providing specific thiol-targeting through chemical reactivity which has tertiary structural constraints [12].
Collectively, the advantage of directed labeling and/or enrichment of targeted moieties is the drastic improvement in sensitivity and in accurate characterization of low stoichiometric thiol modifications.

It is considered that cysteine thiols possess a highly variable pK\(a\) depending on their immediate microenvironment. Most thiols targeted by oxidation exhibit a lower pK\(a\) (~3 - 6) than that of free cysteine (~8). This feature makes them prone to reactivity not only in a physiological context, but during classical labeling and sample preparation steps, ultimately decreasing the accuracy of the intended analytical read-out [7,13]. However, the ability to exploit these pH-dependent chemical characteristics has been hindered by the availability of labeling agents capable of reacting with a thiol at pH values low enough to prevent non-specific oxidation [14,15]. We recently examined the ability of organomercurials to label thiols at low pH and found that not only could these reagents react and enrich for thiols at pH ≤ 4, they could do so in the presence of reductant. Thus we described a method for the rapid labeling and enrichment of peptide thiols at low pH using mercury-immobilized metal affinity chromatography (Hg-IMAC) methods [16]. Others have suggested the use of trichloroacetic acid in the lysis of cells to quench oxidation and disulfide exchange during handling steps, which could be implemented quite readily to this scheme, highlighting the future applicability of this method to those in the field of redox signaling [7,17].

Keeping on the same theme, recent synthesis of a novel reagent capable of reversible thiol labeling at low pH has been described. Built on a glutathione (GSH)-based backbone, Nielsen et al. describe the use of an isothiocyanate (ITC)-based cyclic activated disulfide (ITC-CAD) compound which is able to react with thiols both specifically and rapidly at low pH [18]. Furthermore, through use of fluorescein-isothiocyanate (FITC), they were able to impart fluorescent properties onto this molecule (FCAD) for the sensitive quantification protein thiols through a variety of means. Based on this platform, we have sought to produce a library of ITC-CAD-based, rapid low pH thiol labeling agents which possess versatile analytical properties. We have successfully produced and characterized a phenylisothiocyanate-CAD reagent which possesses similar chemical properties to that of FCAD described by Nielsen et al. [18]. Moreover, we have
optimized the synthetic scheme, reducing time and cost, while increasing throughput and characterization, and also have begun to define both the minimal and optimal reaction characteristics involved in formation of CAD-based reagent design. Through changes to the synthetic scheme, we have uncovered the potential use of a low pH thiol labeling agent as a MS-based reporter ion capable of targeted MS approaches. Taken together, we have fortified and diversified the CAD-based reagent platform, discovering new molecular properties which may allow for future 'tuning' of such reagents chemical reactivity.
5.2 - EXPERIMENTAL PROCEDURES

*Synthesis of isothiocyanate-cyclic activated disulfide (ITC-CAD) labeling agents*

Preparation of CAD reagents was largely based on methods previously described [18]. Key modifications to the synthetic scheme and overall approach were adapted herein due to the formation of appreciable amounts of reaction by-products which reduced experimental yields, the poor adaption to scaling up the synthesis, and the resultant poor cost efficiency using the cited method above.

Stock solutions of reduced glutathione (GSH; Sigma) and tris(2-carboxyethyl) phosphine hydrochloride (TCEP-HCl; Thermo Scientific) were prepared gravimetrically in Burdick and JacksonBrand® High Purity Solvent HPLC Grade water (BJ H₂O). Standardized concentrations of GSH free thiol (-SH) and of TCEP were determined using recrystalized 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB; Sigma) as per Riddles *et al.* (\(\varepsilon = 14 \text{ 150 } \text{L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1} \) @ \(\lambda_{\text{Abs}} = 412\text{nm}\)) [19,20]. Stock solutions of 4,4-dithiopyridine (4-DTP; Acros Organics) were prepared gravimetrically in BJ H₂O, and solubilized by the addition of 12 M HCl in small increments to an approximate final concentration between 20 and 30 mM [15]. Concentration of stock 4-DTP solutions were determined using standardized GSH or TCEP solutions (described above) as per Riener *et al.* (\(\varepsilon = 21 \text{ 400 } \text{L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1} \) @ \(\lambda_{\text{Abs}} = 324\text{nm}\)) using semi-micro acrylic cuvettes with 93 - 97% light transmission between \(\lambda = 313 - 334\) nm (Sarstedt) [15]. Stoichiometry of the GSH:4-DTP reaction was found to be 1:1 as determined by titration of GSH against 4-DTP, with reaction completion occurring within 10 min, consistent with previous findings[15].

Based on this data, in 50 mM sodium acetate pH 4.1, 1 mM GSH was reacted with 1.1 mM 4-DTP shaking at room temperature for 30 - 60 min to ensure complete reaction. As a starting derivative, phenylisothiocyanate (PITC; Sigma) was used based on its intrinsic absorption characteristics (\(\lambda_{\text{Abs}} = 254\text{nm}\)). Two stock solutions of 20.75 mM PITC were prepared, by dilution, in different solvent mixtures, either 1:1 BJ H₂O:pyridine (Sigma) or 2:3:1 BJ H₂O:pyridine:triethylamine (TEA; Sigma). Solvent pH was estimated with pH paper and determined to by approximately 8.5 and 10 respectively. Serial dilutions of each stock into their respective solvent were performed to produce a range of PITC concentrations (1, 2, 3, 4, and 5 mM), with each being mixed 1:1 with the above GSH:4-DTP stock solutions.
DTP mixture to result in molar ratios of 1:1 to 1:5 respectively. Each reaction was performed in duplicate shaking at room temperature allowing for incubation of either 30 or 60 min. At time, reactions were quenched with a final concentration of 10 mM ethanolamine (Sigma) for 30 min at room temperature shaking. Each reaction was transferred to siliconized microcentrifuge tubes containing an equal volume of toluene, mixed by vortex, centrifugally pulsed to collect the solution, then rotary evaporated for 30 min at 30 °C to remove the volatile toluene:pyridine azeotrope formed. This step is repeated one time until all of the organic layer is gone, leaving a volume equivalent to either the 1 part H₂O or 2 parts H₂O and 1 part TEA respectively, denoting that most of the pyridine has been removed. Samples were characterized and analyzed on a Waters nanoACQUITY ultra-performance liquid chromatography® (UPLC) system interfaced into a Micromass Quattro-micro® electrospray ionization triple-quadrupole mass spectrometer (ESI MS) fitted with a Waters nanoESI source as described below.

**Analysis of PITC-CAD synthesis and thiol labeling ability**

To characterize target PITC-CAD containing samples for both disulfide presence and thiol labeling ability, samples were tested for thiol-dependent reactions. Stock solutions of N-ethylmaleimide (NEM; Sigma) and L-cysteine (Cys; Sigma) were prepared gravimetrically in BJ H₂O, while standardized concentrations of Cys free thiol were determined using a DTNB assay as described above. Solutions of 1.1 mM Cys were prepared in 100 mM sodium acetate pH 4 or in 25 mM Tris-HCl pH 8 supplemented with 10 mM N-ethylmaleimide. The latter was incubated at room temperature for 1 h in the dark to block free thiols. Stock samples containing PITC-CAD were diluted 1:5 into either buffer solution, 5 mM NEM, 5 mM TCEP, 5 mM TCEP then 5mM NEM, 100 μM Cys-NEM or 100 μM Cys, incubated for 60 min at room temperature shaking in the dark, then analyzed by LC/MS as described.
**Analysis and characterization of PITC-CAD reaction products by nanoLCMS/MS**

Samples of synthetic PITC-CAD reactions were diluted 1:10 into 0.1% trifluoroacetic acid (TFA; Thermo Scientific) in BJ H₂O, transferred to total recovery, pre-slit PTFE/silicone septa sealed, LectraBond capped, 12x32 mm glass screw neck vials (Waters Corp.) and placed into the autosampler plate at room temperature. The nanoACQUITY UPLC® was equilibrated with 98% mobile phase A consisting of 0.1% formic acid (FA; Thermo Scientific) in Optima® LC/MS grade water (Optima H₂O; Fisher Scientific) and 2% mobile phase B consisting of 0.1% FA in Optima® LC/MS grade acetonitrile (Optima ACN; Fisher Scientific). Samples were injected onto a 180 µm x 20 mm x 5 µm Symmetry® C18 reverse phase nanoACQUITY UPLC trapping column for 3 min at a flow rate of 5 µL/min under equilibration conditions. Samples were then resolved on a 100 µm x 100 mm x 1.7 µm BEH®130 C18 reverse phase nanoACQUITY UPLC analytical column over a 35 min linear gradient of 2 - 55% mobile phase B at 300 nL/min. Timed-ion chromatograms and mass spectra were acquired on a Waters Quattro-micro® nanoESI triple-quadrupole MS. Instrument settings varied based on MS tune requirements for each mode of analysis with tune settings of the quadrupoles being set for wide-range applicability across a diversity of biomolecules. For targeted approaches, tune settings were determined empirically by continuous direct-injection of molecule(s) of interest using the microESI source interfaced onto the above triple-quadrupole MS. This practice is necessary to maximize sensitivity of the MS for the target precursor and/or transition ion(s) during all MS applications. Settings for reverse phase ultra performance liquid chromatography, MS scan, daughter scan, precursor ion scanning and multiple reaction monitoring can be found in appendices [Appendix A].
5.3 - RESULTS AND DISCUSSION

Research on biological thiols continues to reveal their expanding and diverse roles in cellular homeostasis and physiology. Thus, there remains the need to develop improved analytical tools for studying their functional state within the cellular milieu under both normal and conditional states. Based on the preliminary efforts and ideas outlined by Nielsen et al., we sought to initiate the synthesis of a CAD-based library of rapid, low pH thiol labeling agents possessing multifaceted analytical properties, with our main focus being MS-based detection [18]. In our first attempts, we adapted the current CAD synthetic schemes, however found that the time and amount of processing, along with the inherent chemistries within, promoted formation of appreciable amounts of reaction by-products which reduced experimental yields of PITC-CAD. While this scheme produced single isothiocyanate (ITC) modifications of GSSG, we sought the dually modified form to double the yield of reaction product upon TCEP reduction [Fig. 5.1]. Moreover, to purify differentially labeled species, we exploited their UV absorbance property along with suspected differences among their protonated charge states. Thus use of ion exchange chromatography (IEC) as opposed to the original C18 RP-HPLC mode, resulted in much larger injection and loading/binding capacities, which also offered ease of scaling-up the synthesis in multiple downstream applications.

From our accounts, using this slightly modified approach, reaction of PITC with GSSG yielded mixed populations of products over time. As hypothesized, these species could be isolated and resolved by IEC; single amino-terminal modification was the predominate product after 10 min reaction time and eluted first (~ 23 min), whereas dual modification predominated after approximately 40 - 80 min under the given conditions, eluting at higher salt concentration (~ 52 min) as expected [Fig. 5.2 and Fig. 5.3]. Upon MS analysis, we observed the presence of additional molecules among each fraction in addition to the singly or dually modified species, suggesting that appreciable amounts of non-targeted side reactions may have occurred. Intriguingly, in the singly modified fraction, the predominant contaminant was a measured $16 \text{ m/z}$ less than the mass of the PITC-modified GSSG ($\text{m/z} = 746$), whereas in the dually modified fraction, the
Figure 5.1 - Synthetic scheme of isothiocyanate-cyclic activated disulfide reagents using oxidized glutathione. In the first step of synthesis, one amino group of excess GSSG reacts with an isothiocyanate derivative (ITC) to form ITC-GSSG. This product is purified by RP-HPLC, followed by reduction of the disulfide bond by tris(2-carboxyethyl)phosphine (TCEP) generating a free thiol in the glutathione moiety (GSH) to form ITC-GSH. The free thiol is activated by 4,4′-dithiopyridine (4-DTP) forming ITC-GS-4-TP whereby the disulfide is subsequently attacked by the thiol of the thiourea to form the cyclic activated disulfide and release of 4-thiopyridone (4-TP). Adapted from Nielson et al., Anal. Biochem., (2012) 421:1, 115-120.
Figure 5.2 - Separation of phenylisothiocyanate modified oxidized glutathione derivatives by anion exchange chromatography.  

A) Reaction controls from synthetic schemes according to Nielsen et al. were injected and separated to use as comparative references. Reaction solvent (Blank), phenylisothiocyanate (PITC) without oxidized glutathione (GSSG), PITC with GSSG immediately quenched with ethanolamine, and GSSG without PITC were resolved by anion exchange chromatography. 

B) Synthetic reactions of PITC with GSSG were quenched at 10, 40 or 80 min and resolved by anion exchange chromatography. 

C) Structure and chemical characteristic of GSSG along with gradient conditions used in separations. In all cases, absorbance at $\lambda = 254$ nm and conductivity were monitored continuously. Conductivity of linear sodium chloride (NaCl) gradient shown in red.
Figure 5.3 - Mass spectra of differentially resolved fractions by anion exchange chromatography. Synthetic reactions quenched at A) $t = 10$ and B) 80 min were analyzed by liquid chromatography/mass spectrometry (LC/MS) as described in 'Experimental Procedures'.
predominant contaminant was 32 m/z less than the target m/z = 881. [Fig. 5.3].

Upon further inspection, it was not surprising to find that these observed by-products are quite reminiscent of products formed during the first step in the Edman degradation reaction [21]. In this scheme, PITC first reacts with the amino terminus of a protein or peptide under mildly organic alkaline conditions to form a phenylthiocarbamoyl (PTC) adduct, which is then converted by both anhydrous and aqueous acids, and heat, to the phenylthiohydantoin (PTH)-amino acid derivative used in analysis [Fig. 5.4]. *En route* to formation of the PTH derivative, nucleophilic attack of the carbonyl carbon within the first peptide bond is mediated by a formed thiolate resulting from tautomerization of the PTC thiourea. Based on this, we suspect that due to the unique peptide bond formed in GSH between the C_δ carboxylate of glutamate and the α-amino group of cysteine, the free α-carboxyl group of glutamate is the target for nucleophilic attack and loss of an oxygen (m/z = 16) in the form of water [Fig. 5.4A,B]. This is further supported by the evidence of the y1 and y2 fragment ions at m/z = 76 and m/z = 177 of these reaction by-products, which correspond to the intact α-amino acid glycine and dipeptide cysteine-glycine (CG) respectively, as well as the fragment ion at m/z = 423 which is suspected to be a result of breakage about the disulfide forming cyclic PITC-GSH [Fig. 5.5C]. Moreover, if a similar reaction to that of Edman's degradation was occurring, one would expect the unique GSH peptide bond would have to hydrolyze, resulting in decomposition of GSSG which was not detected by LC/MS analysis under the given conditions [Fig. 5.3 and Fig. 5.5].

After the pioneering work by Edman, others had success in gaining sensitivity through the use of fluorescein-isothiocyanate (FITC) as their labeling agent in protein sequencing, which in combination with data presented above, suggests that to some degree, Nielson *et al.* likely saw similar yields of reaction by-products [18,22,23]. From these collective conclusions and datasets, we governed a new synthetic scheme, which would act to minimize not only potential side reactions, but also processing time and costs, while maximizing purification yields of target CAD reagents. In reassessment of the original synthetic scheme for CAD-based reagents, GSSG is modified with an ITC derivative (ITC-GSSG), purified by analytical RP-HPLC, reduced with TCEP (ITC-GSH), then
Figure 5.4 - Synthetic scheme of Edman's degradation. Phenylisothiocyanate (PITC) first reacts with the amino terminus of a protein or peptide under mildly organic alkaline conditions to form a phenylthiocarbamoyl (PTC) adduct. This is then converted by both anhydrous and aqueous acids, and heat, to the phenylthiohydantoin (PTH)-amino acid derivative used in analysis, being accomplished by nucleophilic attack of the carbonyl carbon within the first peptide bond by a formed thiolate resulting from tautomerization of the PTC thiourea. Adapted from Edman, P., Acta Chem. Scand., 4 (1950) 34.
Figure 5.5 - Suspected route and structure of synthetic by-product formation during phenylisothiocyanate reaction with oxidized glutathione.  

A) The unique peptide bond formed in glutathione (GSH) leaves the free α-carboxyl group of glutamate proximally located to the thiourea thiolate for nucleophilic attack and loss of an oxygen, as opposed to attack of the carbonyl and hydrolysis of the amide bond.  

B) Proposed structure of the synthetic by-product cyclic PITC-GSSG-PITC at m/z = 849.  

C) Tandem mass spectrum (MS/MS) of synthetic by-product at m/z = 849 as analyzed by liquid chromatography/mass spectrometry (LC/MS) described in 'Experimental Procedures'.

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reacted with 4-DTP to form a mixed disulfide between ITC-GSH and 4-thiopyridine (4-TP, and thus ITC-GS-4-TP) \([\text{Fig. 5.1}]\). At this point, similar to the Edman degradation reaction, it is thought that tautomerization of the ITC thiourea results in formation of a thiolate capable of nucleophilic attack of the activated mixed disulfide, releasing this efficient 4-TP leaving group, to spontaneously form the ITC-CAD reagent \([\text{Fig. 5.1}]\).

Isothiocyanates can react not only with free amines, but also with sulfhydryls and therefore, use of GSSG affords protection to reactive thiols from ITC-modifications by means of a disulfide bond between two equivalents of GSH. Since the ITC-GSSG is later reduced to ITC-GSH for subsequent reaction with 4-DTP, we presumed that the primary reaction of GSH with 4-DTP would offer the same protection during a subsequent ITC-modification. Moreover, using this as starting material would presumably have multiple benefits both synthetically and analytically. First, the stoichiometry of the reaction between GSH and 4-DTP can be monitored by spectrophotometry, has been found to occur rapidly in a 1:1 ratio at low pH, can be readily scaled-up to > 20 mM concentrations and produces mixed disulfides with efficient leaving groups for cyclization as well as chemically inert thio-ketone (thione) products \([15]\). In addition, this inert product is incapable of further reaction during subsequent thiol or amine modification steps, removing the requirement of a purification step prior to final product formation. Secondly, this would also avoid GSSG and TCEP serving as reactants during the synthesis, significantly reducing costs of both small and large scale applications. Lastly, it is suspected that the PTC-adduct-like reaction by-products observed were a result of kinetic reaction limitations involving the thiourea-based nucleophilic attack of the activated ITC-GS-4-TP mixed disulfides \([\text{Fig. 5.1}]\). The length of processing time to isolate and achieve this activated ITC-GSH-4-TP species would likely kinetically favour attack of the neighbouring \(\alpha\)-carboxyl group of glutamate in both ITC-GSSG or ITC-GSH, resulting in irreversible consumption of the ITC thiolate, and thus, drastic reduction of ITC-CAD yields. We hypothesize that use of the thiol-protected, pre-activated starting material, GS-4-TP, would promote a kinetic battle between reaction of the nucleophilic ITC thiolate and either the activated mixed disulfide or the \(\alpha\)-carboxyl group of glutamate \([\text{Fig. 5.6}]\).
Figure 5.6 - Modified synthetic scheme of isothiocyanate-cyclic activated disulfide reagents using glutathione and 4,4'-dithiopyridine. In the first step of this modified synthesis, glutathione (GSH) is reacted with 4,4'-dithiopyridine (4-DTP) to form GS-4-TP. Next, an isothiocyanate derivative (ITC) is added to react with the free amino group to form ITC-GS-4TP, with subsequent conversion to form either the desired cyclic activated disulfide (ITC-CAD) and 4-thiopyridone (4-TP) or cyclic ITC-GS-4-TP and water.
This modified CAD synthetic scheme, achieved rapidly (1.5 h) and relatively pure synthesis of PITC-CAD (m/z = 441) in a two step process [Fig. 5.6 and Fig. 5.7]. Moreover, although some reactants and by-products were still observed, a far greater proportion of target PITC-CAD reagent was generated [Fig. 5.7]. To begin characterizing the reaction, we performed MS/MS analysis on some of the reactants and products throughout the synthesis. The initial step involves reaction of GSH (m/z = 306) with 4-DTP to form GS-4-TP (m/z = 417), where MS/MS analysis can differentiate among these moieties based not only on their differing precursor ion masses, but also by their uniquely characteristic fragment ion masses. Glutathione exists as a tripeptide with the C-terminal dipeptide CG having the potential to serve as a multifaceted reporter ion system as described above. Fragmentation of GSH results in a prominent y1 ion of m/z = 76 which corresponds to glycine denoting that the species analyzed is constructed of a GSH molecule, while a detectable y2 ion of m/z = 177 corresponds to a CG dipeptide denoting that the species contains a reduced thiol [Fig. 5.8A]. Interestingly, the GS-4-TP sample gave a distinct fragment ion at m/z = 112 which was not observed for GSH. This daughter ion is likely the result of fragmentation of the 4-TP (111 Da) disulfide bond, and thus characteristic of 4-TP containing species [Fig. 5.8B]. Also observed were additional fragment ions corresponding to the neutral loss/gain of sulfur as well as by an ion at m/z = 288 denoting y2 + 4-TP, further suggesting that the GS-4-TP Cys was participating in a disulfide bond [Fig. 5.8B].

Based on the observed fragment ion series [Fig. 5.6], the presence or absence of the m/z 112 fragment ion should be useful to differentiate between the formation of an intact ITC-CAD reagent versus an off-target reaction by-product. To confirm the use of these fragments as characteristic reporter ions for analysis, we performed precursor ion scanning (PIS) MS using either m/z = 76 or m/z = 112 as the target ion and found that the former identified all GSH containing species, including PITC-CAD [Fig. 5.9A], whereas the latter only identified 4-TP containing species in which PITC-CAD was not identified [Fig. 5.9B]. Moreover, PIS MS of m/z = 112 was an order of magnitude more sensitive than that of m/z = 76 under the given conditions, suggesting that 4-TP labeling may be a suitable reporter ion for use in many diverse applications aimed at analysis of biological thiols [Fig. 5.9]. Taken together, we feel that this simplified reaction scheme has
Figure 5.7 - Mass spectral analysis of modified cyclic activated disulfide synthesis products.  

A) Reaction products synthesized in water:pyridine:triethylamine at a 1:4 ratio of activated glutathione (GS-4-TP) to phenylisothiocyanate (PITC) were analyzed by liquid chromatography/mass spectrometry (LC/MS) as described in 'Experimental Procedures'.

B) Tandem mass spectrum (MS/MS) of synthetic PITC-cyclic activated disulfide at m/z = 441 as analyzed by liquid chromatography/mass spectrometry (LC/MS) described in 'Experimental Procedures'.
Figure 5.8 - Tandem mass spectra of modified cyclic activated disulfide starting materials glutathione and glutathione-4-thiopyridine. A) Glutathione at m/z = 306 and B) glutathione-4-thiopyridine at m/z = 417 were analyzed by liquid chromatography/tandem mass spectrometry (LC/MS/MS) as described in 'Experimental Procedures'.
Figure 5.9 - Precursor ion scanning mass spectra for characterizing cyclic activated disulfide synthetic reaction products. Fragment ions at A) $m/z = 76$ and B) $m/z = 112$ were analyzed by liquid chromatography/precursor ion scanning mass spectrometry (LC/PIS/MS) as described in 'Experimental Procedures'.
improved the overall efficiency of CAD-based reagent production, providing a working platform for continued development of a functionalized library of tailored reagents for a variety of thiol-directed conjugation applications.

To utilize the CAD-based reagents as biological thiol labeling tools, they must possess an intrinsic disulfide bond and also react with exogenous thiol-containing moieties rapidly at low pH. Initially, to examine these properties, we took our unpurified reaction mixture and treated it with either N-ethylmaleimide (NEM), TCEP, or TCEP followed by NEM, and compared them to untreated reaction mixture. It was observed that treatment with NEM alone had little effect on the target PITC-CAD precursor ion of \( m/z = 441 \) as denoted by relatively no change in the timed ion chromatogram (TIC) selecting only \( m/z = 441 \pm 1 \) Da [Fig. 5.10A,B]. However, treatment with TCEP or TCEP and NEM resulted in a drastic loss of signal, suggesting that TCEP reduction dissociated the PITC-CAD disulfide bond, thus forming the linearized version which is an estimated +2 Da heavier and misses the TIC mass window, while TCEP reduction followed by NEM treatment formed the alkylated version of \( m/z = 568 \) [Fig. 5.10 C,D]. To confirm this hypothesis, we monitored the TIC of \( m/z = 568 \) of PITC-CAD-NEM and it was observed that under TCEP alone, no ions of this mass were readily detected [Fig. 5.11A]. However, in the samples subsequently treatment with NEM, the formation of a dominant species at \( m/z = 568 \) was observed, confirming that PITC-CAD possesses an intrinsic, reactive disulfide bond moiety and that synthesis was successful [Fig. 5.11B]. To note, dual NEM modification was not observed to an appreciable amount, suggesting that tautomerization prefers the thiourea form under these conditions.

Based on this data, a similar approach was utilized to determine if PITC-CAD was capable of reacting with low molecular weight thiols such as L-cysteine (Cys). Again, we took our unpurified mixture and reacted it with Cys-NEM or with Cys and analyzed the products by LC/MS. We observed that reaction with Cys-NEM had very little effect on PITC-CAD as the TIC of \( m/z = 441 \) did not significantly alter in comparison to that of the starting material alone [Fig. 5.12A and Fig. 5.10A], and that the TIC of \( m/z = 562 \), a PITC-CAD-Cys species was also not detected [Fig. 5.13A]. By contrast, reaction with Cys resulted in a decrease in the \( m/z = 441 \) TIC [Fig. 5.12B] and an increase in the \( m/z =
Figure 5.10 - Timed ion chromatograms of phenylisothiocyanate-cyclic activated disulfide show sensitivity to reduction and alkylation. Unpurified reaction mixture containing phenylisothiocyanate cyclic activated disulfide (PITC-CAD) was reacted with \textbf{A)} water, \textbf{B)} N-ethylmaleimide (NEM), \textbf{C)} tris(2-carboxyethyl)phosphine (TCEP) and \textbf{D)} TCEP then NEM. Samples were analyzed by liquid chromatography/mass spectrometry (LC/MS) as described in ‘Experimental Procedures’, with data representing the timed ion chromatograms of $m/z = 441$ to detect the loss of PITC-CAD.
Figure 5.11 - Timed ion chromatograms of phenylisothiocyanate-cyclic activated disulfide show a positive reaction upon reduction and alkylation with N-ethylmaleimide. Unpurified reaction mixture containing phenylisothiocyanate cyclic activated disulfide (PITC-CAD) was reacted with A) tris(2-carboxyethyl)phosphine (TCEP) or B) TCEP then N-ethylmaleimide (NEM). Samples were analyzed by liquid chromatography/mass spectrometry (LC/MS) as described in 'Experimental Procedures', with data representing the timed ion chromatograms of m/z = 568 to detect the formation of PITC-CAD-NEM.
Figure 5.12 - Timed ion chromatograms of phenylisothiocyanate-cyclic activated disulfide show sensitivity to reduced cysteine not alkylated cysteine. Unpurified reaction mixture containing phenylisothiocyanate cyclic activated disulfide (PITC-CAD) was reacted with A) cysteine alkylated with N-ethylmaleimide (NEM) or B) cysteine. Samples were analyzed by liquid chromatography/mass spectrometry (LC/MS) as described in 'Experimental Procedures', with data representing the timed ion chromatograms of m/z = 441 to detect the loss of PITC-CAD.
562 TIC [Fig. 5.13B], as would be expected for a positive reaction between PITC-CAD and Cys. Upon MS/MS analysis, it was confirmed that an intact PITC-CAD-Cys species was formed, being linked through a disulfide bond, exemplifying the ability of PITC-CAD to react rapidly at low pH with thiol containing biomolecules [Fig. 5.13C]. Furthermore, from the MS/MS data, formation of a y2 + Cys ion at m/z = 298 suggests that it is the Cys thiol from GSH forming the mixed disulfide with free Cys, not that of the ITC thiourea, which also suggests that it is the GSH Cys that NEM is modifying in the characterizations discussed above [Fig. 5.13C and Fig. 5.11B]. These observations suggested that, similar to the prototypical FCAD described by Nielsen et al., PITC-CAD was also effective in rapidly labeling thiols at low pH. Moreover, the ability of these reagents to form reversible disulfide linkages also highlights their versatility for a wide variety of thiol-based applications.

From these observations, we have begun to define both the minimal and optimal reaction characteristics involved in formation of CAD-based reagent design. Although the inaugural scheme involved use of GSH derivatives as a starting material building block, our data suggests that the unique peptide bond formed by glutamate hampers the effective yield of target CAD-reagents. Using a generic peptide moiety may afford less off target reactions, as the neighbouring α-carboxylate will no longer be adjacent to the thiourea for attack, but found opposite to the ITC reactive N-terminus, at the peptides extreme C-terminus. With this, the longer the peptide chain, the farther the C-terminal carboxylate will be from the reactive thiolate granted no intrinsic glutamic or aspartic acid (Glu or Asp) residues are located within the primary sequence. Simple synthesis of peptides increasing in size will define the spatial limits of reactivity, and provide insight to the minimally optimal size of peptide for such reactivity, after which Glu and Asp residues could be added with no potential side reaction susceptibility. Similarly, sequential placement of a Cys residue throughout a series of synthetic peptides would allow for definition of the spatial reactivity limits of the ITC thiourea thiolate in forming the cyclic disulfide. Knowledge of such reaction constraints would allow 'tuning' of CAD-based reagents through manipulation of the primary sequence of the peptide, the ability to impose chemical reactivity past the minimal spatial limits of ITC thiolate, and through functional derivatization of the ITC reagent [Fig. 5.14].
Figure 5.13 - Timed ion chromatograms of phenylisothiocyanate-cyclic activated disulfide show a positive reaction with reduced cysteine not alkylated cysteine. Unpurified reaction mixture containing phenylisothiocyanate cyclic activated disulfide (PITC-CAD) was reacted with A) cysteine alkylated with N-ethylmaleimide (NEM) or B) cysteine. Samples were analyzed by liquid chromatography/mass spectrometry (LC/MS) as described in 'Experimental Procedures', with data representing the timed ion chromatograms of m/z = 562 to detect the formation of PITC-CAD-Cys. C) PITC-CAD-Cys at m/z = 562 was analyzed by liquid chromatography/tandem mass spectrometry (LC/MS/MS) as described in 'Experimental Procedures'.
Figure 5.14 - Theoretical model of the minimal and optimal reaction characteristics involved in the formation of CAD-based reagent design. Reaction constraints would allow 'tuning' of CAD-based reagents through manipulation of the primary sequence of the peptide (R₁, R₂, R₃ etc.), the ability to impose chemical reactivity past the minimal spatial limits of ITC thiolate (Y), and through functional derivatization of the ITC reagent (X).
Future work will involve purifying PITC-CAD and other CAD-based reagents with varying X group [Fig. 5.14] sterics using IEC as described above. As the pKa of the pyridine tertiary amine is ~ 5.01, approximately 60 - 70% of it would be protonated at pH 5 [15], and thus, separating target CAD (-2 @ pH 5) from reaction by-products such as cyclic-ITC-GS-4-TP (~ -0.5 @ pH 5) should be attainable. Furthermore, based on the striking resemblance of a thiol-bound CAD reagent to GSH, we have begun to use α-GSH antibodies in non-reducing immunoblotting as a complimentary screening method during CAD reagent design, and also to test the potential of these reagents for use in alternative analytical readouts. Ultimately, once reaction characterization is complete, we aim to make isotopic versions of CAD library reagents for relative, and potentially absolute, quantification of reactive thiols by MS. In another application, we have begun to attempt immobilization of CAD reagents to isothiocyanate-activated polystyrene resin. Immobilized versions of CAD reagents would allow for reactive thiol enrichment and reductive elution, and since polystyrene has better resin swelling capacity in organic solvents such as acetonitrile, this application should readily interface into MS-based modes of analysis.

One unexpected finding was the ability of 4-TP labeled molecules to readily decompose during MS/MS to form a fragment ion of \( m/z = 112 \) [Fig. 5.8]. This suggested that any 4-TP labeled thiols within a complex mixture could be identified by targeted MS modes such as PIS MS using this \( m/z = 112 \) reporter ion [Fig. 5.9]. We feel that this feature will be very powerful to study biologically relevant thiols in vivo, due to the ability to attain orders of magnitude higher sensitivity using targeted approaches. As described above and by others, this molecule has an abundance of unique properties which make it a very suitable redox-active thiol labeling agent, such as low pH labeling capabilities and inert thione products to name a few [15,16]. Currently the 'gold standard' of monitoring cellular redox homeostasis involves use of this molecule, and can achieve approximately 5 pmol sensitivity through monitoring 4-TP absorbance [14,17]. However, stand-alone, this can not discriminate among many thiol containing moieties within a complex mixture. Replacement of spectrophotometry with targeted quantitative MS will likely improve sensitivity, and offer the ability to identify and monitor many distinct thiol modifications in a given LC/MS run. Nonetheless, our results to date
suggest that this scheme will afford the increased sensitivity, and throughput described, perhaps adding another 'tunable' tool for studying the growing field of cellular thiol redox regulation.
5.4 - REFERENCES


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CHAPTER 6 - PHOSPHORYLATION-DEPENDENT ENDOosomal TARGETING OF THE PI(3)P PHOSPHATASE MTMR2 IS MEDIATED BY AN ERK1/2 NEGATIVE FEEDBACK MECHANISM AND IS REGULATED THROUGH INDIRECT INFLUENCES ON THE PH-GRAM DOMAIN

6.1 - INTRODUCTION

Although the majority of protein tyrosine phosphatases (PTPs) target phosphotyrosine-containing proteins as substrates, the active myotubularin (MTM) family members instead dephosphorylate lipid second messengers phosphoinositides (PI(3)P and PI(3,5)P2) [1-4]. These phosphatidylinositol phosphates mainly reside in endocytic structures, having key roles in membrane targeting, vesicular trafficking, and regulation of signal transduction pathways through recruitment and interactions with distinct signaling proteins containing appropriate phosphoinositide-binding module(s) [5-7]. The importance in regulating phosphorylation of phosphoinositides (PIPs) is highlighted by the discovery that loss of function mutations in three mtmr genes have been associated with distinct neuromuscular disorders [8-10]. For example, the gene encoding myotubularin-related 2 (MTMR2) is mutated in Charcot–Marie-Tooth (CMT) disease 4B, an autosomal recessive demyelinating disorder, characterized by abnormally folded myelin sheaths, inadequate nerve signaling to muscles, and eventual muscle weakness and atrophy [8].

Despite the identification of cellular substrates, elucidation of the three-dimensional crystal structure [11,12], and the well established pathophysiological consequences resulting from loss of MTMR2 function, how MTMR2 actively participates in vesicular trafficking remains poorly understood. Discovering roles for MTMR2 among these events has been further confounded due to the observations that under typical cell culture conditions this enzyme is not widely localized to substrate-rich endosomal structures containing PI(3)P and PI(3,5)P2 even though it possesses a domain thought to be capable of associating with PIPs [11-18]. Recent evidence from our laboratory has begun deciphering this anomaly, in which we have identified a prominent phosphorylation site at Ser58 that dramatically regulates MTMR2 endosomal localization, and thus access to
its lipid substrates [14]. Using phosphomimetic mutagenesis, we observed that a phosphorylation deficient variant (MTMR2 S58A) strongly co-localizes with substrate-rich, Rab5-positive endocytic vesicles, resulting in depletion of PI(3)P, mis-localization of PI(3)P binding proteins [19], and an increase in growth factor receptor signaling pathways, most notably activation of the extracellular signal-regulated kinases 1 and 2 (ERK1/2) [14]. Furthermore, our mass spectrometry analysis suggested that MTMR2 Ser58 phospho-levels may be constitutively high under normal cellular growth conditions, offering an explanation as to the lack of discernable co-localization with substrate-rich vesicles under these conditions. Moreover, since catalytic activity and hetero-dimerization with its inactive partner, MTMR5, were found to be independent of the phospho-status of Ser58, reversible phosphorylation may regulate the endosomal targeting of MTMR2, and thus its physiological catalytic activity through an allosteric mechanism. This has provided valuable insight to the regulated control of cellular PIP levels through direct influences on the spatial and temporal control of PI(3)P phosphatases. However, the exact mechanism of MTMR2 phosphorylation-dependent localization, identification of the responsible kinase(s), and the resultant effects on distinct membrane targeting, vesicular trafficking, and regulation of signal transduction pathways remains to be elucidated.

Although phosphoinositides are commonly regarded as membrane association cues targeting peripheral proteins to cellular membranous surfaces, it is becoming increasingly clear that additional interactions function in unison with PIP recognition to regulate membrane association of PIP-binding proteins [20]. As PIP-binding domains are notoriously low-affinity modules that commonly utilize basic residues for directed association with target PIPs [5,6,18], the combination of PIP-binding with that of a secondary signal (i.e. oligomerization, co-operative binding, multiple domains or binding sites etc.) not only stabilizes interactions, it also affords a far greater diversity of regulatory control over signal manipulation than that achieved through PIP levels alone [18,20]. Many mechanisms of this multivalent avidity-based membrane association exist, being commonly referred to as co-incidence detection [18,20]. One such pertinent example is that of Rab5 and Early Endosomal Antigen 1 (EEA1). The Rab5 GTPase is a master coordinator of early endosome maturation events including homotypic early
endosome fusion as well as heterotypic early to late endosome fusion [21,22]. Rab5 collaborates with numerous peripheral PIP-binding proteins to facilitate fusion of endosome membranes, including Rab5 effectors whose targeting requires association with endosomal PI(3)P [23-26]. The critical endosomal effectors Rabenosyn-5 and EEA1 are essential tethering factors during fusion which require association with Rab5 and PI(3)P simultaneously to stably localize at early endosomes, exhibiting a direct role for endosomal PI(3)P levels in governing proper vesicular trafficking outcomes [24-26]. Taken together, this low affinity, multivalent mode of regulation defines a highly plastic system, exhibiting dynamic instability which allows for rapid re-modeling in response to fluctuations in the level of PIPs and/or secondary signals [20]. In combination with the influences of PI kinases and phosphatases, this can readily translate into pronounced effects on the membrane associations and functions of PIP-binding proteins, to ultimately give rise to greater spatial and temporal regulation of phosphoinositide-mediated cellular processes.

An excellent example of cellular integration amongst these tiers of layered co-incident regulation involves the recent evidence that endosomes rich in the adaptor protein containing a pleckstrin homology domain, phosphotyrosine binding domain (PTB) and a leucine zipper motif (APPL1) represent a critical stage during attenuation of receptor signaling and initiation of early endosome maturation. APPL1 is another Rab5 effector complex, which directly binds Rab5, but also to the lipid bilayer through both membrane and PIP-binding domains, as well as to the cytosolic tail of plasma membrane receptors through a PTB domain [27-31]. It has been demonstrated that when PI(3)P is depleted using the PI(3)K inhibitor wortmannin, Rab5/EEA1-positive early endosomes are converted to Rab5/APPL1-positive early endosomes. Interestingly, the Rab5/APPL1 endosomes displayed a delay in endosomal maturation and an enhanced endosomal growth factor signaling capability [32]. In addition to functioning as a Rab5 effector and as a regulator of endosomal signaling, APPL1 serves as a stage marker for proximal early endosomes [33]. The shedding of APPL1 as vesicles move centrally coincides with increased levels of PI(3)P, recruitment of EEA1, and subsequent maturation into the canonical PI(3)P/Rab5-positive endosomes [32,34]. Since APPL1 vesicles are known to play a functional role in the assembly of signaling complexes for mitogen activated
protein kinase (MAPK) and protein kinase B (Akt) pathways [29,35,36], increased levels of PI(3)P act as a molecular switch to turn off growth factor endosomal signaling through the simultaneous loss of APPL1 on these endosomes [32]. Taken together, regulation of endosomal PI(3)P levels represents a fundamental aspect in achieving proper homeostasis of vesicular trafficking and receptor signaling through the orchestration of a vast array of effector molecules possessing dynamic PIP binding domains.

Here, using in vitro kinase assays, cellular MAPK inhibitors, siRNA and an in-house generated phosphospecific-Ser58 antibody, we now provide evidence that ERK1/2 are kinases capable of phosphorylating MTMR2 at position Ser58. Furthermore, using hydrogen-deuterium exchange mass spectrometry, co-immunoprecipitation and immunofluorescence we have identified candidate residues within the putative PIP-binding domain of MTMR2 potentially involved in regulating endosomal localization/association through transient and competitive interactions between both phospho-Ser58 and membrane components. This strongly suggests that the endosomal targeting of MTMR2 may be regulated through a phospho-dependent, ERK1/2 negative feedback mechanism, which is mediated structurally through indirect influences on the PIP-binding domain of MTMR2. Moreover, we have identified that Ser58 is the master regulator of endosomal localization, whereas another phosphorylation event at Ser631 regulates shuttling of MTMR2 between Rab5/PI(3)P-rich and APPL1 endosomal subtypes in a MAPK-dependent manner [37]. Taken together, our results highlight multiple tiers of functional regulation among cellular activities involving PIPs through direct influences of the receptor-mediated MAPK signaling pathway on PI phosphatases.
6.2 - EXPERIMENTAL PROCEDURES

Plasmids, cell culture and transfections

The synthesis of pCDNA3.1-NF and pET21a vectors containing wild type and mutant FLAG-MTMR2 or MTMR2-His6 constructs have been previously described [2,14]. Generation of mutant variants were performed using PCR based site-directed mutagenesis. The forward and reverse primers for the generation of mutant variants are as follows; K137A forward, 5'-GATAAATAGAGTAGAAGCAATTGGTGCTTCTTAG-3', reverse 5'-CTAGAAGCACCACCAATGCTTCTACTCTATTTATC-3'; R172A forward, 5'-CCTGAGGGGCAGACAGCAAGATCCATATTGAG-3', reverse 5'-CTCAAATATGGATCTTGCTGCCCCTCAGG-3'; R173A forward, 5'-GAGGGGCGGACAAGACATCCATATTGAGAATC-3', reverse 5'-GATTCTCAAATATGGATGCTTTGTCGCCCCTCAGG-3'; E199Stop forward, 5'-GCTTTTGAATACAAATAAGTATTCCCTGAAAATGGG-3', reverse 5'-CCCATTTTCAGGGAATACTTATTTGATTTCAAATAGAATGGG-3'. The pCDNA3.1-NF FLAG-MTMR2 constructs generated include K137A/R172A, K137A/R173A, S58A/K137A/R172A/C417S, S58A/K137A/R173A/C417S, S58A/E199Stop, S58E/E199Stop, and were verified by DNA sequencing (ACGT Corp. and Bio Basic Inc.). The human cell culture lines HEK293 (ATCC) and HeLa (ATCC) were cultured as a monolayer and maintained at 37 °C with 5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM; Sigma) containing 10% (v/v) fetal bovine serum (Gibco), 2 mM L-glutamine (Sigma), supplemented with antibiotics (100 units/ml of penicillin, 100 µg/ml of streptomycin; Sigma). For transient transfection, cells were seeded into antibiotic-free medium 24 h prior to transfection, then transfected using FuGENE® HD (Roche Applied Sciences) at a FuGENE:DNA ratio of 3:1 as per manufacturer’s protocol. HEK293 cells were selected for reproducible transfection efficiency in immunoprecipitation experiments, while HeLa cells were utilized for immunofluorescence microscopy studies to better resolve endosomal compartments. The pEFGP-2xFYVE construct was a generous gift from Harald Stenmark [38].
Cell lysis, immunoprecipitation, immunoblotting and immunofluorescence

At time of lysis, cells were washed 1x with ice-cold HyClone® phosphate buffered saline (PBS; Thermo Scientific) then collected using a rubber scraper and lysed in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 0.1% SDS, 1 mM EDTA, 2.5 mM NaF, 2.5 mM β-glycerophosphate, 1 mM PMSF, 10 µg/mL aprotinin) by pipetting. Lysates were cleared by centrifugation at 15 000 x g for 15 min at 0 °C and protein concentration determined by Bradford assay (Bio-Rad). Lysates were prepared for SDS-PAGE and immunoblot (IB) analysis by the addition of 6x SDS-PAGE loading dye (final concentration of 1x) and boiling at 95 °C for 5 min. Proteins were resolved by 10% SDS-PAGE followed by transfer to polyvinylidene flouride membrane for immunoblot analysis. Immunoblots were routinely processed using Tris-buffered saline supplemented with Tween-20 (25 mM Tris-HCl pH 7.4, 137 mM NaCl, 2.5 mM KCl, 0.1% Tween 20; TBST) containing BSA for probing with phospho-antibodies or skim milk for all other antibodies. Prior to probing targets, membranes were blocked in either 5% BSA or skim milk, respectively. All primary antibody incubations were done overnight at 4 °C, while all secondary antibody incubations were done for 1 h at room temperature, both done under gentle platform rocking. For immunoprecipitation (IP), equal amounts of lysate, as per Bradfords assay, were loaded on the respective antibody-conjugated agarose support as described below. For immunofluorescence analysis (IFA), HeLa cells were pre-treated, fixed, permeabilized, immuno-stained and imaged as previously described [14] and as described below. Prior to probing targets, cells were blocked with 5% BSA/TBST, while all primary antibody incubations were done in 1% BSA/TBST for 1 h at room temperature under gentle rotation. All secondary antibody incubations were done in 1% BSA/TBST for 1 h at room temperature, in the dark, under gentle rotation.

Production of MTMR2 Ser58 phospho-specific antibody

The target peptide epitope was synthesized (NEO BioScience), resuspended in conjugation buffer (100 mM NaPO4, 150 mM NaCl, 5 mM EDTA, pH 7.3), reduced with immobilized TCEP (Pierce Biotechnology) and conjugated to maleimide-activated
Keyhole Limpet Hemocyanin (Pierce Biotechnology) through an engineered thiol terminal. The formulated carrier was then used for immunization of *Gallus gallus* (Amicus Biotech Inc.). Purified polyclonal chicken IgY was isolated by the water dilution method [39], where chicken anti-pSer58 MTMR2 IgY antibodies were isolated sequentially by subtractive affinity chromatography against non-phosphorylated and phosphorylated peptide epitopes immobilized on iodoacetamide-activated agarose (Pierce Biotechnology) conjugated similarly to that described above. Antibody specificity was tested against both MTMR2 peptide and protein epitopes by standard dot and immunoblot procedures. Briefly, peptides or FLAG-MTMR2 immunoprecipitates (as described below) were resuspended in 50 mM Tris-HCl pH 9.3, 1 mM MgCl₂, 0.1 mM ZnCl₂ and treated with or without 2.5 U of calf intestinal alkaline phosphatase (Promega) for 4–6 hours at 25 °C shaking, then spotted on nitrocellulose (peptide dot blot analysis) or prepared for immunoblotting. Alkaline phosphatase activity was monitored using matrix-assisted laser desorption ionization mass spectrometry as previously described [Appendix B] [40]. Membranes were probed with chicken anti-pSer58 MTMR2 IgY at 1:1000 (0.7 µg/µl stock) in 2.5% BSA TBST, washed, then probed with donkey antichicken IgY-HRP conjugate at 1:3000 (Gallus Immunotech) in 2% BSA/TBST for subsequent chemiluminescent imaging. Detailed characterization of antibody synthesis, isolation, purification and characterization can be found in the appendices [Appendix B,D].

**In vitro kinase assay**

*In vitro* kinase assays were performed using 5 µg of bacterial recombinant MTMR2-His₆ proteins purified as described previously [41]. In this study, the activated kinases ERK1, ERK2, p38a, JNK1 (Signal Chem) were tested with all assays being carried out in reaction buffer (25 mM HEPES, pH 7.2, 12.5 mM β-glycerol phosphate, 25 mM MgCl₂, 5 mM EGTA, 2 mM EDTA, 0.25 mM DTT) containing 200 µM ATP and 100 ng of the specified kinase. Reactions were incubated shaking at 30 °C for 30 minutes, terminated with 2x SDS-PAGE loading dye and prepared for immunoblotting as described above. Protein substrate (20 ng) was probed with chicken anti-pSer58 MTMR2 IgY (as above),
rabbit anti-MAPK (Sigma) at 1:10,000 in 1% skim milk/TBST, rabbit anti-p38 (Cell Signaling) at 1:1000 in 5% BSA/TBST, rabbit anti-JNK1 (Santa Cruz Biotechnology) at 1:1000 in 2.5% BSA/TBST and mouse anti-MTMR2 (Santa Cruz Biotechnology) at 1:1000 in 1% skim milk/TBST. Membranes were then probed with donkey anti-chicken IgY-HRP conjugate (as above), goat anti-rabbit IgG-HRP conjugate (Bio-Rad) or goat anti-mouse IgG-HRP conjugate (Promega), both at 1:5000 in 2.5% skim milk/TBST for subsequent chemiluminescent imaging.

**Cell treatment with kinase inhibitors**

HEK293 or HeLa cells were transfected with FLAG-MTMR2 as described above and treated with a JNK inhibitor (SP600125; LC laboratories) at 40 µM for 1 hour, a p38 inhibitor (SB203580; LC laboratories) at 20 µM for 30 minutes or a MEK inhibitor to inhibit ERK1/2 activation (U0126; LC laboratories) at 50 µM for 1 hour. For phosphorylation studies, cells were first serum starved for 30 minutes followed by pre-incubation with U0126, then subsequently stimulated with EGF (5 ng/ml) for 5 or 30 minutes. Cells were lysed as described above and samples purified by FLAG-immunoprecipitation for 3 h rotating at 4 °C using anti-FLAG agarose (Sigma). Resin was washed 3x with IP wash buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% SDS, 0.1% Triton X-100), eluted with 4x loading dye, resolved by SDS-PAGE and subjected to immunoblot analysis. Immunoblots were probed with chicken anti-pSer58 MTMR2 IgY (as above), PathScan® Multiplex Western Cocktail I (Cell Signaling) at 1:200 in 5% BSA/TBST, rabbit anti-MAPK IgG (as above), mouse anti-FLAG IgG (Sigma) at 1:3000, and rabbit anti-actin IgG (Sigma) at 1:1000, all in 2.5% skim milk/TBST. Secondary antibodies included donkey anti-chicken IgY-HRP conjugate, goat anti-mouse IgG-HRP conjugate or goat anti-rabbit IgG-HRP conjugate, with membranes being probed as described above. Data was quantified by densitometry from n = 3 independent experiments using ImageJ, and normalized to FLAG-IP levels. For immunofluorescence studies, cells were prepared as described above and probed with mouse anti-FLAG IgG (Sigma) at 1:500, rabbit anti-Rab5 IgG (Santa Cruz) at 1:80, rabbit anti-Rab7 IgG (Sigma) at 1:100 and rabbit anti-APPL1 IgG (Santa Cruz) at 1:80, as described above.
Secondary antibodies for visualization included goat anti-mouse IgG-Alexa Fluor 568 conjugate (Molecular probes) at 1:500 and goat anti-rabbit IgG-FITC conjugate (Vector Laboratories) at 1:500 as described above. Images were acquired with a Zeiss Axiovert 200M inverted fluorescent microscope with FITC/TRITC/DAPI filter cubes and a LD Plan-APONeofluar 636 0.75 or EC Plan-Neofluor 406 1.3 oil objectives equipped with 103061300 monochrome CCD camera (Axiovision MRM). Captured pictures from both microscopes were processed with Northern Eclipse software 6.0 (Empix, Mississauga, ON) with contrast/brightness and exposure image corrections performed using program processing application software in addition to Adobe Photoshop 7.0.

**siRNA treatment and serum starvation**

HEK293 or HeLa cells were seeded into antibiotic-free medium 24 hours prior to transfection with either 35 nM SignalSilence® control siRNA or SignalSilence® p44/42 MAPK (ERK1/2) siRNA (Cell Signaling Technology) using Lipofectamine® RNAiMAX transfection reagent (Invitrogen) as per manufacturers protocol. Cells were also left untransfected with siRNA as a comparative control for probing of Ser58 phosphorylation in response to ERK1/2 knockdown. After 30 hours, both siRNA treated and untreated cells were transfected with either FLAG-MTMR2 or FLAG-MTMR2 S58A and incubated for another 30–36 hours. Cells were moved into low (0.5%) serum containing medium for another 6–10 hours, followed by a short serum starvation and 10 minute EGF stimulation as described below. Cells were also left untransfected for probing of endogenous MTMR localization and Ser58 phosphorylation in response to serum starvation. Cells were then prepared for immunofluorescence microscopy as described below, or washed, lysed and prepared for immunoprecipitation and/or immunoblot analysis as described above. For endogenous immunoprecipitation, equal amounts of lysate were added to mouse anti-MTMR2 IgG (Santa Cruz Biotechnology) immobilized on protein-A agarose (Thermo Pierce;Invitrogen). Precipitation was allowed to go for approx. 6–8 h, nutating at 4 °C. Resin was washed and prepared for immunoblot analysis similar to that described above for FLAG-immunoprecipitates. Quantitative immunoblotting of both siRNA and serum starvation experiments was
performed using chicken anti-pSer58 MTMR2 IgY at 1:200 in 2.5% BSA/TBST, mouse anti-MTMR2 IgG at 1:1000 in 2.5% skim milk/TBST, mouse anti-FLAG IgG (as above), rabbit anti-phospho-p44/p42 MAPK IgG at 1:200 (pThr20pTyr204 ERK1/2; Cell Signaling) in 2.5% BSA/TBST, rabbit anti-MAPK (as above), and rabbit anti-actin IgG (as above). Secondary antibodies included donkey anti-chicken IgY-HRP conjugate, goat anti-mouse IgG-HRP conjugate or goat anti-rabbit IgG-HRP conjugate, with membranes being probed as described above. Acquired chemiluminescent images from at least n = 3 independent experiments were quantified through densitometry using ImageJ (NIH, http://imagej.nih.gov/ij). Resulting data was normalized to its respective actin loading control and subjected to either a one-way ANOVA with Tukey’s post test or a two-tailed, unpaired t-test statistical analysis both with 95% confidence intervals for relative comparison of related proteins (ie. ERK1/2 versus pERK1/2 and FLAG-MTMR2 versus FLAG-MTMR2 pSer58) using GraphPad Prism version 4.0 for Windows, GraphPad Software, San Diego, California, USA, www.graphpad.com. Relative comparison among all experimental and control samples was performed, with significance differences among data sets being denoted by at least *P < 0.05. Data are represented as the mean value +/- s.e.m. Immunofluorescence of siRNA experiments were performed with rabbit anti-phospho-p44/p42 MAPK IgG (pThr20pTyr204 ERK1/2; Cell Signaling Technologies) at 1:50 and mouse anti-FLAG IgG (as above), using goat anti-rabbit IgG-Alexa Fluor 568 conjugate (Molecular Probes, Invitrogen) and horse anti-mouse IgG-Fluoroscein conjugate (Vector Laboratories) secondary antibodies at 1:500 as described above. Immunofluorescence of endogenous experiments were performed with rabbit anti-MTMR2 IgG (a generous gift from Gregory S. Taylor; University of Nebraska) at 1:50 and mouse anti-Rab5 IgG (BD BioSciences) at 1:50, using goat anti-rabbit IgG-FITC conjugate and goat anti-mouse IgG-Alexa Fluor 568 conjugate as described above. Microscopy image analysis and acquisition is as described below.
**Hydrogen-deuterium exchange mass spectrometry**

Methods of analysis and data interpretation herein were devised based on the merger of multiple previously described techniques and reviews to obtain optimal accuracy throughout sample handling and data acquisition [12,42-50]. Bacterial recombinant MTMR2-His$_6$ and MTMR2 S58E-His$_6$ were purified as previously described [41]. Equal amounts of protein were desalted using Zeba™ Desalting spin columns (Pierce Biotechnology), as per manufacturer's instructions, into 20 mM Tris-HCl pH 7.4, 50 mM NaCl, 1 mM DTT, 20% glycerol. To initiate deuterium exchange, protein was diluted 1:9 in similar buffer (20 mM Tris-HCl pH 7.0, 50 mM NaCl) constituted in deuterium oxide (D$_2$O; Sigma). Buffer pH was adjusted with NaOD (Sigma) and measured with a glass electrode as described by Glasoe et al [51]. Buffer pH values were measured against three-point calibration curves using the appropriate pH standard solutions (pH 1, 4, 7, and 10; Fisher Scientific). All subsequent steps were performed in a walk-in cold room (approx. 4 °C) where all plasticware was pre-chilled for at least 12 h, and all samples/solutions pre-chilled and kept in a circulating ice bath at 0 °C. At defined times, exchange was quenched by 1:1 dilution with quench buffer (500 mM NaPO$_4$ pH 2.25, 1.71 M Gdn-HCl), rapidly digested using immobilized pepsin (Pierce Biotechnology) previously activated by washing 2x with quench buffer. Sample digests were immediately bound to ZipTip® C18 reverse-phase pipette tips (Millipore) for concentration and desalting, eluted in 65% acetonitrile/water containing 0.1% trifluoroacetic acid, and spotted 1:1 with α-hydroxycinnamic acid matrix (Pierce Biotechnology), constituted in elution buffer, by the dried-droplet method. Just prior to spotting, a room-temperature stainless steel target plate was placed under a gentle stream of pre-chilled (4 °C) N$_2$(g) to rapidly chill the metal spots. Pre-chilling target plates (0 - 4 °C) for extended time periods causes condensation to form on plates within MALDI sources during data acquisition, and has been shown to adversely affect back-exchange during analysis, thus resulting in both instrumental and experimental concerns [45]. Spots were rapidly dried under the gentle stream of pre-chilled N$_2$(g) and samples were immediately analyzed on an Applied Biosystems delayed extraction (DE PRO) matrix assisted laser desorption ionization-time of flight mass spectrometer (MALDI-TOF MS) in both linear and reflector acquisition modes. Eight spectra were collected from each
sample (ie. each time point) and this acquired data was calibrated against Sequazyme™ peptide mass standards kit (Applied Biosystems) over an average isotopic $m/z$ range of 905.05 - 5734.59. Extracted average isotopic $m/z$ values from each individual spectra were graphically represented as collective average values and fit to a one-site binding curve using graphpad prism (details above). Primary sequence elucidation was determined by exact mass matching and/or tandem MS using post-source decay as previously described [40,52]. Peptide masses of putative pepsin digestion products were identified with the aid of an in-house generated mathematical matrix capable of calculating every possible peptide mass upon hydrolysis at every combination of peptide bond(s). Further described details can be found in the appendices [Appendix C]. Further confirmation of peptide ID was obtained using nanoLC/MS/MS on an ion trap MS, with a collective sequence coverage of ~65% under the given data analysis conditions.

**Epitope availability immunoprecipitation**

HEK293 cells were transfected for 24 h with FLAG-MTMR2, FLAG-MTMR2 S58A, FLAG-MTMR2 K137A/R172A or FLAG-MTMR2 K137A/R173A as described above. At time, cells were lysed, lysates cleared, prepared for immunoblotting (as described above), and both FLAG (as above) and pSer58 MTMR2 immunoprecipitation. For pSer58 MTMR2 immunoprecipitation, 1.5 µg of chicken anti-pSer58 MTMR2 IgY was added to equal amounts of transfected lysates and precipitation was allowed to go for 6 h, nutating at 4 °C. To isolate the antibody complexes, lysates were then added to donkey anti-chicken IgY-agarose (Gallus Immunotech) for 3 h, nutating at 4 °C. Resin was washed and prepared for immunoblot analysis similar to that described above for FLAG-immunoprecipitates. Quantitative immunoblotting was performed as above, using chicken anti-pSer58 MTMR2 IgY, mouse anti-FLAG IgG, and rabbit anti-actin IgG, while secondary antibodies included donkey anti-chicken IgY-HRP conjugate, goat anti-mouse IgG-HRP conjugate, and goat anti-rabbit IgG-HRP conjugate. Acquired chemiluminescent images from at least $n = 3$ independent experiments were quantified through densitometry using ImageJ (NIH, http://imagej.nih.gov/ij). Data represents
normalized values of the relative comparison among all experimental and control samples being represented as the mean value +/- s.e.m.

**Immunofluorescence analysis**

HeLa cells were transfected for 24 h with FLAG-MTMR2, FLAG-MTMR2 S58A, or FLAG-MTMR2 S58A/K137A/R172A with pEFGP-2xFYVE as described above. Cells were prepared for immunofluorescence as described above, being probed with mouse anti-FLAG IgG. Secondary antibody used for visualization included goat anti-mouse IgG-Alexa Fluor 568 conjugate. Images were obtained using a Leica DMI 6000 inverted fluorescent microscope equipped with FITC/TRITC/DAPI filter cubes and HCX PL APO 40x/1.25-0.75 oil immersion objective with a Leica DFC 425 camera. Post acquisition, brightness-contrast adjustments were made in Adobe Photoshop and were applied equally to all panels of a figure.
6.3 - RESULTS

**ERK1/2 phosphorylates MTMR2 at position Ser58 in vitro**

Phosphorylation of Ser58 strongly correlates to sequestration of MTMR2 away from its physiological endosomal substrate PtdIns(3)P, however, has little influence on catalytic competency or binding with MTMR5 [14]. The ability to monitor phosphorylation levels of MTMR2 Ser58 would then reflect both the pseudo-inactive state of MTMR2 towards this endosomal PtdIns(3)P, and also provide an indirect measure of the targeted endosomal PtdIns(3)P levels. Furthermore, evidence of the responsible kinase(s) would be key information to understanding the mechanisms that underlie this critical regulatory event. To this end, we designed and generated a polyclonal phospho-specific antibody, and using immunoblot analyses, characterized its phospho-specificity towards MTMR2 Ser58. The antibody readily detects both phospho-Ser58 peptide and wild type MTMR2, whereas treatment with alkaline phosphatase completely ablates the signal similar to that of Ser58 peptide or MTMR2 S58A, emphasizing phosphate-dependent recognition [Fig. 6.1A,B]. Interestingly, the phospho-specific antibody also detected MTMR2 S58E [Fig. 6.1B] supporting our previous results that this phosphomimetic mutant of MTMR2 may functionally behave like Ser58-phosphorylated MTMR2 [14].

From primary sequence analysis, Ser58 resides immediately N-terminal to an adjacent proline residue. This, the minimal requirement for proline directed kinase recognition, suggested that phosphorylation of MTMR2 may be mediated through these enzymes, including those members of the mitogen activated protein kinase (MAPK) family. As phosphorylation deficient MTMR2 localizes to endosomes, depletes PtdIns(3)P, and enhances ERK1/2 activation [14], we hypothesized that activated ERK1/2 may, in turn, regulate MTMR2 in a negative feedback mechanism through phosphorylating Ser58 to induce MTMR2 release from endosomes attenuating this signal activation. To test this hypothesis, *in vitro* kinase assays, using our Ser58 phospho-specific antibody, were performed to examine the ability of MTMR2 to be phosphorylated by distinct MAPK family members. Purified bacterial recombinant MTMR2 was assayed using commercially available MAPKs, the proteins were separated by SDS-PAGE, and phosphorylation of MTMR2 Ser58 was examined by immunoblot analysis using our
Figure 6.1 - Characterization of a phospho-Ser58 MTMR2 antibody identifies ERK1/2 phosphorylation of recombinant MTMR2 in vitro.  

**A)** Phospho-Ser58 and nonphospho-Ser58 peptides were incubated with and without alkaline phosphatase, spotted on nitrocellulose membrane and probed with an in-house generated α-phospho-Ser58 MTMR2 (α-pSer58) antibody.  

**B)** HEK293 cells were transfected with empty vector (UT), FLAG-MTMR2, and FLAG-MTMR2 phospho-mimetic variants, FLAG-immunoprecipitated (IP: FLAG), treated with and without alkaline phosphatase, and probed for MTMR2 (IB: α-FLAG) and phospho-Ser58 MTMR2 (IB: α-pSer58).  

**C)** Purified bacterial recombinant MTMR2-His$_6$ was incubated with various MAPKs *in vitro* as described in *Experimental procedures* and phosphorylation of Ser58 MTMR2 was detected using the α-pSer58 antibody. Total MTMR2 and kinase levels were determined with their respective antibodies.
phospho-Ser58 antibody. It was determined that both ERK1 and ERK2 could effectively phosphorylate MTMR2 at Ser58, but in contrast, neither recombinant JNK1 nor p38α were able to detectably phosphorylate MTMR2 Ser58 in vitro under the given conditions [Fig. 6.1C], providing the first evidence that ERK1/2 may represent the MTMR2 Ser58 kinases.

Both knockdown and inhibition of ERK1/2 reduces MTMR2 Ser58 phosphorylation

The in vitro kinase assays demonstrated direct phosphorylation of MTMR2 Ser58 by ERK1/2. To complement these studies, we examined the phosphorylation status of Ser58 in cells whose expression levels of ERK1/2 had been reduced using small interfering RNA (siRNA) and/or whose ERK1/2 phosphorylation had been attenuated. In response to various stimuli such as growth factors in serum (e.g. epidermal growth factor (EGF)), ERK1/2 becomes activated by phosphorylation (pERK1/2), which in turns leads to phosphorylation of downstream target substrates [53]. Conversely, serum starvation has been shown to attenuate MAPK activation in a variety cell types, providing a means for tuning MAPK signaling pathways, and thus, their target substrates [54-56].

Recent evidence has suggested that the steady-state pERK1/2 levels remain largely unaltered upon perturbations in total ERK1/2 protein levels [57]. Since our initial interpretations were largely confounded by this phenomenon, we devised conditions to extend the dynamic range of analysis in response to this robustness of MAPK signaling afforded through sustained pERK1/2 activity. HEK293 cells transfected with ERK1/2 siRNA were grown under low serum conditions for 6 hours followed by a 10 minute EGF stimulation prior to cell lysis. These initial conditions effectively reduced both total ERK1/2 and active pERK1/2 protein levels in the cells [Fig. 6.2A,C], and thus following stimulation, enabled us to examine the direct correlation of these protein levels (both ERK1/2 and pERK1/2) on MTMR2 Ser58 phosphorylation [Fig. 6.2A,B]. This scheme significantly decreased ERK1/2, pERK1/2 and phosphorylation levels of MTMR2 Ser58 as compared to control (~50% reduction) [Fig. 6.2].
Figure 6.2 - siRNA-mediated depletion of ERK1/2 protein levels reduces MTMR2 Ser58 phosphorylation.  A) HEK 293 cells were transfected with control scramble siRNA or ERK1/2 siRNA, followed by FLAG-MTMR2 or FLAG-MTMR2 S58A and cultured as described in 'Experimental Procedures'. Cells were stimulated with 5 ng/ml EGF for 10 minutes, lysed and analyzed by immunoblot analysis.  B) Graphs represent normalized quantitative immunoblot densitometry data of each indicated antibody to their respective α-actin loading control. Data represents mean and SEM values from at least n = 3 independent experiments, with ***P<0.001, **P<0.01 or *P<0.05.
This data was fortified upon observation of a significant decrease in MTMR2 Ser58 phosphorylation levels when cells were grown under low serum conditions, which had not been stimulated with EGF, as compared to their 10% serum containing counterparts [Fig. 6.3A,B]. Importantly, these cells also exhibited low pERK1/2 levels, thus activity, all patterns that were also observed upon analysis of endogenous MTMR2 [Fig. 6.3A,C and Fig. 6.4]. Moreover, HEK293 cells expressing MTMR2 under low serum conditions were pre-treated with or without the MAPK/ERK kinase (MEK) inhibitor U0126, then subjected to short EGF stimulation and subsequently examined for the effects on MTMR2 Ser58 phosphorylation [Fig. 6.3]. EGF stimulation transiently activated ERK1/2 in cells that were void of MEK inhibition (- U0126), as detected by an increase in pERK1/2 levels and of phospho-S6 kinase (pS6K) levels, a downstream ERK1/2 substrate, in comparison to levels from those cells exposed to MEK inhibition (+ U0126) [Fig. 6.3A,C]. Under these same conditions, MTMR2 Ser58 phosphorylation levels also increased ~2-fold compared to the samples treated with the MEK inhibitor [Fig. 6.3A,B]. Importantly, this observed increase in Ser58 phosphorylation was strongly attenuated upon pre-treatment of cells with the MEK inhibitor coinciding with decreased ERK1/2 activity [Fig. 6.3]. These results, taken together with those of our in vitro kinase assays, strongly implicate ERK1/2 as kinases capable of phosphorylating MTMR2 at position Ser58.

**ERK1/2 inhibition and knockdown targets MTMR2 to endosomal structures**

For insight to the potential of ERK1/2 as not only a regulator of MTMR2 Ser58 phosphorylation, but also of MTMR2 endosomal localization, we performed similar experiments in HeLa cells, and through immunofluorescence, examined them to identify if attenuation of ERK1/2 and/or pERK1/2 levels would alter MTMR2 localization patterns. MTMR2 commonly displays a predominantly diffuse cytoplasmic localization, likely due to the high stoichiometry of Ser58 phosphorylation under typical cell culture growth conditions of 10% serum-containing media [13-16]. Dephosphorylation of MTMR2 Ser58 leads to its recruitment to Rab5- and PI(3)P-containing endosomes, as indicated by the formation of a distinctly co-localized punctate distribution [14].
Figure 6.3 - Inhibition of ERK1/2 activity decreases MTMR2 Ser58 phosphorylation.  
A) HEK293 cells were transiently transfected with empty vector (UT) or with FLAG-MTMR2, serum-starved (- serum) for 30 minutes, then treated with the MEK inhibitor U0126 to inhibit ERK1/2 activation (where indicated). Following stimulation with 5 ng/ml EGF for 5 or 30 minutes at 37°C, cells were lysed, FLAG-immunoprecipitated (IP: FLAG), then probed for phosphorylation of MTMR2 Ser58 by immunoblot (IB). ImmunobLOTS of all samples for FLAG was used to confirm equal transfections and loading IP levels. Total ERK1/2 and actin lysate immunoblots served as loading controls, while pERK1/2 immunoblots displayed U0126 efficacy and ERK1/2 activation by EGF.  
B) MTMR2 Ser58 phosphorylation was quantified by densitometry using ImageJ, normalized to FLAG IP levels and represented as fold change relative to serum-starved control without inhibitor.  
C) Changes in ERK1/2 phosphorylation (pERK1/2) thus activation is represented as the relative change in pERK1/2 levels compared to total ERK1/2 levels. All data represents mean and SD values from at least n = 3 independent experiments, with ***P<0.001, **P<0.01 or *P<0.05.
**Figure 6.4 - Endogenous MTMR2 Ser58 and ERK1/2 phosphorylation is influenced by altering serum conditions.** A) HEK 293 cells were cultured and processed as described in 'Experimental Procedures', following either serum (+ 10% (v/v) serum; + serum) or low serum (+ 0.5% (v/v) serum; - serum) treatment for ~ 12 h. Both lysate and immunoprecipitates (IP) were subjected to immunoblot (IB) analysis using the indicated antibodies. B) Graphs represent normalized quantitative densitometry data to the indicated loading control (y-axis). Data represents mean and SD values from at least $n = 3$ independent experiments.
Consistent with the *in vitro* kinase assays, inhibitors to JNK and p38α did not alter the localization pattern of MTMR2 as compared to untreated cells [Fig. 6.5A]. However, when cells were treated with the MEK inhibitor U0126, MTMR2 dramatically re-localized to Rab5-rich endosomal structures, exhibiting a localization pattern analogous to MTMR2 S58A [Fig. 6.5B,C]. Similarly, upon serum starvation conditions we observed significant co-localization of MTMR2 with Rab5 positive endosomes [Fig. 6.6A], with this observation also being recapitulated at the endogenous level [Fig. 6.6B]. Moreover, analysis of MTMR2 localization in HeLa cells subjected to similar conditions of siRNA-mediated ERK1/2 knockdown as those described above, we observed a similar shift of MTMR2 localization to endosomal punctate structures [Fig. 6.7]. Collectively, these results indicate that both direct and indirect attenuation of ERK1/2 signaling promotes MTMR2 endosomal targeting presumably by influences accrued through ERK1/2-mediated phosphorylation of MTMR2 Ser58. Furthermore, our results complement those of MTMR2 phospho-Ser58 immunoblot analyses performed under similar conditions, and together with the *in vitro* kinase assays, begin establishing a working model for the hypothesis that endosomal targeting of MTMR2 is mediated through an ERK1/2 negative feedback mechanism.

**Phosphomimetic mutagenesis of MTMR2 Ser58 alters solvent accessibility in regions of the PH-GRAM domain**

The data presented have provided a role for MAPK-mediated signaling in the potent allosteric regulation of MTMR2 localization. Reports of using hydrogen-deuterium exchange mass spectrometry (H/DX MS) for structural protein analysis are continually growing, with experiments using both point-mutations and *bona fide* phosphorylation events being abundantly and continually reported in the literature [44,47,58-60]. To begin investigation of structural mechanisms controlling MTMR2 phospho-dependent sub-cellular localization, we employed H/DX MS to examine conformational protein dynamics through alterations in protein solvent accessibility upon MTMR2 phosphorylation using our previously characterized phosphomimetic mutant MTMR2 S58E. From this approach, we have identified regions in the putative PIP-binding
Figure 6.5 - Inhibition of the ERK1/2 pathway induces sub-cellular targeting of MTMR2 to Rab5-positive endosomes. A-C) HeLa cells were transiently transfected with FLAG-MTMR2 or FLAG-MTMR2 S58A and treated with the MEK inhibitor U0126 (ERK1/2) at 50 μM for 1 hour, SB203580 (p38) at 20 μM for 30 minutes and SP600125 (JNK) at 40 μM for 1 hour as indicated and described in 'Experimental Procedures'. Cells were analyzed by immunofluorescence for either FLAG-MTMR2 (grayscale or red) alone or with endogenous Rab5 (green). Merged images display regions of co-localization and are represented in expanded views. Images were collected using 40x oil objectives; Scale bars: 15 μm.
Figure 6.6 - Serum starvation induces subcellular targeting of MTMR2 to Rab5-positive endosomes. **A)** HeLa cells were transiently transfected with FLAG-MTMR2 and analyzed by immunofluorescence microscopy following either serum (+ 10% (v/v) serum; + serum) or low serum (+ 0.5% (v/v) serum; - serum) treatment as described in 'Experimental Procedures'. Cells were probed for FLAG-MTMR2 (red) and Rab5 (green). **B)** HeLa cells were treated as above and probed with endogenous MTMR2 (green) and Rab5 (red). Merged images display regions of co-localization and are represented in expanded views. Images were collected using 40x oil objectives; Scale bars: 15 μm.
Figure 6.7 - Depletion of ERK1/2 expression by siRNA induces sub-cellular targeting of MTMR2 to punctate regions.  

**A**) HeLa cells were transfected with control scramble siRNA or ERK1/2 siRNA, followed by FLAG-MTMR2 and cultured as described in 'Experimental Procedures'. Cells were stimulated with 5 ng/ml EGF for 10 minutes then analyzed by immunofluorescence analysis of FLAG-MTMR2 (green) and pERK1/2 (red). Images were collected using 40x oil objectives; Scale bars: 15 μm.  

**B**) Graphs represent the total number of puncta between control scramble siRNA and ERK1/2 siRNA using automated counting in ImageJ. Data represents mean and SD values from at least n = 3 independent experiments counting n = 10 cells per experiment, with ***P<0.001, **P<0.01 or *P<0.05.
pleckstrin homology-glucosyltransferases, Rab-like GTPase activators and myotubularins (PH-GRAM) domain which exhibit decreased solvent accessibility upon N-terminal pseudo-phosphorylation [Fig. 6.8A,B]. Similarly, decreased accessibility was observed within the phosphorylated region itself [Fig. 6.8C], suggesting that these regions may be involved in co-coordinating the allosteric regulation of MTMR2 by phosphorylation. Upon modeling this data onto the available crystal structure and the previous HDX MS studies of MTMR2 [12], it was observed that the three dimensional folding of these regional targets form a highly solvent accessible pocket of positive electrostatic potential on the membrane proximal face of the enzyme [Fig. 6.9A,B] [12]. As many phosphate and PIP binding modules utilize electrostatic interactions to partially stabilize and orient the electronegative charge density of phosphorylated biomolecules, this phosphorylation event could sequester MTMR2 from substrate/membrane association by preventing key residues within the PIP binding domain accessibility to membrane phospholipid ligands.

**Mutation of basic residues in the PH-GRAM domain of MTMR2 influence endosomal localization and accessibility of the Ser58 phosphorylation site**

From our H/DX MS data, we developed a targeted-structural framework for mutagenic studies of candidate residues within the PH-GRAM domain potentially involved in phosphate and/or endosomal interactions. Combining elements from the crystal structure and from primary sequence analysis of our HDX MS data, we made point mutations of Lys137 (K137A), Arg172 (R172A) and Arg173 (R173A) to alanine, as well as the double mutants K137A/R172A and K137A/R173A. We first analyzed these constructs (K137A/R172A MTMR2 and K137A/R172A MTMR2) for Ser58 phosphorylation and found that there was no significant change [Fig. 6.10A,C,D]. From this, we began testing the hypothesis that these PH-GRAM domain mutants increase the accessibility of phospho-Ser58. Using co-immunoprecipitation experiments, we examined the ability of our phospho-specific antibody to enrich for MTMR2, MTMR2 S58A, MTMR2 K137A/R172A and MTMR2 K137A/R172A. We reproducibly observed that both mutants, K137A/R172A and K137A/R172A, exhibited increased enrichment as compared to both MTMR2 and the negative control MTMR2 S58A [Fig. 6.10A,B].
Figure 6.8 - Deuterium exchange of S58E MTMR2 suggests conformational changes may occur in a basic cleft within the PH-GRAM domain. A,B) Purified bacterial recombinant MTMR2-His₆ and MTMR2 S58E-His₆ was analyzed by HDX-MS as described in 'Experimental Procedures'. Peptides of the PH-GRAM domain in MTMR2 S58E (red) showed significant alteration in their relative deuterium uptake compared to wild-type (black). Highlighted is the signature motif KXn(K/R)XR characteristic of high affinity PIP-binding PH domains (increased font size and underlined) as well as neighbouring basic and hydrophobic amino acids (underlined). C) The mutated peptide containing residue S58E also shows decreased uptake (red). The peptide shown has Ser58 for clarity of the mutation site. Graphs represent calibrated average isotopic m/z values from each individual MALDI-TOF MS spectra and are represented as a collective average value. Primary sequence elucidation was determined by exact mass matching and/or tandem MS using post-source decay as previously described and as in 'Experimental Procedures'.
Figure 6.9 - Crystal structure of the lipid phosphatase MTMR2 shows residues of the electropositive PH-GRAM domain regions undergoing changes in solvent accessibility upon Ser58 phosphorylation. A) Crystal structure and B) surface electrostatic potential map of MTMR2 shows the position of target basic peptides found on the membrane proximal face (boxed) with respect to the locale of the active site cleft, both showing a net positive charge, presumably for polyphosphorylated lipid interactions. Inset highlights residues (Lys137, Arg172 and Arg173) responsible for the formation of this basic region within the PH-GRAM domain. Crystal structure and electrostatic potential map of MTMR2 from Begley et al., Proc. Nat. Acad. Sci., 103:4 (2006) 927-932.
Figure 6.10 - Mutation of target basic residues in the PH-GRAM domain leads to increased accessibility of phospho-Ser58.  

A) HEK293 cells were transiently transfected with the indicated FLAG-MTMR2 variants of target PH-GRAM domain residues and analyzed by co-immunoprecipitation (IP) using both α-FLAG and α-pSer58, and by immunoblotting (IB) with α-FLAG, α-pSer58 and α-actin.  

B) FLAG-MTMR2 levels of α-pSer58 IP samples were quantified by densitometry using ImageJ and normalized to FLAG lysate levels.  

C) FLAG-MTMR2 pSer58 levels of α-FLAG IP samples were quantified by densitometry using ImageJ and normalized to FLAG-MTMR2 levels from α-FLAG IP samples.  

D) FLAG-MTMR2 lysate levels were quantified by densitometry using ImageJ and normalized to actin lysate levels.  

All graphs represent normalized quantitative immunoblot densitometry, with data representing the mean and SEM values from at least n = 3 independent experiments.
These observations suggest that endosomal localization may indeed depend on the regulated competition between phosphate and PIP electrostatic interactions with this basic region of the PH-GRAM domain. Furthermore, to test if Ser58 dephosphorylation alone was sufficient in localizing MTMR2 to PI(3)P-rich endosomes, we mutated Ser58 to alanine on these constructs to form MTMR2 S58A/K137A/R172A and MTMR2 S58A/K137A/R173A and analyzed them using immunofluorescence. Comparing to both MTMR2 and MTMR2 S58A, we observed that these mutants displayed the common diffuse, cytosolic staining pattern similar to that of wild type [Fig. 6.11]. Moreover, they did not cause mis-localization of the PI(3)P-binding marker, EGFP-2xFYVE, as is observed for endosomal MTMR2 S58A,suggesting that MTMR2 endosomal localization may require these basic residues for stabilization at the membrane [Fig. 6.11]. Overall, coupling structural mass spectrometry approaches to traditional cell biology techniques has provided mechanistic insight into how reversible phosphorylation so effectively regulates MTMR2 endosomal targeting.
Figure 6.11 - Phospho-dependent endosomal localization of MTMR2 requires basic residues of the PH-GRAM domain.  

A) HeLa cells were transiently transfected with the indicated FLAG-MTMR2 variants of target PH-GRAM domain residues along with the endosomal PI(3)P marker EGFP-2xFYVE, and analyzed by immunofluorescence microscopy as described in 'Experimental Procedures'. Cells were probed for FLAG-MTMR2 (red) and EGFP (green). Merged images display regions of co-localization, while images were collected using 40x oil objectives; Scale bars: 15 μm.
6.4 - DISCUSSION

In recent years, it has become apparent that early endosomes are heterogeneous in terms of both their protein and phosphatidylinositol lipid composition on the vesicular surface [61,62]. This provides layers of functional diversity that likely contribute to the regulated differentiation among endosomal cargo destinations, maturation rates, and/or signaling through the coordinated recruitment of distinct effector proteins. Our discovery that ERK1/2 regulates the phosphorylation of MTMR2 Ser58 is a compelling finding that implicates MAPK signaling in the regulation of endosomal processing events through direct influence on PI(3)P phosphatases. In this regard, we have shown by various means that attenuation of ERK1/2 activity reduces MTMR2 phospho-Ser58 levels leading to MTMR2 co-localization with Rab5/PI(3)P-rich early endosomes. Here, MTMR2 mediates the depletion of PI(3)P, in turn, regulates receptor signaling duration through increased growth factor stimulated ERK1/2 activation. Moreover, we have evidence that phosphorylation of Ser58 results in decreased solvent accessibility in both the intrinsic Ser58 phospho-region and the PH-GRAM domain of MTMR2, acting to potentially shield a poly-basic region from stabilizing endosomal interactions. Similarly, mutations within this basic region do not alter Ser58 phospho-levels, but do increase the accessibility of phospho-Ser58, collectively suggesting a transient interaction may exist between these two moieties. Although dephosphorylation of Ser58 targets MTMR2 to endosomal structures and does not affect interaction with MTMR5 [14], these PH-GRAM domain mutations prevent MTMR2 phospho-dependent endosomal localization, and thus, the mis-localization of PI(3)P-binding proteins when combined with the constitutively dephosphorylated variant MTMR2 S58A (MTMR2 S58A/K137A/R172A), further suggesting a role for both Ser58 phosphorylation and the PH-GRAM domain of MTMR2 in endosomal docking events.

Extending the scope our findings, a recent phosphoproteomic study mapped Ser631 as a phosphorylation site on MTMR2 [63]. Coincidently, as for Ser58, Ser631 also resides immediately N-terminal to an adjacent proline residue typical of a proline-directed kinase consensus sequence suggesting that a MAPK family member may also regulate this
phosphorylation event. Thus in relation to Ser58, we were again interested in examining the endosomal localization pattern of MTMR2 in response to different MAPK inhibitors, alone or in combination. To this end, we have begun to characterize this secondary phosphorylation site with respect to its functional role within the previously established MAPK-mediated Ser58 phospho-dependent endosomal localization of MTMR2. To our surprise, combined inhibition of MEK1/2 and JNK1 displayed a pronounced MTMR2 punctate pattern, however, these vesicles were completely void of the MEK1/2 inhibitor-induced, Rab5-rich, endosomal co-localization described above [Fig. 6.5B,C]. These distinct MTMR2-rich endosomes were also noticeably enlarged when compared to their Rab5-rich counterparts [37].

Recent evidence has defined a subpopulation of enlarged vesicles containing APPL1 as an early and transient endosomal subtype that resides downstream of clathrin-coated pits en route to formation of PI(3)P-positive early endosomes. In addition, PI(3)P depletion has resulted in the reversion of isolated Rab5/PI(3)P-rich endosomes back into their preceding APPL1 progenitors as well as their vesicular re-enlargement and sustained EGF signaling [32], suggesting that under these dual MAPK inhibitory conditions, MTMR2 may be localizing to APPL1-rich vesicles. Thus, using co-immunofluorescence, we found that MTMR2 strongly co-localized with APPL1 under these MEK1/2 and JNK1 inhibitory conditions, whereas inhibition of the MEK1/2 pathway alone resulted in a punctate staining pattern devoid of APPL1 co-staining [37]. To complement this result, we found that the dephosphorylated mutant of MTMR2, S58A/S631A, also co-localized with APPL1, but not with Rab5, suggesting that this doubly dephosphorylated form of MTMR2 localizes to APPL1 endosomes that are Rab5-negative. By contrast, the mutant form MTMR2 S58A/S631E was unable to co-localize with APPL1, but could still target to Rab5-rich early endosomes. Moreover, regardless of the phospho-status of Ser631, phosphorylation of Ser58 sequesters MTMR2 to the cytoplasm away from endocytic structures, suggesting that Ser58 is the master regulator controlling general endosomal targeting, while Ser631 is responsible for regulation of shuttling between endosomal subtypes [37].
Taken together, we have found that MAPK regulated phospho-isomers of MTMR2 localize to different endosomal subtypes, illustrating the important role of reversible post-translational modifications (PTMs) in regulating and achieving early endosome heterogeneity. In the case of MTMR2, our data strongly suggests that Ser58 regulates the general endosomal binding capacity of MTMR2, as the phosphomimetic mutation (S58E) sequestered MTMR2 in the cytoplasm regardless of the phosphorylation status of Ser631. Meanwhile, upon Ser58 dephosphorylation, it is the phosphorylation of Ser631 that seems to regulate early endosomal subtype destination; if phosphorylated, MTMR2 is targeted to PI(3)P-rich Rab5-positive endosomes; if dephosphorylated, MTMR2 is targeted to APPL1-positive endosomes. Understanding the structural mechanisms behind this Ser58-based master regulation of MTMR2 localization, thus catalytic activity and PI(3)P levels, would have deep implications in understanding how PTMs contribute to the orchestration of cross-talk among membrane targeting, vesicular trafficking, and regulation of signal transduction pathways.

Upon solving the crystal structure of MTMR2, it was observed that this enzyme possessed an N-terminal pleckstrin homology (PH)-like domain fold within its suspected glucosyltransferases, Rab-like GTPase activators and myotubularins (GRAM) domain [17], which has since been coined a PH-GRAM domain [11]. Moreover, Begley et al. suggested that of the ~180 suspected GRAM domain containing proteins, all of them would likely adopt a similar fold [11,17]. The PH-GRAM domain of MTMR2 adapts a classical PH domain fold, exhibiting a β-sandwich structure capped by an α-helix, with loops connecting adjacent β-strands. Several other binding modules have been shown to adapt a fold similar to that of PH domains despite having extreme variability in their primary sequence, and while being one of the largest domain families in the proteome, the distinct properties of PH domains in membrane association are yet to be clearly defined [11,18].

Based on sequence homology, crystal structure and binding assays, it has been discovered that a distinctly positioned β-loop within a group of high-affinity, stereospecific PH domains lines a deep binding pocket containing the sequence motif KXn(K/R)XR which functions in the interactions with phosphoinositide headgroups.
The side chains of basic residues form most of the phosphate-group interactions, whereas other complementary residues from elsewhere within the domain provide additional contacts to define the preferred inositol ring orientation and phosphorylation pattern. Defined rules for predicting ligand preference on the basis of sequence have been identified, suggesting that contributions from the intrinsic GRAM domain may dictate the distinct phosphoinositide-binding affinities/specificities and contribute to variations on the above themes [65,66]. Combination of our H/DX mass spectrometry experiments with those of others, and the three-dimensional crystal structure of MTMR2, we observed that mutation of Ser58 to glutamic acid resulted in decreased solvent accessibility of both this pseudophospho-region itself, and a region within the PH-GRAM domain [Fig. 6.8A,B]. Upon closer inspection, we identified that this regional target of the PH-GRAM domain resides in the same loop as that described above, and also possesses the signature motif along with flanking hydrophobic and basic residues [Fig. 6.8A,B]. Moreover, this region lies on the membrane proximal face of the enzyme, adjacent to the active site, closely resembling the electropositive charge density observed within the active site cleft [Fig. 6.8C] [12]. This was compelling due to the fact that this motif has been defined mostly among highly affinitive and specific PIP-binding PH domains, whereas in MTMR2, localization to PIP-containing vesicles, namely substrate containing endosomes, had been elusive for some time.

It is thought that nearly 80% of known PH domain-containing proteins likely do not bind PIPs at all due to the lack of functional redundancy among these similarly folded domains [67]. Additionally, many PIP-associating proteins require multivalent avidity-based interactions to stabilize transient membrane docking [18,20]. Previous studies have shown that the coiled-coil domain of MTMR2 is capable of forming a parallel dimer with itself and/or the inactive myotubularin, MTMR5, which in the case of the former, would double the available PIP-binding PH-GRAM domains on the membrane proximal face which are capable of stabilizing such an interaction [13,15]. Although the inactive binding partner MTMR5 contains a much larger and more diverse domain arrangement, and has been shown to affect the localization of MTMR2, whether it could contribute to stabilizing membrane interactions is yet to be determined [15]. Homology modeling of the electrostatic polarization of inactive members using MTMR2 as a template showed
that the membrane proximal face of the inactive members analyzed (MTMR9 - MTMR12) mostly had an electronegative charge density, suggesting that these binding partners may not be involved in the membrane associations of their respective active binding partners, but perhaps in regulating them [12].

As previously mentioned, MTMR2 seems to possess the sequence motif and structure of high-affinity PIP-binding PH domains [18], but can only localize to vesicular structures when Ser58 is dephosphorylated, which has been found to occur independently of PI(3)P presence (unpublished data). Data has also been suggested that the PH-GRAM domain alone is capable of associating with PIPs in vitro [13], however, in all of the MTMR2 crystal structures to date, the catalytically inactive variant has been used, along with the incorporation of artificial water soluble PI(3)P or PI(3,5)P2 substrate analogs, which did not show any signs of PH-GRAM domain association [11,12]. Furthermore, alignment of putative GRAM domain-containing proteins shows high degree of sequence similarity among this basic motif found within the PH-GRAM domain of MTMR2, with strong conservation of the targeted Lys and Arg residues from our studies [11,17]. From these observations, it is not clear whether effects on these regions within the PH-GRAM domain limit the binding affinity to PIPs or if a multivalent avidity-based mechanism involving membrane and/or protein contacts is necessary to stabilize and/or destabilize interactions, or if it is some combination of the two. Based on these possibilities, we tested the effects of Ser58 phosphorylation and PH-GRAM domain residues on the ability to sequester MTMR2 away from membrane docking, either directly or indirectly. The position of Ser58 is ~30 amino acids upstream of the PH-GRAM domain, is quite flexible and solvent accessible, but has not been resolved in any crystal structures to date [11,12]. We hypothesized that like some other PIP-binding proteins, including another lipid phosphatase, phosphorylation may shield the accessibility of key residues within the PIP-binding domain from membrane association [68-70]. Upon mutation of the PH domain loop motif residues, we found that phospho-Ser58 levels were unaltered, however, the accessibility of this N-terminal phosphosite increased. Similarly, the same mutations on the constitutively dephosphorylated variant prevented localization to PI(3)P-rich vesicular structures and subsequent PI(3)P depletion, suggesting that regulation of MTMR2 localization to substrate-rich membranes may be mediated through competitive
phospho-dependent interactions between Ser58, endosomal PIPs, and residues within the PH-GRAM domain. Ultimately, we have begun to uncover how this master regulator potently regulates the sub-cellular localization of MTMR2, providing future insights to studying the regulation of shuttling between Rab5 and APPL1 subtypes. It will be interesting to test if mutations within the PH-GRAM domain effect the ability of dually-dephosphorylated MTMR2 variants (MTMR2 S58A/S631A) to localize to PI(3)P-poor APPL1 endosomes. This would determine if membrane associations between the two endosomal subtypes are performed similarly or are completely unique, furthering the understanding of phospho-dependent endosomal targeting of PI phosphatases and the regulation of relative PI(3)P levels on each.

We are currently using chemical cross-linking in conjunction with targeted MS applications to examine structural contact constraints between Ser58 and Lys residues within the PH-GRAM domain. Preliminary data using precursor ion scanning MS has provided a means for rapid detection of the targeted, low abundant cross-linked peptides containing Ser58, thus allowing for a comprehensive future analysis of stable contacts of this phospho-region. Alternatively, we have preliminary data suggesting that the PH-GRAM domain alone is capable of endosomal interaction, however, still requires dephosphorylation of Ser58, suggesting that homo/hetero-dimerization is not required for stabilizing membrane associations. This suggests that regions within the C-terminus are required for endosomal shuttling and stabilization at APPL1 containing vesicles, however further evidence is required to rule out any APPL1 localization of these constructs.

There are numerous reports that have described APPL1 and APPL2 as Rab5 effector molecules that co-localize on early endosomes [32,33,71]. The dissociation of APPL1 from early endosomes during the conversion of APPL1 endosomes into Rab5/EEA1 early endosomes is mediated by the accumulation of PI(3)P, and in turn, the recruitment of FYVE domain containing proteins including EEA1. Therefore, the generation and accumulation of PI(3)P is thought to function as the molecular switch that controls maturation into the canonical early endosomes. It has also been shown that EEA1 and APPL1 compete for a limited number of binding sites on Rab5-positive endosomes [32]. When PI(3)P is depleted, the binding of APPL1 to Rab5-positive endosomes is
dramatically increased, whereas in contrast, attenuation of APPL1 association to endosomes by PI(3)P generation allows for proficient stabilization of EEA1 binding to Rab5 endosomes. This competitive scenario is thus mediated by both PI(3)P kinases and phosphatases to ultimately regulate the signaling and maturation properties of early endocytic events transitioning through these particular stages. The direct ability to allosterically control the activities of these families of enzymes provides a potent tier of regulation to achieve proper response to a given stimulus. This effect further transitions to downstream sorting machinery which utilize PI(3)P binding modules for activity. For example, we recently identified a novel PI(3)P binding protein, receptor-mediated endocytosis-8 (RME-8), involved in endosomal sorting decisions and retrograde transport [19]. RME-8 association with early endosomes was dependent on PI(3)P levels, being attenuated by MTMR2 lipid phosphatase activity, exemplifying the importance of phosphatidylinositol levels among a diversity of endosomal activities.

It is also important to note that in all cases it was observed that MTMR2/APPL1-positive endosomes (either MTMR2 S58A/S631A or stimulated equivalents) were reproducibly found enlarged when compared to APPL-positive endosomes alone. Surprisingly, when catalytic activity of MTMR2 was impaired (MTMR2 S58A/C417S/S631A), vesicle size did not change, remaining comparable to sole APPL1 containing endosomes [37]. One possibility is that MTMR2/APPL1 endosomes are a distinct endosomal subtype that function as an initial platform for downstream signaling events regulated in a PI(3)P-dependent fashion. However, a more likely explanation is that these MTMR2/APPL1 endosomes represent a proximal endosomal stage that precedes Rab5/APPL1 early endosome formation, potentially as a means to spatially and temporally control PI(3)P levels which, in part, regulates the linked signaling events. Supporting our and others findings with respect to these endosomal maturation and signaling capabilities, a similar, but prolonged increase in ERK1/2 activation during EGF stimulation was also observed upon expression of the aforementioned MTMR2 S58A/S631A variant as compared to that observed for MTMR2 S58A alone [37]. Since the formation of enlarged MTMR2/APPL1 endosomes is dependent on MTMR2 catalytic competency, these combined findings support the hypothesis that MTMR2/APPL1 endosomes may act to control PI(3)P accumulation rates, to in turn, regulate intrinsic
endosomal signaling events. In this regard, MTMR2-mediated PI(3)P depletion may lead to halting or retardation of endosomal maturation, a phenotype which others have shown to result in endosomal enlargement and continued receptor signaling [72-74].

Further testing of this hypothesis will be needed, as our current results do not directly demonstrate PI(3)P depletion on APPL1-positive endosomes. Nonetheless, our findings are reminiscent of an elegant study by De Camilli and colleagues who used a chimeric MTM1 inducible system to deplete PI(3)P from EEA1/Rab5 vesicles causing reversion back into APPL1-positive endosomes. Importantly in this study, these PI(3)P depleted APPL1 endosomes were also enlarged and displayed increased growth factor signaling, with the enlargement also being dependent on the catalytic activity of MTM1 [32]. However, in the inaugural studies using this chimeric MTM1 inducible system, Larijani and colleagues concluded that PI(3)P depletion resulted in endosomal tubulation rather than enlargement [75]. Both of these studies however, clearly demonstrated that depletion of PI(3)P on early endosome compartments acutely compromises endosome maturation. Analogous to the inducible chimeric MTM1 system, our results highlight how controlling the PI(3)P phosphatase activity of MTMR2 allosterically via reversible phosphorylation serves as a potent mechanism to regulate the residency time of MTMR2 on these various early endosomal subtypes. The importance of these finding is highlighted by the fact that MTM1 and the homologous MTMR1 also possess potential phosphorylation sites within similar N-terminal and C-terminal regions. It stands to reason that this may then be a common mode for the controlled subcellular targeting of other MTM family members through similar reversible phosphorylation-mediated regulatory mechanisms.

Nevertheless, we have uncovered a potential negative feedback mechanism that may have involvement in both the transient and/or sustained activation of ERK1/2 signal transduction events. A long standing dogma is that MAPK signaling duration can dynamically influence cellular fate [76], whereby sustained ERK activation is thought to illicit different biological outcomes compared to transient ERK activation. For example, differentiation of PC12 cells into sympathetic-like neurons requires sustained ERK activation, whereas transient ERK activation induces PC12 cell proliferation [77,78]. As
is seen in PC12 cells, in most cell types, sustained ERK activation induces differentiation. However, in Schwann cells, sustained ERK activation triggers de-differentiation in response to a variety of environmental cues including neuronal injury [79,80]. Schwann cell de-differentiation into a progenitor cell-like stage is critical for axonal re-growth and is highlighted by extensive demyelination [80]. Moreover, Schwann cell demyelination is one of the main hallmarks of CMT diseases, including those devoid in proper MTMR2 functions. Since phosphorylation of MTMR2 at position Ser58 by ERK1/2 results in allosteric inactivation through cytoplasmic sequestration of MTMR2 away from its PI(3)P-rich endosomal substrates, it will be important to investigate if sustained ERK1/2 mediated phosphorylation of MTMR2 plays a role in the pathophysiology of CMT disease(s).

From these collective observations, our working model is that dephosphorylation of MTMR2 increases accessibility of key residues within PH-GRAM which leads to its stabilized localization to Rab5/PI(3)P-rich endosomes, resulting in PI(3)P depletion, mis-localization of PI(3)P-binding proteins, and ERK1/2 activation. This resultant ERK1/2 activation may then lead to Ser58 phosphorylation, destabilizing MTMR2-endosomal interactions through competitive shielding of the PH-GRAM domain, and thus completing a functional negative feedback mechanism to remove MTMR2 from substrate-rich early endosomes. This may, in part, be critical for achieving proper homeostatic endosomal signaling and maturation rates, acting to halt or slow maturation events to allow for increased receptor-signaling responses from these PI(3)P depleted endosomes until an appropriate threshold is met, triggering ERK1/2-mediated attenuation of the stimulus through Ser58 phosphorylation.
6.5 - REFERENCES


the ERK-signaling pathway in controlling Schwann cell plasticity and peripheral nerve regeneration in vivo, Neuron 73 (2012) 729-742.
CHAPTER 7 - GENERAL CONCLUSIONS, OUTLOOK AND FUTURE WORK

7.1 - Regulation of Biological Redox Switches: Novel Labeling and Enrichment Strategies for Monitoring Protein Thiol S-Modifications Using Mass Spectrometry

Research Significance

Cellular oxidants are often considered unfavourable by-products of aerobic respiration, but increasing evidence has highlighted their roles as second messenger molecules in normal signal transduction. Reactive oxygen and nitrogen species provide a tier of transient and reversible post-translational regulation over 'redox switch or sensor' proteins whose activities depend on cysteine thiols. Multiple redox-based S-modification of target proteins exist, occurring rapidly and specifically with susceptible thiols, and it is these characteristics that often hinder isolation, detection, identification and quantification. However, it is also these collective characteristics which highlight the importance of the continued development of methods and reagents for use in their study. In collaborative methods pioneered by our and Dr. Bulent Mutus' lab (University of Windsor), we have used a variety of novel labeling and enrichment strategies allowing for quantitative and/or qualitative identification of protein thiol redox status using mass spectrometry (MS).

Using chemically assisted fragmentation (CAF) technology, we developed a novel, MS-based chemical derivatization method to identify inter-peptidyl disulfide bonding of the tyrosine phosphatase human YVH1. This was one of the first accounts that protein tyrosine phosphatase (PTP) oxidation is not limited to the active site thiol, but capable of modifying other intrinsic low p$\text{Ka}$ thiols which serve to buffer inactivating oxidative stress conditions and preserve catalytic activity. In a separate study, isotope coded affinity tag (ICAT) technology and thiol labeling was used by Kozarova et al., on the oxidoreductase protein disulfide isomerase (PDI) to show some of the first evidence for quantitative analysis of thiol redox status using MS.

In a following proof of concept study, we used these redox regulated proteins as models for a novel approach which combined both thiol isolation and enrichment steps.
Our method exploited the near covalent interaction of gold nanoparticles (AuNPs) with protein thiols providing a simple, single or multiple-step enrichment method of $S$-modified peptides for subsequent MS analysis of the specific site and type of $S$-modification(s). These manuscripts have since fostered an invited article on methods for studying redox regulation of PTPs and an invited review of current methods for identifying protein $S$-glutathionylation and $S$-nitrosylation sites.

Our latest approaches have focused on reversible, low pH labeling of thiols for their enrichment and analysis using MS. We have used Mercury-Immobilized Affinity Chromatography (Hg-IMAC) to specifically isolate reversibly oxidized thiol-containing peptides for subsequent MS analysis. In a similar approach, based on the scheme proposed by Nielson et al., we have began development of a ITC-CAD-based chemical library of low pH thiol labeling reagents in which unique functionality can be built on the designed platform of reagents. Combined with this discovery, the designed reagents act as molecular reporter ions within the MS, having future applications in quantitative MS of a diverse array of biological thiols.

**Practical Application Significance**

Identification of cross-linked species by MS is often hindered by their low abundance, increased size, and the resulting mixture of data arising from two (or more) peptides which are covalently linked. Based on the studies above, differential CAF labeling technology and molecular reporter ions enables one to locate and dramatically improve identification and *de novo* sequencing of labeled and di- or higher order peptides using MS. These characteristics could have profound utility in studying structural protein interaction networks with biochemical cross-linking reagents by improving data acquisition and simplifying interpretation. Moreover, the CAD-based reagent platform can be tailored to impart multiple chemical functionalities, and thus, will serve as a multifaceted MS-labeling agent. Similar to that of the ICAT methodology, both CAF, CAD and AuNP methodologies can be readily applied to high-throughput proteomic-based MS approaches. Our CAD and nanoparticle-based approaches are further highlighted by their inexpensive synthesis, ability to interact with a variety of $S$-modified protein thiols, ease of harvesting by centrifugation (for AuNPs), and elution by thiol
exchange. Most notably, the AuNP enrichment strategy has been recently cited by multiple review articles published in journals such as Cancer Letters, Analytical and Bioanalytical Chemistry, Antioxidants and Redox Signaling, and Proteomics. This exhibits the broad range of future applications this framework may support for end-users among diverse areas of study.

7.2 - Differential Phosphorylation of the Phosphoinositol-3-phosphatase MTMR2 Regulates its association with Early Endosome Subtypes - Journal of Cell Science

Research Significance

Early endosome maturation rates are dependent on their membrane complement of the phospholipid phosphatidylinositol-3-phosphate (PI(3)P), one substrate of the lipid phosphatase myotubulin related protein 2 (MTMR2). Recently we have shown that phosphorylation of MTMR2 on Ser58 is responsible for its cytoplasmic sequestration. A deficient variant, MTMR2 S58A, targets MTMR2 to Rab5-positive endosomes resulting in PI(3)P depletion, mislocalization of PI(3)P binding proteins, and an increase in endosomal signaling [1,2]. Notably was a significant increase in activation of the mitogen activated protein kinase (MAPK), extracellular receptor kinase 1/2 (ERK1/2) [1].

Using in vitro kinase assays, cellular MAPK inhibitors, siRNA and an in-house generated phosphospecific-Ser58 antibody, we have provided evidence that ERK1/2 are kinases responsible for phosphorylating MTMR2 at position Ser58. This strongly suggests that the endosomal targeting of MTMR2 may be regulated through an ERK1/2-mediated negative feedback mechanism. We have further shown evidence that phosphorylation of MTMR2 allosterically regulates its sub-cellular localization through effects mediated by the PH-GRAM domain. In addition, we provided evidence that MTMR2 is dynamically regulated by multiple MAPKs resulting in differential localization to and among endosomal subtypes; phospho-status of Ser58 regulates general endosomal localization, while the phospho-status of Ser631 mediates endosomal shuttling between Rab5 and APPL1 subtypes. Taken together, this illustrates an important role for MAPK-mediated phosphorylation in regulating and achieving early endosomal
heterogeneity and thus aspects of receptor signaling and vesicular trafficking through direct influences on PI(3)P phosphatases.

**Practical Application Significance**

Phospho-specific antibodies offer an economic and safe alternative to radioactive $[^{32}P]$ labeling experiments while still providing adequate sensitivity and specificity for analyzing unique phosphorylation events. Based on the above findings, the ability to monitor phosphorylation of MTMR2 Ser58 would reflect levels of the pseudo-inactive state of MTMR2 towards endosomal PI(3)P. This would in turn provide an indirect measure of endosomal PI(3)P levels, and insight to both the binding events and maturation rates of vesicular trafficking under the tested condition. Since loss of function mutations in the *mtmr2* gene result in Charcot-Marie Tooth disease 4B1, this reagent may find clinical use in discovering functional pathophysiologic roles of MTMR2. This is supported by strong evidence that differential ERK1/2 activity is essential for Schwann cell differentiation and myelination, a main process disrupted by CMT4B1. Monitoring phosphorylation of MTMR2 Ser58 may not only represent a proximal ERK1/2 substrate, but serve as a diagnostic marker for transient upstream ERK1/2 activation events, both in normal and disease models. These notions highlight the future applicability of the designed reagent to those studying among diverse fields ranging from vesicular trafficking to cellular signaling to pathophysiology.
Experimental parameters for reverse phase ultra performance liquid chromatography and mass spectrometry applications

Samples were injected onto a 180 µm x 20 mm x 5 µm Symmetry® C18 reverse phase nanoACQUITY UPLC trapping column then resolved on a 100 µm x 100 mm x 1.7 µm BEH®130 C18 reverse phase nanoACQUITY UPLC analytical column as described in the experimental parameters [Appendix A1]. Timed-ion chromatograms and mass spectra were acquired on a Waters Quattro-micro® nanoESI triple-quadrupole MS. Instrument settings varied based on MS tune requirements for each mode and are described in the experimental parameters [Appendix A2-4]. For targeted approaches (MS/MS and PIS), tune settings were determined empirically by continuous direct-injection of molecule(s) of interest using the microESI source interfaced onto the above triple-quadrupole MS and found to be similar to those used in LC/MS/MS and LC/PIS experiments. Noted differences were a reduction in Cone voltage to ~ 26 V, and increase in collision energy to ~ 31 - 33.
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- Scan duration (secs): 1
- Inter Scan Delay (secs): 0.1
- Retention window (mins): 0.000 to 50.000
- Ionization mode: ES+
- Data type: Accurate Mass
- Function type: Precursor ion scan
- Mass range: 300 to 700
- Collision Energy: 25
APPENDIX B - CHAPTER 6

Production, isolation, purification and characterization of avian α-pSer58 MTMR2 and α-MTMR2 polyclonal antibodies

To construct the pSer58 MTMR2 antigen, we used the Basic Local Alignment Search Tool to perform multiple searches across the human proteome to limit the sequence similarity between the target peptide epitope and other endogenous proteins. Once determined, we checked both the predicted antigenicity and hydrophobicity, and found no limitations on either. From this, we placed an N-terminal cysteine on the peptide and amidated the C-terminus to mask the negative charge. The target peptide was synthesized (NEO BioScience), resuspended in conjugation buffer (100 mM NaPO₄, 150 mM NaCl, 5 mM EDTA, pH 7.3), reduced with immobilized TCEP (Pierce Biotechnology) and conjugated to maleimide-activated Keyhole Limpet Hemocyanin (Pierce Biotechnology) through the engineered thiol terminal. Reduction by tris(2-carboxyethyl) phosphine hydrochloride (TCEP-HCl) was monitored by matrix-assisted laser desorption ionization mass spectrometry (MALDI-TOF MS) and 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB)-based assays. The formulated carrier was then used for immunization of Gallus gallus (Amicus Biotech Inc.). Purified polyclonal chicken immunoglobulin Y (IgY) was isolated by the water dilution method as described below and characterized for efficacy against the target epitope by immunoblot analysis. As observed, only purified bacterial recombinant MTMR2 was readily detected, neither recombinant hYVH1, transiently over-expressed MTMR2 in HEK 293 cells nor endogenous MTMR2 were detected to a significant amount. This highlighted that α-MTMR2 IgY could specifically recognize MTMR2, but in a non-phospho-specific manner, thus required affinity purification to isolate the phospho-specific antibodies [Appendix B1].

Chicken α-pSer58 MTMR2 IgY antibodies were isolated sequentially by subtractive affinity chromatography against non-phosphorylated and phosphorylated peptide epitopes immobilized on iodoacetamide-activated agarose (Pierce Biotechnology) conjugated similarly to that described above [Appendix B2]. Antibodies were first eluted off the column using 175 mM glycine pH 2.5 being immediately neutralized with 1 M
Tris-HCl pH 8, however, it was determined by enzyme-linked immunosorbant assays that during the course of elution, IgY, not IgG-HRP secondary, loses nearly 50% of its binding activity under these conditions, while addition of a stabilizer, sorbitol, improved this to 75%. Using 50 mM diethylamine, no significant loss of binding activity was
observed, thus this eluant was continued throughout subsequent affinity purification of avian IgY antibodies [Appendix B3,4].

Antibody specificity was tested against both MTMR2 peptide and protein epitopes by standard dot and immunoblot procedures. Briefly, peptides or FLAG-MTMR2 immunoprecipitates (as described in Chapter 6 'Experimental Procedures') were resuspended in 50 mM Tris-HCl pH 9.3, 1 mM MgCl₂, 0.1 mM ZnCl₂ and treated with or without 2.5 U of calf intestinal alkaline phosphatase (Promega) for 4–6 hours at 25 °C shaking, then spotted on nitrocellulose (peptide dot blot analysis) or prepared for immunoblotting. Alkaline phosphatase activity was monitored using MALDI-TOF MS [Appendix B6]. Membranes were probed with chicken anti-pSer58 MTMR2 IgY at 1:1000 (0.7 µg/µl stock) in 2.5% BSA TBST, washed, then probed with donkey anti-chicken IgY-HRP conjugate at 1:3000 (Gallus Immunotech) in 2% BSA/TBST for subsequent chemiluminescent imaging [Appendix B5,6].
Mathematical matrix calculating peptide masses upon hydrolysis at every possible combination of peptide bond(s).

Using Microsoft Excel 'IF' functions, three consecutive formulae were constructed to convert primary amino acid sequences (one-letter code) into their respective residue masses. Summation of these formulae align the masses of each residue within the protein of interest. Transposition of the primary amino acid sequences (one-letter code) and their calculated respective masses down the left column sets up the starting amino acid (n) for a given peptide of interest. A given mass is located within the matrix using the 'find' function, whereby the horizontal axis denotes the amino-terminal peptide residue (n), while the vertical axis denotes the carboxyl-terminal peptide residue (x), giving the corresponding theoretical peptide. Detailed descriptions of the mathematical formulae are outlined in the matrix [Appendix C1].
The formula:  

Formula 1 =IF(C14="G",57.02,IF(C14="A",71.04,IF(C14="V",99.07,IF(C14="L",113.08,IF(C14="I",113.08,IF(C14="S",87.03,IF(C14="T",101.05)))))))))  

Formula 2 =IF(C14="Y",163.06,IF(C14="F",147.07,IF(C14="W",186.08,IF(C14="D",115.03,IF(C14="E",129.04,IF(C14="Q",128.06,IF(C14="N",114.04)))))))))  

Formula 3 =IF(C14="K",128.09,IF(C14="R",156.10,IF(C14="H",137.06,IF(C14="C",103.01,IF(C14="M",131.04,IF(C14="P",97.05,"")))))  

Formula is broken into three sets of amino acids due to calculation constraints.

Primary Seq. M A M E K S S C E S L  
15 Formula 1 FALSE 71.04 FALSE FALSE FALSE 87.03 87.03 87.03 FALSE FALSE 87.03 113.08  
16 Formula 2 FALSE FALSE FALSE 129.04 FALSE FALSE FALSE FALSE FALSE FALSE 129.04 FALSE  
17 Formula 3 131.04 FALSE 131.04 FALSE 128.09 FALSE FALSE FALSE 103.01 FALSE FALSE  
18 F1+F2+F3 = 131.04 71.04 131.04 129.04 128.09 87.03 87.03 87.03 129.04 87.03 113.08  
19  
20 Where n is the first residue in the transposed peptide sequence (left column) and x is the last amino acid in the theoretical peptide (across the row of n)  
21 Primary Seq. Residue Mass =SUM(F1,F2,F3)+x(F1+F2+F3)) + Mass of residue x + 19 = Peptide Mass  
22 M 131.04 221.08 352.12 481.16 609.25 696.28 793.31 870.34 973.35 1102.39 1189.42 1302.5  
23 A 71.04 221.08 350.12 478.21 565.24 652.27 739.3 842.31 971.35 1058.38 1171.46  
25 M 131.04 279.08 407.17 494.2 581.23 666.26 771.27 900.31 987.34 1100.42  
26 E 129.04 276.13 363.16 450.19 537.22 640.23 769.27 856.3 966.38  
27 K 128.09 234.12 321.15 408.18 511.19 640.23 727.26 840.34  
28 S 87.03 193.06 280.09 383.1 512.14 599.17 712.25  
29 S 87.03 193.06 296.07 425.11 512.14 625.22  
30 S 87.03 209.04 338.08 425.11 538.19  
31 C 103.01 251.05 338.08 451.16  
32 E 129.04 235.07 348.15  
33 S 87.03 219.11  
34 L 113.08
APPENDIX D - CHAPTER 6

Isolation, purification and characterization of avian polyclonal antibodies

Prior to immunization, ~ 6 eggs were collected as 'pre-immune' comparative controls. After immunization, eggs were collected and processed similarly to the water-dilution method described by Akita and Nakai, *Journal of food science*, 57, (1992) 629-634 [Appendix D1]. Briefly, after letting eggs settle, they were cracked open to separate the vitellus (yolk; IgY) from the albumen (egg white; IgA and IgM). Yolk sacs are rinsed with water, then with 5% glacial acetic acid directly over collected albumen to remove any residual traces left on the sac. Vitellus is then drained into a large secondary vessel by puncturing the sac, ensuring not to rip or drop the membrane into the vessel. The collected vitellus is diluted 1:10 (~ 135 mL/yolk) in slightly acidified water (with acetic acid) and gently mixed by magnetic stir bar, with the pH being adjusted to ~ 5.1. Solution is poured equally into oak-ridge centrifuge tubes, tubes are balanced, then left still overnight at 4 °C. The egg albumin is treated similarly, being diluted 1:4 (~ 35 mL/egg) in slightly acidified water, gently mixed as above, with the pH being adjusted to ≤ 5. Solution was partitioned and let stand overnight as above.

The soluble fraction of egg albumen is filtered through coarse filter paper to separate the soluble immunoglobulin A (IgA) containing fraction from the insoluble immunoglobulin M (IgM) containing fraction. Insoluble IgM can be stored as is at 4 °C for extended time periods, or can be resolubilized by neutralizing the system. The soluble IgA fraction (WSF IgA) is gently stirred at 4 °C, slowly being brought to 40% ammonium sulfate (AMS). Once reached, sample is stirred for ~ 1 h, then let stand overnight at 4 °C. The samples containing the diluted vitellus are spun at 6000 x g for 20 min at 4 °C to separate the lipids and lipoproteins from the soluble immunoglobulin Y (IgY). The soluble supernatant is collected (WSF IgY), then treated similarly to that of soluble IgA, being brought to 40% AMS and let stand overnight.

Insoluble Ig pellets are centrifuged at 6000 x g for 20 min at 4 °C, washed 2 - 3x with cold 100% AMS, collected and stored at 4 °C in 100% AMS until use. At time of use, pellet is resuspended, an aliquot taken which is centrifuged to collect pellet. The 100% AMS storage solution is removed, antibodies are resuspended in sterile phosphate
**D1**

**IgY, IgA, IgM**

- Four-fold dilution
  - O/N 4°C
  - Centrifuge
  - Collect S/N (IgA WSF)
  - 40 % AMS O/N 4°C
  - Centrifuge
  - Collect S/N Wash ppt 100 % AMS
  - Centrifuge
  - Collect S/N Store ppt 100 % AMS
  - Resuspend ppt Dialyze (IgA AMS ppt)

- Ten-fold dilution
  - O/N 4°C
  - Centrifuge
  - Collect S/N (IgY WSF)
  - 40 % AMS O/N 4°C
  - Centrifuge
  - Collect S/N Wash ppt 100 % AMS
  - Centrifuge
  - Collect S/N Store ppt 100 % AMS
  - Resuspend ppt Dialyze (IgY AMS ppt)
buffered saline and dialyzed against ≥ 10 volumes of PBS 2 - 3x until AMS is removed as determined by a BaSO₄ turbidity assay (detection limit ~ 10 - 20 µM). Antibodies (AMS ppt IgY or IgA) are empirically tested for efficacy among immunological assays of interest and compared to pre-immune antibody controls.

To characterize the purification, samples were resolved by 10% SDS-PAGE and visualized by coomassie stain or by immunoblot analysis against the target immunoglobulins using the appropriate horserasdish peroxidase conjugated secondary [Appendix D2]. As observed, this two step purification method is capable of achieving near 80 - 90% purity of target immunoglobulins, with yields of ~ 100 mg total IgY/yolk and 15 mg total IgY/egg. Membranes were blocked with 5% skim milk in tris-buffered saline/0.1% Tween-20, then probed with donkey anti-chicken IgY-HRP conjugate at 1:3000 or donkey anti-chicken IgA-HRP conjugate (Gallus Immunotech) in 2.5% skim milk/TBST for subsequent chemiluminescent imaging.
APPENDIX E

Production, isolation, purification and characterization of avian α-hYVH1 polyclonal antibodies

Recombinant bacterial human YVH1 was purified by affinity chromatography then anion exchange chromatography to produce a high purity antigen for immunization. A 1 mL EconoPac High Q Cartridge (Bio-Rad) was equilibrated with degassed, 0.2 µm filtered mobile phase A of 25 mM Tris-HCl pH 8 and mobile phase B of 25 mM Tris-HCl, 1 M NaCl pH 8 as per manufacturer instructions using a Bio-Logic Low Pressure Liquid Chromatography (LPLC; Bio-Rad) system monitoring $\lambda_{abs} @ 280$ nm and conductivity (mS/cm). After zeroing absorbance, sample was injected directly on the column at 500 µL/min using an off-line syringe pump luer-fitted to the column inlet. The system flow was then re-connected and the column equilibrated at 1 mL/min with mobile phase A until near baseline absorbance was achieved. Samples were eluted using a linear gradient of 0 - 100% mobile phase B over 60 min at 1 mL/min and collected in 1 mL fractions [Appendix E1]. Fractions were resolved using 12% SDS-PAGE and purity was determined by silver staining [Appendix E1]. Target fractions 1 thru 5 were pooled, concentrated using an Amicon centrifugal concentration device, and compared directly to an equal amount of starting material (prior to anion exchange as per Bradford assay) by 12% SDS-PAGE analysis as above [Appendix E2]. This highly purified protein was tested for phosphatase activity using the artificial substrate analog DiFMUP, and for identity using in-gel trypsin digestion as described [Appendix E3,4]. The confirmed antigen of interest was used as an immunogen for the immunization of Gallus gallus (Amicus Biotech).

Target antibodies were purified as described [Appendix D] and IgY was tested for efficacy in the detection of hYVH1 by immunoblot (IB) and immunoprecipitation (IP) analysis. As observed, both transiently over-expressed hYVH1 in HEK 293 cells and purified bacterial recombinant hYVH1 were readily detected, however, endogenous hYVH1 was not detected to a significant amount [Appendix B1]. This highlighted that α-hYVH1 IgY required affinity purification.
**Econo-Pac High Q Cartridge**

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<td>Dynamic binding capacity</td>
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- **Buffer A**
  25 mM Tris-HCl, pH 8
- **Buffer B**
  25 mM Tris-HCl, pH 8
  1 M NaCl
- **Linear gradient**
  1 mL/min; 1 mL fractions
Chicken α-hYVH1 IgY antibodies were isolated by affinity chromatography against purified bacterial recombinant hYVH1 immobilized on iodoacetamide-activated agarose conjugated similarly to that described above [Appendix B]. Target antibodies were again tested for efficacy in the detection of hYVH1 by immunoblot (IB) and immunoprecipitation (IP) analysis. As observed, affinity purified α-hYVH1 IgY was able to detect endogenous protein levels in both HeLa and HEK293 cells as directed compared to α-hYVH1 IgG. Moreover, this antibody was able to effectively IP endogenous hYVH1 from the cell lines, as detected by both α-hYVH1 IgY and IgG. Affinity purification, immunoprecipitation and immunoblotting conditions are currently being optimized to reduce the non-specific interactions. To note, thick bands in the α-hYVH1 IgY IP and α-hYVH1 IgY IB blots is the heavy chain of IgY (~ 65 kDa).
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§These individuals contributed equally to this manuscript as co-corresponding authors

¶This manuscript was an invited article for a *Methods* special issue on studying of protein tyrosine phosphatases


*These individuals contributed equally to this manuscript in co-authorship


*These individuals contributed equally to this manuscript in co-authorship

¶Protected under Provisional Patent #61 / 246, 270


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Research in Chapter 4 was originally published and has been reprinted from Methods, August 23, [epub ahead of print] http://dx.doi.org/10.1016/j.ymeth.2013.08.014, ‡†Bonham, C.A., ‡Steevensz, A.J., Geng, Q., †Vacratsis, P.O., Investigating Redox Regulation of Protein Tyrosine Phosphatases using Low pH Thiol Labeling and Enrichment Strategies Coupled to MALDI-TOF Mass Spectrometry ‡Co-authorship, †Co-corresponding authorship. © 2013 with permission from Elsevier.