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Structure-Function Characterization of the Human Dual Specificity Phosphatase hYVH1

Colleen Mailloux
University of Windsor

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Structure-Function Characterization of the Human Dual Specificity Phosphatase hYVH1

by

Colleen Mailloux

A Thesis
Submitted to the Faculty of Graduate Studies through the Department of Chemistry and Biochemistry in Partial Fulfillment of the Requirements for the Degree of Master of Science at the University of Windsor

Windsor, Ontario, Canada
2010
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Author’s Declaration of Originality

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Abstract

YVH1 is a highly conserved dual specificity phosphatase that possesses a novel zinc-binding domain. Although studies implicate hYVH1 in cell survival and cell cycle progression, it remains poorly characterized. In this study, association of hYVH1 with the 60S subunit was demonstrated. Oxidative stress inhibits this association, with the appearance of a 25kDa hYVH1 fragment. Domain deletion studies reveal that regions of the catalytic and zinc-binding domains facilitate ribosomal binding. Collectively, our results lead to a proposed mechanism whereby structural rearrangements in the zinc-binding domain mediate dissociation of hYVH1 from the ribosome and exposure of a proteolytic cleavage site.

We have also purified several hYVH1 variants for X-ray crystallography. To date, we have obtained a low resolution solution structure of full length hYVH1 representing the first structure of any YVH1 orthologue. We anticipate that structural analysis will offer invaluable insights concerning the regulation, mode of action, and substrate specificity of hYVH1.
This paper is dedicated to my family.
Acknowledgements

After all the time and energy that has gone into this thesis, it is a pleasure to thank those who made possible this feat. First and foremost I would like to express immense gratitude to my supervisor Dr. Vacratsis. His enthusiasm for the sciences influenced my enrolment into the Master’s program, and his ceaseless guidance and support throughout are greatly appreciated. He was an excellent mentor, and his teachings were key in my scientific development.

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Katie, Chris, Norah and Besa, thank you for your help, for your friendship, and for putting up with me in the months leading up to my defense. We definitely have some unforgettable memories.

To my family and my husband. My personal support team. Thank you for picking up the slack when I was overwhelmed with my studies, not only throughout my time as a Master’s candidate, but throughout my entire 21 years as a student. You are the best family anyone could ask for. But you already knew that.
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<th>Description</th>
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<tbody>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>ATM</td>
<td>ataxia telangiectasia mutated</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>ATR</td>
<td>ATM-related</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>Cal A</td>
<td>calyculin A</td>
</tr>
<tr>
<td>CDC</td>
<td>cell division cycle</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin-dependent kinase</td>
</tr>
<tr>
<td>DAMMIN</td>
<td>dummy atom model minimization</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate</td>
</tr>
<tr>
<td>DiFMU-</td>
<td>6,8-difluoro-4-methylumbelliferyl</td>
</tr>
<tr>
<td>DiFMUP</td>
<td>6,8-difluoro-4-methylumbelliferyl phosphate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DSP</td>
<td>dual specificity phosphatase</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>DUSP12</td>
<td>dual specificity phosphatase 12</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetracetic acid</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FPLC</td>
<td>fast pressure liquid chromatography</td>
</tr>
</tbody>
</table>
GSH  glutathione
GST  glutathione S-transferase
HEK 293 human embryonic kidney
HeLa Henrietta Lacks (cervical cancer cell line)
HPLC high performance liquid chromatography
IB  immunoblot
IP  immunoprecipitation
IPTG isopropyl β-D-1-thiogalactopyranoside
kDa kilodaltons
MALDI TOF matrix-assisted laser desorption ionization-time of flight
MAPK mitogen-activated protein kinase
MKP mitogen-activated protein kinase phosphatase
mRNA messenger ribonucleic acid
m/z mass to charge ratio
OD optical density
P∗ inorganic phosphate
PBS phosphate buffered saline
PCR polymerase chain reaction
PEI polyethylenimine
PMSF phenylmethysulphonyl fluoride
pNP- para-nitrophenyl
pNPP para-nitrophenyl phosphate
PTP protein tyrosine phosphatase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVDF</td>
<td>polyvinylidene fluoride</td>
</tr>
<tr>
<td>rDNA</td>
<td>ribosomal deoxyribonucleic acid</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>RPL26</td>
<td>ribosomal protein large subunit 26</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>SDS PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SER</td>
<td>surface entropy reduction</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering ribonucleic acid</td>
</tr>
<tr>
<td>snoRNA</td>
<td>small nucleolar ribonucleic acid</td>
</tr>
<tr>
<td>TAP</td>
<td>tandem affinity purification</td>
</tr>
<tr>
<td>TBH</td>
<td>tert-butyl hydroperoxide</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline-Tween 20</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>ZBD</td>
<td>zinc-binding domain</td>
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CHAPTER 1

Introduction

1.1 Cellular Phosphorylation

Protein phosphorylation is a highly relevant and ubiquitous post-translational modification which serves as a key regulatory mechanism of several biological pathways including cell cycle, cellular differentiation, growth, apoptosis and metabolism [1, 2]. It is estimated that more than 30% of eukaryotic proteins are phosphorylated; with these phosphorylation events most commonly occurring on the hydroxyl group of serine, threonine, and tyrosine residues [3]. Introduction of a negatively charged phosphate group onto one or several of these residues generally elicits a conformational change which can alter protein activity, stability, localization, affinity for binding partners, or allow it to adopt an entirely different function [4, 2]. Phosphorylation levels throughout the cell are tightly regulated through the antagonistic action of protein kinases and protein phosphatases [1]. As shown in Figure 1.1, protein kinases phosphorylate proteins by mediating the transfer of the gamma phosphate from ATP, while protein phosphatases catalyze the hydrolysis of a phosphate group from phosphotyrosine, phosphothreonine, or phosphoserine residues, releasing inorganic phosphate as a biproduct of the dephosphorylation reaction [2].

Specific phosphorylation and dephosphorylation events can be triggered by numerous intracellular or extracellular stimuli, initiating signal transduction pathways, and ultimately producing a specialized response. As phosphorylation participates in the regulation of various cellular activities, aberrant phosphorylation has implications in many human diseases such as cancer and Alzheimer’s disease [5,6].
Figure 1.1: Regulation of Cellular Phosphorylation Levels. Protein kinases phosphorylate target proteins by mediating the transfer of the gamma phosphate from an ATP molecule to a target protein. Protein phosphatases remove a phosphate molecule from the target protein, releasing inorganic phosphate. [Figure from reference 39]
1.2 Protein Phosphatases

Protein phosphatases are a diverse family of proteins that remove a phosphate group from a phosphotyrosine, phosphothreonine, or phosphoserine residue on its substrate protein. Protein phosphatases are classified by their mode of action, by their dependence on metals, and by their substrate specificity. The serine/threonine phosphatases function as metalloproteins and dephosphorylate phosphoserine and/or phosphothreonine residues [7]. A second group of phosphatases, the protein tyrosine phosphatases (PTPs), or cysteine dependent PTPs, act through the formation of a thiol-phosphate enzyme intermediate in the removal of a phosphate. All PTPs possess the consensus sequence HC(X)₃RS/T (also termed P-loop), as well as an aspartic acid on one of the protein’s outer loops which acts as a general acid/base in the phosphatase mechanism. The conservation of this characteristic P-loop suggests that these phosphatases proceed through a similar catalytic mechanism as outlined in Figure 1.2 [8].

The PTP subfamily (summarized in Figure 1.3) consists of the tyrosine specific phosphatases, the dual specificity phosphatases (DSPs), and the low molecular weight phosphatases [7]. While the tyrosine specific phosphatases dephosphorylate exclusively phosphotyrosine residues, the dual specificity phosphatases can dephosphorylate both phosphotyrosine and phosphothreonine/phosphoserine residues. Although the DSPs show limited sequence homology with other PTPs, they do share the consensus D…HC(X)₃RS/T catalytic cysteine sequence, and proceed through a parallel catalytic mechanism [9]. It is postulated that the broader substrate specificity of these phosphatases is derived from the depth of their catalytic cleft as well as the presence of non-catalytic, terminal sequences, which often act in substrate binding [1, 10].
Figure 1.2: General Mechanism of Dephosphorylation Employed by Protein Tyrosine Phosphatases. The first step in protein dephosphorylation by PTPs involves the formation of a thiol-phosphate intermediate. The active-site cysteine performs a nucleophilic attack onto the electron-deficient phosphate center. An upstream aspartic acid residue then donates a proton, allowing for release of the dephosphorylated substrate. The second catalytic step employs this same aspartic acid residue to render a water molecule nucleophilic, allowing for the hydrolysis of the cysteinyl-phosphate intermediate, and hence enzyme regeneration. Throughout the dephosphorylation reaction, an arginine residue stabilizes the phospho-substrate within the catalytic cleft, and a serine residue stabilizes the nucleophilic thiolate ion. A glutamine residue (not shown here) allows proper positioning of the water molecule within the active site. [Figure from reference 40]
Figure 1.3: Classification of the Protein Tyrosine Phosphatases. The protein tyrosine phosphatases can be classified into three major groups: the tyrosine specific phosphatases, the low molecular weight phosphatases, and the dual specificity phosphatases (DSPs). One group of DSPs, the atypical DSPs, consists of a variety of poorly characterized enzymes which are thought to target substrates other than MAPKs or CDKs. Among these is the dual specificity phosphatase hYVH1, or DUSP12.
Among the dual specificity phosphatases are the mitogen-activated protein kinase phosphatases (MKP), the cell division cycle (CDC) phosphatases, and the atypical DSPs [11]. The MKPs act to dephosphorylate the mitogen-activated protein kinases (MAPK), thereby deactivating them. The MAPK signalling pathways regulate several cellular functions including cell proliferation, differentiation, and stress response [2]. In addition to their catalytic domain, MKPs possess an N-terminal substrate-binding domain. Only upon binding to a substrate MAPK molecule is the MKP catalytic domain active, hence enhancing substrate specificity [2].

The atypical, or VH1-like DSPs are a poorly characterized group of phosphatases. These phosphatases lack the MAPK recognition motifs, and hence dephosphorylate substrates other than the MAPKs [11]. In addition to their VH1-like catalytic domain, the atypical DSPs often possess additional domains or short sequences that participate in substrate identification, localization, and protein-protein interactions [10]. While this group of DSPs consists primarily of protein phosphatases, one of them (DUSP11) has been shown to dephosphorylate mRNA [12].

1.3 The Dual Specificity Phosphatase YVH1

The first discovered eukaryotic atypical dual specificity phosphates was YVH1 in yeast, which displays high evolutionary conservation with orthologues present in species ranging from yeast to human [13]. YVH1 contains the P-loop consensus sequence, characteristic of all PTPs, in its N-terminal catalytic domain. However, YVH1 is particularly unique in that it is the only PTP family member that possesses a highly
conserved C-terminal zinc-binding domain in addition to its N-terminal catalytic domain [7]. This cysteine-rich domain is able to coordinate two moles of zinc per mole of protein. Through the use of truncated mutant variants, it has been found that the zinc finger domain is essential for *in vivo* function of YVH1 [7,14,15]. This novel zinc-binding domain is highly conserved throughout evolution amongst YVH1 orthologues, suggesting that the domain is critical for proper protein function [7].

In yeast, it has been found that transcription of *yvh1* is induced by nitrogen starvation and low temperature [16]. Yeast with *yvh1* gene knocked out display a slow growth phenotype, sporulation defects and defective glycogen accumulation (all independent of catalytic activity) [17].

To date, no substrates have been identified, however a study conducted by Sakumoto et. al in 2001 showed interaction between YVH1 and YPH1 by the yeast two-hybrid method. YPH1 is a dynamic protein that is critical for DNA replication, cell cycle control, and biogenesis of the 60S ribosomal subunit [18]. Multicopy *yph1* was able to rescue both the slow growth defect and recover transcript levels of sporulation-specific genes associated with *yvh1* disruption mutants. Additionally, deletion studies showed that the catalytic domain of YVH1 was sufficient for interaction with YPH1. It was therefore suggested that YPH1 could be a candidate substrate; however, no further evidence has been presented to support this hypothesis [13]. Interestingly, YVH1 has recently been found to play a role in ribosome biogenesis in yeast, specifically, in the maturation of the 60S ribosomal subunit [14,15,19].
1.4 The Human Dual Specificity Phosphatase hYVH1

The human orthologue, hYVH1 (also termed DUSP12), shares approximately 30% sequence identity with YVH1 and possesses the characteristic DSP domain and C-terminal zinc-binding domain (Figure 1.4). hYVH1 is located on the chromosomal region 1q21-q22, which is amplified in human liposarcomas and a variety of other solid tumours, including ovarian cancer and hepatocellular carcinomas [7,20]. It has been found that the human orthologue possesses the ability to rescue the slow growth defect caused by yeast yvh1 disruption mutants [7]. The Vacratsis lab has identified HSP70 as a binding partner of hYVH1. Further, our lab has recently shown that hYVH1 acts as a cell survival phosphatase: its overexpression protects cells from apoptosis induced by heat shock, oxidative stress and Fas receptor activation [21]. The notion of hYVH1 as a cell survival phosphatase was preceded in a study conducted by MacKeigan et al., in which systematic knockdown of phosphatases by transfection with siRNAs identified hYVH1 as potential anti-apoptotic protein [22]. Notably, this was the first physiological role of hYVH1 shown to necessitate its catalytic activity: substitution of the catalytic cysteine residue with serine was unable to protect cells [21].

Interestingly, the catalytic activity of hYVH1 toward artificial substrates in vitro at high temperature or in non-reducing conditions was affected to a lesser extent compared to other PTPs, suggesting that hYVH1 may be resistant to inactivation under these conditions [21]. This hypothesis was further investigated by Bonham et al. who showed that the zinc-coordinating cysteines in the zinc-binding domain were able to protect the active-site cysteine of hYVH1 from inactivation by oxidation [23]. Under conditions of mild oxidative stress in vitro, which generally leads to inactivation of PTPs,
Figure 1.4: Schematic of hYVH1 Protein Sequence. The C-terminal catalytic domain and N-terminal zinc-binding domain are depicted. The highly conserved D…HC(X)₅RS/T motif, characteristic of all PTPs, is emphasized and the catalytic cysteine and arginine residues underlined. The catalytic domain of hYVH1 shares 30% sequence homology with the classical DSP VHR.
the coordinating cysteine residues in the zinc-binding domain are oxidized, triggering the release of zinc. When the levels of oxidative stress exceed the redox buffering range of the coordinating cysteines, the active site cysteine forms intramolecular disulfide bonds with nearby cysteine residues. Upon return to normal cellular redox conditions, this zinc ejection is readily reversed, and the active site returned to its active, reduced state. This supports a mechanism in which the active site cysteine is capable of eluding irreversible oxidation through the formation of intramolecular disulfide bonds [23].

It has been shown that hYVH1 is itself regulated by phosphorylation. Through the use of phosphatase inhibitors to boost cellular phosphorylation levels, followed by the subsequent analysis of hYVH1 peptides by mass spectrometry, it has been found that hYVH1 can be phosphorylated at three sites: serine 14, threonine 252 and serine 335, located near the N-terminus, within the zinc-binding domain, and near the C-terminus, respectively [24]. When mutating these residues to alanine or glutamic acid, hence mimicking the non-phosphorylated and phosphorylated forms, respectively, it was observed that phosphorylation may affect subcellular localization of hYVH1. Typically, hYVH1 exhibits a nuclear, perinuclear, and cytoplasmic localization. However, overexpressed S14A or S335A mutants in HeLa cells displayed nuclear localization, while overexpressed S14E or S335E mutants resided mainly in the cytoplasm. This distinction in localization was accompanied by changes in cell cycle profiles as analyzed by flow cytometry.

When comparing HeLa cells overexpressing wild type hYVH1 to cells expressing normal levels of endogenous hYVH1, a marked decrease in cells in G0/G1, coupled to an increase in G2/M and polyploid cells was observed [24]. While overexpression of the
S14A mutant produced cell cycle profiles comparable to that of cells overexpressing wild type hYVH1, the overexpression of S14E had an augmented response with an even greater decrease in G0/G1 cells and an increased number of polyploid cells when compared to wild type. Similarly, overexpression of S335E resulted in an increased number of cells in G2/M. This study, briefly summarized in Figure 1.5, showed that human YVH1 may be involved in the control of cell cycle progression, and that this function may be regulated through phosphorylation.

When investigating the effect of domain-deletion constructs on the cell cycle profiles, it was found that the catalytic domain alone was unable to significantly affect the cell cycle profile in comparison to untransfected cells. When expressing constructs consisting of the catalytic domain and segments of the zinc-binding domain, increasing amounts of cells in G2/M and decreasing amounts of cells in G0/G1 were observed, culminating with the zinc-binding domain alone, which had the greatest amount of cells in G2/M and least amount in G0/G1, as compared to wild type. And finally, overexpression of a catalytically dead variant of hYVH1 did not have an effect on cell cycle compared to overexpression of wild type hYVH1. This study suggests that the zinc-binding domain alone is likely sufficient to elicit changes in cell cycle [24].

1.5 Ribosome Biogenesis

As mentioned previously, YVH1 in yeast has recently been identified as a trans-acting factor in the biogenesis of the 60S ribosomal subunit, and it was suggested that hYVH1 may fulfill a similar role in humans [14,15]. Ribosomes are the machinery responsible for the synthesis of proteins using mRNA as a template. Eukaryotic
Figure 1.5: Summary of Effect of hYVH1 Mutants on Cell Cycle. Overexpression of hYVH1 in HeLa cells causes a decreased number of cells in G\textsubscript{0}/G\textsubscript{1} and an increase of cells in G\textsubscript{2}/M and polyploid cells compared to empty vector, as determined by flow cytometry. Further, phosphomimetic mutants S14E and S335E cause more pronounced increases in percentage of polyploid cells, and cells in G\textsubscript{2}/M, respectively. Deletion studies show that the catalytic domain alone (CT1) is unable to complement the phenotype displayed by wild type hYVH1. However, mutants comprised of the catalytic domain and fragments of the zinc-binding domain (CT2, CT3) are able to surpass polyploid cell numbers seen with overexpression of wild type hYVH1, while the zinc-binding domain alone has an ever increased number of polyploid cells and cells in G\textsubscript{2}/M. [Figure from reference 24]
ribosomes consist of four ribosomal RNAs (25S/28S, 5.8S and 5S in the 60S subunit, and 18S in the 40S subunit) and approximately 80 ribosomal proteins [25]. Biogenesis of the ribosomal 60S and 40S subunits (outlined in Figure 1.6) requires a considerable amount of coordination between RNA polI for synthesis of 25S/28S, 5.8S and 18S rRNA, RNA polII for synthesis of mRNA of the various ribosomal proteins, and RNA polIII for the synthesis of 5S rRNA [26]. Additionally, ribosome biogenesis demands strict temporal and spatial coordination of a vast range of trans-acting factors that facilitate the process, which in yeast results in the synthesis of approximately 40 ribosomes per second [27]. These trans-acting factors include greater than 150 non-ribosomal proteins, and approximately 70 small nucleolar RNAs (snoRNAs), which guide modifying factors and ribonucleases via complementary base-pairing with rRNA [26,27]. It is therefore logical that such a complex and dynamic process would consume a significant amount of the cells resources, and hence require meticulous regulation [28]. Mutations in this intricate process are often associated with human disease such as bone marrow failure syndromes and tumorigenesis [29,30].

1.5.1 Ribosome Biogenesis: From Nucleolus to Cytoplasm

The first step in ribosome biogenesis is the transcription of ribosomal RNA and the transcription and translation of ribosomal proteins. The nucleolus is the central location for transcription of ribosomal genes. Here, ribosomal genes are organized in tandem arrays, termed nuclear organizer regions, along chromosomes [26,31]. The 25S/28S, 5.8S and 18S rRNAs are transcribed as a single precursor transcript in the nucleolus, while the 5S rRNA must be transported into the nucleolus following nuclear
Figure 1.6: Ribosome Biogenesis in Mammalian Cells. Once ribosomal RNA is transcribed within the nucleolus, various ribosomal proteins and trans-acting factors are imported into the nucleus to mediate rRNA processing events and assembly of the ribosomal subunits. Following several highly coordinated maturation events, pre-40S and pre-60S ribosomal particles are exported from the nucleus to the cytoplasm, where the final stages of maturation occur. 40S ribosomal subunits are then free to bind mRNA, followed by association with the 60S subunit, forming the translationally competent 80S ribosome.
transcription [29]. A high degree of regulation is observed at the transcriptional level, where chromosomal modifications can render genes of essential ribosomal components inactive, thereby inhibiting the entire biogenesis pathway [28].

Once transcribed, rRNA transcripts must be modified, cleaved by exonucleases and endonucleases, folded, and assembled with ribosomal proteins [31]. Several of the ribosomal proteins are imported from the cytoplasm into the nucleolus where they are able to associate with rRNA. The earliest reported pre-ribosomal complex is the 90S particle. This complex includes the uncleaved 35S/47S precursor rRNA with various associated ribosomal and non-ribosomal proteins [32]. Cleavage of the rRNA then yields two separate complexes: the 43S particle and the 66S particle, which are precursors to the 40S and 60S ribosomal subunits, respectively [27,29,33]. The 43S subunit is almost immediately exported to the cytoplasm, where the few final stages of maturation occur, including a final rRNA cleavage event. The pre-66S subunit however, has a considerably higher ratio of protein:RNA than the mature 60S particle, and must reside in the nucleus for an extended period of time. During this time, the pre-60S particle must undergo several processing steps in which association and dissociation with various trans-acting factors must occur prior to becoming competent for nuclear export [27,33]. While the majority of ribosomal proteins are assembled onto the 60S subunit in the early steps of maturation, there are a few that are presumed to associate with the complex following export of the 60S subunit into the cytoplasm [14,15].
1.5.2 Ribosome Biogenesis is Sensitive to Intra and Extracellular Environment

Because of the taxing demand imposed on cellular resources by ribosome biogenesis, it is essential that this process be highly regulated. As mentioned previously, a significant amount of regulation is observed at the level of transcription. Ribosomal genes can be completely silenced by structural rearrangement of genes into transcriptionally inactive heterochromatin [31]. This inactivation of ribosomal genes is poorly understood and remains relatively constant within a cell. Alternatively, transcription levels can be adjusted through the modulation of the transcriptional machinery to provide a more immediate and transient response to cell cycle and growth, as well as cellular environment. For example, rRNA production in metabolically active, proliferating cells is quite high compared to the significantly reduced levels of rRNA synthesis that can be observed in fully differentiated cells. Additionally, ribosome biogenesis is closely coordinated with the cell cycle, with highest levels of rRNA transcription occurring in S and G2 phases of the cell cycle (in mammalian cells) and suppressed during mitosis [31]. The mitotic repression is due to phosphorylation of the transcription factors SL1 and TTF-1 by cdk1/cyclin B, consequently inhibiting RNA polymerase I activity and therefore hindering rRNA synthesis. Similarly, a drastic reduction of rRNA production is observed in response to cellular insults such as deficient nutrients and drug treatment [31]. In both yeast and mammalian cells, transcription of ribosomal genes is closely coupled to cell cycle and growth in response to environmental conditions via the TOR signalling pathway [34]. Further, the RAS-cAMP protein kinase A (PKA) pathway has been shown to affect levels of ribosomal protein transcripts [28].
Although regulation of ribosome biogenesis is well characterized at the transcriptional level, little is known concerning the various aspects of regulation throughout the process of maturation. For one, it is clear that the regulation of ribosome biogenesis by TOR extends beyond transcriptional control. TOR is responsible for the activation of P70 S6 kinase. Phosphorylation of ribosomal protein S6 by S6 kinase is required for recognition of ribosome protein mRNA by the translational machinery, hence allowing for the synthesis of relevant ribosomal proteins [34]. Additionally, TOR participates in the later stages of ribosome biogenesis in the nucleoplasm, as inhibition of TOR in yeast causes defects in processing of the 35S rRNA precursor [34,35]. Furthermore, it is thought that there are several proteins that act as a surveillance mechanism throughout ribosome biogenesis. These “quality control” proteins ensure that pre-ribosomal subunits do not continue in the biogenesis pathway if improper assembly has occurred. Because ribosome synthesis is a spatially ordered event, a common mechanism of control is thought to be the inhibition of transportation of maturing ribosomal particles through the requirement of proteins that function either as active transport signals or to overcome retention signals [27]. When triggered, these surveillance mechanisms may also result in the degradation of non-functional ribosomes in response to improperly assembly, or damage due to insult by UV radiation or oxidative stress [29, 36]. Studies in yeast have presented evidence of crosstalk between ribosome biogenesis and other major synthetic pathways throughout the cell, such as the secretory pathway [28]. While it is speculated that the plethora of trans-acting factors that mediate this process provide several layers of regulation throughout the ribosome biogenesis
pathway and allow for its coordination with intra- and extracellular environmental cues, the specifics concerning these mechanisms have yet to be unveiled [27,28].

1.5.3 Trans-Acting Factors Involved in Ribosome Biogenesis

The maturation of ribosomal subunits is a highly ordered event, enabled by several trans-acting factors (non-ribosomal proteins and snoRNPs), which transiently associate with various pre-ribosomal complexes along the pathway from nucleolus to cytoplasm, but are not a part of the final, mature 60S or 40S subunits. The trans-acting factors may fulfill roles such as acting as endo/exonucleases, participating in ribosomal transportation, mediating the association and dissociation of ribosomal and non-ribosomal proteins, recycling of export proteins or functioning in signal transduction pathways to communicate with other cellular processes [29,36]. Several of these enzymes are energy consuming GTPases and ATPases that, upon NTP hydrolysis, undergo conformational changes that can trigger the desired response [27,29,36]. While in many cases their role may seem trivial, their concerted action is essential for normal cellular function, and even the smallest defect may alter biogenesis or translational efficiency. For example, a defective ATPase involved in the release of certain trans-acting factors from the ribosome may result in the inhibition of 40S and 60S subunit association, and hence inhibit translation [29]. Although a significant number of these trans-acting factors have been identified, in many cases their function and means of regulation remain elusive.
1.5.4 Tools to Identify and Analyze Trans-acting Factors in Ribosome Biogenesis

Despite our comprehensive understanding of the translational mechanism exercised by the mature ribosomal machinery, the process of ribosomal biogenesis and the role of several of the involved non-ribosomal proteins in this very dynamic process are poorly understood. Most of the studies of eukaryotic ribosomal biogenesis to date were performed in yeast, and mammalian studies are still very premature [26,27]. Nonetheless, the yeast studies performed offer valuable insight into the mechanism of ribosomal biogenesis in higher eukaryotes, as several of the ribosomal proteins and the identified trans-acting factors are conserved between yeast and higher eukaryotes [31].

There have been various approaches taken in the investigation of the numerous premature complexes associated with ribosome biogenesis. One such method involves the use of tandem affinity purification (TAP) tagging of proteins known for their involvement in the maturation pathway to isolate various intermediate, pre-ribosomal complexes [27,29,36,37]. Once isolated, proteins within the complex are identified via the use of mass spectrometry. In conjunction with TAP tagging, reverse tagging of the identified proteins, assessment of rRNA content within the complex, and the use of GFP fusion proteins to observe the subcellular localization of these intermediates allows for the establishment of a timeline of sequential maturation events [27,29,36,37]. Because ribosome biogenesis is spatially and temporally regulated, it can be concluded that pre-ribosomal complexes in close proximity to the nucleolus are more premature than those found within the nucleoplasm, which are more premature than the late complexes which have been exported to the cytoplasm [27].
Another method used to investigate the role of non-ribosomal proteins in ribosome biogenesis is ribosome profiling. Because ribosome particles sediment differently, ribosomes can be fractionated on a density gradient, and levels of subunits observed through the detection of rRNA. Therefore, alteration of expression of a protein involved in the maturation pathway may consequently affect the levels of mature 60S, 40S, 80S (complexed 60S and 40S), polysomes (several 80S ribosomes complexed to a single mRNA transcript), or halfmers (several 40S subunits and associated translational machinery bound to a single mRNA transcript). These observations can lend insight into the function of the protein under study as it pertains to ribosome synthesis [14,15,19,26].

In many cases, yeast and mammal protein orthologues involved in ribosome biogenesis fulfill very similar roles in their respective organisms [27]. Despite the numerous similarities, there are marked discrepancies in the function of these orthologues, which stress the importance of studying this process in mammals and higher eukaryotes in conjunction with the yeast model. The eukaryotic initiation factor (eIF6), shares 77% sequence identity between its orthologues in yeast and humans [32]. This protein has been shown to be involved in ribosome biogenesis in yeast, presumably in stabilization of the 60S subunit. Although it is not characterized as well in humans, studies have also shown eIF6 to be implicated in maturation of the 60S subunit in humans, presumably fulfilling a similar role as in yeast, considering human eIF6 is able to recover normal biogenesis in eIF6 deletion mutants in yeast. However, in contrast to yeast, mammalian eIF6 has been shown to have an additional role in anti-association of the 60S and 40S subunits, preventing the formation of the 80S particle in the absence of an mRNA transcript, which otherwise requires an energy-consuming reaction to
disengage [32]. In addition to illuminating the potential differences between proteins in this process in yeast and higher eukaryotes, these studies highlight the dual role that may be fulfilled by several proteins involved in ribosome biogenesis, which could provide means for crosstalk with other cellular processes.

1.5.5 YVH1 is a Trans-acting protein in Ribosome Biogenesis in Yeast

Recently in yeast, YVH1 has been found to play an important role in maturation of the 60S ribosomal subunit. Although yeast two hybrid studies have shown YVH1 to interact with YPH1/ Nop7, a known factor in ribosome biogenesis, the first strong indication that YVH1 may be a trans-acting factor in ribosome assembly was in a study that showed that yvh1 deletion strains containing a misfolded mutant form of the membrane protein pma1-10 were still able to grow [19,38]. Upon further investigation into the properties of yvh1 that were responsible for suppressing this mutant pma1-10, it was found that HA-tagged YVH1 co-fractionated with the 60S subunit, and deletion of the yvh1 gene caused defects in 60S subunit biogenesis [19]. Interestingly, this phenotype was paralleled in yeast containing deletions of large (60S) subunit proteins RPL19 and RPL35. Ribosome profiling revealed decreased levels of free 60S and 80S ribosomal subunits and an increase in free 40S subunits as well as an accumulation of halfmer polysomes (43S particles which consist of the 40S subunit, and associated translation initiation factors stalled at the start codon) [14,15]. Complementation studies using a YVH1 truncated mutant, containing the zinc-binding domain alone, were able to rescue the yvh1 deletion phenotype [14,15,19]. This study was followed by two others in
which further investigation into the function of YVH1 in ribosome biogenesis not only revealed that ribosome profiles change in response to \textit{yvh1} deletion, but also used Northern blotting to detect an accumulation of early pre-60S rRNA (i.e. 27S rRNA) and a reduction in late 60S rRNA (i.e. 25S rRNA) in response to \textit{yvh1} deletion, further supporting its involvement in maturation of the 60S subunit. A reduction in 60S export was also observed in these studies [14,15,19]. Interestingly, the zinc-binding domain alone was found to be capable of restoring normal ribosome profiles, suggesting that the catalytic domain is not required for ribosome biogenesis. Through this observation, the slow growth phenotype described in \textit{yvh1} deletion yeast was attributed to the observed defect in ribosome biogenesis. Co-purification of YVH1 with late pre-60S particles using the TAP method further validates the implication of YVH1 in ribosome biogenesis [14].

The ribosome stalk is an important structure of the ribosome, which is required for the recruitment/association of several translation factors. P0 is a protein that forms the base of the stalk and associates with the 60S subunit only in the cytoplasm. A highly homologous protein, Mrt4, associates with the ribosome only localized in the nucleolus and nucleoplasm. Because the association of Mrt4 and P0 with the 60S subunit is mutually exclusive, it has been suggested that Mrt4 and P0 bind the same site on the 60S ribosome, hence the association of P0 with the 60S subunit requires that Mrt4 and the pre-60S subunit have dissociated from each other. However, upon deletion of \textit{yvh1}, GFP tagged Mrt4 is specifically mislocalized to the cytoplasm and co-fractionated with the 60S subunit on a sucrose gradient, suggesting its inability to dissociate from the 60S pre-subunit [14,15]. Further, this localization could be restored in the presence of the zinc-
binding domain of YVH1, but not the catalytic domain alone [14,15]. The human orthologue hYVH1 was able to complement the yvh1 deletion in yeast, and knockdown of hYVH1 in both HeLa and HEK 293 cells caused aberrant localization of human MRTO4, suggesting a similar role in humans [15]. The proposed role for YVH1 in yeast ribosome biogenesis is shown in Figure 1.7. Despite these observations, further investigation of the role of hYVH1 in ribosomal biogenesis in human cells is required with respect to evidence of 60S subunit association and characterization of the interaction between hYVH1 and the ribosome, and is a major objective of the proposed research.

1.6 Methods to Explore Structural/Functional Features of Protein Tyrosine Phosphatases

1.6.1 In Vitro Phosphatase Assays Exploit Artificial Substrates

The first discovered dual specificity phosphatase is VH1 from the vaccinia virus [9]. Its human orthologue, VHR, shares 30% sequence homology with the N-terminal catalytic domain of human phosphatase hYVH1. It was therefore estimated that VHR would serve as a good model for the catalytic activity of hYVH1. However, our lab has found that the in vitro phosphatase activity exhibited by GST-VHR fusion protein toward artificial substrates is 453 times greater than that exhibited by GST-hYVH1 fusion protein [40]. This observation evokes interest as to the structural features responsible for such a marked decrease in activity and to the functional significance of these discrepancies.
Figure 1.7: Proposed Role of YVH1 as a Trans-Acting Factor in Ribosome Biogenesis. In yeast, YVH1 is required for normal maturation of the 60S ribosomal subunit. A linear series of events is proposed, in which binding of YVH1 to the pre-60S particle allows for the removal of the trans-acting factor Mrt4. This is necessary for the association of the ribosomal stalk protein, P0, with the 60S subunit. [Figure from reference 15]
In vitro phosphatase assays are useful tools that can lend insight into the function or regulation of protein phosphatases, and assess the consequence of effector molecules and protein modifications on phosphatase activity [41]. Because of the challenges associated with phosphatase substrate identification, artificial substrates are often employed in these assays. There has been much emphasis placed on the design of artificial substrates, and a researcher may choose from an assortment of substrates to best suit the specific phosphatase. These substrates include peptides or single amino acids that can accurately mimic natural substrates. In such cases, hydrolysis of the phosphoester bond can be observed through the use of radioactive assays or non-radioactive assays in which released phosphates are detected either through the use of phospho-specific antibodies or phosphomolybdate colorimetry. Alternatively, spectrophotometric substrates can be used, which absorb light or fluoresce upon hydrolysis. These substrates are advantageous as their hydrolysis can be directly and immediately detected, and although they are not phosphorylated amino acids, their structures generally mimic that of a phosphorylated amino acid residue, facilitating recognition by the protein phosphatase.

Para-nitrophenyl phosphate (shown in Figure 1.8) is an artificial substrate that consists of a phenyl ring linked to a phosphate through a phosphoester bond, accurately mimicking a phosphotyrosine residue. Upon hydrolysis of the phosphor-ester bond, para-nitrophenyl absorbs light at a wavelength of 450 nm. The p-nitrophenyl product has a pKa of 7.2, and therefore produces a more intense signal at alkaline pH. If the phosphatase under study requires reaction conditions in a lower pH range, pNPP can be
used in a discontinuous assay: quenching the reaction with a base can allow for detection of para-nitrophenyl [41].

Another spectrophotometric substrate is 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP) (shown in Figure 1.8). Upon incubation with DiFMUP, the phosphatase under study catalyzes the hydrolysis of DiFMUP to its hydrolysis product 6,8-difluoro-4-methylumbelliferyl (DiFMU). The generation of the fluorogenic product DiFMU, allows for the rate of formation of DiFMU, and hence the rate of catalysis, to be monitored [42]. DiFMUP presents advantages over other spectrophotometric substrates as it has a high fluorescent quantum yield, which allows for greater sensitivity than can be achieved with alternative artificial substrates (such as OMFP and pNPP). Further, DiFMU has a lower pKa than do the hydrolysis products of other artificial substrates such as pNPP, making it a better candidate for continuous phosphatase reactions at a reduced pH [41,43].

1.6.2 X-ray Crystallography and Supporting Techniques to Elucidate Protein Structure

X-ray crystallography is a powerful technique that allows for the acquisition of a protein structure by obtaining resolution at the atomic level [44]. Proteins suitable for analysis by this method must be able to form highly ordered crystals. The formation of suitable crystals has been a major obstacle faced by researchers. Various techniques have been developed in an effort to promote crystallization of resistant proteins. Traditionally,
Figure 1.8: Hydrolysis of the Artificial Phosphatase Substrates DiFMUP and pNPP. The hydrolysis of the artificial phosphatase substrate pNPP yields a product that can be colorimetrically detected, allowing for phosphatase activity to be monitored. The hydrolysis of the artificial substrate DiFMUP by a protein phosphatase yields the fluorescent product DiFMU, allowing for the rate of DiFMU production, and hence phosphatase activity, to be monitored. The lower pKa of DiFMUP compared to other artificial substrates allows for this substrate to be used in continuous phosphatase assays when a lower reaction pH is required [41,43].
a screening process is established in which various buffers are tested in order to
determine optimal conditions for crystallization of the protein in question. The
conditions tested may include variations in types of buffer and pH, temperature and
presence of ligands (which often enhances protein stability) [45,46].

In addition to these screening methods, crystal engineering is becoming a popular
field of study. Crystal engineering involves the modification of proteins to increase
propensity to form crystal contacts. This may be done through the fusion of the proteins
of interest to a readily crystallized carrier protein or through construct optimization in
which flexible regions/loops are excluded from the protein. Another emerging technique
is surface entropy reduction (SER), which involves site-directed mutagenesis of certain
high entropic residues (such as arginine, glutamine, glutamic acid, lysine) to residues of
lower entropy (such as alanine). This is based on the fact that crystallization is driven by
very small changes in Gibb’s free energy and that reduction in the thermodynamic cost of
packing high entropy side chains into crystals increases propensity for crystallization
[47]. Therefore, it has been found that these regions of lower entropy are more suitable
to mediate crystal contacts [47,48]. Surface entropy reduction has been experimentally
validated as these mutations are often found to be involved in crystal interfaces [48].

Crystallographic analysis has proven to be important in determining the structural
features of PTPs that mediate kinetic properties of the phosphatase. In particular, through
the determination of the crystallographic structure, the reason for the low activity of some
phosphatases has been discovered by aligning the elucidated structure (of the active site)
of the low activity phosphatase (such as PrI1) with that of DSPs with a higher activity
(such as VHR), and identifying major differences [49]. In the case of the MKPs,
crystallography has proven to be important in studying the substrate-induced activation mechanism practiced by this class of phosphatases [50,51]. It is predicted that analysis of hYVH1 via crystallography could reveal fundamental information as to the structural relationship between the catalytic domain and the novel zinc-binding domain, as well as provide structural insights regarding the regulation of this enigmatic phosphatase and clues concerning substrate identification.

In addition to X-ray crystallography, there are other techniques available that can be used to obtain protein structure, or complement that observed through X-ray crystallography. One such technique is protein NMR. This technique has the added advantage of allowing for the determination of protein structure in solution, as crystal structures, while generally accurate, occasionally prove to be unfaithful to the protein’s native form. However, in the structure determination of large proteins, NMR data analysis can become extremely complex, and in such cases NMR is more effectively used for the structural determination of individual domains rather than full length proteins [52].

Yet another technique that allows for the observation of protein structure in solution is small angle x-ray scattering (SAXS). While this method reveals a very low resolution image of the protein structure (protein envelope), it does not require isotopic labelling and is often sufficient in the validation of protein structure as determined by X-ray crystallography [53,54]. SAXS is of particular use as this technique does not have molecular weight limitations as do other techniques, such as NMR, and may lend structural insight into regions that are too flexible to be resolved using X-ray crystallography [55,54]. Furthermore, SAXS allows for the determination of overall
three-dimensional protein shape and domain arrangement under a variety of conditions, ranging from near physiological to denaturing [55]. This allows for the exploration of structural changes in response to external conditions, affording the observation of global changes in protein folding from one state to another [56,57]. Additionally, SAXS can be used for the visualization of global conformational changes in the presence of effector molecules or ligands, as well as the study of oligomeric complexes in solution. [55,57,58]

In most cases, structural information obtained from SAXS and X-ray crystallography have been highly concordant [54]. However, while most crystal structures are validated against those structures determined by SAXS, there are several cases, particularly in the determination of quaternary structure of protein complexes, that revealed large discrepancies in the crystallographic models, highlighting the importance of verifying crystal structures with structures obtained from particles in solution [54].

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1.7 Objectives

This study focuses on elucidating novel structural features of the human dual specificity phosphatase hYVH1. Particular emphasis is placed on the relationship between the catalytic domain and novel zinc-binding domain as well as their relevance in the proposed function of hYVH1 as a trans-acting factor in ribosome biogenesis.

The specific aims are as follows:

1) Confirm the interaction of hYVH1 with the ribosomal subunits and, in the event that hYVH1 does interact with the ribosome, further characterize this interaction by:
   a. Investigating the domains or regions of hYVH1 required for this interaction;
   b. Evaluating the effects of phosphorylation and oxidation of hYVH1 on ribosomal binding.

2) Prepare samples for structural analysis of hYVH1 by X-ray crystallography as well as explore conditions in vitro that may enhance activity of hYVH1 toward artificial substrates with the common goal of elucidating information concerning modes of regulation, physiological function, and potential substrates of hYVH1.
CHAPTER 2

Materials and Methods

2.1 Plasmids

The synthesis of pGEX-4T1 vector containing wild type hYVH1 has been previously described [40]. This plasmid was harvested from a 5mL overnight culture as described by the manufacturer using GenElute Plasmid Miniprep Kit (Sigma-Aldrich, Inc.). To generate pGEX-4T1 ZnΔhYVH1, PCR-based site directed mutagenesis was performed on purified pGEX-4T1 plasmid containing the wild type hYVH1 insert. Oligonucleotide primers (synthesized by Invitrogen Corp.) were designed to carry out the mutation Y191Stop. The forward primer used is 5’-GGTTACAGAGAAGTAGCCA GAATTGC–3’, while the reverse primer is 5’-GCAATTCTGGCTACTTCTCTGTAA CC–3’. Following PCR, DpnI (Invitrogen Corp.) digestion was performed for 1hr at 37°C. Competent DH5a cells were then transformed with PCR products (that were subjected to DpnI digestion) as described below. Plasmids were harvested using GeneElute Plasmid Miniprep kit and plasmids verified by automated sequencing (ACGT Corp.).

The truncated hYVH1 derivative lacking the first 28 N-terminal amino acid residues, E29 hYVH1, and that lacking the first 45 N-terminal amino acid residues, D46 hYVH1, were obtained by PCR using wild type pGEX-4T1 hYVH1 as a template. The forward primer used to generate E29hYVH1 is: 5’-GCAGAATTCGAAGGTCAGCCAG GATTGTATTTCCG-3’, and the forward primer used to generate D46 hYVH1 is: 5’-GC
GAATTGCATCACCTGAGGGAAGCGGGC-3’, both of which contain an Eco-RI restriction site. The reverse primer used for both mutants was: 5’-CGATGCGCGCCGCTCTAGAAGTGAG-3’, which contains a NotI restriction site. PCR products were then subjected to DpnI digestion to degrade template DNA, verified on a 1% agarose gel, and purified using QIAquick® PCR Purification Kit (Qiagen, Inc.), according to manufacturer’s instructions. Subcloning of the purified inserts into the pGEX-4T1 vector was achieved using the restriction enzymes EcoRI and NotI, and the ligated plasmids transformed into highly competent DH5a cells. Plasmids were harvested using Sigma miniprep kit, and the sequence verified by automated sequencing (ACGT Corp.).

Surface entropy reduction (SER) mutants were predicted by submission of primary sequence of hYVH1 to http://nihserver.mbi.ucla.edu/SER/ and the top 5 clusters of mutations were generated by site directed mutagenesis, using pGEX 4T1 containing wild type hYVH1 as a template. SER1, which consists of the mutations of the glutamic acid residues at positions 62 and 63 to alanines, was generated using a single set of primers: sense 5’-CAGTGACTCGGCGGCGCCAGCTTC-3’, and antisense 5’-GAAGCTGGGCGCCGCCGAGTCCACTG-3’. All other SER mutants were generated through two (for SER3, SER4, SER5 variants) or three (for SER2 variant) separate rounds of site directed mutagenesis, each round employing a new set of primers to introduce a new mutation. The primers used to construct SER2 are as follows: SER2, round 1 (E151A): Sense, 5’-CCAGATTCTCAAACCAGCGGCTAAGATGAATGAG-3’ Antisense, 5’-CTCATTTCCTTACGGCGCTGTGTTTGAGAATCTGG-3’; SER2 round 2 (E151A, K152A) Sense, 5’-CTCAAAACCAGCGGCTGAGATGAATGAGG-3’, Antisense, 5’-CCCCTCATTTCATCGCAGCCGCTGGTTTGAAGT-3’, SER2 round
3(K149A, E151A, K152A) Sense, 5’-GCTCCAGATTCTCGACCCAGCCAGCCTGCG-3’, Antisense, 5’-CGACGCGCTGGTGAGAATCTGGGACGC-3’. The primers used to generate SER3 are as follows: SER3 round 1 (E189A) Sense, 5’-CAAAAGGTTACAGCAGATATTCCAGAATTGCGCAG-3’, Antisense 5’-CTGCAACGTCTGGATACTCGCCTGAACTTTTG-3’; SER3 round 2 (E189A, K190A) Sense, 5’-CAAAAGGTTACAGCAGATATTCCAGAATTGCGCAG-3’, Antisense, 5’-CTGCAACGTCTGGATACTCGCCTGAACTTTTG-3’. Primers used to generate SER4 are as follows: SER4 round 1 (E305A) Sense, 5’-CTGGTATTGTTGCACAGTGCACCTGTCTTTTGGTGGGATACCCGCTGTAACCCCTTGTGAG-3’, Antisense 5’-CACCTACCCACAAAGAGCAGCCTGTGGGACTCTTTTGGTGGATACCCGCTGTAACCCCTTGTGAG-3’; SER4 round 2 (E305A, Q306A) Sense, 5’-CAACTGGGATTGTTGCACAGTGCACCTGTCTTTTGGTGGGATACCCGCTGTAACCCCTTGTGAG-3’, Antisense, 5’-CCACCTACCCACAAAGAGCAGCCTGTGGGACTCTTTTGGTGGGATACCCGCTGTAACCCCTTGTGAG-3’.

Primers used to generate SER5 are as follows: SER5 round 1 (E200A) Sense, 5’-GCAGAATTTCCTCAAGGACTCTTTTGGTGGACCC-3’, Antisense 5’-GGGTCAACAGCAAAGAGTGCTTGAGGTAAATTCTGC-3’; SER5 round 2 (Q199A, E200A) Sense, 5’-GCAGAATTTCCTCAAGGACTCTTTTGGTGGACCC-3’, Antisense 5’-GGGTCAACAGCAAAGAGTGCTTGAGGTAAATTCTGC-3’.

Following each round of site directed mutagenesis, the PCR product was subjected to DpnI digestion, followed by transformation into highly competent DH5a cells. The generated plasmids were then harvested using GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich, Inc.), verified by agarose gel electrophoresis and automated sequencing (ACGT Corp.), and used as a template for the following round of site directed mutagenesis.
pCMV2-flag DThYVH1 was generated by site directed mutagenesis using the singly mutated pCMV2-flag D84AhYVH1 as a template. Sense and antisense primers were designed to flank glutamine at amino acid position 161 and mutate this residue to an alanine. Sense: 5’-GGGGTTTGAGTGGGCACTGAAATTATACC-3’, and Antisense: 5’-GGT ATAATTTCAAGTGCCCACTCAAACC-3’. PCR products were subjected to DpnI digestion and plasmids transformed, harvested, and verified as described above.

Construction of mammalian DNA plasmids used (pCMV2-flag hYVH1, pCMV2-flag C115 S, pCMV2-flag CT1 hYVH1, pCMV2-flag CT2 hYVH1, pCMV2-flag CT3 hYVH1, pCMV2-flag S14A hYVH1, pCMV2-flag S14E hYVH1, pCMV2-flag S335E hYVH1) have been previously described [24,40,60].

2.2 Cell Culture and Transfections

HEK 293 cells were obtained from American Type Tissue Culture Collection. HEK 293 cells were cultured in Dulbecco’s Modified Eagle’s Medium/Nutrient F-12 HAM supplemented with 8% fetal bovine serum, 2mM glutamine, 100U/mL penicillin and 100µg/mL streptomycin at 37°C, 5% CO2. Approximately 2x10^6 cells were seeded into 100mm cell culture plates 24 hours prior to transfections. Cells were grown to approximately 70-80% confluency and subsequently transfected using polyethyleneimine (PEI)-mediated transfection. Fresh media was replaced 4 hours prior to transfection. PEI transfection proceeded as follows: 50µL of a 150mM solution of NaCl was mixed and incubated with 5 to 20µg of DNA, while simultaneously 50µL of 150mM NaacCl was mixed and incubated with 15 to 30µL of a 1mg/mL solution of PEI (pH 7.4) for 10
minutes at room temperature. Following incubation, solutions containing DNA and PEI were mixed and incubated at room temperature for an additional 10 minutes. DNA/PEI transfection mixtures were then added dropwise to tissue culture plates. Plates were gently mixed, and subsequently incubated at 37°C, 5% CO₂ for 24 to 36 hours. For tert-butyl hydroperoxide treatments, cells were treated 24hrs after transfection with concentrations of 200µM, 500µM, or 2mM tert-butyl hydroperoxide and incubated for 4 hours at 37°C, 5% CO₂.

2.3 Ribosome Profiling

All solutions used for ribosome profiling were made using DEPC treated water. DEPC treated water was prepared by adding DEPC (Sigma-Aldrich, Inc.) to millipore water to a final concentration of 0.1% (vol/vol). The DEPC solution was mixed thoroughly, incubated at 37°C for at least 18hours and subsequently autoclaved. All sucrose solutions had a buffer composition of 80mM NaCl, 5mM MgCl₂, 20mM Tris-HCl pH 7.4, 1mM DTT. Sucrose gradients were made by carefully layering 2mL of a 5% sucrose buffer on top of 2mL of a 60% sucrose buffer in a Beckman Coulter Ultracentrifuge tube (Beckman Coulter, Inc.) and incubating the tube on its side for 2.5 to 3hrs at room temperature.

Lysates were prepared for fractionation according to a modified version of the protocol described by Idol et al. [26]. Approximately 1x10⁷ cells were washed in phosphate buffered saline and were incubated in trypsin for 1 minute to facilitate dislodging of cells from cell culture dish. Cells were suspended in 10mL of DMEM/F12
and collected by centrifugation at 1100xg for 7.5min at 4°C. Following centrifugation, cell pellets were gently resuspended in ice cold PBS containing 100µg/mL cycloheximide and incubated for 10min on ice. Following a second centrifugation step, cell pellets were resuspended in 100µL cold hypotonic buffer (1.5mM KCl, 2.5mM MgCl₂, 5mM Tris-HCl pH 7.4) supplemented with 4µL RNaseOUT™ Ribonuclease Inhibitor (Invitrogen Corp.) before adding 100µL cold hypotonic lysis buffer (1.5mM KCl, 2.5mM MgCl₂, 5mM Tris-HCl pH 7.4, 2% sodium deoxycholate, 2% Triton X-100, 2.5mM DTT). Cells were lysed by 40 strokes in a pre-chilled Dounce homogenizer (Kontes) and centrifuged at 8000xg for 10min at 4°C to remove cellular debris. Total protein concentration was determined by the Bradford method, and lysates were supplemented with 1.7mg/mL heparin (Sigma-Aldrich, Inc.). Lysate volumes corresponding to equal amounts of total protein (approximately 1.5mg) were loaded onto the sucrose gradient and fractionated by ultracentrifugation (Beckman Coulter Optima MaxE, Beckman Coulter, Inc.) at 245 000xg for 3hours at 4°C on a swinging bucket rotor (MLS-50).

Following centrifugation, a 50% sucrose cushion was carefully layered on top of the gradient and an 80% sucrose cushion was injected through the base of the polyallomer centrifuge tube. The tube was capped and plumbed in line with a UV detector with filter set to 254nm. A 60% sucrose chase solution was then continuously pumped into the centrifuge tube at a flow rate of 1mL/min using a BioLogic LP Chromatography System (Bio-Rad Laboratories). Fractions corresponding to cytoplasmic RNA, intermediate fractions, 40S, 60S, 80S and polysomes were collected manually.
2.4 Immunoprecipitation

Approximately 2x10⁷ cells were washed in ice cold PBS and lysed as described in Section 2.3. Anti-FLAG M2-Affinity Gel (Sigma-Aldrich, Inc.) was equilibrated using a 50/50 solution of cold hypotonic buffer and hypotonic lysis buffer (see previous Section 2.3). Lysates were loaded onto agarose and gently mixed for 3hrs at 4°C. Following incubation, tubes were centrifuged at 2500xg for 1min and the flow through aspirated to waste. Resins were washed three times in IP wash buffer (150mM NaCl, 50mM Tris-HCl pH 7.4, 0.1% Triton, 0.1% SDS). Following washes, resin was resuspended in 2xSDS loading dye (0.12M Tris-HCl pH 6.8, 4% SDS, 0.3% β-mercaptoethanol, 10% glycerol, 0.004% bromophenol blue, 3% DTT) and boiled for 5 minutes. Boiled resins were vortexed and centrifuged at 2500xg for 2 minutes and stored at -20°C until analysis by SDS PAGE.

2.5 Immunoblotting

Lysate and ribosomal profiling fractions were denatured in 6XSDS loading dye (0.350M Tris-HCl pH 6.8, 10% SDS, 0.3% β-mercaptoethanol, 30% glycerol, 0.12% bromophenol blue, 3% DTT) and boiled for 5 minutes. Lysates, immunoprecipitations, or fractions from ribosomal profiling were resolved via SDS PAGE and transferred to a polyvinylidene difluoride membrane (Millipore Corp.). Membranes were blocked in Tris-buffered saline, Tween-20 (TBST; 0.068M NaCl, 8.3mM Tris-base, 0.1% Tween-20 pH 7.6) containing 5% milk for 1 hour at room temperature (in the case of anti-hYVH1 blots) or overnight at 4°C (for anti-flag and anti-RPL26 blots). hYVH1 was detected
using a polyclonal hYVH1 antibody raised in rabbit at 1:3000 in 2.5% milk in TBST buffer, overnight at 4°C. Secondary antibody for hYVH1 blots was goat anti-rabbit IgG HRP conjugate (Bio-Rad Laboratories), used at 1:5000 in 2.5% milk in TBST. For anti-flag blots (Sigma-Aldrich, Inc.), primary antibody was used at a dilution of 1:5000 in 2.5% milk TBST for 1 hour at room temperature, and anti-mouse secondary antibody (Promega Corp.) was used at a dilution of 1:5000. Following visualization of bands by chemiluminescence using SuperSignal® West Femto Maximum Sensitivity Substrate (Pierce Biotechnology), blots were washed thoroughly in TBST and a second blocking stage of 30 minutes performed. Blots were then incubated in anti-RPL26 antibody raised in rabbit at a dilution of 1:2000 in 5% milk in TBST, followed by washes, and incubation in a 1:2000 dilution of goat-anti rabbit in 2.5% milk in TBST. Bands were again visualized by chemiluminescence. All secondary antibodies were incubated in 2.5% milk in TBST for 45 minutes. All blots were washed 3 times for 5 minutes each in TBST following incubation with primary and secondary antibodies, with the exception of anti-L26 blots, which required three 10 minute washes following incubation in anti-RPL26 primary antibody.

2.6 Protein Expression and Purification

Proteins intended for X-ray crystallography, activity assays and limited proteolysis were purified in the same manner, with the exception that glycerol was excluded from all buffers when purifying protein for use in limited proteolysis experiments. Highly competent BLR cells were transformed with a pGEX-4T1 vector
containing either wild type, E29Start, or SER mutant hYVH1, plated on ampicillin-agar plates (0.1mg/mL) and incubated overnight at 37°C. A 5mL solution of Luria Broth containing 0.1mg/mL ampicillin was inoculated with a single bacterial colony and incubated at 37°C overnight with 250rpm shake. This entire 5mL culture was then used to inoculate 500mL of 2xYT media supplemented with 1.0% glycerol and 0.1mg/mL ampicillin. The large culture was incubated at 37°C with 250rpm shake for approximately 4 hours, until reaching an optical density of 1.0 to 1.5 at a wavelength of 600nm.

Upon reaching the desired OD, the large scale culture was supplemented with an additional 1% glycerol. Expression of the recombinant GST fusion protein was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.8mM and shaken at 250rpm at room temperature for 16 hours. Following incubation, cultures were pelleted by centrifugation at 4500xg for 25 minutes at 4°C. The supernatant was discarded and the pellets were stored at -20°C until protein purification. Successful induction was verified by SDS PAGE and visualized by Coomassie stain.

Induced pellets were thawed on ice and resuspended in 20mL of lysis buffer (150mM NaCl, 10mM Tris, 10% glycerol, 1% Triton x-100, 1mM PMSF, 0.1% β-mercaptoethanol, pH 7.3). The resuspended cells were lysed at 4°C by french press (2 passes at approximately 1.5mL/min). Lysates were centrifuged at 14000xg for 20min at 4°C to pellet cell debris.

Lyophilized Glutathione-Agarose (Sigma-Aldrich, Inc.) was swollen and washed according to manufacturer’s protocol. Prior to addition of cellular lysates, 2mL of
glutathione agarose was equilibrated with 10mL of binding buffer (150mM NaCl, 10mM Tris-base, 10% glycerol, pH 7.3). The equilibrated resin was centrifuged on a swinging bucket rotor at 1600xg for 1.5 minutes at 4°C, and the buffer solution discarded. The cellular lysate (supernatant) was incubated with 2mL of glutathione-agarose for 2 hours at 4°C on a nutator.

Following incubation, the resin-protein mix was centrifuged in a swing bucket rotor at 1600xg for 1.5 minutes at 4°C. The supernatant (flow through) was aspirated into waste. In order to remove non-specific proteins, the protein-bound resin was washed three to five times by the addition of buffer (150mM NaCl, 10mM tris-base, 10% glycerol, pH 7.3) followed by a 10 minute incubation at 4°C on a nutator, and centrifugation at 1600xg for 1.5 minutes at 4°C. The supernatant was aspirated into waste. Aliquots of flow through, washes, and bound resin (2µL of bound resin diluted with 8µL binding buffer) were collected for SDS PAGE.

Lyophilized thrombin (Promega Corp.) was resuspended in phosphate buffered saline to a final concentration of 1U/µL and stored at -80°C. GST-hYVH1 bound to the GSH-agarose was cleaved by the addition of 2mL of thrombin cleavage buffer (50mM Tris-HCL, 150mM NaCl, 2.5mM CaCl₂, 10% glycerol, pH 8) containing 80U of thrombin to 2mL of protein-bound agarose. The mixture was incubated for 4 to 5 hours at 4°C with gentle agitation. Following this cleavage step, the mixture was centrifuged at 1600xg for 1.5 minutes and the supernatant, containing the purified protein, was supplemented with 1mM PMSF and 1mM DTT, and stored at -80°C (contains purified protein). A final wash was performed in which 2mL of ice cold thrombin cleavage buffer was added to the resin and incubated with gentle agitation for 10 minutes. Again, the
mixture was centrifuged at 1600xg for 1.5 minutes at 4°C, the supernatant supplemented with 1mM DTT and 1mM PMSF and stored at –80°C (recovery). Aliquots of the purified protein, recovery sample, and cleaved resin were collected for SDS PAGE. Following protein purification, the GSH-agarose resin was regenerated and stored as described by manufacturer. Protein concentrations were determined by the Bradford assay using BSA as a standard. All steps were visualized using SDS PAGE and Coomassie stain.

Proteins intended for analysis by X-ray crystallography or small angle X-ray scattering were subjected to buffer exchange into a solution containing 20mM Tris pH8, 150mM NaCl, 1mM DTT, 10% glycerol, using Millipore Ultra Centrifugal Filter Devices (maximum 15mL sample volume) as described by manufacturer at a temperature of 4°C. Samples were concentrated until reaching desired protein concentration as determined using the Bradford method.

2.7 DiFMUP Assays

For each DiFMUP assay performed, a calibration curve was constructed using 100µL solutions of DiFMUP buffer (150mM NaCl, 50mM Tris, 50mM Bis-Tris, 5mM DTT, pH 7) and DiFMU at final concentrations of 0, 1, 2.5, 5, 10, 20, and 40µM. In the case of experiments containing glycerol or DMSO, calibration curves were constructed using 0%, 15%, and 30% glycerol or DMSO in the buffer described above. Buffer solutions were incubated at 30°C for 10 minutes prior to addition of DiFMU. DiFMU was then added, and the fluorescence of the standard solutions was measured on a fluorometer (Molecular Devices, SpectraMax Gemini XPS) using excitation/emission wavelengths of 358nm/450nm.
In the analysis of kinetic parameters, hydrolysis of DiFMUP by hYVH1 and ZnΔhYVH1 was carried out in a 100µL reaction volume containing 50mM Tris pH 7, 50mM Bis-Tris, 150mM NaCl, 5mM DTT, and the indicated amount of glycerol or DMSO with an enzyme concentration of 0.6µM. Blanks were also prepared which contained no enzyme. The reaction mixture was incubated at 30°C with gentle agitation for 10min. Serial amounts of DiFMUP were then added to the reaction mixtures to final DiFMUP concentrations of 25, 50, 150, 300, 400, 500, 600, 900, 1200, and 2400µM. The samples were mixed well and loaded onto a Nunc® FluoroNunc 96 well plate. Changes in relative fluorescence units over time (using ex/em wavelengths of 358/450) were monitored for 1 hour. Initial velocities were obtained using the standard curve to convert slope values from RFU/time to µM DiFMU produced/time, and plotted against substrate concentrations to obtain kinetic parameters.

DiFMUP assays verifying activity of proteins purified for analysis by X-ray crystallography were performed in a similar manner to that described above, with the exception that only a single substrate concentration of 900µM DiFMUP was used.

2.8 pNPP Assays

Reaction buffers used in pNPP activity assays are as described for DiFMUP assays, with specified concentration of DMSO. Reaction mixtures were prepared in a 96-well clear-bottom plate (Sarstedt, Inc.). In a final reaction volume of 110µL, 1µM enzyme was incubated with 20mM of pNPP at 30°C for 30 minutes, 70 minutes, or 130 minutes. The reaction was subsequently quenched with 50µL of 1M NaOH and
absorbance at 405 nm read. Blanks lacking pNPP or hYVH1, or both were also read for each reaction condition. To correct for spontaneous hydrolysis of pNPP under specific reaction conditions, absorbance of blanks containing pNPP were subtracted from absorbance of reaction mixtures containing both pNPP and hYVH1 to yield final absorbance values plotted.

2.9 Limited Proteolysis

Bacterially purified full-length hYVH1 (containing no glycerol) was subjected to proteolysis under non-denaturing conditions (2.5mM CaCl₂, 150mM NaCl, 50mM Tris-HCl, pH 8 in Burdick and Jackson, High Purity Water), using ratios of proteinase K: hYVH1 of 1:1, 1:100 and 1:1000 for 15 minutes at 37°C with gentle agitation. The resulting solution containing proteolytic fragments was spotted (1µL) on a MALDI sample plate followed by 1µL of a freshly prepared solution of a UV absorbing matrix (1% α-cyano-4-hydroxycinnamic acid (Sigma), 60% acetonitrile (HPLC grade, Burdick and Jackson), 0.01% formic acid). Peptide fragments were analyzed by MALDI TOF mass spectrometry in linear mode using Voyager DE Pro Biospectrometry Workstation (Applied BioSystems). Spectra were annotated manually. Protein fragments obtained by limited proteolysis were resolved by SDS PAGE and visualized by Coomassie stain.
CHAPTER 3

Results

PART I) Characterization of the hYVH1:Ribosome Interaction

3.1 Human YVH1 Interacts With the 60S Ribosomal Subunit

Recently, yeast YVH1 has been identified as a novel trans-acting factor involved in the late stage maturation of the 60S ribosomal subunit [14,15]. Since human YVH1 shares approximately 30% sequence identity, we were interested in determining if the human orthologue can also interact with the 60S ribosomal subunit. In order to verify the interaction between hYVH1 and ribosomal complexes, HEK 293 cells expressing endogenous hYVH1 were lysed using a dounce homogenizer. Ribosomal subunits were sedimented on a 10% - 50% sucrose gradient by ultracentrifugation. Following ultracentrifugation, gradients containing sedimented particles were displaced by injection of a 60% sucrose chase solution through an 80% sucrose cushion (Figure 3.1). RNA absorbance at 254nm was monitored, revealing peaks corresponding to small RNA species, 40S, 60S, and 80S ribosomal subunits, as well as polysomes and halfmers. As shown by the dotted lines in Figure 3.2A, fractions were collected by peak. Immunoblot analysis was then performed on lysates and fractions to determine the presence of hYVH1. The presence of the 60S ribosomal subunit protein RPL26 was used as a fractionation control. As shown in Figure 3.2B, endogenous hYVH1 (37kDa) co-fractionates with the 60S and 80S subunits (Figure 3.2B, upper panel). These fractions were confirmed by the presence of the large ribosomal subunit protein RPL26 (17kDa) in
Figure 3.1: Schematic of Experimental Set-up for Ribosome Profiling. Following ultracentrifugation, sedimented lysates were displaced via injection of a 60% sucrose chase solution through an 80% sucrose cushion using a BioRad Biologic LP Chromatography System. Absorbance was monitored at a wavelength of 254nm to allow for detection of RNA-containing fractions.
Figure 3.2: Fractionation of Endogenous hYVH1 with Respect to Ribosomal Subunits. HEK 293 cells were lysed and sedimented on a 10-50% sucrose gradient. (A) Monitoring absorbance at 254nm yielded peaks pertaining to small RNA species (fraction 1), rRNA complexed in the 40S, 60S and 80S subunits (fractions 4, 5 and 6, respectively) and polysomes and/or halfmers (fraction 7). (B) Lysates and fractions were resolved by SDS PAGE and immunoblotted for hYVH1 and RPL26. Fraction boundaries indicated by the dotted lines in (A) are representative of the fraction boundaries in all ribosome profiling experiments, unless otherwise stated.
lanes 5 and 6 (Figure 3.2B, lower panel), corresponding to the 60S and 80S ribosomal complexes, respectively.

In order to characterize the structural features mediating the interaction between hYVH1 and ribosomal subunits, it was important to determine if ectopically expressed hYVH1 can also associate with the 60S ribosomal subunit. HEK 293 cells were thus transfected with N-terminally flag-tagged hYVH1 and ribosomal profiling was performed. As shown in Figure 3.3A, no appreciable change occurs in the ribosome profiles in the presence of overexpressed flag-hYVH1. Furthermore, flag-hYVH1 displays a similar pattern of co-fractionation as endogenous hYVH1 with respect to the 60S and 80S subunits (Figure 3.3B).

In order to determine whether the detection of flag-hYVH1 in the 80S fraction was due to an association with the 80S complex, or due to low resolution between the 60S and 80S peaks, the experiment described above was conducted collecting fractions of small and equal intervals (approximately 50µL). This analysis revealed that RPL26, a 60S ribosomal protein that remains associated with the 80S ribosomal subunit, persists throughout the 60S and 80S fractions (Figure 3.4, lower panel), whereas flag-hYVH1 fractionation is most intense at the 60S peak, with diminishing intensity as the peak tails off (Figure 3.4, upper panel). This supports the notion that hYVH1 interacts with the 60S complex (or pre-complex species) exclusively, and not the 80S particle.

While both endogenous and overexpressed hYVH1 co-fractionate with the 60S subunit, as assessed by sucrose gradient density fractionation, we wanted to support these findings using a complimentary technique. Therefore, a co-immunoprecipitation
Figure 3.3: Fractionation of Overexpressed hYVH1 with Respect to Ribosomal Subunits. HEK 293 cells were transfected with flag-hYVH1 and cellular lysates fractionated on a 10-50% sucrose gradient by ultracentrifugation. (A) Absorbance at 254nm was monitored allowing for the visualization of ribosomal subunit peaks. Fractions were numbered and collected as depicted by the dotted lines. (B) Lysates and fractions were resolved by SDS PAGE and immunoblotted with anti-flag and anti-RPL26. Both western blot panels shown were visualized on a single PVDF membrane.
Figure 3.4: Focus on Co-fractionation of Overexpressed hYVH1 with 60S or 80S Ribosomal Subunits. Lysates from HEK 293 cells transfected with flag-hYVH1 were fractionated on a sucrose gradient. Absorbance at 254nm allowed for the detection of ribosomal subunit peaks. Fractions of only 2 drops (approximately 50µL) were collected in the 60S and 80S peak ranges. Fractions were resolved by SDS PAGE and immunoblotted with anti-flag and anti-RPL26. Both panels shown in were visualized on a single PVDF membrane.
experiment was performed in which untransfected HEK 293 cells or cells expressing flag-hYVH1 were subjected to immunoprecipitation using anti-flag agarose (Figure 3.5). Immunoblot analysis shows flag-hYVH1 in the lysates of transfected cells, but not of the untransfected, negative control lysates (Figure 3.5, upper panel). Furthermore, while equal levels of RPL26 are observed in the lysates of both untransfected and transfected lysates, only the flag-immunoprecipitation of cells expressing flag-hYVH1 shows detectable levels of RPL26 (Figure 3.5, lower panel). Collectively, these studies strongly support the conclusion that hYVH1 can interact with the 60S ribosomal subunit.

3.2 Effect of Substrate Trap Mutants on Co-fractionation of hYVH1 With the Ribosome

In order to assess the influence of the phosphatase activity of hYVH1 on the association of hYVH1 and ribosomal subunits, the catalytically inactive flag-C115S hYVH1 was expressed in HEK 293 cells. Cell lysates were fractionated by ultracentrifugation and ribosomal profiling was performed. Lysates and fractions were subjected to western blot analysis. As shown in Figure 3.6, flag-C115S hYVH1 (Figure 3.6B, upper panel) exhibits similar fractionation with respect to ribosomal particles (Figure 3.6B, lower panel), compared to wild type flag-hYVH1 (Figure 3.6A).

Further active site mutations can be performed on catalytically significant residues of protein tyrosine phosphatases to generate substrate traps. These mutant substrate trap proteins are able to form a complex with their respective substrates, but are incapable of executing the complete hydrolysis mechanism, thereby forming a relatively stable enzyme-substrate complex [61]. One of the most sophisticated traps (termed the
**Figure 3.5: Co-Immunoprecipitation of Overexpressed hYVH1 and RPL26.** HEK 293 cells were untransfected or transfected with flag-hYVH1. Lysates were immunoprecipitated using anti-flag agarose. Lysates and immunoprecipitates were resolved by SDS PAGE and immunoblotted using anti-flag or anti-RPL26 antibodies.
double trap, DT) involves two mutations: the invariant aspartic acid which acts as the catalytic acid/base in the dephosphorylation mechanism is mutated to an alanine residue, and the glutamine which is responsible for proper positioning of a water molecule required for hydrolysis, is also mutated to an alanine. This double trap is capable of performing the nucleophilic attack on the substrate molecule, but is the unable to hydrolyze the phosphatase-substrate covalent bond.

To determine if the double trap affects co-sedimentation of hYVH1 with the ribosomal subunit, flag-DT hYVH1 was expressed in HEK 293 cells and ribosome profiling performed (Figure 3.6C). While the anti-flag western blot shows a potential increase in levels of flag-DThYVH1 in the 80S fraction compared to that of wild type flag-hYVH1, small fraction collection in the region of the 60S and 80S subunit peaks reveals no significant difference between the fractionation patterns of wild type and flag-DT hYVH1 (Figure 3.6D). To further support the suggestion that the trap does not form a stable complex with a substrate molecule tethered to the ribosomal subunits, a co-immunoprecipitation was performed in which untransfected cells or cells transfected with flag-hYVH1 or flag-DT hYVH1 were immunoprecipitated using anti-flag agarose. Lysates and immunoprecipitates were analyzed by western blot (Figure 3.6E). While similar levels of flag-hYVH1 and flag-DT hYVH1 were seen in the immunoprecipitates (Figure 3.6E, upper panel), no increase in the amount of co-precipitated RPL26 was observed (Figure 3.6E, lower panel). These results indicate that phosphatase activity is not a pre-requisite for association with the 60S ribosomal subunit.
Figure 3.6: Fractionation of Catalytically Significant Point Mutants. (A,B,C) HEK 293 cells were transfected with wild type flag-hYVH1, flag-C115S hYCH1 or flag-DT hYVH1. Cell lysates were fractionated on a sucrose gradient and ribosomal profiling performed. Lysates and fractions were resolved by SDS PAGE and immunoblotted using anti-flag and anti-RPL26 on a single membrane, allowing for the visualization of bands corresponding to flag-hYVH1 (A), flag-C/S hYVH1 (B), flag-DT hYVH1 (C) and RPL26 (A, B, C). (D) HEK 293 cells were transfected with flag-DT hYVH1, and ribosome profiling performed collecting peaks of only two drops (50µL) to better visualize the fractionation patterns of flag-DT hYVH1 and RPL26 in the 60S and 80S regions. Fractions were immunoblotted with using anti-flag and anti-RPL26 antibodies. (E) HEK 293 cells overexpressing wild type or flag-DT hYVH1 were incubated with anti-flag agarose. Lysates and immunoprecipitates were probed using anti-flag and anti-RPL26 antibodies.
3.3 Effect of Phosphomimetic Mutants on Fractionation of hYVH1

Recently, phosphorylation of hYVH1 has been shown to play a role in its subcellular localization as well as augmenting hYVH1-mediated changes in cell cycle progression [24]. In order to determine the effect of the phosphorylation state of hYVH1 on its association with the ribosomal subunit, phosphomimetic and non-phosphorylation mutants were overexpressed in HEK 293 cells and their sedimentation with respect to ribosomal subunits observed. Both flag-S14E hYVH1 and flag-S14A hYVH1 (the phosphomimetic and non phosphomimetic forms of serine 14, respectively) displayed similar fractionation patterns to each other as well as to wild type hYVH1 (Figure 3.7A, 3.7B). Similarly, flag-S335E hYVH1, which mimics phosphorylation at serine 335, sedimented in fractions 5 and 6, corresponding to peaks from 60S and 80S subunits, respectively (Figure 3.7C). These results suggest that phosphorylation of hYVH1 does not affect its association with the 60S ribosomal subunit.

Calyculin A, a serine/threonine phosphatase inhibitor, is often used to increase the stoichiometry of cellular protein phosphorylation levels [62]. Further, this treatment has been used to enhance phosphorylation levels of hYVH1 in cell culture [24]. To further investigate the effect of phosphorylation on the fractionation pattern of hYVH1, HEK 293 cells expressing flag-hYVH1 were treated with Calyculin A and ribosome profiling was subsequently performed. Again, no change in the fractionation pattern of flag-hYVH1 from calyculin A treated cells was observed compared to untreated HEK 293 cells (Figure 3.7A, 3.7D), supporting the independence of the hYVH1:60S subunit interaction from the phosphorylation state of hYVH1.
Figure 3.7: Fractionation of Phosphomimetic hYVH1 mutants. HEK 293 cells were transfected with wild type flag-hYVH1 (A, D), flag-S14EhYVH1 (B, upper two panels), flag-S14AhYVH1 (B, lower two panels), or flag-S335E hYVH1 (C). Cells in (D) were treated with Calyculin A, while all other cells were left untreated. Lysates were fractionated on a sucrose gradient and ribosome profiling performed. Lysates and fractions were resolved by SDS PAGE and immunoblotted using anti-flag and anti-RPL26 antibodies. Each set of anti-flag and anti-RPL26 blots were probed and visualized on a single PVDF membrane.
3.4 Effect of tert-Butyl Hydroperoxide on hYVH1: Ribosome Interaction

Studies *in vitro* suggest that hYVH1 undergoes dynamic conformational changes in its zinc-binding domain in response to oxidative stress. This conformational change is accompanied by intramolecular disulfide formation and ejection of zinc from the zinc-binding domain of hYVH1 [23]. Tert-butyl hydroperoxide (TBH) is an oxidant that has been shown to elicit mass zinc ejection throughout the cell [63]. In order to assess the effect of oxidative stress (and possibly zinc ejection) on ribosomal binding of hYVH1, HEK 293 cells overexpressing flag-hYVH1 were treated with 0, 200µM, 500µM, and 2mM TBH for 4 hours and subsequently lysed for analysis by ribosome profiling. As shown in the profiles in Figure 3.8, levels of 40S, 60S, and 80S subunits undergo only mild changes with increasing TBH concentrations. Fractions were collected and probed for flag-hYVH1 and the 60S ribosomal protein RPL26 (Figure 3.9). Interestingly, ribosomal binding of flag-hYVH1 was significantly diminished in fractions 5 and 6 with increasing concentrations of TBH. Only a slight decrease in the 200µM sample was detected while the majority of hYVH1 was no longer detected co-sedimenting with the 60S fraction at 500µM and 2mM concentrations of TBH. It is important to note that levels of the internal control, RPL26, in these fractions remain constant or even increase. This effect is further demonstrated in Figure 3.10B, in which equal volumes of fractions 5 and 6 (corresponding to 60S and 80S peaks, respectively) were loaded onto a single gel and immunoblot analysis performed. Intriguingly, immunoblot analysis of lysates from 500µM and 2mM TBH treated samples (Figure 3.10A) shows an approximately 25kDa N-terminal flag-hYVH1 fragment in the anti-flag immunoblot. The absence of this fragment band in the untransfected control samples supports this
Figure 3.8: Ribosomal Profiles in Response to tert-Butyl Hydroperoxide Treatment. Cells transfected with wild type flag-hYVH1 were treated with indicated concentration of tert-butyl hydroperoxide for 4 hours. Cells were then lysed and lysates fractionated on a 10-50% sucrose gradient by ultracentrifugation. Sedimented samples were displaced using a 60% sucrose chase solution and absorbance at 254nm monitored.
Figure 3.9: Co-fractionation of Overexpressed hYVH1 in response to TBH Treatment. Fractions from experiment described in Figure 3.8 were resolved by SDS PAGE and immunoblotted using anti-flag and anti-RPL26 antibodies.
Figure 3.10: Fractionation of Overexpressed hYVH1 in Response to TBH Treatment. (A) HEK 293 cells were left untransfected or were transfected with flag-hYVH1. 24 hours after transfection, cells were treated with indicated concentration of TBH for 4 hours. Cell lysates were resolved by SDS PAGE and immunoblotted for flag-hYVH1 and RPL26 on a single PVDF membrane. (B) HEK 293 cells transfected with flag-hYVH1, and treated with TBH 24 hours after transfection, were lysed and lysates subjected to ribosomal profiling. Only fractions corresponding to the 60S and 80S peaks were resolved by SDS PAGE and immunoblotted using anti-flag and anti-RPL26 antibodies.
band being a flag-hYVH1 cleavage product suggesting that the mechanism of oxidative stress induced ribosomal dissociation may involve hYVH1 cleavage.

3.5 Effect of tert-Butyl Hydroperoxide on C115S hYVH1: Ribosome Interaction

Our laboratory has previously shown that the active site Cys115 of hYVH1 is required for its cytoprotective function in response to oxidative stress. Therefore, we examined the effect of TBH on the co-fractionation of the catalytically inactive C115S hYVH1 variant with the 60S subunit. HEK 293 cells expressing flag-C115S hYVH1 were treated with 0, 200µM, 500µM, and 2mM TBH for 4 hours. Immediately following treatments, cells were lysed and ribosome profiling was performed to observe the fractionation pattern of the catalytically dead C115S hYVH1 with respect to ribosomal subunits. Similar to flag-hYVH1, flag-C115S hYVH1 levels decrease in the ribosomal fractions with increasing concentrations of TBH, while RPL26 levels remain relatively consistent (Figure 3.11A, B). A cleavage fragment also is detected in the lysates in the 500µM and 2mM TBH treated samples (Figure 3.11A). These results suggest that phosphatase activity is not required for stress mediated inhibition of hYVH1 ribosomal targeting.
Figure 3.11: Fractionation of Catalytically Inactive C115S hYVH1 in Response to TBH Treatment. HEK 293 cells were transfected with the catalytically inactive flag-C115S hYVH1. 24 hours after transfection, cells were treated with 0, 200µM, 500µM or 2mM TBH for 4 hours. Lysates were fractionated on a sucrose gradient and ribosome profiling performed. (A) Lysates and fractions were resolved by SDS PAGE and immunoblotted using anti-flag and anti-RPL26 antibodies. (B) Fractions from each treatment corresponding to 60S and 80S peaks were run on a single gel and immunoblotted using anti-flag and anti-RPL26 antibodies.
3.6 Investigation of Regions Required for Interaction of hYVH1 with Ribosomal Subunits

To elucidate specific regions that facilitate interaction of hYVH1 with the ribosome, the N-terminal phosphatase domain (flag-CT1 hYVH1) and C-terminal zinc-binding domain (flag-Zn hYVH1) were expressed separately in HEK 293 cells. Cells overexpressing domain-deletion mutants were lysed and ribosome profiling was performed. Immunoblot analysis of lysates and fractions reveals complete loss of co-fractionation of flag-CT1 hYVH1 with the ribosomal subunits (Figure 3.13A) and partial loss of co-fractionation of the zinc-binding domain (flag-Zn hYVH1) with the 60S ribosomal subunit (Figure 3.13B), suggesting that both domains, or regions of both domains, are required for optimal ribosomal interaction.

To further investigate regions required for this interaction, mutants consisting of the catalytic domain and portions of the zinc-binding domain were expressed in HEK 293 cells and ribosome profiling was performed (see Figure 3.12 for schematic of domain deletion constructs). Similar to flag-CT1 hYVH1, flag-CT2 hYVH1 displayed no co-fractionation with the ribosomal particles, fractionating at lower sedimentation layers than the ribosomal subunits (Figure 3.14A). Interestingly, flag-CT3 hYVH1, displayed an entirely unique pattern of sedimentation, fractionating predominantly in the 80S and polysome/halfmer fractions (Figure 3.14B). However, preliminary experiments show no appreciable levels of RPL26 in anti-flag immunoprecipitation of cells overexpressing flag-CT3 hYVH1 (Supplementary Figure 4). These results suggest that the second set of zinc-coordinating residues is necessary for efficient ribosome binding.
Figure 3.12: hYVH1 Domain Deletion Constructs. Human YVH1 consists of two major domains, the catalytic domain, shown in white, and the zinc-binding domain, depicted in various shades of grey. N-terminal to the catalytic domain is a short sequence that displays limited conservation between YVH1 orthologues. The zinc-binding domain includes a short, hydrophobic region which falls between the zinc-coordinating residues and is highly conserved between YVH1 orthologues.
Figure 3.13: Effect of Domain Deletion on Co-fractionation of hYVH1 with Ribosomal Subunits. HEK 293 cells were transfected with flag-CT1 hYVH1 (A), or flag-ZnhYVH1 (B). Lysates were subjected to ribosomal profiling. Lysates and fractions were resolved by SDS PAGE and immunoblotted using anti-flag and anti-RPL26 antibodies on a single membrane.
Figure 3.14: Effect of Partial Domain Deletion on Co-fractionation of hYVH1 with Ribosomal Subunits. HEK 293 cells were transfected with either flag-CT2 hYVH1 (A), or flag-CT3 hYVH1 (B). Cells were lysed and lysates sedimented through a 10-50% sucrose gradient. Fractionated lysates were displaced using a 60% sucrose chase solution and absorbance at a wavelength of 254nm monitored, mediating the collection of fractions. Lysates and fractions were resolved by SDS PAGE and immunoblotted using anti-flag and anti-RPL26 antibodies on a single PVDF membrane.
PART II) *In Vitro* Structure Function Analysis of hYVH1

### 3.7 Effect of Zinc-binding Domain on Activity of hYVH1 Toward DiFMUP

It has been previously observed that hYVH1 exhibits a significantly lower phosphatase activity toward artificial substrates compared to its yeast orthologue [17,40]. Furthermore, Vacratsis lab members have shown that VHR, a DSP that shares 30% sequence homology with the catalytic domain of hYVH1, but possesses no additional domains, has a phosphatase activity 453 times greater than that of hYVH1 toward artificial substrates [40].

In order to investigate the effect of the zinc-binding domain on the *in vitro* phosphatase activity of hYVH1, full length recombinant hYVH1 was purified and a mutant construct of hYVH1 lacking the zinc-binding domain (ZnΔhYVH1), was designed and purified from BLR *E. coli* cells (Figure 3.15). A thick protein band at 65kDa and a band at 48kDa in the induced GST-hYVH1 and GST-ZnΔhYVH1 lanes, respectively, indicate successful induction. The N-terminal GST-tagged proteins were immobilized on a glutathione agarose (*GSH* resin), and subsequently subjected to thrombin cleavage to remove the GST tag and elute the purified protein of interest (*Elution*).

Phosphatase activities of wild type hYVH1 and ZnΔhYVH1 toward the artificial substrate DiFMUP were compared at pH 7. Through the construction of Michaelis-Menten plots (Figure 3.16A, 3.16B), the $K_m$ of wild type hYVH1 was determined to be approximately 780µM while that of ZnΔhYVH1 mutant is approximately 900µM. Further, the $k_{cat}$ could be determined (using the relationship $v_{max} = k_{cat} [E_T]$) to be 0.0047s⁻¹ for wild type hYVH1 and 0.0038 s⁻¹ for ZnΔhYVH1. The catalytic efficiencies
Figure 3.15: Purification of Recombinant hYVH1 and Zn∆hYVH1. IPTG induction was used to induce protein expression in BLR cells transformed with PGEX-4T1 vector containing hYVH1 or Zn∆hYVH1. Cells were collected before (uninduced) and after (induced) induction. Protein purification was achieved by immobilization of recombinant GST-tagged proteins onto glutathione agarose (GSH resin), followed by thrombin-cleavage to remove the GST tag. The supernatant, containing the purified protein of interest was collected (elution). Molecular weight ladder is indicated in kDa.
Figure 3.16: Activity of Full Length and Zn\( \Delta \)hYVH1 Toward DiFMUP. Michaelis-Menten plot displaying activity (initial rates) of hYVH1 (A), and Zn\( \Delta \)hYVH1 (B), toward DiFMUP at various initial concentrations of DiFMUP (pH 7). From the plot, the \( K_m \) of hYVH1 is estimated to be 780\( \mu \)M, while the \( v_{max} \) is estimated to be 0.0028\( \mu \)M/s, producing a \( k_{cat} \) of 0.0047 s\(^{-1}\). The \( K_m \) of Zn\( \Delta \)hYVH1 is estimated to be 900\( \mu \)M, while the \( v_{max} \) is estimated to be 0.0022\( \mu \)M/s, producing a \( k_{cat} \) of 0.0038 s\(^{-1}\).
(k_{cat}/K_m) toward DiFMUP are therefore $5.9 \times 10^{-6} \text{ s}^{-1} \cdot \text{µM}^{-1}$ and $4.2 \times 10^{-6} \text{ s}^{-1} \cdot \text{µM}^{-1}$ for hYVH1 and ZnΔhYVH1, respectively. These results indicate that deletion of the zinc-binding domain does not significantly affect the intrinsic catalytic activity of hYVH1.

3.8 Effect of Mutating Conserved Residues on In Vitro Phosphatase Activity of hYVH1

In an effort to determine structural characteristics that may explain the lower activity exhibited by hYVH1 toward exogenous substrates compared to other DSPs, we first examined the primary sequence within and surrounding the active site cysteine. Human YVH1 possesses all of the invariant phosphatase residues important for catalysis: HCX₃RS/T accompanied by an upstream aspartic acid residue and a downstream arginine, important for stabilizing the phosphatase-substrate interaction (Figure 3.17A). However, it has been reported that certain conserved residues are important in stabilizing the active site conformation, although they do not directly participate in the DSP catalytic mechanism [49,59,66]. One such residue is a threonine corresponding to position 124 in hYVH1. In other DSPs that lack a threonine at this position, mutation of this residue to a threonine has caused substantial gain in activity [49]. While various YVH1 orthologues have a threonine in this position, hYVH1 has an alanine at position 124. A recombinant GST-tagged mutant form of hYVH1 containing the A124T back mutation was expressed in E.coli cells using the IPTG induction system and purified through the use of glutathione agarose and thrombin for cleavage of the GST tag as previously described. The activity of this mutant was compared to wild type hYVH1, however no
Figure 3.17: Effect of Point Mutation of Conserved Residues on Catalytic Activity.

(A) Certain non-catalytic residues which are generally highly conserved and have been shown to important for phosphatase activity of YVH1 orthologues or other DSPs are not conserved in hYVH1. Specifically, a threonine residue (corresponding to residue position 124 in hYVH1) following the consensus catalytic sequence is substituted with an alanine in hYVH1 and a tyrosine residue (corresponding to residue position 129 in hYVH1) is replaced by a phenylalanine residue in hYVH1. Catalytic residues are highlighted. (B) Catalytic activity of hYVH1 variants containing mutations for these residues (A124T and F129Y) was evaluated in vitro. Activity of wild type hYVH1, A124T hYVH1 and F129Y hYVH1 are all comparable at 0.144 ± 0.010 µmol/min-µmol enzyme, 0.176 ± 0.012 µmol/min-µmol enzyme, and 0.168 ± 0.009 µmol/min-µmol enzyme, respectively.
appreciable difference in phosphatase activity toward DiFMUP was observed (Figure 3.17B).

Another residue that was found to confer stability of the active site pocket is a tyrosine corresponding to position 129 of hYVH1 [59,66]. Again, while earlier YVH1 orthologues contain a tyrosine in this position, in hYVH1 this residue is substituted with a phenylalanine. This mutant (F129Y hYVH1) was purified in the same manner as described above, and activity toward DiFMUP evaluated. Similarly, no change in phosphatase activity compared to wild type hYVH1 was observed in vitro (Figure 3.17B). Therefore, it can be concluded that the low in vitro hYVH1 phosphatase activity is not due to the aforementioned conserved DSP residues.

3.9 Effect of DMSO and Glycerol on In Vitro Phosphatase Activity of hYVH1

Previous lab members have found that the phosphatase activity of hYVH1 could be detected in vitro toward the artificial substrate DiFMUP, but not toward pNPP [40]. The pKa of pNPP is higher compared to that of DiFMUP, thus the dephosphorylation of pNPP is more heavily dependent on the catalytic acid step [41,43]. It was therefore hypothesized that the inability of hYVH1 to hydrolyse pNPP is due to an enzyme conformation in which the catalytic acid-containing loop is not in the proper position, but rather strayed predominantly out of range of the active site. Others have reported that DMSO and glycerol are able to promote closed conformation of enzymes, thereby increasing in vitro phosphatase activity toward artificial substrates [64,65].

When performing kinetic analysis in the presence of glycerol, significant increases in hydrolysis of DiFMUP by hYVH1 were observed, with a 1.4 fold increase
and a 1.9 fold increase in activity in the presence of 900µM substrate in 15% and 30% glycerol, respectively (Figure 3.18). Although catalytic efficiency, $k_{\text{cat}}$, and $K_m$ values could not be determined from Michaelis-Menten plots for 15% and 30% glycerol assays due to no plateau ($V_{\text{max}}$) being observed, it is clear that $k_{\text{cat}}$ value at only 15% glycerol increases by greater than 2 fold compared to 0% glycerol. However, this increase in $k_{\text{cat}}$ is accompanied by an increase $K_m$, which for the 0% glycerol sample is approximately 780µM. Therefore, catalytic efficiencies ($k_{\text{cat}}/K_m$) likely remain unchanged or are reduced in the presence of glycerol.

When performing kinetic analysis of activity of hYVH1 toward DiFMUP in the presence of 0%, 15% or 30% DMSO, a more profound increase in activity was observed compared to assays performed in the presence glycerol. At 900µM substrate, there is a 3.2 fold increase and a 5.9 fold increase in activity in the presence of 15% and 30% DMSO, respectively (Figure 3.19). In contrast to the glycerol analysis, the Michaelis-Menten plots obtained in the DMSO kinetic analysis show characteristic plateaus in catalytic rate, allowing for the estimation of the catalytic parameters $k_{\text{cat}}$ and $K_m$. Furthermore, in the case of increasing concentrations of DMSO, $K_m$ seems to decrease slightly (to approximately 330µM in the case of 30% DMSO). More prominent, however, is the change in $k_{\text{cat}}$ which increases approximately 4 fold when increasing DMSO concentration from 0% to 30%. Catalytic efficiency ($k_{\text{cat}}/K_m$), therefore increase with increasing concentrations of DMSO. Catalytic efficiency in the presence of 0% DMSO is approximately 5.8 x10^{-6} \text{s}^{-1} \text{µM}^{-1}, in the presence of 15% DMSO is approximately 2.2 x10^{-5} \text{s}^{-1} \text{µM}^{-1}, and in the presence of 30% DMSO is approximately 5.4 x10^{-5} \text{s}^{-1} \text{µM}^{-1}.
Michaelis-Menten plots were constructed for the activity of hYVH1 (0.6µM) toward DiFMUP in the presence of 0, 15%, and 30% glycerol. The $k_{cat}$ and $K_m$ for wild type hYVH1 in no glycerol are, as previously stated, approximately $0.0047 \text{ s}^{-1}$, and $780 \mu\text{M}$ respectively. Because of the absence of a plateau in the 15% and 30% glycerol plots, the kinetic parameters cannot be estimated, although it is clear that both $k_{cat}$ and $K_m$ increase with increasing concentrations of glycerol.
Figure 3.19: Effect of DMSO on Catalytic Activity of hYVH1 Toward DiFMUP. Michaelis-Menten plots were constructed for the activity of hYVH1 (0.6µM) toward DiFMUP in the presence of 0, 15%, and 30% DMSO. The $k_{cat}$ values for hYVH1 in 0, 15% and 30% DMSO are estimated to be $0.0045 \text{s}^{-1}$, $0.013 \text{s}^{-1}$, and $0.018 \text{s}^{-1}$, respectively. Therefore, the $K_m$ values for hYVH1 in 0, 15% and 30% DMSO are estimated to be 780 µM, 600 µM, and 330 µM, respectively.
Because of the observed increase in activity in the presence of glycerol and DMSO, it was of interest to determine if these same conditions could allow for a detectable signal for pNPP hydrolysis. Bacterially purified hYVH1 was incubated with pNPP in the presence of increasing concentrations of glycerol or DMSO. After 30 min, 70 min, or 130 min, the reaction was quenched with sodium hydroxide, and colorimetric detection of pNP$^-$ at a wavelength of 405nm recorded. In the presence of increasing concentrations of glycerol, as in the case of no glycerol present, no absorbance due to pNP$^-$ could be detected (data no shown). Interestingly however, increasing concentrations of DMSO yielded increased absorbance at 405nm, corresponding to an increase in hydrolysis of pNPP to pNP$^-$ (Figure 3.20). This finding represents the first condition found to induce hYVH1 pNPP phosphatase activity and coupled with the DiFMUP suggest that hYVH1 can exist in a more catalytically competent conformation.

3.10 Preparation of Protein for Analysis by X-ray Crystallography

The physiological substrates for all YVH1 orthologues remain a black box. The above enzyme assays in the presence of DMSO hint that hYVH1 can adopt different catalytically active conformations. Thus, three-dimensional structural data may help guide further mechanistic studies and possibly shed light on possible classes of phospho-substrates. In order to elucidate the structure of hYVH1, full length, GST-tagged hYVH1 was expressed in E. coli cells using an IPTG induction system. Protein purification was achieved by immobilizing the protein on glutathione agarose and subsequent thrombin cleavage to remove the GST tag (as previously described). Furthermore, a truncated
Figure 3.2: Effect of DMSO on Activity of hYVH1 toward pNPP. hYVH1 (1µM) was incubated with 20mM pNPP at 30°C for 30 min, 70 min, or 130 min, followed by quenching of reaction with NaOH. Absorbance at a wavelength of 405nm was recorded, indicating pNPP hydrolysis to pNP⁻.
form of hYVH1 containing only the catalytic domain, ZnΔhYVH1, was designed, ectopically expressed, and purified in a similar manner as full length.

Protein crystallization requires concentrations of proteins near saturation, to better mediate crystal formation [47]. However, it is also vital that proteins are not precipitated out of solution. Therefore, several purifications, yielding protein concentrations between 0.5 and 1.5mg/mL were pooled and concentrated up to 7mg/mL. Furthermore, because it is important to reduce the amount of excess salt, a buffer exchange step was performed.

Following purification, concentration, and buffer exchange, it was important to ensure that purified proteins were still active. This was achieved via in vitro phosphatase assays using DiFMUP as an artificial substrate. Protein samples that had undergone concentration and buffer exchange steps showed no loss in activity toward DiFMUP compared to samples collected immediately following purification (data not shown). These samples were sent to Michael Airola, under the supervision of Dr. Brian Crane at Cornell University for analysis by X-ray crystallography. There, the proteins were further purified via FPLC and crystallization trials initiated.

3.11 Small Angle X-ray Scattering Reveals Low Resolution Structure of hYVH1

Although no crystals were obtained from the initial full length and ZnΔhYVH1 trials, a low resolution protein structure of hYVH1 was obtained using small angle X-ray scattering. Dammin was used to generate 12 ab initio model structures as shown in Figure 3.21A. These models were then averaged to avoid overfitting data (Figure 3.21A, right-most structure). This low-resolution solution structure represents the first ever structure to be solved for any YVH1 orthologue. This structure strongly suggests that
Human YVH1 was purified from bacterial sources, concentrated and buffer exchanged. Small angle X-ray scattering was performed by Michael Airola at Cornell University, allowing for the acquisition of a low-resolution image of hYVH1 in solution. (A) Results were analyzed using Dammin and 12 ab initio models generated. The image on the right represents an average of the 12 models. (B) A classical DUSP catalytic domain crystal structure was overlaid onto the SAXS structure. (Figure Generated by Michael Airola, Cornell University)
hYVH1 exists as a monomeric protein. A crystal structure of the classical DSP domain was overlaid onto the SAXS structure, allowing for the prediction of the domain arrangement within the SAXS structure of hYVH1 (Figure 3.21B). This shows a linear domain arrangement, in which the phosphatase and zinc-binding domains occupy distinct regions within the protein. Additionally, this structure revealed the presence of a potentially flexible N-terminal tail, which may have hindered crystal formation.

3.12 Limited Proteolysis Confirms Boundaries of Flexible N-terminal Region

In order to determine the boundaries of the potentially flexible N-terminal tail of hYVH1, limited proteolysis was performed. This involved the proteolytic degradation of native hYVH1 by proteinase K. Any flexible regions are more accessible to the protease and are therefore more susceptible to proteolytic cleavage. hYVH1 was incubated with proteinase K in various ratios for 15 minutes. As an additional control, proteinase K was incubated alone, in the same amounts as used in mixtures containing hYVH1. While cleavage at ratios of proteinase K : hYVH1 of 1:10 to 1:1000 yielded few cleavage fragments, a 1:1 ratio shows a very distinct cleavage as seen by SDS PAGE (Figure 3.22). The mixture of proteolytic fragments was then spotted onto a MALDI sample plate along with a UV absorbing matrix. Spots were analyzed by MALDI TOF mass spectrometry in linear mode. As shown in Figure 3.23, several fragments corresponding to N-terminal segments are observed, supporting a flexible N-terminal region. The fragments are indicated by “p” followed by the number of amino acid residues belonging to the primary sequence of hYVH1 that precede the cleavage site. Note that GSPEF are not included in the numbering scheme, as these are residual residues from the GST-tag.
Figure 3.22: Limited Proteolysis of hYVH1. Bacterially purified hYVH1 was incubated in various ratios with proteinaseK for 15 minutes. hYVH1 not subjected to proteolysis (hYVH1), mixtures containing proteolytic fragments (ProK : hYVH1), and mixtures containing proteinaseK alone, in same concentrations as used for proteolysis reactions (ProK) were resolved by SDS PAGE and protein bands visualized by Coomassie stain. Full length hYVH1 is indicated on the gel, and proteolytic fragments are indicated by (*). Molecular weight ladder is indicated in kDa.
Figure 3.23: Mass Spectrometry of Peptide Fragments Obtained Using Limited Proteolysis. Solutions containing proteolytic fragments were analyzed by MALDI TOF mass spectrometry. Through analysis of fragments in linear mode, a series of peaks is observed corresponding to various N-terminal fragments. Spectra are annotated, denoting peaks due to N-terminal proteolytic fragments with a “p”, followed by the number of hYVH1 residues N-terminal to this cleavage site. The sequence “GSPEF” is not included in the numbering scheme, as these are not native residues to hYVH1, but are residual residues from the GST tag.
From the flexible regions determined through limited proteolysis, two mutant constructs of hYVH1 were designed, the first starting at the glutamic acid residue 29 of hYVH1 (termed E29 hYVH1), and the second starting at the aspartic acid at residue position 46 in the full length sequence of hYVH1 (termed D46 hYVH1). While the limited proteolysis experiment implies N-terminal flexibility beyond the glutamic acid at residue position 29, this mutant was designed to include the entire catalytic domain of hYVH1. Alternatively, it is possible that the D46 hYVH1 mutant cuts into the catalytic domain, but excludes the highly solvent-accessible region determined by limited proteolysis. Both of these mutants were cloned into the pGEX-4T1 vector, containing an N-terminal GST tag. Protein expression was induced using an IPTG induction system. A broad band in the induced lanes corresponding to the molecular weights of E29 hYVH1 (63kDa) and D46 hYVH1 (62kDa) indicates successful induction (Figure 3.24A). Lysates containing truncated GST-tagged hYVH1 variants were incubated with glutathione agarose and subjected to thrombin cleavage to remove the GST tag and elute the purified protein. E29 hYVH1 was successfully purified (Figure 3.24B), however D46 hYVH1 remained predominantly in the insoluble pellet, and thus was not suitable for purification using this method (data not shown). Following purification, buffer exchange and concentration, phosphatase activity of E29 hYVH1 was assessed in vitro toward DiFMUP (Figure 3.24C). A significant loss (≈70%) in activity was observed for E29 hYVH1 (0.036 ± 0.008 µmol DiFMU/min·µmol enzyme) compared to wild type hYVH1 (0.12 ± 0.01 µmol DiFMU/min·µmol enzyme) indicating that this N-terminal region of hYVH1, not predicted to be a part of the phosphatase domain, is important for hYVH1 phosphatase activity. Despite this loss of activity, purified E29 hYVH1 was sent to
Figure 3.24: Purification of Deletion Mutants As Determined Using Limited Proteolysis. Two mutants were designed to exclude the flexible N-terminal region as determined via limited-proteolysis experiments. (A) These mutants (starting with residues E29 and D46 in the primary sequence of hYVH1) were ectopically expressed in E. coli using an IPTG induction system. E. coli were labelled as uninduced prior to induction and induced following IPTG induction. (B) E. coli cells expressing GST-tagged E29 hYVH1 were lysed by French press, and the lysates incubated on a glutathione agarose. Following incubation, the resin was pelleted and the flow through collected. The resin was washed three times prior to thrombin cleavage of the GST-tagged protein, allowing for the release of E29 hYVH1 from the agarose (elution), while the GST tag remained bound to the resin (resin post-cleave). (C) Activity of E29 hYVH1 compared to wild type was assessed toward the artificial substrate DiFMUP. The activity of E29 hYVH1 is approximately 30% that of wild type hYVH1 (0.036 ± 0.008 µmol DiFMU/min·µmol enzyme and 0.12 ± 0.01 µmol DiFMU/min·µmol enzyme, respectively).
Dr. Brian Crane at Cornell University for attempts at crystallization.

3.13 Design and Purification of Mutants Predicted by Surface Entropy Reduction

An emerging technique in crystal engineering is surface entropy reduction (SER), a method in which high entropy surface residues are mutated to lower entropic residues, which are likely to better mediate crystal contacts. Candidate high entropic sites were predicted by submission of the full length protein sequence into the surface entropy reduction prediction server http://nihserver.mbi.ucla.edu/SER/. This algorithm predicts candidate sites based on their likelihood of being surface residues and their entropy, while mutations of highly conserved residues are disfavoured. Further, it has been shown that mutating clusters of high entropic residues has proven to be more effective in enhancing crystallizability [67]. The top 5 clusters of mutations (Figure 3.25) were chosen for attempts at crystallization.

Five SER variants were generated by multiple rounds of site-directed mutagenesis using the pGEX-4T1 hYVH1 as a template. The mutants were expressed and purified as previously described (shown in elution lanes of Figure 3.26). Several purification elutions were pooled and subjected to concentration and buffer exchange (shown in conc. lanes of Figure 3.26). Activities of purified proteins were assessed by DiFMUP assay. As shown in Figure 3.27, the activity of SER mutants are comparable to that of wild type, (SER1: $0.12 \pm 0.01 \, \mu\text{mol DiFMU/min} \cdot \mu\text{mol enzyme}$, SER3: $0.178 \pm 0.001 \, \mu\text{mol DiFMU/min} \cdot \mu\text{mol enzyme}$, SER4: $0.10 \pm 0.01 \, \mu\text{mol DiFMU/min} \cdot \mu\text{mol enzyme}$, and SER5: $0.14 \pm 0.01 \, \mu\text{mol DiFMU/min} \cdot \mu\text{mol enzyme}$) with the exception of SER2, which displays a much reduced activity of $0.018 \pm 0.002 \, \mu\text{mol DiFMU/min} \cdot \mu\text{mol enzyme}$. 
Figure 3.25: **Surface Entropy Reduction Mutants Predicted In Silico for Crystallization Trials.** The primary sequence of hYVH1 was submitted to http://nihserver.mbi.ucla.edu/SER/, a server which is able to predict sites of high entropy that may be mutated to residues of lower entropy and consequently promote protein crystallization. The top five clusters of SER mutations were selected for attempts at crystallography (highlighted in grey). Catalytically significant residues are outlined in a box.
Figure 3.26: Purification and Preparation of SER mutants for X-ray Crystallography. SER mutant constructs were designed by site-directed mutagenesis using pGEX-4T1 hYVH1 as a template. Competent BLR cells were transformed with these constructs and protein expression induced using IPTG. Cells were lysed and the GST fusion proteins isolated on glutathione agarose. Following elution of SER mutants by thrombin cleavage, purified proteins were subjected to concentration and buffer exchange in preparation for X-ray crystallography. Aliquots of purified proteins (elution) and proteins after concentration (conc.), collectively referred to as “SERhYVH1”, were resolved by SDS PAGE and protein bands visualized by Coomassie stain.
Figure 3.27: Activity of Purified and Concentrated SER mutants Toward DiFMUP. The activity of hYVH1 SER mutant proteins prepared for analysis by X-ray crystallography was assessed toward the artificial substrate DiFMUP \textit{in vitro}. SER hYVH1 mutants displayed similar phosphatase activity toward DiFMUP, with the exception of SER2 hYVH1, for which a drastic decline in activity is observed (SER1: 0.12 ± 0.01 μmol DiFU/min·μmol enzyme, SER2: 0.018 ± 0.002 μmol DiFU/min·μmol enzyme, SER3: 0.178 ± 0.001 μmol DiFU/min·μmol enzyme, SER4: 0.10 ± 0.01 μmol DiFU/min·μmol enzyme, and SER5: 0.14 ± 0.01 μmol DiFU/min·μmol enzyme).
CHAPTER 4

Discussion

PART I) Characterization of the hYVH1: Ribosome Interaction

4.1 hYVH1 Interacts With Particles of the 60S Subunit

In yeast, it has been found that YVH1 participates in late stages of ribosome maturation. Because \( yvh1 \) deletion results in the persistence of Mrt4 on immature 60S particles and is consequently mis-localized into the cytoplasm, it was concluded that YVH1 is required for the release of Mrt4 from the immature 60S subunit. This event allows the association of the ribosomal stalk protein, P0, with the ribosome [14,15]. In this same study, preliminary results suggest that hYVH1 may fulfill a similar role in humans as knockdown of hYVH1 by siRNA in HeLa cells caused mis-localization of the human Mrt4 orthologue, MRTO4, into the cytoplasm. However, in these cells a depletion of MRTO4 in the nuclear and nucleolar regions was not observed. Further, while expression of hYVH1 or MRTO4 alone was able to recover growth defects observed in \( yvh1 \) deletion yeast and \( mrt4 \) deletion yeast, respectively, their co-expression was unable to recover double deletion yeast strains [15]. Apart from these experiments, no other evidence was presented to implicate hYVH1 in ribosome biogenesis in human cells, and no experiments were done to prove the association of hYVH1 with the 60S subunit. Therefore, these studies require direct validation and investigation concerning the role of hYVH1 in ribosome biogenesis in humans. In the study presented, interaction of hYVH1 with particles of the 60S subunit was shown through co-fractionation of
endogenous hYVH1 with the 60S subunit (Figure 3.2), as well as co-fractionation and co-immunoprecipitation experiments using overexpressed flag-hYVH1 (Figures 3.3-3.5).

The role of various ribosomal proteins and trans-acting factors involved in ribosome biogenesis is often paralleled in yeast and humans. However, as previously discussed, not all proteins fulfill the same function in yeast and mammalian ribosomal biogenesis. It was therefore of significance to decipher the true fractionation pattern of hYVH1 with respect to the 60S and 80S ribosomal particles. A macroscopic view of fractions obtained from ribosomal profiling suggested that hYVH1 may interact with particles of both the 60S and 80S subunits (Figures 3.2, 3.3). It is evident that the profiling method used in this study provides incomplete resolution between the 60S and 80S peaks, and that proteins detected in the 80S peak could be from residual, unresolved 60S leaching into the 80S fraction. Therefore, fractions of equal and small intervals were collected. The collection of smaller fractions gave a more accurate insight into the fractionation pattern of hYVH1, showing the decline of hYVH1 following fractions corresponding to the 60S peak, while levels of the internal control, RPL26, persist throughout the 60S and 80S peak fractions (Figure 3.4). This supports interaction of hYVH1 exclusively with the 60S or pre-60S particles, and not the 80S ribosome.

Notably, the co-fractionation experiments do not identify the exact pre-mature or mature 60S particle with which hYVH1 associates, as the profiling used does not differentiate between immature or mature subunits [14,15]. Again, although yeast studies suggest that YVH1 interacts with certain late pre-60S particles, the role of hYVH1 in humans may differ from its yeast orthologue, or hYVH1 may fulfill multiple roles in ribosome biosynthesis. It is therefore necessary to conduct further investigation
pertaining to the particles with which hYVH1 associates, which could aid in deciphering its role in ribosome biogenesis in mammalian systems. In addition to profiling experiments, TAP tagging pulldowns are commonly used to determine the specific particles with which a trans-acting factor associates. While this was done in yeast, showing that YVH1 associates with particles intermediate to the Mrt4 and P0-associated particles, these studies in humans have not yet been conducted [15]. TAP tagging pulldowns in humans could prove useful not only in establishing the role of hYVH1 in ribosome biogenesis, but also evaluating potentially altered binding-specificities of mutant forms of hYVH1 to the pre-60S particles compared to that of wild type hYVH1.

4.2 Overexpression of hYVH1 Does Not Affect Ribosome Profiles

Extensive studies of ribosome biogenesis have been done in yeast. Changes in ribosome profiles in response to gene deletions can lend insight into the role of a specific protein in ribosome biogenesis. For example, knockout of *yvh1* in yeast causes reduction in levels of 60S subunits and polysomes, which is accompanied by an increase in the number of 40S subunits as well as the appearance of halfmers [14,15]. Additionally, variations in these peaks can yield information pertaining to altered export competence or susceptibility to degradation in response to genetic deletions [14]. In this way, ribosome profiling has served as a useful tool in providing preliminary indications as to the nature of a protein’s involvement in ribosome biogenesis. Recent studies in human cell culture use ribosome profiling in a similar manner to observe the effect of depletion of ribosomal proteins on ribosome profiles [26, 68].
While profiles often yield important information pertaining to the function of a ribosomal protein or trans-acting factor in ribosome biogenesis, in the study presented very little change in ribosome profiles was observed in response to overexpression of hYVH1. This could be due to a number of possibilities. One possibility is that only a certain number of rRNA and ribosomal proteins are synthesized. It is possible that while its knockdown may have a significant effect as hYVH1 becomes a limiting factor in ribosome synthesis, its overexpression may have a minimal effect as other ribosomal proteins become limiting in the biogenesis pathway. Consequently, overexpression of hYVH1 may bear an insignificant effect on overall ribosome profiles due to saturation of ribosomal constituents. It is therefore likely that knockdown of hYVH1 would yield more informative profiles. However, preliminary transient transfection results using siRNA to knockdown hYVH1 shows very little modification in ribosome profiles (data not shown). This is likely due to masking of the effect of knockdown by untransfected cells expressing normal levels of endogenous hYVH1. Therefore, it is likely that a better knockdown efficiency (i.e. stable hYVH1 siRNA cell lines) or cell gating is required to observe effects of decreased expression of hYVH1 on levels of ribosomal subunits.

Moreover, while normal ribosome profiles are still seen from the cell populations overexpressing wild type and mutant forms of hYVH1, it is possible that any changes from these mutants are masked due to the presence of endogenous hYVH1. It would be useful to produce a cell line which is stably expressing mutants, coupled with efficient knockdown of endogenous hYVH1. This could further reveal deviations in ribosome maturation in response to hYVH1 constructs (i.e. catalytic mutants, domain deletions, and phosphomimetic mutants).
4.3 Catalytic Mutants Do Not Affect hYVH1:60S Interaction

Various enzyme families have shown to be important for ribosome biogenesis. The best characterized consist primarily of ATPases and GTPases that power the release and association of trans-acting factors from the ribosomal complex [25,29]. Less is known regarding the role of phosphorylation related enzymes in ribosome biogenesis. One specific protein phosphorylation event is involved in the maturation of the 40S subunit. The phosphorylation of a trimeric complex by Hrr25p triggers the dissociation of this complex from the pre-40S subunit, and a subsequent dephosphorylation event allows for proper incorporation of ribosomal proteins onto the subunit [25,29].

Previously, yeast studies have shown that a catalytically inactive form of YVH1 is able to restore normal ribosome profiles in yvh1 deletion strains. While this supports the insignificance of the catalytic activity of YVH1 in ribosome biogenesis, these papers were not readily rejecting the possible requirement of the phosphatase activity of YVH1 in the dephosphorylation of a trans-acting factor, a ribosomal protein, or even in rRNA processing to mediate ribosome maturation [15,19]. In order to assess the importance of the phosphatase activity of hYVH1 in its association with the ribosomal subunit, a catalytically inactive form of hYVH1 was overexpressed in HEK 293 cells. In this variant, the catalytic cysteine is substituted with a serine, hence rendering the phosphatase incapable of nucleophilic attack, which is the first step in the dephosphorylation mechanism. C115S hYVH1 co-fractionated with the ribosome, demonstrating its competence in the association with the 60S subunit (Figure 3.6B).

While this suggests that catalytic activity is not required for the association and subsequent dissociation of the hYVH1:60S complex, it is important to note that this does
not prove that phosphatase activity is not involved in 60S ribosome biogenesis. Any possible differences in the ribosome profile resulting from incomplete maturation of C115S hYVH1-bound particles may be saturated by normally maturing particles which have associated with endogenous hYVH1. Because of this possible saturation effect and the inability to obtain high resolution in the polysome fractions, it is unclear as to whether the association between C115S hYVH1 produces competent ribosomal particles. Therefore, this experiment does not assess the implication of the phosphatase activity in ribosome biogenesis, but simply evaluates the ability of catalytically inactive C115S hYVH1 to interact with the 60S particle.

Assessment of the ability of C115S hYVH1 and other hYVH1 mutants to mediate ribosomal maturation may be achieved through the use of pulse chase experiments. Pulse chase experiments involve the brief exposure of cells to radioactively labelled constituents such as [³H]uridine or L-[methyl³H] methionine, which allow for the incorporation of radioactive labels into rRNA through rRNA synthesis, or rRNA methylation events, respectively [14,26,68,69]. Following this pulse, cells are then incubated with excess non-radioactive constituents for various amounts of time. In this way, rRNA synthesis and processing may be monitored, and can give insight as to whether ribosome biogenesis is proceeding normally. For example, in yvh1 deletion yeast strains, a delay in the processing of 27S rRNA to 25S rRNA can be visualized through pulse chase experiments using [³H]uridine labelling [14]. This is a relatively sensitive technique and may allow for the detection of defects in ribosome maturation where ribosome profiling experiments are of insufficient sensitivity.
Perhaps a more informative catalytic mutant is that in which the catalytic aspartic acid as well as the invariant glutamine necessary for positioning of the water molecule in the phosphatase mechanism, are substituted with alanine residues. This hYVH1 variant (termed DT hYVH1) is able to perform the nucleophilic attack on the substrate molecule, but is incapable of subsequent substrate release and enzyme regeneration, hence capturing the substrate in a covalently bound enzyme-substrate complex. It was hypothesized that if the phospho-substrate of hYVH1 resided on the premature 60S complex, then hYVH1 would be trapped on the complex throughout maturation and thereby become incorporated into the 80S ribosome, and/or polysome. Fractions probed for DT hYVH1 show possible co-fractionation with the 80S subunit, however, upon closer investigation by collection of smaller fractions, it becomes evident that DT hYVH1, like wild type hYVH1, co-fractionates only with the 60S particle (Figure 3.6C, D). Furthermore, immunoprecipitation experiments of flag-hYVH1 and flag-DT hYVH1 did not show significantly different levels of the co-immunoprecipitated large subunit protein, RPL26, suggesting that the substrate trap, DT hYVH1, is not being tethered to the ribosome (Figure 3.6E). Again, it is uncertain as to whether this mutant is able to assist in the synthesis of competent mature 80S ribosomes, or if pre-60S particles associated with DT hYVH1 go on to form inactive ribosomal subunits, although there is no increase in halfmers observed that would support the latter.

And while these results suggest that the hYVH1 substrate does not reside on the ribosome, the possibility remains that one of the functions of hYVH1 in ribosomal maturation is to facilitate the removal or inactivation of a substrate protein from the pre-60S complex. In such a case, DT hYVH1 may still be able to bind its substrate, hence
sequestering the phosphorylation site, and thereby mimicking a dephosphorylated or inactive substrate. In this situation, hYVH1 and its substrate may in fact form the “substrate trap” complex, however this complex is able to be released from the ribosome, and a functional 80S ribosome is still produced. In such a case, the phosphatase activity is important for ribosome biogenesis, but not required for the release of hYVH1 from the ribosome.

4.4 hYVH1:Ribosome Interaction is Not Regulated Through Phosphorylation of hYVH1

hYVH1 has been shown to be regulated by phosphorylation. Specifically, phosphorylation of serine 14 and serine 335 has been shown to affect localization of hYVH1 as well as have effects on cell cycle progression [24]. However, ribosome profiles followed by immunoblot analysis of fractions showed no change in the association of S14A hYVH1, S14E hYVH1, or S335E hYVH1 with the ribosome compared to wild type hYVH1 (Figure 3.7), suggesting that ribosome biogenesis is independent of the phosphorylation state of hYVH1. It remains puzzling that the change in localization observed with these phosphomimetic mutants does not result in or is not a result of changes in the ability of these mutants to associate with the ribosome. Perhaps slight alterations do occur in the association and dissociation of hYVH1 mutants to and from the ribosome, but are overlooked in the method employed, which is incapable of differentiating between the various types of 60S particles. TAP tagging could allow for the determination of a potential shift in the interaction of hYVH1 mutants with nuclear or cytoplasmic 60S particles. Again, it is also important to keep in mind that while no
apparent changes are seen in ribosome profiles, it is unclear as to whether these mutant-associated particles are in fact forming mature 60S subunits.

In yeast, a slow growth defect is observed in yvh1 deletion strains. In a study conducted by Lo et al this slow growth defect is attributed to a possible inability to recycle Mrt4 into the nucleolus to mediate production of 60S subunits [15]. A mutant form of Mrt4 which has lower affinity for the ribosome is able to recover the slow growth defect as well as normal ribosome profiles in yvh1 deletion strains, suggesting that these two effects are closely related [15]. Interestingly, a previous study showed that the sporulation defects associated with yvh1 deletion yeast are separate from the vegetative growth defects [17]. Therefore, since the sporulation process requires proper cell cycle progression, this may suggest that hYVH1 plays separate roles in cell cycle progression and ribosome biogenesis. Because of the energetic demand imposed by the synthesis of ribosomes, cell cycle and ribosome biogenesis are tightly coordinated, and studies support the existence of various signalling and crosstalk mechanisms that afford this coordination [28,70,71]. It is possible that hYVH1 is a novel mediator between the two processes, playing distinct roles in cell cycle progression as well as ribosome biogenesis.

Furthermore, cell cycle analysis of cells overexpressing wild type hYVH1 and various hYVH1 deletion constructs by flow cytometry show a decreased number of cells in G0/G1 and an elevated number of cells in G2/M as well as polyploid cells compared to control cells expressing endogenous levels of hYVH1 [24, Figure 1.5]. While overexpression of the catalytic domain alone (CT1 hYVH1) is unable to produce this phenotype, cell cycle profiles of cells overexpressing constructs consisting of the catalytic domain and portions of the zinc-binding domain (CT2 hYVH1 and CT3
hYVH1) closely resemble that of overexpressed wild type hYVH1. Notably, overexpression of the zinc-binding domain alone produces an amplified phenotype, in which an even greater number of polyploid cells and cells in G2/M is observed as compared to cells overexpressing full length hYVH1 [24, Figure 1.5]. In contrast, overexpression of hYVH1 domain deletion constructs show that the zinc-binding domain alone, as well as the CT2 hYVH1 and CT3 hYVH1 deletion constructs, are insufficient for efficient ribosomal association (Figure 3.13). The ability of the zinc-binding domain to produce this amplified effect in cell cycle progression while unable to efficiently bind the ribosome supports the hypothesis that hYVH1 plays separate roles in cell cycle progression and ribosome biogenesis.

4.5 Tert-Butyl Hydroperoxide Treatment Results in Dissociation of the hYVH1: Ribosome Complex

In addition to its N-terminal phosphatase domain, hYVH1 has a novel zinc-binding domain. This domain has been shown to bind two moles of zinc per mole of protein [7]. It is presumed that the first set of zinc-coordinating residues consists of two cysteines, separated by only two residues, as well as a downstream histidine and cysteine, which are separated by 27 residues. The second, most C-terminal set of zinc-coordinating residues are thought to form a traditional “zinc finger motif”, consisting of two sets of cysteines, each in which the cysteines are separated by only one or two amino acid residues (see Figure 4.1 for a sequence alignment of the zinc-binding domain of YVH1).
Figure 4.1: Alignment of Zinc-binding Domain of YVH1 Orthologues. Alignment of the zinc-binding domain of YVH1 orthologues depicts the conservation of the zinc-coordinating residues (shown by an arrow). The first set of zinc-coordinating residues consists of two cysteines separated by only two amino acid residues, followed by a histidine and cysteine residues which are some distance apart in the primary sequence. The second set of zinc-coordinating residues consists of two sets of two cysteines, each separated by only one or two amino acid residues. [Figure from reference 7]
The microenvironment within the PTP catalytic cleft lowers the pKa of the nucleophilic cysteine, thereby rendering the active site more susceptible to oxidation than most other cysteinyl residues [72]. In nature, this is often used as a regulatory mechanism to inactivate PTPs [72]. However, it has been found by our laboratory that in vitro hYVH1 is less susceptible to inactivation by oxidation compared to VHR, a DSP which shares high homology with the catalytic domain of hYVH1 [23]. Studies suggest that this effect is a consequence of preferential oxidation of the zinc-coordinating cysteine residues, which is accompanied by zinc ejection [23]. Some zinc-binding domains are referred to as “redox sensors”: changes in protein folding and function can occur in these domains as a result of sensitivity to the redox environment [73]. In the case of the zinc-binding domain of hYVH1, the possible formation of disulfide bonds in vitro in response to oxidative stress suggests that hYVH1 may undergo dynamic conformational changes under these conditions.

Tert-butyl hydroperoxide (TBH) is a cell permeable oxidant that has been reported to elicit mass zinc-ejection throughout the cell, and was therefore used to treat cells in an attempt to investigate the effect of zinc-ejection on the association of hYVH1 with the ribosomal subunit [63]. Interestingly, when treating HEK 293 cells with increasing concentrations of TBH, the association of hYVH1 with the ribosomal subunits declined in a dose-dependent manner (Figures 3.9, 3.10B, Supplementary Figure 1). A similar pattern seen in C115S hYVH1 suggests that this effect is not caused by oxidation of the catalytic cysteine and is altogether independent of phosphatase activity (Figure 3.11). It is of importance to note that while TBH may be triggering oxidation and zinc ejection within the zinc-binding domain of hYVH1, therefore eliciting a possible redox...
switch response of this novel domain, it is also likely that several other factors are involved in the cellular response to TBH-induced oxidative stress. For example, TBH is known to activate ATM/ATR DNA damage checkpoints, ultimately resulting in G1 and G2 arrest, presenting several means by which the hYVH1:60S association may be disrupted [74,75,76]. In addition to the activation of cell cycle checkpoints, it is apparent that this effect may be due to the activation of certain pro-apoptotic or necrosis factors as discussed below.

Surprisingly, 500µM and 2mM TBH-treated lysates revealed a flag-hYVH1 cleavage fragment presenting proteolytic cleavage as a possible step in the mechanism for the dissociation (or inhibition of association) of hYVH1 from the ribosome (Figure 3.10A). Notably, the use of the flag antibody to detect N-terminally tagged hYVH1 only allowed for the detection of the N-terminal fragment by western blot analysis. Therefore, although it is likely that this fragmentation event signifies the release of hYVH1 from the 60S subunit, it cannot be ruled out that the C-terminal fragment portion is also unable to associate with the 60S subunit. The 25kDa fragment corresponds to a cleavage occurring near or within the first set of zinc-coordinating residues. As previously stated, the first set of zinc-coordinating residues is spread over a much larger range within the primary sequence of hYVH1 compared to the second set of coordinating residues. It is therefore hypothesized that this first cluster of residues loosely coordinates zinc and undergoes preferential zinc ejection, suggesting an increased susceptibility to oxidation and subsequent structural rearrangements.

Furthermore, cells treated with 500µM or 2mM TBH showed significant levels of lifting off the cell culture plate 24 hours post-treatment, likely signifying cell death
initiation, while culture plates harbouring 200μM TBH treated cells were indistinguishable from untreated cells 24 hours following TBH treatment (unpublished observation). It appears then, that the observed cleavage fragment occurs when cells are committed to necrosis or apoptosis (the mechanism of cell death was not investigated). Various proteases are activated in response to oxidative stress and cell death (necrosis or apoptosis) [77-81]. It is necessary to first decipher the pathway by which cell death is elicited, which may then allow for the construction of a candidate list of proteases responsible for this cleavage event.

In addition to zinc ejection, in vitro studies have shown that disulfide bond formation occurs in the zinc-binding domain. This often leads to dynamic conformational changes, altering protein structure and function. For this reason, conformational rearrangements triggered by cysteiny1 oxidation remains an attractive medium by which the release of hYVH1 from the ribosome transpires. Moreover, domain deletion studies show the requirement of the zinc-binding domain for association with the ribosome. It is therefore logical that structural changes in this domain arising from zinc ejection and rearrangements in disulfide bond formation could alter the proteins ability to bind the 60S particle. A model is proposed in Figure 4.2 in which the proteolytic cleavage site is not accessible under normal cellular condition, hence further protecting hYVH1 from this cleavage event. However, in the event of sufficient oxidative stress, zinc may be ejected from the protein, coupled with cysteiny1 oxidation, and thereby effecting protein conformational changes. This conformational change may then expose the cleavage site, allowing for the proteolytic fragmentation of hYVH1.
Figure 4.2: Proposed Mechanism of hYVH1:Ribosome Dissociation In Response To Oxidative Stress. Under normal reducing conditions, hYVH1 associates with the 60S particle through regions of both its N-terminal phosphatase domain and C-terminal zinc-binding domain (ZBD). Oxidative stress triggers disulfide bond formation and cysteinyl oxidation, which results in zinc ejection and structural rearrangement in the ZBD. This rearrangement either directly results in dissociation of the hYVH1:ribosome complex, or exposes the proteolytic cleavage site (★). Proteases (★★) are then free to cleave hYVH1, thereby inhibited its association or reassociation with the 60S particle.
To further investigate the proposed mechanism, a variation of the biotin switch assay was attempted [82]. In this method, cells were lysed and free thiols immediately alkylated using iodoacetamide. Following this blocking step, all reversibly oxidized thiols (including those involved in disulfide bonds, or have been oxidized to the cyclic sulfenamide, and sulfenic acid, but not sulfinic or sulfonic acid) are reduced in DTT [83]. The reduced thiols are then labelled with an alkylating reagent that is conjugated to biotin. Biotin-labelled proteins can then be immunoprecipitated using streptavidin-conjugated sepharose beads, or can be detected by immunoblot analysis using a streptavidin-conjugated HRP, hence allowing for comparison of levels of oxidized cysteine residues between samples (see Supplementary Figure 2 for an experimental schematic). Attempts using this method showed no difference in oxidized thiol levels of hYVH1 (Supplementary Figure 3), although it is important to note that the 0 to 2mM TBH treated lysates, which acted as a positive control, also showed no change in levels of biotinylated proteins (Supplementary Figure 3). Therefore, optimization of this experiment is necessary prior to drawing conclusions pertaining to the oxidation of hYVH1 thiols in response to TBH treatment and their role in dissociation of the hYVH1: ribosome complex. It is important to note that the biotin switch method has inherent issues with obtaining results from in vivo sources due to issues of sensitivity and complexity [84]. However, our laboratory was part of a collaborative study with Dr. Mutus’ group, in the Department of Chemistry and Biochemistry at the University of Windsor, that developed a novel technique for enriching modified thiols prior to mass spectrometry analysis [85]. Therefore, we plan to apply this approach to determine if
release of hYVH1 from the 60S subunits involves disulfide bond formation during oxidative stress.

Another approach to evaluating the impact of zinc ejection on the dissociation of the hYVH1: ribosome complex is to assess the ability of zinc-finger mutants to form complexes with the ribosomal subunits. Specifically, mutation of the zinc-coordinating cysteine residues to serines could mimic oxidized cysteinyl residues and would be unable to coordinate zinc. The shortcoming of this approach is that the substituted serine residues lack the ability to accurately mimic the electronic environment generated from fully oxidized cysteinyl residues and to participate in disulfide bonds. Therefore, if protein conformational change and subsequent dissociation of hYVH1 from the ribosome mandates the formation of disulfide bonds, then the Cys to Ser mutants would be unable to evaluate this hypothesis.

Furthermore, hydrogen-deuterium exchange coupled with mass spectrometry allows for the evaluation of solvent-accessible regions. \textit{In vitro} HDX experiments can be performed in reducing and oxidative environments. Conformational changes that result in alterations of solvent accessible regions can be detected in the form of shifts in deuterated and non-deuterated peptides. Thus, HDX experiments may allow for the elucidation of local regions of hYVH1 that undergo structural rearrangements in response to zinc ejection triggered by oxidative stress. These experiments are currently being conducted by other members of the Vacratsis laboratory.
4.6 Ribosome Biogenesis and the Cell Survival Effect

hYVH1 was recently shown to be a cell-survival phosphatase as overexpression of hYVH1 protects HeLa cells from apoptosis induced by hydrogen peroxide, Fas receptor activation and heat shock [21]. It was therefore hypothesized that this survival effect may be related to the ability of hYVH1 to produce an increased number of functional ribosomes under stress conditions. However, this effect necessitated the catalytic activity of hYVH1 as C115S hYVH1 was not able to protect cells. In contrast, experiments done in yeast suggest that the catalytic activity of YVH1 is not essential for ribosome biogenesis, and experiments in this study suggest that the catalytic activity is not required for association of hYVH1 with the ribosome. Furthermore, C115S hYVH1 behaved similarly to wild type hYVH1 in its dissociation from the ribosome in a dose-dependent manner (Figures 3.9-3.11). And while hYVH1-mediated cell protection was not demonstrated against TBH-induced oxidative stress, this is nonetheless a form of oxidative stress and may further support a role of hYVH1 in ribosome biogenesis independent of its role in cell survival.

Although studies presented suggest that the role of hYVH1 in ribosome biogenesis does not require its phosphatase activity, it is possible that the trap forms a complex with its substrate and the enzyme-substrate complex is subsequently released from the ribosome, as previously discussed. Alternatively, DT hYVH1 and C115S hYVH1 may produce non-functional 60S complexes, but no changes in ribosomal profiles are observed (i.e. an accumulation of 40S subunits or an accumulation of halfmers) because the effect is masked by the presence of endogenous hYVH1. In both cases, DT hYVH1 and C115S hYVH1 would only co-fractionate in the 60S fraction as
seen experimentally. In the case that catalytic activity is important for ribosome biogenesis, it remains possible that the protective effect is in fact directly related to the cells ability to produce mature and competent ribosomes. An alternative hypothesis we are interested in testing is that the cell survival role requires release of hYVH1 from the ribosome. In this scenario, hYVH1 serves a redox sensor role in which cellular stress causes the phosphatase to release from the ribosome and is free to dephosphorylate substrate targets to protect cells from cell death.

4.7 Domain Deletion Studies Suggest Regions of Both Domains are Necessary for Complete Ribosome Binding

Because of the effect of TBH treatment on the association of hYVH1 with the 60S subunit as well as the fact that zinc finger domains often bind nucleic acids, it was relevant to evaluate the domains or regions responsible for ribosome binding. Expression of the catalytic domain alone results in complete loss of co-fractionation with the ribosomal subunit (Figure 3.13A). Interestingly, expression of the zinc-binding domain alone resulted in a significant, but not complete, loss of co-fractionation with the ribosomal subunit (Figure 3.13B). These results suggest that parts of the catalytic domain (or the short N-terminal fragment) and the zinc-binding domain are necessary for efficient ribosome binding.

Further investigation of the specific regions that enable ribosome binding was conducted using variants of hYVH1 consisting of the N-terminal phosphatase domain and parts of the C-terminal zinc-binding domain (see Figure 3.12 for construct maps).
Similar to CT1 hYVH1, CT2 hYVH1 displayed complete loss of co-fractionation with ribosomal subunits (Figure 3.14A). Intriguingly, CT3 hYVH1, a mutant that contains the first zinc finger along with an additional highly conserved hydrophobic sequence that is flanked by the two zinc-binding regions, exhibits a distinct co-fractionation pattern, being detected in fractions corresponding to 80S ribosome and polysome/halfmers, but not the 60S subunit (Figure 3.14B). Initially, it was hypothesized that this could indicate the requirement of this short hydrophobic sequence for ribosomal binding, and imply that the second zinc finger, which is absent in CT3, is required for dissociation of hYVH1 from the ribosome after it has completed its function. However, preliminary co-immunoprecipitation experiments suggest that CT3 hYVH1 does not directly interact with the 60S subunit (Supplementary Figure 4). It is therefore likely that the co-fractionation of CT3 with the 60S and polysomes is a non-specific effect, perhaps due to its sticky hydrophobic terminus complexing with high sedimentation complexes other than the ribosome.

From the domain deletion experiments, it is inferred that regions of the zinc-binding domain (including the second zinc finger), along with parts of the N-terminal domain are both required for ribosome binding. In contrast, studies in yeast conclude that the zinc-binding domain alone is sufficient to recover ribosome biogenesis in yvh1 deletion mutants, suggesting variations in the interaction specificities between yeast and human YVH1 with the ribosome, and presenting another dimension by which this interaction can be regulated. While it would be interesting to evaluate the effect of deletion of the short non-conserved N-terminal sequence of hYVH1 on ribosomal binding, it is likely that expression without this region would cause significant loss in
protein stability and/or activity, as bacterially-expressed mutants lacking portions of the N-terminal sequence displayed significant decreases in solubility or phosphatase activity toward artificial substrates (Figure 3.24). Therefore, alanine scanning mutagenesis of this N-terminal variable region will be a more feasible approach to determining residues that participate in ribosome association.

PART II) *In Vitro* Structure Function Analysis of hYVH1

4.8 Structural Effects on *In Vitro* Catalytic Activity of hYVH1

The phosphatase domain of hYVH1 contains all of the invariant PTP residues required for catalysis. This domain shares high homology with the yeast orthologue, YVH1, as well as other DSPs, such as VHR. Despite the similarities in primary sequence, hYVH1 displays significantly lower activity toward artificial substrates compared to other DSPs, suggesting fundamental structural differences in the catalytic domain of these phosphatases. This observation raises question as to the relationship between the phosphatase and zinc-binding domains of hYVH1, and the possible effect of this novel domain on the catalytic activity of hYVH1.

The activity of full length hYVH1 and a mutant lacking the zinc-binding domain was assessed *in vitro* toward the artificial substrate DiFMUP (Figure 3.16). This revealed no appreciable difference in phosphatase activity, suggesting that the zinc-binding domain does not significantly affect the catalytic activity of hYVH1. It is important to consider that although the zinc-binding domain does not alter the activity of hYVH1 toward artificial substrates, it remains possible that in the presence of the physiological
substrate of hYVH1, the zinc-binding domain participates in substrate-induced activation. This mechanism involves the formation of a tertiary structure upon substrate binding that promotes more efficient hydrolysis of the phosphoester bond. Substrate-induced activation is not unusual among the dual specificity phosphatases, as it is a common mechanism exercised by the MKPs, which have a MAPK-binding domain (MKB) in addition to their catalytic domain [64,65,86].

While hYVH1 contains the invariant residues required for phosphatase activity, there are certain non-catalytic residues that have been shown to be important for conformational stabilization of the catalytic cleft. One such residue is a threonine, corresponding to position 124 in the primary sequence of hYVH1 (which is an alanine in hYVH1). It has been found that back mutation of this residue from an alanine to a threonine in the DSP Prl1 has resulted in significant increase in phosphatase activity toward artificial substrates [49]. Similarly, a tyrosine conserved in the majority of DSPs corresponding to position 129 in hYVH1 which is postulated to confer stabilization of the catalytic pocket is substituted for a phenylalanine in hYVH1 [59,66]. However, back mutations of the corresponding residues in hYVH1 yield no change in activity toward DiFMUP, suggesting that some other feature is responsible for the reduced activity of this phosphatase (Figure 3.17).

As previously stated, various MKPs have been shown to exhibit substrate-induced activation. In these cases, binding of the endogenous substrate promotes conformational changes (often in the positioning of the loop which houses the catalytic aspartic acid), hence allowing for enhanced phosphatase activity. An example of this effect is presented in the case of MKP3. This enzyme, which dephosphorylates and activates ERK2 in vivo,
has demonstrated poor *in vitro* phosphatase activity toward artificial substrates. However, in the presence of ERK2, MKP3 is not only able to efficiently dephosphorylate ERK2, but its activity toward artificial substrates is also stimulated. It is found that binding of ERK2 promotes active site rearrangements and closure of the general acid-containing loop [64,65]. Furthermore, in solution structural information obtained by NMR revealed that ERK2 binding altered the interaction between the substrate binding domain and the catalytic domain of MKP3, resulting in structural reconfiguration of active site residues. [86]

In addition to activation by ERK2, MKP3 has been shown to be activated by glycerol and DMSO: solvents referred to as chemical chaperones due to their ability to stabilize certain conformations of enzymes [64]. Although the physiological substrate of hYVH1 remains unknown, these solvents were used to evaluate the potential of a more active enzyme conformation. In the presence of 30% glycerol, activity of hYVH1 toward DiFMUP increased substantially (Figure 3.18). Catalytic parameters could not be determined as a maximum rate of catalysis was not attained for assays containing glycerol in the range of substrate concentrations used (up to 40µM). It is estimated that \( k_{\text{cat}} \) and \( K_m \) increase by similar factors, resulting in very little change in catalytic efficiency (\( k_{\text{cat}}/K_m \)). In contrast, the use of DMSO in reaction buffers resulted in a significant increase in \( k_{\text{cat}} \) and a slight decrease in \( K_m \), producing a 10 fold increase in catalytic efficiency in the presence of 30% DMSO (Figure 3.19). While this does not confirm that hYVH1 exercises substrate-induced activation, it does suggest the ability to form a closed enzyme conformation for efficient substrate dephosphorylation and supports the possibility of a more active enzyme conformation.
It has been previously shown that hYVH1 is unable to dephosphorylate artificial substrates such as pNPP. Because pNPP has a higher pKa than that of DiFMUP, it is more dependent on the catalytic acid step for efficient substrate release. It is therefore postulated that the loop which contains the catalytic acid, is held away from the active site in the absence of a physiological substrate, forming a loose enzyme conformation which hinders phosphatase activity. Furthermore, the ability to detect the dephosphorylation of pNPP in the presence of DMSO but not in its absence suggests that the catalytic acid is generally positioned out of range of the active site cleft, and is stabilized near the cleft in the presence of DMSO, allowing for efficient hydrolysis of pNPP by hYVH1 (Figure 3.20). It was previously reported that mutation of the catalytic acid Asp84 to an alanine residue resulted in loss of pH dependence of in vitro activity of hYVH1 [40]. Although this implies that the catalytic acid participates in catalysis in the absence of chemical chaperones, the addition of these agents to the reaction buffer may induce structural rearrangements that result in a more favourable positioning of this catalytic residue.

To understand the mechanism of hYVH1 activation by DMSO, hydrogen-deuterium exchange experiments can be conducted. As previously explained, HDX experiments allow for the detection of solvent accessible regions based on the degree of incorporation of heavy deuterium into a peptide due to the exchange of protons from the protein with solvent deuterons. Therefore, HDX experiments can be performed in the absence and presence of DMSO, allowing for the identification of peptides that have differentially incorporated deuterium under the various conditions. In this way, structural
rearrangements that contribute to increased catalytic activity in the presence of DMSO may be unveiled.

4.9 Elucidating the Structure of hYVH1

hYVH1 is emerging as a dynamic protein that may satisfy several, possibly distinct, functions throughout the cell. Moreover, several potential layers of regulation have been proposed. In spite of recent progress, the physiological substrate of hYVH1 remains elusive, and to date the only function that demonstrates a necessity for the catalytic activity of hYVH1 is its role in cell survival. Although \textit{in vitro} catalytic activity is unaffected by deletion of the zinc-binding domain, it remains unclear as to whether the zinc-binding domain and the phosphatase domain act as two separate entities, or if their functions are intertwined. To elucidate the structural features that allow hYVH1 to fulfill its physiological roles, attempts at X-ray crystallography are underway with the collaboration of crystallographer, Dr. Brian Crane, at Cornell University.

Obtaining a crystal structure of hYVH1 would lend invaluable insight into the relationship between the phosphatase and zinc-binding domains and could give indication as to the local regions required for ribosome binding. Additionally, by aligning the structure of the catalytic domain with DSPs of high intrinsic phosphatase activity, differences between the positioning of important catalytic residues, or the geometry of the active site pocket could reveal vital information pertaining to the low \textit{in vitro} phosphatase activity of hYVH1. This same approach has allowed identification of structural characteristics responsible for poor activity or differential substrate specificity.
in other DSPs [86,87]. Further, X-ray crystallography has often been used to demonstrate substrate-induced activation practiced by various MKPs [49].

As previously stated, there is no known physiological substrate for hYVH1, and very little evidence available to aid in the construction of a candidate substrate list. Substrate trapping experiments have been attempted with little success. However, because of the recently presented functions of hYVH1, it is becoming apparent that precise temporal or spatial control may be required for substrate identification. Crystal structures of enzymes have often allowed careful examination of surfaces that could be responsible for substrate binding, and hence provide clues as to potential substrates [87]. And while hYVH1 contains all of the invariant residues required for PTPs, it is possible that the structure of hYVH1 may reveal that it is in fact not a protein phosphatase, but rather acts to bind and dephosphorylate other types of molecules, such as nucleic acids or carbohydrates. This could allow for the construction of a candidate substrate list that could then be tested through \textit{in vitro} phosphatase assays as well as \textit{in vivo} experiments.

In the event of obtaining a crystal structure, examination of regions containing residues known to undergo post-translational modifications would be of particular interest. This would allow for the prediction of the effects of these post-translational modifications on protein structure and function. Further, it would be of interest to pursue structures of hYVH1 variants, such as phosphomimetic mutants, which could show structural changes that may occur in response to modification of these residues. Additionally, various crystal structures of PTPs have shown disulfide bond formation in response to oxidative environments. For example, in the case of CDC25B, the formation of disulfide bonds between the active site and a nearby cysteine residue (commonly
termed the backdoor cysteine) mediates the folding of the P-loop over the active site, providing a means of reversible inactivation of the phosphatase as well as protecting the active site cysteine from further oxidation [88]. It would be of interest to observe structural changes in response to oxidative stress, which could complement the studies done in vitro (and currently underway HDX experiments) in showing dynamic conformational changes of hYVH1 upon exposure to an oxidative environment.

Although a crystal structure of hYVH1 has not been obtained from initial trials, a low-resolution three-dimensional structure of full length hYVH1 was acquired via small-angle X-ray scattering (Figure 3.21). While structures have been solved for several of the PTPs, this study presents the first three-dimensional structure obtained for any YVH1 orthologue. This structure reveals a monomeric protein and a relatively linear domain arrangement, with no significant overlap between the phosphatase and zinc-binding domains under normal buffer conditions. This supports the observation that the zinc-binding domain does not affect catalytic activity of hYVH1 in vitro.

Details concerning specific residues are not resolved by SAXS, however global structural rearrangements in response to environmental effects can be readily observed using this method. Therefore, structural changes in hYVH1 in response to oxidative stress or zinc ejection may be observed if the changes are substantial enough to be detected with the 10-20 angstrom resolution afforded by SAXS [89]. This same principle may be applied to the phosphomimetic mutants of hYVH1 to observe universal structural rearrangements of these mutants compared to wild type hYVH1. Furthermore, SAXS may yield valuable information concerning the mechanism of DMSO-stimulated
activation of hYVH1, which could complement any information obtained by the previously proposed HDX experiments [57].

Because of the flexible N-terminal tail of hYVH1 revealed in the SAXS structure and limited proteolysis experiments (Figures 3.22-3.23), it is possible that this region may inhibit crystallization of hYVH1. Although constructs excluding this flexible region were designed, these mutants were predominantly insoluble (in the case of D46 hYVH1), or showed significant loss in activity (in the case of E29 hYVH1) (Figure 3.24). In the event that a crystal structure is obtained, it is still unlikely that this flexible N-terminus will be resolved by X-ray crystallography. Therefore, structures obtained using SAXS may help to fill in the missing gaps corresponding to these flexible regions in addition to validating any structural information obtained by X-ray crystallography. Additionally, efforts to purify protein for NMR are underway. Although analysis of large proteins by NMR is extremely complex, this technique has the advantage of obtaining high resolution structures of proteins in solution, without the added challenge of obtaining viable crystals.

Surface entropy reduction mutants have been designed and purified, and crystallization trials are currently underway at Cornell University (Figures 3.25, 3.26). Although four of the five SER mutants retained catalytic activity toward DiFMUP, SER2 hYVH1 exhibited very poor phosphatase activity (Figure 3.27). This is likely due to the proximity of this mutation to the active site residues. In DSPs for which the structure has been solved, the region corresponding to that in which the SER2 mutation resides is part of a random coil which lies in close proximity to the active site [87]. This mutation could perturb some secondary structure near the active site, compromising the catalytic capacity
of hYVH1. Despite the decline in activity, this mutant, along with the other SER mutants, is currently undergoing crystallization attempts.

4.10 Concluding Remarks

YVH1 is a highly conserved DSP present in species ranging from yeast to humans. YVH1 has no known homologues, and in humans, hYVH1 is expressed in a variety of cell types, suggesting that this unique phosphatase fulfills a fundamental cellular function. While hYVH1 seems to play a role in various biological pathways, it remains unclear as to the specific mode of action of hYVH1 as well as the relationship between its observed physiological roles.

To date, hYVH1 has been implicated in cell survival and cell cycle progression. In this study, the association between hYVH1 and the 60S ribosomal subunit is displayed, implicating hYVH1 in maturation of the 60S particle in human cells. Further, this study suggests that hYVH1 plays a separate role in cell cycle progression and ribosome biogenesis. Importantly, this dynamic protein is overexpressed in various cancer tissues, in particular late stage metastatic tumours. This expression pattern is significant since hYVH1 plays a role in cellular pathways which are often upregulated during carcinogenesis and therefore hYVH1 may be considered a putative oncogene [30]. Thus, much emphasis will be placed on the detailed characterization of the function of hYVH1 in ribosome biogenesis, cell cycle progression and its role in tumour growth/survival. Furthermore, investigation into the molecular mechanism by which cellular stress triggers dissociation of the hYVH1:ribosome interaction, may yield critical
information as to how hYVH1 regulates these essential cellular processes under normal and disease conditions.

To better understand the mode of action of hYVH1 and the relationship between its various alleged biological roles, it is important to elucidate the structural features necessary for protein function. In the future, HDX and complimentary SAXS experiments in which hYVH1 is subjected to a range of conditions may reveal structural rearrangements that allow for regulation of this redox-sensitive, zinc binding DSP. Moreover, despite the various attempts at hYVH1 substrate discovery, its substrate remains unidentified. Analysis of hYVH1 by X-ray crystallography or NMR may yield invaluable insight into structural features that confer substrate specificity, essential details that may assist in establishing future directions for substrate identification and enzyme inhibition.
Supplementary Figure 1: Co-fractionation of Endogenous hYVH1 in Response to TBH Treatment. To complement the studies done using overexpressed flag-hYVH1, the effect of TBH treatment on co-fractionation of endogenous hYVH1 was investigated. Untransfected HEK 293 cells were treated with indicated concentration of TBH for 4 hours and lysed. Lysates were sedimented through a 10-50% sucrose gradient. Fractionated lysates were displaced via a 60% sucrose chase solution and absorbance at 254nm was monitored to mediate fraction collection (as described in section 2.3). Lysates and fractions were resolved via SDS PAGE and immunoblotted using anti-hYVH1 and RPL26 antibodies. Similar to overexpressed hYVH1, endogenous hYVH1 levels decrease in the 60S and 80S fractions in response to increasing concentrations of TBH. Although the cleavage fragment observed with TBH treatments of cells overexpressing flag-hYVH1 is not apparent in the case of endogenous hYVH1, it is likely that the sensitivity of the anti-hYVH1 immunoblot is insufficient to detect such a cleavage fragment.
Supplementary Figure 2: Approach to Elucidate Mechanism of Disruption of hYVH1:Ribosome Interaction in Response to TBH. HEK 293 cells overexpressing flag-hYVH1 were treated with various concentrations of TBH for 4 hours (as described in section 2.2). Cells were washed in ice cold, degassed PBS and subsequently lysed in degassed lysis buffer (50mM TrisHCl pH 8, 150mM NaCl, 1% Triton-X100, 0.1% SDS), containing protease inhibitors (PMSF, EDTA and aprotinin), catalase (0.1mg/mL) and iodoacetamide (50mM). Lysates were incubated for 1 hour at room temperature under steady argon flow to allow IAM-alkylation of all reduced thiols. Following incubation, lysates were cleared by centrifugation (16000xg, 5 minutes), and cleared lysates were desalted (Zeba Spin Desalting Columns, Pierce Biotechnology), as per manufacturer’s protocol. Desalted lysates were then supplemented with 5mM DTT and incubated for 30 minutes at room temperature to allow for reduction of all reversibly oxidized thiols. A second desalting step was performed to eliminate excess DTT. Lysates were then supplemented with 1.14mM EZ-Link® Iodoacetyl-LC-Biotin (Pierce Biotechnology) and incubated at room temperature with vigorous agitation for 90 minutes. Protein concentrations were normalized by Bradford assay and 1mg of protein was loaded onto 20μL of equilibrated anti-flag agarose. Lysate/agarose slurries were incubated for 3 hours, washed, and eluted as previously described (see section 2.4 for immunoprecipitation protocol). [Protocol and figure modified from reference 82]
Supplementary Figure 3: Attempt at Elucidating Mechanism of TBH-Induced hYVH1:Ribosome Dissociation. HEK 293 cells overexpressing flag-hYVH1 were treated with 0µM, 500µM, or 2mM TBH for 4 hours. Cells were lysed (lysates pre-biotin) and reversibly oxidized thiols were biotin-labelled (lysates post-biotin) as outlined in Supplementary Figure 2. Lysates were then incubated on an anti-flag conjugated resin and immunoprecipitation (αflag IP) performed as described in section 2.4. (A) Lysates and immunoprecipitates were immunoblotted using anti-flag antibody. The presence of hYVH1 in lysates and IP lanes demonstrates successful overexpression and enrichment of hYVH1, respectively. (B) Lysates prior to biotinylation, lysates following biotinylation, and anti-flag IPs were probed using HRP-conjugated streptavidin (Pierce Biotechnology), allowing for the detection of biotinylated proteins.
Supplementary Figure 4: RPL26 Does Not Co-Immunoprecipitate with flag-CT3 hYVH1. HEK 293 cells were left untransfected, transfected with flag-hYVH1 or transfected with flag-CT3 hYVH1. Cells were lysed and immunoprecipitation were performed using anti-flag agarose as described in section 2.4. Lysates and immunoprecipitates were immunoblotted (IB) with anti-flag and anti-RPL26 antibodies. Although flag-hYVH1 and flag-CT3 hYVH1 were successfully immunoprecipitated (upper panel), RPL26 does not co-enrich with flag-CT3 hYVH1 (lower panel).
References


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Vita Auctoris

Name: Colleen Mailloux

Place of Birth: Windsor, Ontario, CA

Year of Birth: 1985

Education: St. Thomas of Villanova Secondary School, LaSalle, Ontario, CA

1999-2003

University of Windsor, Windsor, Ontario, CA
2004-2008 B.Sc.

University of Windsor, Windsor, Ontario, CA
2008-Present M.Sc