Exploring the Impact of Src-Directed Phosphorylation on hYVH1’s Intracellular Function

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Exploring the Impact of Src-Directed Phosphorylation on hYVH1’s Intracellular Function

By

Griffin Lotze

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

2023

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Exploring the Impact of Src-Directed Phosphorylation on hYVH1’s Intracellular Function

by

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May 12, 2023
DECLARATION OF CO-AUTHORSHIP / PREVIOUS PUBLICATION

I. Co-Authorship

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

Chapter 3 of the thesis includes the outcome of publications which have the following other co-authors: DaDalt AA, Bonham CA, Luiso AA, and Vacratsis PO. In all cases only my primary contributions towards these publications are included in this thesis and include mainly supplemental supporting data developed to satisfy reviewer concerns. The main findings of the paper were contributed primarily by co-authors DaDalt AA and Bonham CA and the work included in this thesis acts to support their discoveries by expanding on their original findings utilizing different techniques/approaches. The contribution of co-author Luiso AA was in also providing additional supplementary data independent of the work included in this thesis. Co-author Vacratsis PO contributed through providing assistance in experimental design and data analysis.

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Human hYVH1, also referred to as DUSP12, is a dual specificity protein tyrosine phosphatase that is highly conserved across species and has been demonstrated to be overexpressed in various cancerous states.\(^1\)–\(^4\) Prior investigation of hYVH1 has implicated it in numerous cellular functions including stress response, cell survival, and cell cycle modulation.\(^5\)–\(^7\) Recently, novel evidence has suggested that hYVH1 also plays a role in ribosome biogenesis, functioning in the maturation of the pre-60S ribosomal subunit by mediating its nuclear export and the ejection of Mrt4.\(^8\)\(^,\)\(^9\) Additionally, the well characterized tyrosine kinase Src has been suggested to trigger hYVH1 to localize into the nucleus through strategic phosphorylation, but this event has not yet been confirmed directly through \textit{in vitro} studies.\(^10\)

In this study, we unambiguously demonstrate that Src kinase can directly target hYVH1 for phosphorylation at the critical phosphorylation site Tyr179 through the development of an \textit{in vitro} kinase assay and mass spectrometry. Further investigation into the impact of the Src-directed phosphorylation event suggested that the post-translational modification of hYVH1 triggers a decrease in hYVH1’s catalytic activity as well as an increase in hYVH1’s ribosomal biogenesis capabilities. Additionally, application of a mild hydrogen peroxide induced oxidative stressor led to the alteration of hYVH1’s ribosome biogenesis function and its ability to bind with Hsp70, a known binding partner.\(^5\) Collectively, these findings led us to propose that Src-directed phosphorylation of unbound hYVH1 is necessary for proper ribosome biogenesis to occur and that mild oxidative stress can modulate this by altering the ability of Src kinase to attenuate the formation of the hYVH1-Hsp70 complex.
ACKNOWLEDGEMENTS

This thesis is the culmination of over two years of work that would not have been possible without the help of numerous peers and colleagues. I would like to start by firstly acknowledging my thesis supervisor, Dr. Panayiotis Vacratsis, for his continued support and instruction over the course of this project. Not only was Dr. Vacratsis’s help instrumental in simply completing this thesis by providing the guidance necessary to conduct the research and interpret the data included in this paper but also in advancing my understanding of the field of biochemistry as a whole. His experience and knowledge of science goes well beyond his own area of research and greatly enhanced my appreciation for and my expertise in the field of scientific research. It was a pleasure to work with him and learn from him.

I would also like to give my deepest gratitude to the entirety of the Vacratsis lab. No one could have asked for a better group of colleagues to work and learn with, and I am confident when I say that the completion of this project would have more difficult and significantly less enjoyable without them. Dr. Ashley “Lab Mom” DaDalt, while she graduated from our lab early into my degree, was still always around to provide guidance and friendship. She was directly responsible for the advancement of my competence within the lab, and I could not have asked for a better teacher to help me develop my abilities. Adrian Luiso, one of the best friends a guy could ask for, was there in the trenches with me the entire way. Due to the similarity of our research, we were able to work together to solve problems and help each other with experiments. He was always there to provide another perspective on things and to just have fun with. Kaitlyn Hand, the newest member of the Vacratsis lab graduate family, was also a great friend. Her outgoing nature made it easy to get to know her and it was a pleasure to experience graduate school with her. I would also like to acknowledge the various undergraduate members we had
over the years. From Alex Colak and Lauren Pupulin to our newer members Lithmi Jayasinghe and Grace Querbach. They were always fun to talk with and it made the day more enjoyable when they were around.

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Figure 3.11 - Puromycin incorporation of cells differentially over-expressing hYVH1 and Hsp70. A) Immunoblots were first generated by probing cell lysates of HeLa cells expressing FLAG-hYVH1 and His-Hsp70 with an α-Puromycin antibody to detect any changes in puromycin incorporation that were occurring between samples. The resultant blots illustrated an
increase in incorporation in both singly transfected samples (lanes 2 and 3), however, co-expression resulted in enhanced band intensities (top). Control blots were also produced to control for protein expression levels by probing the blots using α-FLAG (top-middle), α-His (bottom-middle), and α-Actin (bottom) antibodies. B) A scatterplot was generated to visualize the trends occurring due to overexpression of either DNA construct. The Western blot band intensities were normalized to actin levels and plotted based on their relative fold increases when compared to the EV control. All experimental samples illustrated an increase in puromycin incorporation with hYVH1+Hsp70 showing the most intense levels.

**Figure 3.12 – Src-mediated phosphorylation leads to dissociation of the hYVH1-Hsp70 protein complex.** HeLa cells prepared by co-expressing FLAG-hYVH1 and His-Hsp70 were compared to cells triple transfected with FLAG-hYVH1, His-Hsp70, and Src following a FLAG-IP. Subsequent immunoblotting using both an α-FLAG and α-His antibody showed that when co-expressed with Src kinase, hYVH1 levels following the IP were unaltered, but Hsp70 levels were drastically lower (top). Further analysis using an α-pTyr antibody showed that in the presence of Src, bands appeared at the molecular weights of ~70kDa and ~37kDa (second from top). Control blots were also produced to control for protein expression levels by probing the immunoblots using α-FLAG (third from top), α-His (second from bottom), and α-Actin (bottom) antibodies.

**Figure 3.13 – Src-mediated phosphorylation of Hsp70 is inhibited by low-level oxidative stress.** A) HeLa cells prepared by co-expressing FLAG-hYVH1 and His-Hsp70 were compared to cells triple transfected with FLAG-hYVH1, His-Hsp70, and Src following 2h exposure to a low-level hydrogen peroxide induced oxidative stress and a FLAG-IP. The generated cell lysates were analyzed by Western blot and probed using an α-His antibody. In the presence of Src, significantly less Hsp70 is present, but when the stress is present, the levels of partially rescued (top). Subsequent probing using an α-pTyr antibody showed that the oxidative stress was also altering the phospho-states (second and third blots from top). Control blots were also produced to control for protein expression levels (bottom two blots). B) Normalization of the His band intensities following the IP to FLAG-hYVH1 post-IP and His-Hsp70 protein lysate levels was first conducted to control for expression levels. The resulting relative fold changes for Hsp70
present after IP were graphed and T-testing was conducted. This data visualized a statistically significant decrease in Hsp70 due to Src when stress was absent but saw the loss of that significance when stress was present. C) Normalization of the level of phosphorylation of hYVH1 to FLAG IP levels and Src levels showed a reproducible decline in phospho-hYVH1 under stress, but also had of relatively significant variation in fold change between replicates. D) Normalization of the level of phosphorylation of Hsp70 to His-Hsp70 post-IP levels and Src levels showed a statistically significant decrease in phosphorylation of Hsp70 under stress conditions.

Figure 4.1 – Current working model of hYVH1’s function cellular function. A) Under basal cell conditions, the hYVH1-Hsp70 complex forms and localizes to the perinuclear region. Upon arrival, Src-mediated phosphorylation of Hsp70 leads to dissociation of the complex and Src-directed phosphorylation of hYVH1 at Tyr179 triggers it to shuttle into the nucleus. Once in the nucleus, hYVH1 binds the pre-60S ribosomal subunit aiding in nuclear export. Following export, hYVH1 induces the ejection of Mrt4 from the subunit allowing Rpp0 to replace it and leading to ejection of hYVH1. The hYVH1-Hsp70 complex then reforms allowing hYVH1 to be recycled back to the perinuclear region for another round of ribosome biogenesis. B) Under mild oxidative stress, some of the hYVH1-Hsp70 complex dissociates allowing free Hsp70 and free hYVH1 to participate in independent stress response pathways. The remaining complexes localize to the perinuclear region where Src-mediated dissociation of the complex still occurs, but to a much lesser extent. The un-dissociated complexes are then able to function in stress response.
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>DUSP</td>
<td>Dual Specificity Phosphatase</td>
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<tr>
<td>DUSP12</td>
<td>Dual Specificity Phosphatase 12</td>
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<tr>
<td>hYVH1</td>
<td>Human Yeast Vaccina Homolog 1</td>
</tr>
<tr>
<td>phYVH1</td>
<td>Phospho-Human Yeast Vaccina Homolog 1</td>
</tr>
<tr>
<td>W.T.</td>
<td>Wild type</td>
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<td>Post Translational Modification</td>
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<td>pTyr</td>
<td>Phospho-Tyrosine</td>
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<tr>
<td>mRNP</td>
<td>messenger ribonucleic particle</td>
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<tr>
<td>YB-1</td>
<td>Y Box-1</td>
</tr>
<tr>
<td>FMRP</td>
<td>Fragile X Mental Retardation Protein</td>
</tr>
<tr>
<td>CML</td>
<td>Chronic Myelogenous Leukemia</td>
</tr>
<tr>
<td>SUnSET</td>
<td>Surface sensing of translation</td>
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<tr>
<td>DiFMUP</td>
<td>6,8-difluoro-4-methylumbelliferyl phosphate</td>
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<tr>
<td>DiFMU</td>
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<tr>
<td>HDMSE</td>
<td>High definition mass spectrometry</td>
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<td>DIA</td>
<td>Data independent acquisition</td>
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<td>SRM</td>
<td>Select-ion reaction monitoring</td>
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<td>Hck</td>
<td>Hemopoietic cell kinase</td>
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<td>SFK</td>
<td>Src Family Kinase</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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CHAPTER 1:
INTRODUCTION

1.1 Post-Translational Protein Modifications: A Brief Overview of Protein Phosphatases

1.1.1 Signal Transduction Cascades: A Critical Element of Cellular Function

Signal transduction is a critical mechanism used by the cell to respond to extracellular stimuli in order to maintain homeostasis and adapt to the dynamic cellular environment. \(^{17}\) Classic receptor mediated signal transduction pathways involve an extracellular ligand binding to and activating a cell-surface transmembrane receptor, often by inducing oligomerization and conformational alterations in their intracellular domains. \(^{17}\) This activating event results in strategic changes such as the phosphorylation of Tyr residues within the intracellular domains of the receptor that serves as recruitment signals for a variety of signaling proteins containing phosphotyrosine binding domains. \(^{18}\) Subsequently, this leads to a series of protein-protein interactions and post-translational modification events that activate downstream protein kinase cascades. \(^{18}\) The activation of these signaling cascades can lead to diverse changes in cellular function often through an alteration of gene expression and regulatory pathways in order to properly respond to the original extracellular signal. \(^{17,18}\)

One of the central tenets of signal transduction pathways is the direct modification of intracellular proteins through post-translational modifications (PTMs). PTMs can take many forms including ubiquitination, acetylation, and methylation to name a few. \(^{18}\) However, the most prevalent PTM is thought to be reversible phosphorylation of Ser, Thr, and Tyr residues. \(^{19,20}\) Two enzyme superfamilies that mediate cellular phosphorylation are the protein phosphatases and the protein kinases which act to mediate the de-phosphorylation and phosphorylation of other proteins respectively. Due to the prevalence of reversible phosphorylation in cell biology, it
is logical that a sizable portion of the human genome is comprised of kinase encoding genes
(>500) and phosphatase encoding genes (>200)\textsuperscript{12,21} Moreover, due to phosphorylation’s integral
function in maintaining cellular homeostasis, the activities of kinases and phosphatases are
tightly regulated. Breakdown of this regulation leads to aberrant phosphorylation events that
results in cellular dysfunction and innumerable human disorders\textsuperscript{12,21} Therefore, it is imperative
to comprehensively understand the mechanisms that regulate kinases and phosphatases.

1.1.2 Protein Phosphatases

The protein phosphatase superfamily consists of a class of enzymes that catalyze the
removal of a phosphate moiety from a diverse range of biomolecules, including proteins, lipids,
and carbohydrates\textsuperscript{12,22,23} They are the direct antagonists of protein kinases and are often
identified to function within the same pathways as many prominent kinases. Until relatively
recently, many scientists considered phosphatases as “housekeeping” proteins that displayed
minimal specificity and as such were not highly regulated. As such, the focus of much of the
scientific community was on kinases due to their prominent role in many disease states, namely
cancer\textsuperscript{12} Since the late 20\textsuperscript{th} century and early 21\textsuperscript{st} century however, it has become clear that
phosphatases are highly specific and regulated by analogous mechanisms as kinases.
Additionally, the pharmaceutical industry is beginning to appreciate the potential of targeting
phosphatases in human disease including metabolic disorders, neurodegenerative diseases, and
cancer\textsuperscript{24,25}
Figure. 1.1 – Categorical classification of the human phosphatase superfamilies. The human genome encodes for more than 200 phosphatases than can be divided into two main superfamilies.\textsuperscript{11,12} Further subdivision of each superfamily is possible based on structural similarity and mechanisms of action.

Protein phosphatases can be broadly classified based on the phosphorylated biomolecules they specifically target and by the enzymatic mechanisms they employ.\textsuperscript{11,12,22} Using this classification method, two main superfamilies exist being the Serine/Threonine Phosphatases (STPs) and the Protein Tyrosine Phosphatases (PTPs) (Fig. 1.1). As their names imply, STPs remove a phosphate moiety from either serine or threonine amino acid residues using a metal-based catalyst and distinct regulatory subunits to achieve specificity.\textsuperscript{18,19} The PTP family is
structurally and mechanistically distinct from the STPs and remove phosphate groups predominately from tyrosine residues using a cysteine-based mechanism.\textsuperscript{11,12,19}

PTPs can be further classified into 4 main subfamilies based on the structural similarity of their catalytic domains. Class I PTPs make up the largest subgroup containing 111 of the \textasciitilde 125 known PTP phosphatases and are all cysteine-based similar to classes II and III.\textsuperscript{11} Further classification of these PTPs results in the dual specificity phosphatase (DUSP) group which consists of enzymes that can dephosphorylate not only tyrosine, but serine and threonine residues as well.\textsuperscript{26,27} Within this group also exists the atypical DUSPs, which are a relatively poorly characterized subfamily of dual specificity phosphatases that lack the structural motifs characteristic of other DUSP subfamilies.\textsuperscript{23}

Of the \textasciitilde 200 phosphatases discovered in the human genome, over half of these proteins are classified as PTPs and as such act to dephosphorylate tyrosine residues.\textsuperscript{11,23} Despite the majority of protein phosphatases being classified as PTPs, tyrosine phosphorylation is regarded as the rarest form of phosphorylation with serine followed by threonine being the most common.\textsuperscript{20} Despite the relatively rare nature of tyrosine phosphorylation, it tends to play major roles in many signaling pathways with receptor tyrosine kinases often being critical components of cascade pathways.\textsuperscript{28} The relatively low levels of tyrosine phosphorylation coupled with the relatively large number of PTPs suggest that cells require precise regulation of these phosphorylation events, signifying the importance of PTPs in the proper function of the cell.
PTPs are predominately characterized by the existence of a so-called CX_{5}R motif, with the exception of class IV PTPs.^{22,23} N-terminal to the CX_{5}R motif is the WPD-loop which contains a catalytic Asp residue that participates in the dephosphorylation mechanism.^{23}

The catalytic mechanism of PTPs is a highly conserved process in which the aforementioned motifs and an ordered water molecule collaborate to remove a phosphate molecule from the target substrate (Fig. 1.2). The first step in the phosphatase mechanism is the entering of the substrate into the binding pocket of the enzyme. This process is coordinated by the conserved Arg residue, who’s positively charged side chain forms electrostatic interactions with the negatively charged oxygen molecules of the phosphate group properly orienting the substrate and neutralizing some of the negative charge. A conserved Histidine also plays an important role in the formation of the active site’s unique microenvironment by decreasing the
pKa value of the sulfur atom on the catalytic cysteine resulting in it possessing a default state of deprotonation. Once the substrate has entered the active site, the catalytic cysteine’s sulfur launches a nucleophilic attack on the phosphorus atom forming a covalent thiol phosphate intermediate. The conserved aspartic acid of the WPD-Loop donates a proton to the side chain of the Tyr residue thus regenerating the residue and allowing the dephosphorylated product to be ejected from the active site. An ordered water molecule, coordinated by an invariant Gln residue is deprotonated by the same Asp residue in a catalytic base reaction to generate a hydroxyl nucleophile that regenerates the catalytic Cys residue via a nucleophilic attack on the phosphorus atom. The final product is a dephosphorylated product, a free inorganic phosphate, and a regenerated PTP enzyme.

1.2 DUSP12 and Its Importance in Various Cellular Function

1.2.1 A Brief History of DUSP12

Human YVH1, also known as DUSP12 is an enzyme that belongs to the protein tyrosine phosphatase superfamily. After its discovery in 1999, it was further subclassified as a class I cysteine-based phosphatase, more specifically an atypical DUSP.\(^1\) The first discovered DUSP that was described as a Tyr/Ser phosphatase was the vaccinia virus phosphatase VH1 in 1991 by Kun-Liang Guan and Jack Dixon.\(^2\) Due to VH1 being the first identified DUSP, the DUSP subfamily is often also referred to as the VH1-like subfamily. Not long after the discovery of VH1, the first eukaryotic DUSP was discovered in yeast and subsequently christened as YVH1 (Yeast VH1). YVH1 was discovered to play a significant role in the development of a “slow growth” phenotype in yeast as the knockout of the \(yvh1\) gene resulted in a significant increase to yeast doubling time.\(^3\) These findings spurred an interest in YVH1 and the human ortholog was
soon discovered being coined DUSP12 or hYVH1 (human-YVH1).\textsuperscript{1} YVH1 is highly conserved throughout the eukaryote kingdoms suggesting YVH1 orthologs play a fundamental role in cell biology. Furthermore, a surprising lack of evolutionary complexity has been observed as only one YVH1 family member is present in all species examined, including humans, suggesting there is evolutionary pressure to limit the level of YVH1 activity in the cell.\textsuperscript{5–7,31} It is of interest to note that amplification of the \textit{hyvh1} gene or overexpression of the protein has been recorded in many late-stage cancers.\textsuperscript{32,33}

1.2.2 Structural Features of hYVH1

The \textit{hyvh1} gene is located on chromosome 1 at Cytoband q21-q22 and encodes a 340 amino acid long protein that is comprised of two domains connected by a short linker region (Fig. 1.3).\textsuperscript{1}

![Figure 1.3 – Structural Architecture of hYVH1.](image)

The catalytic DUSP domain is located in the N-terminus of the protein. This domain contains the characteristic HCX\textsubscript{3}R motif that is characteristic of PTPs and is the domain required for DUSP12 to dephosphorylate its substrate molecules. This domain encompasses amino acid residues 1-175 with the crucial catalytic motif located at amino acid residues 114-121. The
conserved Asp residue in the WPD-loop can be located at residue 84 and has its R-group extending into the active site.

The C-terminal domain is a unique zinc-binding domain and YVH1 orthologs are currently the only known phosphatases to possess such a domain. The zinc-binding domain is 120 amino acids long encompassing residues 220-340. It is connected to the N-terminal catalytic domain by a linker region of approximately 45 amino acids. The zinc-binding domain is coordinated by two zinc ions and is predicted to adopt a "criss-cross" formation whereby the first and third Cys pairs coordinate one zinc ion while the second and fourth coordinating residues associate with the second zinc ion. Interestingly, the C-terminal zinc-binding domain has also been proposed to function as a redox sink capable of protecting hYVH1 from oxidative inactivation of the catalytic domain. Under reducing conditions, these cysteines are coordinated by the two zinc molecules. However, under oxidating conditions, the zinc atoms can be ejected allowing the coordinating Cys residues to be preferentially oxidized before the catalytic Cys residue, preventing oxidative inactivation of the phosphatase. As oxidative conditions become more severe and the C-terminal domain reaches its upper limit of oxidative protection, it was observed that the catalytic active site cysteine can form a disulfide bond with the N-terminal proximal cysteine resulting in temporary inactivation of the enzyme but preventing irreversible oxidation of the active site. The ability of hYVH1 to protect itself from oxidative inactivation through a mixed disulfide mechanism has been demonstrated to result in a reduction to activity loss under oxidative stress conditions compared to other Cys based phosphatases. Furthermore, the ability to restore catalytic activity during stress recovery conditions suggests hYVH1 may represent a novel redox sensor during the cell stress response.
1.2.3 Cellular Functions Regulated by hYVH1

As previously mentioned, the atypical dual specificity phosphatase hYVH1 is an evolutionary conserved enzyme possessing an N-terminal phosphatase domain and a unique C-terminal zinc binding domain. Although the precise mechanistic role of hYVH1 remains poorly characterized, recent evidence from our laboratory and others suggests that hYVH1 may be a critical mediator of ribosome biogenesis, protein translation, cell cycle progression, and cell survival.5–7,10

Initial studies on the yeast ortholog discovered that YVH1 plays a key role in the growth of yeast. Knocking out the yvh1 gene in yeast resulted in a stark increase to the doubling time of the affected yeast cells. While the cells were still able to survive and grow, a “slow-growth” phenotype was observed.30 Curiously, when the human ortholog was expressed in these same cells, the slow-growth phenotype was rescued. A more recent study identified YVH1 as a novel ribosome biogenesis factor in yeast and implicated it in proper maturation of the pre-60S ribosomal subunit. When YVH1 is absent in cells, a decrease in mature 60S and 80S subunits and an increase in 40S subunits is observed.8,9,34 YVH1 associates with the nuclear pre-60S subunit, and this interaction is required for the dissociation of the ribosomal trans acting factor Mrt4 from the subunit. The dissolution of Mrt4 is a critical step in the maturation process of the 60S subunit and leads to the formation of the translationally competent 80S ribosome. Knock out of the yvh1 gene in yeast led to a defective nuclear export mechanism resulting in the accumulation of pre-60S subunits in the nucleus, and an increase in ribosomal half-mers which are all indicative of a defective ribosome biogenesis pathway. Additionally, the absence of YVH1 also appeared to result in a failure of Mrt4 to dissociate which typically occurs in the final stages of pre-60S maturation.8,9
Investigation of hYVH1 discovered that similar to the yeast ortholog, hYVH1 displays both nuclear and cytoplasmic localization and the ability to associate with the 60S ribosomal subunit.\textsuperscript{7–10} Interestingly, much of the observed cellular activities attributed to hYVH1 are mediated by the presence of its unique C-terminal zinc-binding domain as mutational studies of the protein have found that this domain is often sufficient and required for the majority of hYVH1’s identified functions. One of the earliest mutation studies found that the zinc-binding domain is absolutely required for the ability of hYVH1 to rescue yeast from their slow-growth phenotype but, interestingly, the catalytic activity of hYVH1 is not.\textsuperscript{1}

Despite hYVH1’s classification of a phosphatase, it exhibits very low enzymatic activity against exogenous substrates \textit{in vitro} compared to prototypical DUSPs such as VHR.\textsuperscript{31} The reason behind this low level of activity is unknown, however, cellular substrates of YVH1 have not been confidently elucidated suggesting that YVH1 orthologs may be highly specific to their target substrates, and/or require a cellular environment for maximum catalytic activity. Mutation studies of hYVH1 have also suggested that while the zinc binding domain is required for proper \textit{in vivo} function of the protein, the presence or absence of the entire C-terminal domain has no significant effect on the phosphatase activity against artificial substrates \textit{in vitro}.\textsuperscript{1} This suggests that the zinc-binding domain likely functions as a tether bringing the catalytic domain into proximity of its intracellular substrate under physiological conditions.

In spite of hYVH1’s apparently poor phosphatase function, the protein has still been implicated in numerous critical cellular mechanisms. Expression levels of hYVH1 was revealed to impact the cell cycle identifying it as a novel modulator of cellular DNA content.\textsuperscript{6} When hYVH1 was overexpressed in cells, a decrease in the G0/G1 population and an increase in the G2/M population was observed. Additionally, polyploidy cells due to a significant increase in
multinucleated cells confirmed by fluorescent microscopy was also documented. Reciprocal studies using siRNA to knock down hYVH1 saw the opposite effects with an increase in cells in the G0/G1 stages and a decrease in cells in the S/G2/M stages along with a decrease in multinucleated cells. Subsequent mutation studies identified the zinc-binding domain as the portion of hYVH1 critical for its role as a novel modulator of DNA and suggested that the catalytic phosphatase domain is not required for this function. The discovery of hYVH1’s function as a novel modulator of cellular DNA content serves to signify the importance of the protein and spurred further investigation into the physiological function of the protein.

In an attempt to further elucidate the functions of hYVH1, immunoprecipitation and mass spectrometric analysis were used to probe the cellular proteome for novel binding partners. Heat Shock Protein 70 (Hsp70) was identified as the first novel binding partner of hYVH1 in human cells. Hsp70 is a well characterized molecular chaperone and as such, plays a significant role in protein folding and cell survival. Mutation studies of both proteins revealed that Hsp70’s ATPase domain directly binds with hYVH1 highlighting that the interaction is not simply due to the molecular chaperone properties of Hsp70, but rather a functional protein-protein interaction. Human YVH1’s zinc-binding domain was also suggested to be critical in this interaction, however, the results were inconclusive as to the requirement of the catalytic domain. Recent research by our lab, however, has suggested the extreme termini of both of hYVH1’s domains bind to the ATPase domain of Hsp70 (unpublished). Subsequent cell survival assays exposing cells to various cellular stresses including heat shock and hydrogen peroxide induced oxidative stress demonstrated hYVH1’s ability to protect cells resulting in a reduced number of cells undergoing apoptosis. Co-expression of hYVH1 and Hsp70 resulted in the amplification of hYVH1’s cell survival function suggesting that hYVH1 and Hsp70 may form a novel protein-
protein complex that can enhance the cell’s capacity to resist stress induced cell death. Importantly, mutation studies of the protein domains critical for hYVH1’s ability to protect the cell from oxidative stress have demonstrated that the zinc-binding domain was required. Interestingly, catalytic activity was also required as substitution of the nucleophilic Cys residue attenuated the ability of hYVH1 to protect cells from oxidative stress.

Follow-up studies exploring hYVH1’s role during stress response elucidated a novel role in stress granule disassembly during stress recovery within human cells. Knock down studies showed that in the absence of hYVH1, stress granules failed to be disassembled properly and were significantly larger than when hYVH1 was present. Interestingly, siRNA resistant variants of hYVH1 could rescue this phenotype. In contrast, increased hYVH1 levels resulted in association with stress granules and accelerated stress granule disassembly. Being that stress granules are formed during stress as a specialized messenger ribonucleoprotein particle (mRNPs) to protect cellular assets and slow translation as a means of energy conservation, mass spectrometry analysis was utilized to probe hYVH1 for novel ribonucleoprotein binding partners. This analysis identified the 60S ribosomal complex as well as the RNP modulators Y Box-1 (YB-1) and Fragile X Mental Retardation Protein (FMRP) as novel hYVH1 associating molecules. YB-1 and FMRP are two RNP associating proteins known to function in regulating transcription, mRNA processing, translation, and, under stress, it was observed that hYVH1 co-localised to stress granules with these molecules. Unsurprisingly, the zinc-binding domain was shown to coordinate the interaction between hYVH1 and the various RNPs as well as being a critical component in stress granule disassembly.

With the novel discovery that hYVH1 can associate with various RNPs including the 60S ribosomal subunit and the previously established findings that the yeast ortholog can function in
60S ribosomal subunit maturation, our lab was interested in mechanisms regulating hYVH1 ribosomal association. We were thus intrigued when during our interactome study we acquired preliminary evidence that Src kinase, a well-known oncogene, was detected in hYVH1 interactome isolations. Investigation of this interaction resulted in the discovery that co-expression of Src in HeLa cells led to the phosphorylation of hYVH1 at amino acid residue Tyr179.  

Figure 1.4 – Src-mediated localization of hYVH1 and the impact on ribosomal structures. A) IFA analysis of the cellular localization patterns of hYVH1 showing that in the presence of Src kinase, hYVH1 depicts a majority nuclear localization pattern. B) Ribosomal profiling of HeLa cells following ribosomal fractionation using a 40% sucrose gradient. An increase in monosome and polysomes coupled to a decrease in disomes can were identified in cells co-expressing hYVH1 and Src. Figure from DaDalt AA, Bonham CA, Lotze GP, Luiso AA, Vacratsis PO. Src-mediated phosphorylation of the ribosome biogenesis factor hYVH1 affects its localization, promoting partitioning to the 60S ribosomal subunit. J Biol Chem. 2022 Dec;298(12):102679.
Subsequent analysis of the importance of this phosphorylation event led to the discovery that phosphorylation of this site has a significant impact on the localization of hYVH1, shifting it from a predominantly cytoplasmic localization to more nuclear localization (Fig. 1.4, A). It was suggested that Src-mediated phosphorylation of Tyr179 functions to trigger the shuttling of hYVH1 into the nucleus where it participates in late stage pre-60S ribosomal subunit maturation.\textsuperscript{8–10} To support this hypothesis, ribosomal subunits levels were analyzed using a sucrose gradient and it was observed that co-expression of hYVH1 and Src led to a significant increase in the levels of monosomes and polysomes and a decrease in the levels of disomes (Fig. 1.4, B). Quantitative proteomic analysis was performed to attempt to identify protein alterations at the monosome/disome in response to co-expression of Src and hYVH1. An increase in the levels of proteins critical for translational efficiency including EF2, EIF6, and RACK1 was confidently measured. Furthermore, a significant decrease in the protein levels of a number of translational repressors including Nucleolin and YB-1 were observed at the monosome/disome.\textsuperscript{10} Collectively, these findings suggest that co-expression of hYVH1 and Src kinase leads to an increase in the amount of translationally competent ribosomes. This provided some of the first evidence that in addition to ribosome biogenesis, hYVH1 may also positively regulate translational fitness.

1.2.4 Overexpression of hYVH1 in Cancer

With our understanding of cancer continuing to improve due to the attention it gets within the scientific and medical fields, more and more genes are being identified to be aberrantly expressed in different cancer disease states. Genomic screening studies have recently illustrated that the \textit{hyvh1} gene (usually identified as dusp12) is significantly amplified in a variety of late-
stage cancers including malignant schwannomas, dedifferentiated malignant liposarcomas, leiomyosarcomas, chronic myelogenous leukemia (CML), and more recently, retinoblastoma.\textsuperscript{2–4} While \(hyvh1\) was initially suggested as a possible oncogene, further characterization did not support this.\textsuperscript{33} Interestingly, characterization of hYVH1 in CML suggested it as a novel antigen target during donor lymphocyte infusion therapy. As such, hYVH1 holds significant promise for being used as an immunogen for CML vaccination therapy or as a biomarker for monitoring CML. Furthermore, our results showing that hYVH1 can function in cell survival and translation, coupled with \(hyvh1\) being located within a gene region prone to amplification,\textsuperscript{1} tempts us to speculate that its overexpression may impart on these tumour cells the ability to survive unfavourable microenvironments and support metastasis more readily. This suggestion is further supported by the observation that \(dusp12\) regulates tumorigenesis in hepatocellular carcinomas with knockdown of hYVH1 leading to reduced levels of proliferation and migration.\textsuperscript{32}

1.3 The Tyrosine Kinase Src

1.3.1 Cellular Activities of Src

c-Src, also referred to simply as Src, is a non-receptor protein tyrosine kinase that has been the focus of profound scientific research for the better part of the last few decades owing to Src’s significant role in many highly regulated cellular mechanisms and in oncogenesis.\textsuperscript{38,39} Src kinase is the prototypical member of the Src family of kinases and the gene coding for Src, was the first discovered oncogene in a chicken virus with the human ortholog having since been discovered to be mutated in a number of different oncogenic disease states.\textsuperscript{40} The Src protein is ubiquitously expressed in human tissues with cells such as neurons and osteoblasts showing significantly higher basal levels of Src.\textsuperscript{41}
Src has been implicated in a wide variety of critical cellular mechanisms, functioning in cell growth, division, migration, and cell survival. It is unsurprising that for this reason Src is a highly regulated protein and that when mutated, has a significant impact in supporting the progression of various disease states.\textsuperscript{38,40} Src is primarily regulated by phosphorylation and dephosphorylation at two primary sites within its structure. The first site occurs at tyrosine 419 and acts as an activating phosphotyrosine.\textsuperscript{40,42} Tyrosine 419 is located within the activating loop of Src and when it is phosphorylated, it promotes kinase activity. This residue is autophosphorylatable by adjacent Src molecules and is required for proper Src function. The second site is tyrosine 530 which acts in an inhibitory manner when in its phospho-state.\textsuperscript{40,42} The phosphorylation of this site is controlled primarily by the kinase Csk and when in its phospho-state results in the stabilization of the inactive form of the enzyme by forming an intramolecular bond with the SH2 domain. While both sites are critical for Src regulation, Tyr530 has been observed to be the dominant residue in ensuring Src is only active when physiologically required and as such, it has been observed that under basal conditions, 90-95\% of Src is inactivated by the phosphorylation of this site.\textsuperscript{42}

1.3.2 Structural Features of c-Src

Src is encoded for by the \textit{src} gene which is located on chromosome 20 at cytoband q11.23. This gene encodes for a 536 amino acid protein which is divided into four functional domains.
Figure 1.5 - Simplified Structural Schematic of Src Kinase. Src kinase is a well characterized enzyme that contains four functional domains. The poorly understood unique domain, the regulatory SH2 and SH3 domain, and the catalytic kinase domain. Src also contains N-terminal and C-terminal tail groups that function in mediating function. Figure created with BioRender.com.

Starting N-terminally, Src contains a myristoyl group, a unique domain, a SH3 domain, a SH2 domain, the catalytic SH1 kinase domain, and a regulatory tail region (Fig. 1.5). The myristoyl group, functions in cell adhesion facilitating the attachment of Src to target cell membranes prior to phosphorylation. The so-called “unique” domain is an intrinsically disordered region that is used to classify different Src family members. Collectively, the SH3 and SH2 domains primarily participate in target recognition and docking. These two domains recognize specific amino acids sequences providing Src with an increased level of target specificity. The largest domain, being the SH1 domain, functions as the catalytic center of the protein and provides Src with its kinase activity. The final important region of Src kinase, is the C-terminal tail. This region primarily functions in regulation of Src and contains the critical regulatory residue Tyr530.
1.4 Heat-Shock Protein 70

1.4.1 Cellular Activities of Hsp70

Heat Shock Protein 70 (Hsp70) is a well characterized member of the 70-kDa heat shock protein family. In particular, Hsp70 is a ubiquitous molecular chaperone, and has been illustrated to play a critical role in proper cellular function.\textsuperscript{44-46} As a molecular chaperone, Hsp70 primarily functions to assist nascent proteins in proper protein folding. While there are many types of molecular chaperones, the HSP family has been demonstrated to bind exposed hydrophobic regions of misfolded proteins and promote refolding under both basal cell conditions, and during cellular stress.\textsuperscript{44,46} Due to the ability of Hsp70 to promote protein refolding, it has been implicated in several different critical cellular mechanisms including cell survivability and stress response, signal transduction, and protein degradation.\textsuperscript{44-46} Hsp70-dependent folding occurs through the repetition of multiple bind-release cycles. During these cycles, the misfolded protein is brought into Hsp70’s binding pocket, and ATP is hydrolyzed resulting in the “capping” of the binding pocket by the C-terminal lid domain. The release of ADP and rebinding of ATP opens the lid, releasing the protein and allowing the cycle to being again. It is the repeated act of binding and release of the misfolded protein that promotes refolding. Interestingly, despite the reliance Hsp70 has on the hydrolysis of ATP, the intrinsic ability of Hsp70 do this is low. This dramatically limits the functional rate of Hsp70 and has been suggested as a means of regulating the chaperone.\textsuperscript{44,46}

1.4.2 Structural Characteristics of Hsp70

Hsp70 is only one member of the heat shock family of proteins and as such the genes coding for the various members have been mapped to different chromosomes.\textsuperscript{47} The gene coding
for Hsp70 in particular, is located on chromosome 6 at cytoband q21.33 The protein itself is 641 amino acids and is divided into three functional domains (Fig. 1.6).

**Figure 1.6 – Simplified Structural Schematic of Hsp70.** Hsp70 is one member of the larger HSP family of proteins and primarily functions as a molecular chaperone. Hsp70 is comprised of three functional domains with the largest domain, the ATPase domain, participating in the binding and hydrolysis of ATP. The substrate binding domain and lid domain function in protein binding and refolding. Figure created with BioRender.com.

The first domain is the N-terminal ATPase domain and functions in the binding and hydrolysis of ATP which provides the energy required to promote protein trapping by altering the structural conformation of Hsp70. The second domain is the substrate binding domain which contains the binding pocket that misfolded proteins enter and bind within. The final domain is the C-terminal lid domain. The lid domain serves to “cap” the binding pocket and trap the misfolded protein substrate within the binding pocket. For the proper mechanism of action to occur, Hsp70 requires the coordinated action of all three domains and as such has been observed to possess a highly conserved structure across species.44,46

### 1.5 Ribosome Biogenesis and Protein Translation

Protein synthesis is a tightly regulated mechanism, and the cell directs a large quantity of energy and assets to their production.48 Cellular protein synthesis is a complicated multi-step process that follows the central dogma of molecular biology. Genetic information contains the instructions the cell needs to synthesize new proteins and is transcribed by RNA polymerase into
complementary precursor RNA strands within the nucleus. These pre-RNA strands then undergo pre-translational modification in which they are 1) differentially cleaved and recombined depending on the protein they are fated to code for and 2) modified with a 5’-guanine cap and a 3’ poly (A) tail. These pre-translational modifications serve to protect the newly synthesized messenger RNA from degradation and provide the site of attachment to the ribosome. Following this modification, the mRNA is exported from the nucleus to the cytoplasm where it can bind to ribosomes and be translated.

Translation is the process where mRNA is used to synthesize new proteins. During this process, the ribosome reads the mRNA codons and catalyzes the addition of new amino acids to a growing polypeptide chain. The ribosome consists of three sites, 1) the aminoacyl site which receives charged aminoacyl-tRNA molecules, 2) the peptidyl site which contains the growing polypeptide chain, and 3) the exit site where tRNA molecules that are no longer charged are released from the ribosome. Upon completion of the protein, a release factor binds the ribosome, the ribosome disassembles into its corresponding subunits, and the protein is released. Following release, the protein can undergo post-translational modifications such as methylation, ubiquitination, and often phosphorylation and dephosphorylation.

Ribosomes are macromolecular complexes compromising of RNA and protein and play an essential role within the cell. Ribosome biogenesis is a tightly regulated process as the number of translationally competent ribosomes within the cell directly influences protein levels and thus cell growth. Ribosome biogenesis is also one of the most energetically demanding cellular processes. Formation and maturation of the ribosome occurs mainly within the nucleolus and the nucleoplasm, but assembly of the mature subunits occurs primarily in the cytoplasm. The ribosome consists of two major subunits, the 40S subunit and the 60S subunit. Synthesis of these
two subunits requires multiple rRNA molecules, dozens of ribosomal proteins, and >200 transient ribosomal assembly factors, all coordinated by three primary RNA polymerases.\textsuperscript{48–50} RNA polymerase I transcribes the polycistronic 47S pre-rRNA molecule, which is then cleaved into the 25S, 5.8S, and 18S rRNA molecules. RNA polymerase II transcribes the required ribosomal proteins, and RNA polymerase III transcribes the 5S rRNA molecule. Maturation of each subunit is a multi-step process and involves nucleolytic cleavage of the polycistronic 47S pre-rRNA, the combination of the 25S, 5.5S and 5S rRNA molecules, and the recruitment of the various required ribosomal proteins.\textsuperscript{48,49} Following nuclear maturation steps, each subunit is independently exported into the cytoplasm where maturation completes and the 60S and 40S subunits can combine to form a translationally competent 80S ribosome.\textsuperscript{48}

1.5.1 SUnSET: A Means to Qualify Cellular Protein Translation

Due to the importance of protein translation in almost every aspect of cell biology it is important that researchers have a reliable way to quantify the level of translation within the cell. Classically, translational rates were quantified using \textsuperscript{35}S pulse chase radioactive labeling experiments.\textsuperscript{14} However, it was desired by the field to develop non-radioactive methods to monitor translational alterations due to the inherent complexity and risk associated with radioactive techniques. Recently, a new method was developed known as surface sensing of translation (SUnSET).\textsuperscript{15} This technique utilizes the naturally occurring aminonucleoside antibiotic puromycin which is structurally analogous to 3' adenosine of a tyrosyl-tRNA.\textsuperscript{16} This structural similarity can be exploited by exposing growing peptide chains to puromycin resulting in premature termination of the growing polypeptide chain due to the inability of the ribosome to catalyze the removal of the nucleoside (Fig. 1.7). This inability stems from the presence of a
peptide bond rather than an ester bond in puromycin when compared to charged tRNA molecules.
Figure 1.7 – Structural difference between puromycin and tyrosyl-tRNA and its ability to terminate translation. A) Structural schematic visualizing the similarity in molecular structure between tyrosyl-tRNA and puromycin. Puromycin mimics the 3’ adenosine of the Tyr-tRNA but contains a peptide bond between the tyrosine and nucleoside as well as not containing the remaining segment of tRNA. This results in non-specific incorporation into growing peptide chains.\textsuperscript{13} B) Non-specific incorporation of puromycin during protein synthesis results in the transfer of the nascent polypeptide to puromycin resulting in spontaneous dissociation of the ribosome and the production of tagged but truncated proteins.\textsuperscript{13–16} Figure from Aviner, R. The science of puromycin: From studies of ribosome function to applications in biotechnology. Comput Struct Biotechnol J 18, 1074–1083 (2020).

The inability of the ribosome to cleave this peptide bond results in stalling of the ribosome and premature dissociation of the nascent peptide chain from the ribosomal subunits. Subsequent analysis of the cells can be conducted to detect the puromycin-tagged polypeptide chains with the use of an anti-puromycin antibody and Western blotting. This allows researchers to indirectly measure the levels of cellular translation occurring under different conditions based on the level of integration of puromycin.\textsuperscript{13} It is important to note that at high concentrations, puromycin exhibits cytotoxic effects due to its ability to essentially prevent the formation of functional proteins and as such, the SUnSET technique is a concentration dependent method.\textsuperscript{15}

1.5.2 Phosphatase Assays Employing Exogenous Substrates

With the well-established importance of enzymes such as kinases and phosphatases, it is often important to employ techniques that can elucidate the level of activity of these proteins and the conditions that alter them. A common means of determining enzymatic activity is through \textit{in vitro} assays utilizing artificial substrates. Chemical substrates such as 6,8-difluoro-4-
methylumbelliferyl phosphate (DiFMUP), are reactive and sensitive artificial substrates for phosphatases that are often exploited for this purpose due to its fluorescent properties.

**Figure. 1.8 – Hydrolysis of DiFMUP to its product DiFMU.** Enzymatic hydrolysis of DiFMUP produces the fluorescent molecule DiFMU and a free inorganic phosphate.

The hydrolysis of DiFMUP by a phosphatase results in the removal of the phosphate moiety and the formation of 6,8-difluoro-7-hydroxy-4-methylcoumarin (DiFMU) (Fig. 1.8). While DiFMUP is not fluorescent, the resulting product, DiFMU, is and has a characteristic excitation/emission maxima of ~358/450nm. Through the use of spectrophotometry, the amount of product produced by the enzyme can be measured and the activity of the protein can be extrapolated. The use of an artificial substrate and *in vitro* phosphatase assays is a viable means of determining the effect of post-translational modifications on the activity of a specific phosphatase generated by recombinant protein production or immunoprecipitated from cellular material.
1.5.3 Mass Spectrometry: A Critical Technique in Biochemistry

Mass spectrometry is a versatile technique in biochemistry that can be utilized for several different purposes such as to elucidate the identity of unknown proteins or to map post-translational modifications on a known protein. Due to the modularity of this technique, a mass spectrometer can be setup in numerous different configurations for different biological applications. One such combination that can be used in proteomic studies due to its established efficiency in peptide analysis is electrospray ionization quadruple time-of-flight (ESI-Q-TOF) mass spectrometry (Fig. 1.9).

Figure 1.9 – Schematic diagram of a general ESI-Q-TOF MS setup for proteomic analysis. Samples are ionized using ESI prior to passing through two quadrupoles and a TOF tube. Depending on the settings used, analysis of the precursor ions can produce a MS spectrum and analysis of the fragment ions can produce an MS/MS spectrum. Figure from Allen DR,

This form of mass spectrometry is advantageous in that it combines two different mass analyzers exploiting the high fragmentation efficiency of quadruple mass spectrometry and the accurate and rapid analysis ability of time-of-flight mass spectrometry.\textsuperscript{51} Prior to peptide sequence determination, electrospray ionization is utilized to desorb the peptide molecules into the gas phase. This ionization method employs electrical energy to aid in the soft transfer of sample ions from a liquid phase to the gaseous phase before they enter the mass spectrophotometer directly for analysis.\textsuperscript{52} Following ionization, the sample passes through two quadrupoles and a TOF tube. The first quadrupole acts as a mass filter and as such determines which ions pass to the second quadrupole based on their corresponding mass to charge ratios (m/z). The second quadrupole acts as a collision cell, and it is here that the peptides can be bombarded with an inert gas resulting in fragmentation of the peptide into their constituent ions in a process known as collision induced dissociation (CID). Following CID, the resultant ions are accelerated into the TOF tube where they first pass through an electric field giving all the ions equal kinetic energy before passing into the tube itself. Once in the tube, they separate based on their m/z ratio and the individual ions can be classified. Depending on the settings applied to the quadrupoles, two scanning methods can be used to identify the sample ions.\textsuperscript{51} The first method, referred to as MS mode or the mass fingerprint, is utilized to provide information about the unfragmented precursor ion and does not utilize CID. The second method, referred to as MS/MS mode, utilizes CID to provide a mass scan of the product ions after fragmentation of the precursor ions. The MS/MS data can be used to directly determine the sequence of a peptide and the presence of post-translational modifications made to individual amino acid residues. Moreover, processing
software can be employed to compare high throughput observed datasets with annotated theoretical databases in order to achieve proteome scale measurements.

1.6 Objectives

Human YVH1, an evolutionarily conserved protein, has been implicated in a number of key cellular mechanisms including the cellular stress response and ribosome biogenesis. Furthermore, research into the function of this protein has provided evidence that Hsp70, and more recently, Src kinase modulate the activities of hYVH1.

The objectives of this project serve to provide a more in-depth analysis of the effect Src-mediated phosphorylation has on the function of hYVH1 including its ability to participate in cellular ribosome biogenesis/translation regulation and complex formation with Hsp70. Specifically, my thesis is focused on the following:

i) Determine direct phosphorylation of hYVH1 by Src and the effects it has on phosphatase activity using enzyme assays and mass spectrometry.

ii) Examine the translational rate of cells co-expressing hYVH1 and Src or Hsp70 using the SUnSET technique.

iii) Investigate the role of Src-mediated phosphorylation on hYVH1-Hsp70 complex formation under various cellular conditions.
CHAPTER 2: MATERIALS AND METHODS

2.1 Cell Culture

All cell culture work conducted in this thesis utilized adherent HeLa cells (ATCC®, CCL-2™) maintained as a monolayer on Sarstedt tissue culture plates in Dulbecco’s Modified Eagles Medium Nutrient Mixture F12-HAM (DMEM). DMEM media was supplemented with 10% (v/v) fetal bovine serum (FBS) to supplement cell growth, and 1% (v/v) penicillin-streptomycin (P/S) to protect against bacterial contamination. To prevent the HeLa cells from reaching complete confluency, cell passages were conducted every 48-72h and the cells were maintained at 37˚C and 5% CO₂. Cell lines were terminated following approximately 30 passages and HeLa cells, stored at -80˚C, were thawed and re-plated.

2.2 Mammalian Cell Transfection

HeLa cells were split 24h prior to transfection in antibiotic free DMEM media and experimental tissue culture plates were prepared with 1-3 million cells as required for individual experimental designs. Following preparation of the tissue culture plates, respective plasmid cDNA constructs were introduced to cells through the formation of cationic nanoparticles and subsequent endocytosis utilizing linear polyethylenimine (PEI) as the transfection reagent. Transfection was conducted in low light due. The plasmids exploited include wild-type (WT) FLAG-hYVH1, constitutively active myc-Src Y530F, WT Hck, and His-Hsp70. Regarding the amount of each DNA construct used, FLAG-hYVH1, myc-Src, and His-Hsp70 were all expressed in a 1:3:1 µg ratio per million cells. Cells were allotted 24h to incorporate and express the DNA constructs they were exposed to at which time any relevant treatments were applied,
and the cells were subsequently lysed. Cold (4°C) phosphate-buffered saline (PBS) was used to wash the cells immediately prior to the addition of lysis buffer (1% Triton X-100, 150mM NaCl, 0.1% SDS, 50mM Tris-HCl; pH=7.4). Addition of the protease inhibitors Aprotinin (Apr; 10µg/mL) and phenylmethylsulfonyl fluoride (PMSF; 1mM) to the lysis buffer was used to minimize protein loss due to degradation. Cells were detached from the culture plates utilizing Sarstedt cell scrapers and incubated on ice for 5 minutes. Subsequent clean-up of the cell lysates was conducted through a 10min, 24 000 x g “hard spin” at 4°C to remove large cellular debris and organelles from the soluble lysate. Removal of the resultant protein-containing supernatant into new tubes for immunoprecipitation or storage followed.

2.3 Immunoprecipitation

Following cellular lysis, immunoprecipitation was often conducted to further purify a specific protein. For experiments aimed at elucidating the presence Src-directed phosphorylation of hYVH1, immunoprecipitation involved the use of α-myc monoclonal IgG1 antibodies conjugated to magnetic beads (Santa Cruz, #9E10) while all other experiments requiring immunoprecipitation utilized Anti-FLAG M2 monoclonal magnetic beads (Sigma, #M8823-5ML). Following the final centrifugation step of cell lysis, the resulting cellular lysate was added to 10µL of IP beads per plate. The magnetic beads were first equilibrated through two washes with lysis buffer prior to addition of lysates. Tubes containing lysates and IP beads were incubated at 4°C for 1.5 hours with gentle agitation. Upon completion of said incubation, the beads are collected through magnetization at which time the supernatant was removed, and the beads were washed three times using IP wash buffer (0.1% Triton X-100, 150mM NaCl, 0.1% SDS, 50mM Tris-HCl; pH=7.4). Subsequently, 40µL of 6x bromophenol blue loading dye
(0.2mg/mL bromophenol blue, 70% v/v 0.5M Tris, 30% v/v glycerol, 100mg/mL SDS; pH=6.8) was added and the samples were boiled at 100˚C for 5 minutes in preparation for SDS-PAGE.

### 2.4 SDS-PAGE and Western Blotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was utilized to separate proteins in preparation for Western blot analysis (also referred to as immunoblot analysis). SDS-PAGE gels were run at 125V for approximately two hours immediately followed by transfer of the protein from the gels onto polyvinylidene difluoride (PVDF) membranes using BioRad’s Mini-PROTEAN apparatus. The protein transfer was conducted at 100V for one hour. Subsequent protein blocking of the PVDF membranes used either 5% skim milk or 5% bovine serum albumin (BSA) in 1x Tris-buffered saline, 0.1% Tween (1x TBST). Blocking was done for one hour at room temperature with gentle agitation. Upon completion of the blocking step, primary antibodies solutions were prepared in solution containing a 1:1 ratio of blocker and 1x TBST. The primary antibodies and concentrations used include mouse $\alpha$-FLAG (1:8000; Millipore, #F3165-2MG), rabbit $\alpha$-hYVH1 (1:4000), mouse $\alpha$-His (1:2000; Santa Cruz, #sc-8036), mouse $\alpha$-Src (1:2000; Millipore, #05-184), mouse $\alpha$-pTyr clone 4G10 (1:4000; Millipore #05-321), mouse $\alpha$-pTyr (1:2000; Cell Signaling #9411S), mouse $\alpha$-puromycin clone 12D10 (1:6000; Millipore #MABE343), and rabbit $\alpha$-actin (1:4000; Sigma, #A2066-.2ML). Antibody solutions were added to corresponding membranes and incubated overnight at 4˚C with gentle agitation. The membranes were then washed three times using 1x TBST and secondary antibodies (goat $\alpha$-mouse-HRP (Sigma #A4416) and goat $\alpha$-rabbit-HRP (BioRad #170-6515)) were added at standard concentration of 1:4000 and incubated for one hour at room temperature with gentle agitation. Three successive washes of the membranes were completed again
following secondary antibody incubation prior to final chemiluminescent imaging using BioRad’s ChemiDoc Imaging system and Thermo Fisher’s SuperSignal™ West Femto Maximum Sensitivity Substrate (Catalog #34095).

2.5 Src-Directed Kinase Assay

The Src-directed kinase assay first involved obtaining Src kinase and proceeded by one of two methods. Method one, referred to as the HeLa cell derived Src kinase approach, involved the partial purification of myc-Src from HeLa cells using immunoprecipitation. HeLa cells were transfected with myc-Src following the procedure outlined in section 2.2 and subsequent immunoprecipitation was conducted, as previously described in section 2.3, utilizing α-c-myc magnetic beads (MCE, #HY-K0206). Method two, referred to as the commercially derived Src kinase approach, involved the use of commercially purchased active WT c-Src kinase (SignalChem, #S19-18G-10). The kinase assay solution was then prepared with a total reaction volume of 35µL in microcentrifuge tubes. HeLa cell derived Src reaction tubes contained the Src bound α-c-myc beads in 10µL of kinase buffer (50mM HEPES free acid, 5mM MgCl₂, 150mM NaCl, 1mM DTT; pH=7.8), 10µL of 10mM ATP, and 15µL of purified hYVH1 C115S. Commercially derived Src reaction tubes contained 8µL of kinase buffer, 2µL of Src (0.1µg/µL), 10µL of 10mM ATP, and 15µL of purified hYVH1 C115S. The kinase mixture was then incubated at 37°C for one hour. Upon completion of the incubation, the reaction was terminated by the addition of 40µL SDS-PAGE loading dye.
2.6 Human YVH1 Phosphatase Activity Assay

Investigation of hYVH1’s activity utilized an *in-vitro* DiFMUP assay. Partially purified hYVH1 was obtained following overexpression of FLAG-hYVH1 and phYVH1 was obtained through co-expression of FLAG-hYVH1 and myc-Src in HeLa cells coupled with FLAG-immunoprecipitation as previously described in sections 2.2 and 2.3. Following FLAG-IP, the hYVH1-bound beads were washed three times using IP wash buffer and the beads were subsequently resuspended in 91μL of phosphatase assay buffer (50mM Tris, 50mM Bis-Tris, 150mM NaCl, 5mM DTT; pH=6.5) and 9μL of 10mM DiFMUP. The reaction was then allowed to proceed for one hour at 37°C with moderate agitation. Upon completion of the activity assay, the beads were magnetized, and the supernatant was removed and loaded onto a 96-well plate (100μL per well). Fluorescent readings were obtained using BioTek’s Cytation5 Cell Imaging Multimode Reader immediately following a brief (one minute) liner shake at an excitation/emission wavelength of 358/450 and an instrumental gain value of 46.

2.7 Mass Spectrometric Analysis

Peptide samples were prepared though in-gel digestion of an SDS-PAGE gel generated as described in section 2.4. Following the completion of the two-hour run, gels were stained using Coomassie Brilliant Blue R-250 (Thermo Fisher, #24615) for 24 hours, then de-stained using MilliQ water for a second 24-hour period. Resultant bands were excised and cut into smaller pieces before being fully de-stained using a 1:1 v/v 50mM ammonium bicarbonate (AB): 100% acetonitrile (ACN) solution. Subsequent treatment with ACN was utilized to dehydrate the gel pieces and a speed vacuum was used to dry the gels prior to addition of the trypsin protease buffer (13ng/μL mass spectrometry grade trypsin in 50mM AB) and an overnight digestion with
agitation. Digested peptides were extracted through two 15 minutes cycles at 37°C using a 1:2 v/v solution of 5% formic acid (FA) and ACN. Excess solution was removed by speed vacuuming the gel pieces and peptides were resuspended in 0.15% trifluoroacetic acid. The digested peptide samples were de-salted using an Oasis column and following elution, were dried and resuspended in 20μL 0.1% FA.

Tryptic peptides were loaded onto a 1.8 μm HSS T3 75 μm x 150 mm reverse-phase column (Waters) at a flow rate of 0.3 μL/min via the nanoAcquity UPLC autosampler. Peptide separation was achieved using a gradient consisting of mobile phase A (0.1% FA in water) and mobile phase B (ACN with 0.1% FA). Equilibration and loading conditions used was a 97:3 solvent ratio (mobile A:B). Peptide elution was achieved using a 90 min gradient (3-30% B for 55 min, 30-50% B for 25 min, 85% B for 10 min) and directly sprayed into a SYNAPT G2-Si mass spectrometer (Waters, Milford, MA) operating with a 3 kV capillary voltage and a 30 V cone voltage. The high definition mass spectrometry (HDMSE) operating mode was utilized consisting of data independent acquisition (DIA) with ion mobility separation activated using a wave speed of 650 m/s. HDMSE data was measured using low energy scans at 4 eV and high energy scans at 20-45 eV in positive high resolution mode, scanning from 50 to 2000 m/z at a rate of 0.8 s. Calibration was using [Glu1]-fibrinopeptide B (50 fmol/μL) in the lock mass channel at m/z 785.8427 for a doubly-charged positive ion. Raw data was collected using Mass Lynx (version 4.1). Putative phosphopeptides were reanalyzed by selected-ion reaction monitoring (SRM) whereby the parent ion mass of interest was selected in the quadrupole and fragmented in the transfer following ion mobility separation. The TOF pusher frequency was synchronized to enhance sensitivity of fragmentation. Phosphorylation site mapping was assigned by manual analysis of the fragment pattern.
2.8 HeLa Cell Treatments

2.8.1 Puromycin

A 3.6mM stock solution of puromycin (Sigma, #P8833-10MG) was first prepared by dissolving 10mg in 5.1mL of MilliQ H2O. Following preparation, the stock solution was stored away from light at -20°C and retrieved when needed. Twenty-four hours post translation, 50.1µL of media was removed from each tissue plate being treated and replaced with 50.1µL of 3.6mM puromycin to attain a final concentration of 10µg/mL. Addition of puromycin was done in low light due. Cells were then incubated at 37°C for 20 minutes to allow puromycin incorporation. Immediately following the treatment, the media containing excess puromycin was removed and the cells were washed using cold PBS in preparation for cellular lysis as described in section 2.2. This method was adapted from the paper “SUnSET, a nonradioactive method to monitor protein synthesis” published in NatureMethods by Schmidt EK et al.

2.8.2 Low-Level Hydrogen Peroxide Induced Stress

A stock solution of H2O2 was first prepared by diluting 2.72µL of 30% (v/v) hydrogen peroxide in 1mL of DMEM media supplemented with FBS and P/S as previously described. Tissue culture plates were then retrieved and 12.5µL of media was removed and replaced with 12.5µL of the 26mM stock H2O2 solution to obtain a final concentration of 33µM. The cells were then incubated for either two or three hours at 37°C. Upon completion, the DMEM media was removed from each plate and cells were either lysed or the media was replaced with fresh antibiotic containing media in preparation for an additional treatment with puromycin as outlined in section 2.8.1.
CHAPTER 3:  
RESULTS AND DISCUSSION

3.1 Src Directly Phosphorylates Amino Acid Residue Tyr179 on hYVH1

Human YVH1 is a unique protein phosphatase that has been implicated in various cellular processes including cell survival, stress response, and ribosome biogenesis. Among the most recent findings regarding hYVH1’s role in ribosome biogenesis was the ability of Src-mediated phosphorylation to alter the cellular localization of hYVH1 from a majority cytoplasmic localization pattern to one that involved a significant increase in nuclear hYVH1 levels. This correlated to higher association with the 60S ribosomal subunit and ribosomal proteome alterations consistent with increased translational fitness.

Previous findings have implicated amino acid residue Tyr179 on hYVH1 as a critical phosphorylation site that dramatically alters the cellular localization of hYVH1, increasing the nuclear concentration of the enzyme, when phosphorylated. This change in localization is currently believed to be a key factor in hYVH1’s ribosome biogenesis activity as it allows the protein to interact with the pre-60S subunit of the ribosome. The site has been confirmed to be in its phospho-state when co-expressed with Src in HeLa cells suggesting that the presence of Src is required for the phosphorylation of Tyr179 to occur. While the effect of Src on the ability of hYVH1 to participate in ribosome biogenesis is becoming clearer through each novel finding, it has thus far been unknown if Src kinase is acting directly to target Tyr179 of hYVH1 as a substrate, or if Src is merely acts within a larger cascade that ends in hYVH1 being phosphorylated by another kinase.
To answer this question, we first wanted to confirm that when co-expressed, hYVH1 does undergo Src-mediated tyrosine phosphorylation. We first began by co-expressing myc-tagged Src and FLAG-tagged hYVH1 in HeLa cells and performing a FLAG-immunoprecipitation (FLAG-IP) to isolate our overexpressed hYVH1 protein. Tyrosine phosphorylation of hYVH1 was measured through Western blot analysis using an antibody specific to pTyr amino acid residues (α-4G10).

**Figure 3.1. Src- and Src-family Mediated Phosphorylation of hYVH1.** A) Immunoblot analysis of HeLa cells co-expressing hYVH1 and Src. Probing of samples post-IP was conducted with an α-4G10 antibody to visualize phospho-tyrosine levels of hYVH1 (top). Control blots for FLAG-hYVH1 post-IP (middle) and Src lysates (bottom) were also developed to ensure efficient pull down and transfection. B) Co-expression of hYVH1 and Src family member Hck in HeLa cells to investigate SFK specificity to hYVH1. Immunoblot analysis using an α-4G10 antibody was utilized to visualize the presence of Hck-mediated tyrosine phosphorylation on FLAG-hYVH1 post-IP (top). A control blot for FLAG-hYVH1 post-IP (middle) is also included to show equal pull down of hYVH1.
Subsequent analysis of the resulting Western blots of the samples collected post-IP (Fig. 3.1, A) using an α-4G10 antibody showed the presence of a band at the appropriate molecular weight for hYVH1 in the lane corresponding to co-expressed Src and hYVH1 that was absent in the lane containing over-expressed hYVH1 alone. The presence of a clearly noticeable band only when co-expressed with Src demonstrates the Src-mediated change hYVH1 undergoes regarding its phosphoTyr (pTyr) state when compared to hYVH1 being overexpressed independently. This Western blot serves to confirm what has been previously seen by previous lab members exploring the interaction between Src kinase and hYVH1 and further supports the existence of Src-mediated phosphorylation of hYVH1.

In an attempt to also explore the specificity of Src to hYVH1, we also attempted to explore if the Src family member Hemopoietic cell kinase (Hck) could also mediate phosphorylation of hYVH1. To accomplish this, we conducted a similar experiment and co-expressed hYVH1 and Hck utilizing two varying DNA concentrations of Hck (1µg and 3µg). The resulting Western blot (Fig. 3.1, B), when probed using the same α-4G10 antibody, similarly showed a lack of tyrosine phosphorylation when hYVH1 was over-expressed independently, but tyrosine phosphorylation of hYVH1 was again detected in the lane containing co-expressed Hck. While tyrosine phosphorylation of hYVH1 could be observed in both Hck containing samples, the phospho-levels were significantly reduced when compared to the Src samples (Fig. 3.1, A). This suggests that Src kinase mediates phosphorylation of hYVH1 more readily but that other Src family kinase (SFK) members may also be able to target hYVH1. This is not entirely unexpected as Src and its family members share significant structural similarity with regard to their domain structure. While each SFK member does possess a so-called “unique” domain which has been suggested to play a critical role in mediating the function and regulation of each
SFK, the domain organization is highly conserved. In fact, interchanging of the unique domains of Src and Yes (an SFK) results in the swapping of each kinase’s specificity further supporting the similarity between family members outside of the unique domain. Of note, while the Western blot results (Fig. 3.1) do suggest that Src-mediated phosphorylation of hYVH1 is more abundant than Hck-mediated, we could not generate a Hck control blot on cell lysates as we did with Src cell lysates due to the lack of a protein tag on our Hck construct and the lack of a Hck specific antibody. For this reason, while it is highly possible that Src-mediated phosphorylation is more prevalent, the possibility that the differences in phosphotyrosine levels is due to lower expression of Hck cannot be ruled out despite the use of three times the cDNA transfected (1µg Src vs 1µg and 3µg Hck). Further research involving a more comprehensive analysis of all SFK member will be needed to explore and compare the specificity of SFK members phosphorylating hYVH1 in various cell types and conditions.

Although the Western blot analysis using a pTyr-specific antibody suggested the presence of phosphorylated tyrosine residues within hYVH1, one concern remained regarding the possibility of non-specific detection of a thiol-phosphate intermediate form of hYVH1 by the α-4G10 reagent. Due to hYVH1’s classification as a PTP member, catalysis involves a transient thiol-phosphate intermediate formed during the dephosphorylation mechanism. This results in a brief moment where a phosphate moiety is trapped within the active site of hYVH1 covalently bound to the catalytic cysteine. While the phospho-antibody we employed is specific to phosphorylated tyrosine residues, it has been observed that in rare cases, these antibodies can bind non-specifically to the intermediate formed during the catalytic mechanism. To eliminate this possibility, we repeated the previous experiment, but introduced the catalytically dead cysteine to serine mutant of hYVH1 (hYVH1 C115S) and compared it to the wild-type form.
Figure 3.2. Ruling out the non-specific detection of an hYVH1 thiol-phosphate intermediate. A) HeLa cells were transfected with either the C/S mutant or WT hYVH1 and Src kinase prior to cell lysis and immunoprecipitation. Western blot analysis was conducted on the resulting samples utilizing an α-4G10 antibody to visualize tyrosine phosphorylation. Chemiluminescent bands were present in both lanes involving Src regardless of the hYVH1 form used (top). Control blots were also generated using α-FLAG (top-middle), α-Src (bottom-middle), and α-Actin (bottom) antibodies to ensure efficient IP and transfection. B) Normalization of the resultant α-4G10 band intensities to FLAG-hYVH1 and Src levels was conducted to ensure that the visualized differences between phosphor-tyrosine band intensities were not due to differing levels of DNA expression. The relative fold changes were then plotted on a bar graph.

Ensuing analysis of the samples using Western blot analysis, probing again using the α-4G10 antibody, produced a chemiluminescent band in both the catalytically dead and wild-type containing lanes (Fig. 3.2, A). While the band in the hYVH1 C115S lane appears to show more significant pTyr detection, normalization of this band to the levels of FLAG-tagged hYVH1 isolated following immunoprecipitation shows a relative fold change less than 0.02% (Fig. 3.2,
B). This would suggest that the difference in pTyr levels is negligible and likely due to the variability inherent to intracellular environments. While some variability may exist between the exact levels of tyrosine phosphorylation of the constructs, the most notable finding of this experiment is the presence of bands in both lanes. This finding provides strong evidence that the bands observed on our previous α-4G10 blot (Fig. 1.1, A) are not likely to be due to non-specific detection of a hYVH1 thiol-phosphate intermediate. Had the antibody been detecting the formation of the thiol-phosphate intermediates rather than Src-mediated phosphorylation of tyrosine residues on hYVH1, we would have expected to see this band completely disappear when utilizing the catalytically dead mutant due to inability to form these transient complexes. The fact that the band persisted even in the presence of our hYVH1 C115S construct, strongly supports the existence of Src-mediated phosphotyrosine residues within hYVH1.

Having illustrated that Src-coexpression in HeLa cells results in the tyrosine phosphorylation of hYVH1, supporting previous findings by our lab, and eliminating the possibility of the non-specific detection of a thiol-phosphate intermediate due to enzymatic activity, we sought to determine whether Src was directly phosphorylating hYVH1, specifically at the critical Tyr179 site. To resolve this, we shifted our focus to take a more in vitro proteomic approach.
Figure 3.3 – Workflow of the methods used to generate phosphorylated hYVH1 classified by the origin of Src kinase. Two methods were used to generate the phosphorylated hYVH1 needed to determine if Src kinase was directly phosphorylating hYVH1 and if Tyr179 was a direct phospho-site. The first method (top) derived the necessary Src kinase from transfected HeLa cells using a myc-IP. Purified hYVH1 C115S was then incubated with the Src to allow for phosphorylation and subsequent MS analysis. The second method (bottom) utilized commercially purchased WT Src and a similar kinase assay followed by MS. Figure created with BioRender.com.

We first began by developing two kinase assays to produce phosphorylated hYVH1 (Fig. 3.3). The first assay involved an \textit{in vitro} approach which utilized HeLa cells overexpressing myc-Src Y530F coupled with an ensuing myc-IP to derive our Src enzyme. Following isolation of the kinase, recombinant hYVH1 C115S purified from E. coli, was added to the kinase assay as the putative substrate. The second kinase assay utilized a full \textit{in vitro} approach with commercially purchased purified wild type Src and the same recombinant hYVH1 C115S. The resulting
samples were analyzed via Western blot analysis to determine if Src was directly phosphorylating hYVH1 and mass spectrometry was used to identify the phosho-state of the Tyr179 residue.
Figure 3.4 - Confirmation of direct phosphorylation of hYVH1 by Src kinase and of Tyr179 as a Src phosphorylation site. A) Immunoblots were obtained following analysis of samples following a 1h kinase assay using HeLa cell derived myc-Src Y530F. Probing of the generated blots using a α-4G10 was done to determine if Src-directed tyrosine phosphorylation was occurring and produced a band only in the presence of Src (top). Control blots were also produced to ensure hYVH1 was added to the appropriate samples (middle) and to ensure proper pull down of myc-Src following immunoprecipitation (bottom). B) Immunoblots were also obtained for samples following the 1h kinase assay utilizing commercially purchased WT Src. Probing of the blots using an α-4G10 antibody resulted in a chemiluminescent band at ~37 kDa, consistent with the molecular weight of hYVH1 (top). A control blot was also produced using an α-FLAG antibody to ensure hYVH1 was present (bottom). C) MS analysis of the generated phosho-hYVH1 using both kinase methods was subsequently conducted using an ESI-Q-TOF setup. Data independent select-ion reaction monitoring produced the above MS/MS spectrum for the tryptic peptide containing Tyr179 (164-180). The spectrum shows an ~80Da mass increase in the ion representing amino acid residue 179 which is consistent with the addition of a phosphate moiety. This figure is published in DaDalt, A. A., Bonham, C. A., Lotze, G. P., Luiso, A. A. & Vacratsis, P. O. Src-mediated phosphorylation of the ribosome biogenesis factor hYVH1 affects its localization, promoting partitioning to the 60S ribosomal subunit. Journal of Biological Chemistry 298, 102679 (2022).

The resulting Western blot membranes and mass spectrometry spectra are illustrated in figure 4. The in vitro kinase assay utilizing HeLa derived Src Y530F produced an α-4G10 band at hYVH1’s molecular weight in the lanes containing purified hYVH1 C115S (Fig. 3.4, A) and this was mirrored by the results obtained from the second in-vitro kinase assay using all recombinant purified proteins (Fig. 3.4, B). Tyrosine phosphorylation of hYVH1 as seen in Figure 4, provides strong supporting evidence to conclude that Src kinase can directly phosphorylate hYVH1. It was important to conduct both types of in vitro kinase assays to examine Src-mediated hYVH1 phosphorylation. While the results of the in-vitro kinase assay
from HeLa cells did point to Src’s ability to directly phosphorylate hYVH1, the intracellular origin of Src coupled to immunoprecipitation, risked the presence of other non-specific kinases being in co-IP’ed with Src. Due to the intrinsic ability of enzymes, such as kinases, to be recycled and to rapidly catalyze many reactions, even a small concentration of non-specific kinases in the immunoprecipitant could result in phosphorylation of hYVH1. Therefore, utilization of a second in-vitro kinase assay using only purified recombinant proteins complemented our cellular in vitro kinase results (Fig. 4, B). Taken together, these results demonstrate that hYVH1 is a novel substrate of Src and that both mutant Src Y530F and WT Src can direct this event.

We were also interested in mapping the Src-mediated phosphorylation site utilizing ESI-Q-TOF mass spectrometry. While the previous experiments (Fig. 3.4, A and 3.3, B) demonstrated Src’s direct role in hYVH1 phosphorylation, we wanted to compare known hYVH1 phospho-sites to those that occur due to Src directly, namely at Tyr179. As previously mentioned, hYVH1 amino acid residue Tyr179, which is located within the intra-domain linker region, has been established as a major phospho-site of hYVH1 and plays a pivotal role in subcellular localization of hYVH1. Previous work has shown that Tyr179 is phosphorylated when co-expressed with Src.10 To map the phosphorylation site, hYVH1 was proteolytically digested with trypsin following both types of in vitro kinase assays. The samples were then subjected to a so-called select-ion reaction monitoring (SRM) experiment to map the phosphorylation site. SRM is a type of targeted mass spectrometry measurement where the instrument settings are set to focus on a particular parent ion of interest. This greatly increases sensitivity and fragmentation needed to resolve the specific hYVH1 amino acid residue modified by Src. Utilizing this approach, an MS/MS spectrum was produced that corresponded to the
specific tryptic peptide fragment containing amino acid residue Tyr179 (Fig. 3.4, C). The MS/MS spectrum generated corresponds to amino acids 164-180 and subsequent comparison of the resultant fragment ions illustrated a mass shift of ~80Da on the C-terminal y2 fragment ion that unambiguously mapped the phosphorylation site to Tyr179. This ~80Da shift is consistent with the addition of a phosphate moiety to an amino acid residue. Collectively, these results not only provide strong evidence that Src can directly target and phosphorylate hYVH1, but it also unambiguously identifies Tyr179 as a Src phosphorylation site.

3.2 Alteration of Human YVH1’s Phosphatase Activity Due to Src-Directed Phosphorylation

With definitive evidence illustrating that hYVH1 is a substrate to Src kinase and contains at least one phospho-site, namely at residue Tyr179, the question became what effect this phospho-event has on the function of hYVH1. Human YVH1 has been implicated in a number of different cellular mechanisms, many of which are consistent with pathways that Src kinase has been implicated in as well including cell survivability, stress response, and DNA content modulation.5–7,10 Due to the ability of Src to directly phosphorylate hYVH1 and the similarity of implicated cellular function, it is tempting to imply that Src-directed phosphorylation may have far reaching effects on hYVH1. It has already been demonstrated that phosphorylation of residue 179 can dramatically alter hYVH1’s localization pattern, increase its association with the 60S ribosome subunit, and alter the ribosomal proteome.10 We were also interested in determining if Tyr179 phosphorylation affects the intrinsic phosphatase activity of hYVH1. Our lab and others have shown that recombinant hYVH1 exhibits poor in vitro phosphatase activity against artificial substrates.31 A number of possible explanations exist for this apparent low level of catalytic
activity, one of which is that hYVH1 requires a post-transcriptional modification to alter its phosphatase activity. For this reason, we set out to explore the impact Src-directed phosphorylation has on the catalytic phosphatase activity of hYVH1.

To explore if any changes to hYVH1’s phosphatase activity were occurring due to Src, we designed a kinase-coupled activity assay using the artificial substrate DiFMUP. The first step of this experiment was to generate our phosphorylated hYVH1. Our experimental design utilized an in vivo approach to phosphorylation which involved co-transfected HeLa cells with FLAG-hYVH1 and Src Y530F followed by isolation and purification of the tagged hYVH1 via a FLAG-IP. Following generation of phosphorylated hYVH1, we performed an end-point fluorescent activity assay by measuring the level of product DiFMU produced by phosphorylated hYVH1 when compared to hYVH1 transfected alone (Fig. 3.5).
Figure 3.5 - Effect of Src-directed phosphorylation of hYVH1 on catalytic activity against the exogenous phosphatase substrate DiFMUP. HeLa cells were transfected with FLAG-hYVH1 and Src and subsequent immunoprecipitation was used to isolate phospho-hYVH1. The resulting protein then underwent a 1h DiFMUP phosphatase activity assay and fluorescent intensity using an excitation/emission spectrum of ~358/450 was measured to qualify the amount of product formed. The collected data from eight replicates was processed and normalized to FLAG-hYVH1 levels. The relative fold changes in fluorescent intensity relative to the EV control were plotted. Statistical analysis using the student’s T-test illustrated that there is a statistically significant decrease in hYVH1’s catalytic activity when co-expressed with Src Y530F.

Utilizing eight replicates of the activity assay where all phosphorylated hYVH1 was synthesized by in vivo phosphorylation, we obtained the results outlined in figure 3.5. Statistical analysis of the changes in activity in the presence or absence of Src-directed phosphorylation showed a statistically significant increase in enzymatic activity when hYVH1 was present at increased concentrations when compared to our negative control. This was to be expected as the samples containing over-expressed hYVH1 consequently contained more enzyme to catalyze the formation of product. While the increase in activity between endogenous levels of hYVH1 and overexpressed levels only average to about ~50%, which is relatively low for a phosphatase, it is consistent with previous observations that hYVH1 has low in vitro catalytic activity. Consequently, this allows use to use this as an internal standard that serves to validate the reliability of the experiment as a whole. Further, statistical examination of the results also showed a statistically significant decrease in activity when we compared hYVH1 alone to hYVH1 + Src. A decrease in activity while Src is present was a somewhat unexpected finding, due to the fact that Src co-expression increased hYVH1’s ribosome activities and in many cellular contexts is considered a cell survival kinase. Also, while it had been hypothesized that
hYVH1 required some sort of post-translational modification to alter its activity, it was believed that the alteration would increase hYVH1’s catalytic activity, not further reduce it. This finding suggests that Src-directed phosphorylation of hYVH1 results in a proteoform with reduced catalytic function. Despite this finding being counterintuitive we propose that this finding makes mechanistic sense regarding hYVH1’s role in ribosome biogenesis.

Prior investigation of hYVH1’s role in yeast ribosomal biogenesis has suggested that YVH1 interacts with the pre-60S ribosomal subunit and is critical in the proper nuclear export of the subunit as well as the release of the biogenesis factor Mrt4 once the subunit has entered the cytoplasm. Domain deletion mutation studies illustrated that for YVH1 to properly execute its function in yeast ribosomal biogenesis, only the C-terminal zinc-binding domain is required and that both catalytic activity and the catalytic domain as a whole are ultimately dispensable for ribosome binding. While this observation was made in yeast and can therefore not be definitively applied to human cells, it has been shown that the human ortholog is structurally similar and can complement yvh1−/− strains in terms of ribosomal biogenesis in yeast suggesting that both orthologs of the protein likely function in a highly similar manner. Furthermore, the primary function of Src-directed phosphorylation of the human ortholog at Tyr179, which is just outside the catalytic domain, has been shown to be the change in localization of hYVH1, presumably recycling it back into the nucleus to participate in another round of 60S ribosomal biogenesis. Therefore, the observation that Src-directed phosphorylation of hYVH1 is causing a reduction in catalytic action points to a model where phosphorylation of hYVH1 may facilitate the shedding of hYVH1 from the 60S subunit (or prevent the rebinding) once it has displaced Mrt4 to increase the efficiency of loading the P0/P1/P2 stalk complex. This would also support a higher rate of hYVH1 recycling and efficient 60S subunit production, higher ribosome
biogenesis and translation. Moreover, the phosphorylation mediated decrease in phosphatase activity may be necessary to keep hYVH1 from dephosphorylating non-specific targets when off the ribosome during the translocation from the cytoplasm to the nucleus.

**Figure 3.6 - Crystal Structure of hYVH1’s N-terminal catalytic domain and the most proximal portion of the linker region.** Structural analysis of hYVH1 shows that Tyr179 (red) is contained within the initial portion of the linker region and is in very close proximity to the catalytic domain. The phosphorylatable hydroxyl group of Tyr179 is pointed inwards toward the N-terminal domain and is surrounded by several polar charged amino acids (light green) that would likely experience electrostatic repulsion/attraction caused by the introduction of a negatively charged phosphate group.

Furthermore, Tyr179 is located in close proximity to the catalytic domain, being found in the linker region just outside the boundary of the N-terminal domain. This region of hYVH1 is rich in both negatively and positively charged polar amino acid residues as outlined by figure 3.6. Examination of Tyr179 in the crystal structure shows that its phosphorylatable hydroxyl
group extends toward the catalytic domain and is consequently surrounded by several charged polar groups. The addition of a negatively charged phosphate group onto Tyr179 could likely result in a significant change to the overall confirmation of this domain and could explain the statistically significant loss of catalytic activity that was observed in the DiFMUP assay (Fig. 3.5).

Taken together, we propose that a phosphorylation-induced conformational change due to Src-directed phosphorylation of Tyr179, is a likely cause for the reduction in catalytic activity observed through the in vitro phosphatase activity assay. While it is possible that other Src-directed phosphorylation sites exist within hYVH1 which also serve to contribute to the reduction in phosphatase action, due to the purposed function of Src in altering hYVH1 localization mainly through phosphorylation of Tyr179, it stands to reason that alterations at this site would have the greatest impact on hYVH1. Furthermore, due to the observed role of hYVH1 in ribosome biogenesis and the necessary recycling of hYVH1 between the cytoplasm and nuclear compartments, a low activity proteoform of hYVH1 may be important to facilitate Src-directed shuttling of hYVH1 into the nucleus to participate in another round of late stage pre-60S ribosomal subunit maturation.

3.3 The Impact of Src-Directed Phosphorylation of hYVH1 on Cellular Translation

With the confirmation of direct hYVH1 phosphorylation at site Tyr179 by Src and a statistically significant decrease in catalytic activity following phosphorylation, we wanted to continue to explore the impact of this event. A reduction in catalytic activity of a phosphatase is of immense importance as it nearly always indicates a shift in the physiologically effect the enzyme has on the cell. This is even more true when it comes to hYVH1 due to its already low
basal levels of activity. Our current hypothesis suggests that Src-directed phosphorylation of Tyr179 shifts hYVH1’s focus toward ribosome biogenesis. This hypothesis is further supported by previous quantitative proteome experiments that has shown that co-expression of hYVH1 and Src in HeLa cells resulted in a statistically significant increase with respect to the levels of monosome and polysome complexes (Fig. 1.4, B). An increase in these structures along with the proteome alterations observed suggests a possible increase in translational fitness and provides evidence that Src-mediated phosphorylation of hYVH1 leads to an increased ability of the protein to function in ribosome biogenesis by increasing the supply of translationally fit ribosomal structures in the cytoplasm.

While our previous work did suggest that hYVH1 was impacting translational efficiency, the resolution of polysome structures observed in our sucrose gradient ultracentrifugation experiments was relatively low in both control and experimental samples and thus it was important to provide complementary evidence to whether Src-mediated phosphorylation of hYVH1 resulted in a significant physiological change to translational levels of the cell.

To confirm that the increased levels of these monosome and polysome ribosome complexes resulted in an overall increase to the translational fitness of the cell implying physiological relevance to the Src-mediated phosphorylation of hYVH1, we utilized the SUnSET method. This method utilizes the tRNA analog puromycin to prematurely terminate polypeptide synthesis and allow for the subsequent qualification of the level of translation within the cell. We utilized this technique to determine if the Src-mediated phosphorylation of hYVH1 resulted in a physiological change to the cells translational fitness and if the observed patterns of translation correspond to the changes in the levels of ribosome structures previously observed.
**Figure 3.7 - Determining the impact of a Src-mediated phosphorylation of hYVH1 on cellular translation through SUnSET.**

**A)** HeLa cells were transfected with FLAG-hYVH1 and Src Y530F and treated with 10µg/mL puromycin for 20 minutes. HeLa cells were then lysed and analyzed by Western blot. Probed of the resulting blots using an α-Puromycin antibody resulted chemiluminescence bands in all lanes with the most intense bands occurring in the lane containing co-expressed FLAG-hYVH1 and Src (top). Control blots were also produced to ensure proper transfection and similar protein expression levels using α-hYVH1 (top-middle), α-Src (bottom-middle), and α-Actin (bottom) antibodies. **B)** Subsequent normalization of the chemiluminescent intensity of each lane to Actin levels and graphing of each sample’s relative fold change relative to the EV control showed a reproducible trend in increasing puromycin incorporation. Overexpression of all three proteins resulted in a statistically significant increased to puromycin incorporation relative to the control sample with hYVH1+Src samples demonstrating the largest relative fold change. This figure is published in DaDalt, A. A., Bonham, C. A., Lotze, G. P., Luiso, A. A. & Vacratsis, P. O. Src-mediated phosphorylation of the ribosome biogenesis factor hYVH1 affects its localization, promoting partitioning to the 60S ribosomal subunit. Journal of Biological Chemistry 298, 102679 (2022).
By comparing the changes in puromycin integration between our control and experimental samples, we made a number of interesting observations that supported the previous findings regarding increasing ribosomal complexes depending on the overexpressed proteins present. Following transfection of HeLa cells with the desired DNA constructs, we incubated the cells with puromycin for twenty minutes before lysing the cells and examining the entire cellular proteome via Western blot analysis. Through the use of an α-Puromycin antibody, we were able to qualify the level of cellular translation of the cells in each sample by comparing the intensity of bands in the resulting immunoblot. The intensity of the bands present is directly proportional to the amount of puromycin integrated and consequently the level of translation. We observed a statistically significant increase in puromycin incorporation between all experimental samples when compared to our empty vector control samples. Furthermore, we noticed that while overexpressed Src and overexpressed hYVH1 positive control samples both exhibited increased puromycin integration, cells that had increased levels of hYVH1, repeatedly showed a larger increase in translation when compared to overexpressed Src kinase. Interestingly, cells co-expressing both hYVH1 and Src displayed the greatest increase in puromycin incorporation compared to control. Subsequent, normalization and statistical analysis, illustrated a replicable increase in puromycin integration when compared to any of the previous samples. When compared to the empty vector control, we observed a statistically significant increase in translation which provides strong evidence that Src-mediated phosphorylation of hYVH1 is resulting in an increased level of cellular translation. While the difference in puromycin integration is statistically significant when compared to the negative control sample, it must be noted that the difference in fold change between either protein alone or co-overexpression is not statistically significant. While figure 3.7 clearly outlines the trend illustrating that co-
overexpression is resulting in a replicable increase in translation, there is relatively high variability in the degree of the increase between experimental replicates. We suggest that the level of variability between replicates is due to the inherent volatility associated with intracellular environments and that while the normalized data may not be statistically significant between all samples, these results still strongly support the impact of Src-mediated phosphorylation of hYVH1 due to the reproducibility of the observed trends present within each replicate. Furthermore, the findings on translational level presented in figure 3.7, strongly mirror the results published previously regarding higher polysome levels when Src and hYVH1 are co-expressed (Fig. 1.4, B). Our puromycin integration experiment supports this as co-overexpression resulted in the most integration followed by hYVH1 alone. Taken together, these results strongly reinforce our current hypothesis regarding the influence Src-mediated phosphorylation of hYVH1 has on ribosome biogenesis and indirectly, cellular translation.

Following our observations regarding the impact of Src-mediated phosphorylation of hYVH1 on cellular translations, we wanted to next explore cellular conditions where this modulation would be relevant. One such cellular status that we hypothesized might have an effect on the prevalence of this phosphorylation event was oxidative stress. We hypothesized that this condition may alter hYVH1’s shuttling mechanism due to a number of previously described characteristics of both Src and hYVH1. The most significant contributing factor to our decision to utilize hydrogen peroxide induced stress was its previously described impact on hYVH1 function. Prior research has shown that hYVH1 functions to increase cell survivability under different forms of oxidative stress but the mechanism through which it does this is still unknown. Under stress, the cell typically reduces overall translation levels as to maintain energy and protect cellular assets. However, equally important to cellular survival is restoration of
ribosome biogenesis and protein translation following recovery from cellular stress. Knowing that hYVH1 functions in ribosome biogenesis and has been shown to be a potential redox sensor through its redox active Cys residues, we hypothesized that this regulatory mechanism may be important during stress responsive conditions. Furthermore, Src-focused research has suggested that oxidative stress can actually increase the activity of Src kinase by inducing a conformation change within its domain organization.

**Figure. 3.8 – Workflow of our preparation and treatment of HeLa cells with both oxidative stress and puromycin.** In order to investigate the impact low-level oxidative stress has on translation, HeLa cells were first transfected with FLAG-hYVH1 and Src. Following an allotted 24h growth period, the cells were first treated with 33µM H₂O₂ for 2h immediately followed by the removal of the stress and addition of puromycin for 20 minutes. Cells were then lysed and the whole cell proteome was collected. Figure created with BioRender.com.

To investigate Src-mediated phosphorylation of hYVH1 during oxidative stress we utilized the SUnSET technique. Cells were first exposed to two hours of low level (33µM) hydrogen peroxide induced oxidative stress prior to treatment with puromycin that allowed for a 20 min recovery period from the oxidative stress (Fig. 3.8). Following the antibiotic treatment, the cells were lysed and analyzed via Western blot in the same manner as previously described.
The resultant immunoblots were further processed to generate normalized scatterplots to control for differential cell growth using actin protein levels.
Figure 3.9 - Puromycin incorporation following 2h of hydrogen peroxide induced oxidative stress. A) HeLa cells were first transfected with FLAG-hYVH1 and Src Y530F. Subsequent Western blot analysis of the cell lysates following both a 2h hydrogen peroxide and a 20min puromycin treatment was next conducted to visualize any changes to cellular translation. Probing of the immunoblots using an α-Puromycin antibody illustrated a replicable increase in puromycin integration among samples exposed to oxidative stress (top). Moreover, samples containing FLAG-hYVH1 and Src appeared to show the most significant increase in puromycin integration (top). Control blots were also generated to ensure that equal protein expression was present, and that cell stress was not resulting in significant cell death by probing the blots with α-FLAG (top-middle), α-Src (bottom-middle), and α-Actin (bottom). B) Normalization of band intensities to actin levels, and conversion of the intensity values to relative fold changes resulted in the generation of the included scatterplot. Subsequent T-testing showed some statistical significance between stress positive samples however significant variability between replicates was observed.
Figure 3.10 - Puromycin incorporation following 3h of hydrogen peroxide induced oxidative stress. A) An additional stress experiment was conducted utilizing a more prolonged (3h) stressor to support our initial observations on the replicability of the impact stress had on translation. HeLa cells were again transfected with FLAG-hYVH1 and Src Y530F. Subsequent Western blot analysis of the cell lysates following both a 3h hydrogen peroxide and a 20min puromycin treatment was conducted. Immunoblot analysis using an α-Puromycin antibody showed a similar pattern in Src+hYVH1 samples experiencing increased puromycin integration when exposed to stress (top). Control blots were also produced to ensure efficient transfection (bottom three blots) B) Subsequent normalization of our band intensities to control for varying protein expression levels visualized the same trends observed through the immunoblots.

Following immunoblotting and data normalization we observed a similar pattern of puromycin incorporation in the non-stressed samples as our original efforts. During that original experiment, we observed a consistent increase in translational levels when hYVH1 was overexpressed in HeLa cells alone, and we observed a further increase in translation when hYVH1 and Src were co-overexpressed (Fig. 3.7). The data collected during this experiment continued to support this finding being clearly illustrated by figure 3.9 which visualizes the
observed translational trends though the plotted averages of each experimental sample’s replicates. Similar to in our initial experiments, we saw that while there was a relatively large amount of variation between replicates regarding the exact fold change in translation, the general trend outlines a clear increase in translation with the overexpression of each subsequent protein. Interestingly, the trends observed following hydrogen peroxide induced oxidative stress outlined the same trend in increasing translation, but to a more significant degree than when stress was absent. Generated immunoblots probed using the same α-Puromycin antibody showed that in the presence of stress, a slight but still noticeable increase in translational fitness could be observed in cells with hYVH1 overexpressed alone, but a more pronounced fold increase in puromycin integration was present when both proteins were co-expressed (Fig. 3.9, A). Normalization of the immunoblots to total cell levels based on actin protein expression, confirmed this observation (Fig. 3.9, B). This finding was somewhat unexpected as cells typically reduce cellular translation under stress. Subsequent comparative analysis of the relative fold change in puromycin incorporation between unstressed and stressed samples, outlined a replicable trend illustrating an increase in translation between each experimental sample. Regarding expression of hYVH1, two of our three stress positive replicates demonstrated a slight increase in puromycin integration compared to the stress negative ones with the average fold change showing a ~14.5% increase of translation when in the presence of stress. Similarly, we saw the same trend when examining the impact of Src-directed phosphorylation with all three stress positive replicates co-overexpressing hYVH1 and Src demonstrating an increase in translational fitness. Comparison of the average fold change of these sample pairs resulted in a ~11% increase in puromycin integration in the presence of stress relative to in the absence. Interestingly, the relative fold change caused by
stress appeared to be more significant for hYVH1 alone then when both proteins were co-expressed.

In addition to our thrice replicated two-hour stress experiments, we also conducted an additional experiment that exposed our HeLa cells to the same low-level oxidative stress for a longer period in an attempt to support our previous observations. Following a near identical experimental method, with exception of the time the stress was applied for (three hours rather than two), we again obtained an immunoblot (Fig. 3.10, A) and subsequent normalized scatterplot (Fig. 3.10, B) for an individual attempt. The resulting data illustrated the same trends that we observed following two hours of stress. We again saw the same characteristic changes to translation previously observed in the absence of stress and again we saw the same trend of stress positive experimental samples possessing additionally enhanced translational fitness. This data serves to further support our initial findings on the impact of stress on the incorporation of puromycin as alteration to how we applied the initial stress still produced comparable trends.

One possible explanation for our results lies in the protein structure of hYVH1, specifically with regard to the zinc-binding domain which has been suggested as the critical domain for ribosome association. Prior research into the behavior of hYVH1 in an oxidizing environment has shown that preferential oxidization of the cysteine residues in hYVH1’s C-terminal zinc-binding domain can occur. This was originally suggested as a means to protect the enzymatic activity of the protein by preventing oxidation of the catalytic cysteine, however, due to the capability of proteins to possess multiple functions it is plausible that this process can also impact hYVH1’s ability to function in ribosome biogenesis. Moreover, prior observations regarding hYVH1’s capability to improve cell survivability under oxidative stress also establishes the precedence that hYVH1 is a stress response protein. This implies the possibility
that hYVH1 is stress resistant and could function as a redox sensor for ribosome biogenesis and translation. We suggest that the preferential oxidation of cysteines in the C-terminal zinc-binding domain may double to alter hYVH1’s structural conformation in such a way that it is more readily phosphorylated by Src at Tyr179 or is more readily transported across the nuclear envelope. Knowing that phosphorylation of this site triggers hYVH1 to localize to the nucleus and participate in ribosome biogenesis, an increase in either the Src-directed phosphorylation of this site or the ability of it to trigger nuclear import could reasonably explain the observed increase in translation occurring. Additionally, due to the intrinsic link between translation and cell survival, an increased in hYVH1’s role in ribosome biogenesis could also partially explain the cytoprotective effect hYVH1 imparts in the presence of stress. One possible issue with this explanation is the lack of translational pausing among the + EV control samples. We propose that a possible reason for the lack of translational pausing observed is due to the relatively low concentration of hydrogen peroxide used. It is plausible that the low level of stress used in this experimental method was insufficient to trigger a significant stress-induced reduction in protein translation, but as evidenced by the results, was sufficient to illustrate the impact it can have on altering hYVH1’s ability to function in ribosome biogenesis.

While more investigation is required to elucidate the effect, oxidative stress has on hYVH1’s structure and how it impacts the level of Src-directed phosphorylation, the physiological relevance of our finding may be in the context of oncogenic tumorigenesis. Src kinase is a potent oncogene and is often constitutively active due to a mutation of its key regulatory residue, Tyr530, while human YVH1 has been observed to be over-expressed in a number of late-stage cancers. Cancer cell growth is characterized by increased ribosome biogenesis and translation, two mechanisms that both aforementioned proteins have been
illustrated to participate in. Moreover, another commonly observed characteristic of cancer cells, is increased oxidative stress due to exposure to hypoxic environments. Reactive oxidative species (ROS) have been shown to be pro-tumorigenic when present in low non-cytotoxic concentrations. Investigation into the impact of ROS in cancer has suggested that ROS can stimulate the growth of cancer cells which is intrinsically linked to ribosome biogenesis and protein translation. With evidence supporting the role of ROS in oncogenic cell growth, and the documented overexpression of both hYVH1 and Src in late-stage cancers, it is reasonable to suggest that an increase in translation could be occurring due to the presence of the oxidative stress despite the typical decrease experienced by non-cancerous cells.

The second explanation to the observed trends is that the increase in translation suggested by the fold change in puromycin integration, is not a direct result of the stress itself, but rather occurs following the removal of the stress due to the cells stress recovery process. Following oxidative stress, it is common to observe an increase in translation to occur as ribosome biogenesis begins to ramp back up following stress-induced pausing. The more translation that occurs following the removal of the stress, the more puromycin-tagged proteins that would be produced and the more significant the fold change would be. Due to the experimental design including a twenty-minute puromycin incubation following the removal of the oxidative stress, the cells were allowed a brief period of time to initiate stress recovery. Based on this, it is plausible that the increase in puromycin incorporation observed in this experiment is not due to hYVH1 and Src functioning to increase ribosome biogenesis during stress, but rather is due to an increased demand for hYVH1 in ribosome biogenesis during stress recovery. Human YVH1 has already been previously implicated in stress response through its role in stress granule disassembly setting a precedence for it to function in the cell’s recovery process, possibly by
participating in the formation of new ribosomes to help resume proper translation. Based on the experimental design allowing for a brief recovery period, we suggest that this explanation is the more likely of the two, but additional research would be required to determine this.

Subsequent to our observation of oxidative stress increasing hYVH1’s function in ribosome biogenesis characterized by an increase in translation, we wanted to continue to explore the impact of stress on hYVH1. It has been determined that Hsp70 is a prevalent binding partner of hYVH1 which interacts with the ATPase domain of Hsp70, implying the interaction is not the result of Hsp70 acting as a chaperone. Furthermore, it was shown that this interaction resulted in a significant increase in cell survival. Considering the intrinsic link between cell survival and ribosome biogenesis, we sought to investigate whether interaction with Hsp70 could alter hYVH1’s behavior in translation. To accomplish this, we replicated our original puromycin experiment, however, we substituted our Src kinase construct out for a His-Hsp70 DNA containing vector. This would allow us to investigate the individual impacts of each construct on cellular translation as well as to examine the impact caused by co-overexpression.
Figure 3.11 - Puromycin incorporation of cells differentially over-expressing hYVH1 and Hsp70. A) Immunoblots were first generated by probing cell lysates of HeLa cells expressing FLAG-hYVH1 and His-Hsp70 with an α-Puromycin antibody to detect any changes in puromycin incorporation that were occurring between samples. The resultant blots illustrated an increase in incorporation in both singly transfected samples (lanes 2 and 3), however, co-expression resulted in enhanced band intensities (top). Control blots were also produced to control for protein expression levels by probing the blots using α-FLAG (top-middle), α-His (bottom-middle), and α-Actin (bottom) antibodies. B) A scatterplot was generated to visualize the trends occurring due to overexpression of either DNA construct. The Western blot band intensities were normalized to actin levels and plotted based on their relative fold increases when compared to the EV control. All experimental samples illustrated an increase in puromycin incorporation with hYVH1+Hsp70 showing the most intense levels.
Western blot analysis of the resulting cell lysates procured from both the control and experimental samples using the same α-Puromycin as previously discussed, resulted in a similar pattern as our Src-hYVH1 experiment (Fig. 3.7) in that we saw that either protein alone resulted in a noticeable increase in puromycin incorporation and thus translation, with the sample co-expressing hYVH1 and Hsp70 showing the most significant change. Unlike when Src was present however, which showed that overexpression of hYVH1 was more impactful than its companion protein, examination of the resultant immunoblots here suggested that the impact on translation of hYVH1 was not significantly different from the alterations caused by Hsp70. This was further supported by figure 3.11, B which visualizes the normalized relative fold change of puromycin incorporation values relative to our empty vector control. While both individually expressed proteins showed a relatively similar increase to puromycin incorporation, it was evident from both figure 3.11, A and 3.11, B that the level of integration was markedly higher when the proteins were co-expressed. Comparison of the normalized relative data elucidated an average increase of 67% when we compare the doubly transfected experimental sample to the EV control and a ~15% average increase compared to either singly transfected experimental sample.

Similar to our previous SUnSET experimental data, we saw a significant level of variation in the observed fold changes resulting in a lack of statistical significance between some of the samples. Despite this, we still saw a statistically significant increase between hYVH1 alone and hYVH1 + Hsp70. Additionally, the replicability of the trends observed across all three replicates still allows us to make conclusions regarding the impact these proteins play in translation and the interplay between them. Consistent with what we have observed previously, every replicate of this experiment demonstrated an increase in the level of incorporation
associated with the over-expression of hYVH1 with the increase ranging from 7% to 116%. While this variability makes it difficult to assign statistical significance to these replicates when compared to the control sample due to the large calculated standard deviation, we can conclude that based on the replicability of the trend, that the overexpression of hYVH1 does cause an increase in cellular translation that we hypothesize is due to its role in ribosome biogenesis.

Of interest was the increase in puromycin integration observed in the samples containing overexpressed Hsp70 alone. Classically, Hsp70 has not been directly suggested to function in ribosome biogenesis, and as such was not expected to have had a major effect on puromycin incorporation. We suggest two possible explanations to this unexpected observation, both of which occur due to the molecular chaperone capabilities of Hsp70. It has been suggested that the absence of Hsp70-family proteins can directly alter the functionality of the translation factors Pab1 and eIF4F in yeast leading to a reduction in translation. Other research has also suggested that a lack of availability of HSPs can result in a reduction of polysomes and protein translation in yeast. Due the significant similarities between heat shock proteins in yeast and humans, it suggests the possibility of a similar function in humans. Moreover, HSP family members have also been suggested to directly alter transcriptional levels through their function in nascent protein folding. The ability of Hsp70 family members to alter cellular translation could explain the increases in puromycin integration observed here (Fig. 3.11) and would occur independent of hYVH1.

While it is completely plausible that the observed integration pattern is due to Hsp70 independently, a second possible explanation could be related to the readout assay. As previously discussed, the SUnSET method operates by utilizing puromycin, a tRNA analog, which results in the formation of truncated polypeptides due to premature termination of elongation. These
proteins are, at their core, defective which will greatly affect their ability to properly fold. Typically, proteins that cannot fold properly are degraded by the cell, however, due to Hsp70’s role as a chaperone, it is possible that it protects these puromycin-containing proteins from degradation by attempting to aid them in re-folding. Theoretically, this could result in an increase in the perceived level of puromycin compared to the control sample and explain the increase observed by Hsp70 overexpression.

The uncertainty of the cause of the increase in puromycin integration observed makes interpreting the observed increase in the doubly transfected experimental sample difficult. The simplest explanation is that the increase is simply the additive effect of both proteins independently. We have thus far provided significant evidence that hYVH1 alone can increase puromycin incorporation presumably through its role in ribosome biogenesis. The impact Hsp70 has on translation is still unclear, but we did reproducibly observe an increase in puromycin incorporation when Hsp70 was similarly expressed alone. Consequently, the observed increase when both proteins are present could be explained due to both proteins independently effects on translation. While this is the simplest explanation, due to the intrinsically linked nature of many biochemical mechanism, we suggest a second possibility in that the observed change could also be due to the interaction between hYVH1 and Hsp70. As previously mentioned, prior investigation of hYVH1 has elucidated Hsp70 as a prevalent binding partner with some data suggesting that a significant amount of cytoplasmic hYVH1 is bound to Hsp70 (DaDalt AA, unpublished). It has also been demonstrated that complex formation of hYVH1 and Hsp70 results in the co-localization of both proteins to the perinuclear region of the cells. Moreover, Src kinase has also been demonstrated to be present in the perinuclear region as well. The presence of both hYVH1 and Src in the perinuclear region and the ability of Hsp70-bound
hYVH1 to localize to this region tempts us to speculate that Hsp70 may play a role in shuttling hYVH1 to the perinuclear region, bringing it into proximity of Src and allowing Src-directed phosphorylation to occur. The increased amount of phosphorylated hYVH1 would result in an expected increase in puromycin integration as observed in figure 3.11. While we cannot definitively conclude which of the aforementioned explanations is the most accurate without further investigation, we can conclude that co-expression of hYVH1 and Hsp70 results in an increased level of puromycin integration and suggest the possibility that this occurs due to a protein-protein interaction between the two.

3.4 The Impact of Hydrogen Peroxide Mediated Oxidative Stress on the hYVH1-Hsp70 Protein Complex

Through the use of the SUnSET technique, we observed that the interaction between Src and hYVH1 resulted in an increase to protein translation, indicated by increased puromycin integration. We have suggested that this increase is likely due to Src-directed phosphorylation of hYVH1 driving the localization of hYVH1 into the nucleus for ribosome biogenesis. Additionally, we have also seen that low-level hydrogen peroxide induced oxidative stress appears to enhance the impact of Src-mediated phosphorylation of hYVH1, possibly by affecting hYVH1’s structural conformation allowing for it to be more readily phosphorylated. Subsequent experiments also illustrated a similar increase in puromycin integration in the presence of both hYVH1 and Hsp70. Previous research has led us to suggest that Hsp70 may aid hYVH1 in localization to the perinuclear region through the formation of a protein-protein complex which encourages Src-directed phosphorylation of hYVH1. To further explore the possibility that this
was occurring, we set out to investigate the interplay between the hYVH1-Hsp7- complex and Src kinase.

Previous work conducted by our lab has observed that immunoprecipitation of FLAG-tagged hYVH1 results in a significant amount of co-precipitation of Hsp70. Interestingly, it has been observed that the presence of Src kinase results in the attenuation of this complex (DaDalt AA, unpublished). To further support this finding and ensure its validity, we first replicated the original experiment and examined both the levels of Hsp70 co-precipitating and the phosphotyrosine levels. HeLa cells expressing FLAG-hYVH1, His-Hsp70, and Src were subjected to a FLAG-IP and analyzed through immunoblotting using appropriate antibodies to examine the effect Src had on the formation of the complex.
Figure 3.12 – Src-mediated phosphorylation leads to dissociation of the hYVH1-Hsp70 protein complex. HeLa cells prepared by co-expressing FLAG-hYVH1 and His-Hsp70 were compared to cells triple transfected with FLAG-hYVH1, His-Hsp70, and Src following a FLAG-IP. Subsequent immunoblotting using both an α-FLAG and α-His antibody showed that when co-expressed with Src kinase, hYVH1 levels following the IP were unaltered, but Hsp70 levels were drastically lower (top). Further analysis using an α-pTyr antibody showed that in the presence of Src, bands appeared at the molecular weights of ~70kDa and ~37kDa (second from top). Control blots were also produced to control for protein expression levels by probing the immunoblots using α-FLAG (third from top), α-His (second from bottom), and α-Actin (bottom) antibodies.
Probing of the generated immunoblot for FLAG-hYVH1 showed even precipitation of hYVH1 but differing levels of Hsp70 band intensity depending on if Src was present (Fig. 3.12). The presence of Hsp70 in readily detectable levels both in the presence and absence of Src supported the previously characterized interaction between hYVH1 and Hsp70 as co-precipitation of Hsp70 implying the existence of a strong and direct protein-protein interaction. Additionally, Src kinase’s ability to trigger a significant decrease in Hsp70 following immunoprecipitation, again supporting prior observations by members of our lab, suggests that Src-mediated phosphorylation causes dissociation of the hYVH1-Hsp70 complex and may have important consequences on hYVH1’s role as a ribosome biogenesis factor. This hypothesis is further supported by our previously discussed observations that overexpression of hYVH1 with either of the other two proteins can have a noticeable and reproducible effect in altering puromycin integration.

With these novel findings regarding puromycin integration and the previously proposed impact of Src-mediated phosphorylation on the formation of the hYVH1-Hsp70 complex, we set out to further explore the ability of Src to disrupt this complex under oxidative stress. Having observed that oxidative stress can influence the apparent level of translation within cells co-expressing hYVH1 and Src, and the impact Src-mediated phosphorylation has on the complex, it was reasonable to propose that stress may additionally augment the protein-protein complex. Similar to our previous efforts, the stress positive samples were subjected to two hours of low-level hydrogen peroxide induced oxidative stress. However, following the two-hour incubation the cells were directly lysed without any stress recovery period. Subsequently, the samples were processed by FLAG immunoprecipitation and analyzed by Western blotting.
Figure 3.13 – Src-mediated phosphorylation of Hsp70 is inhibited by low-level oxidative stress. A) HeLa cells prepared by co-expressing FLAG-hYVH1 and His-Hsp70 were compared to cells triple transfected with FLAG-hYVH1, His-Hsp70, and Src following 2h exposure to a low-level hydrogen peroxide induced oxidative stress and a FLAG-IP. The generated cell lysates were analyzed by Western blot and probed using an α-His antibody. In the presence of Src, significantly less Hsp70 is present, but when the stress is present, the levels of partially rescued (top). Subsequent probing using an α-pTyr antibody showed that the oxidative stress was also altering the phospho-states (second and third blots from top). Control blots were also produced to control for protein expression levels (bottom two blots). B) Normalization of the His band intensities following the IP to FLAG-hYVH1 post-IP and His-Hsp70 protein lysate levels was first conducted to control for expression levels. The resulting relative fold changes for Hsp70 present after IP were graphed and T-testing was conducted. This data visualized a statistically significant decrease in Hsp70 due to Src when stress was absent but saw the loss of that significance when stress was present. C) Normalization of the level of phosphorylation of hYVH1 to FLAG IP levels and Src levels showed a reproducible decline in phospho-hYVH1 under stress, but also had of relatively significant variation in fold change between replicates. D) Normalization of the level of phosphorylation of Hsp70 to His-Hsp70 post-IP levels and Src

n/s – not significant
* P < 0.05
levels showed a statistically significant decrease in phosphorylation of Hsp70 under stress conditions.

Figure 3.13 contains the resulting data collected from three biological replicates of this experiment with figure 3.13, A being a representative Western blot of the generated samples and figures 3.13 B, C, and D being graphical representatives of the normalized trends observed regarding Hsp70 co-precipitation, and tyrosine phosphorylation levels of hYVH1 and Hsp70. By comparing the amount of Hsp70 present in each sample following the FLAG immunoprecipitation, we were able to investigate the effect low-level oxidative stress has on mediating Src’s ability to cause the complex to dissociate. Initial observations of the representative immunoblot (Fig. 3.13, A), showed that in the absence of Src and stress, Hsp70 readily purified with hYVH1 supporting our previous observations (Fig. 3.12). As expected, the addition of Src in the absence of stress consistently resulted in a decrease in the intensity of the chemiluminescent band representing Hsp70 following the IP. This again confirmed that Src-mediated phosphorylation causes the protein complex to dissociate. These observed trends, visualized in figure 3.13 A, were further confirmed following statistical processing. Subsequent normalization and confidence testing using the T-test confirmed that there was a statistically significant decrease in Hsp70 levels following IP when Src was overexpressed in the sample. With Src’s ability to attenuate the formation of the hYVH1-Hsp70 complex in the absence of stress clear, we next turned to the stress positive samples. Interestingly, we did see a noticeable change regarding Hsp70’s levels in the presence of stress relative to non-stress. Surprisingly, the immunoblots showed that in the presence of the low-level stress, the Src-mediated decrease in Hsp70 present following immunoprecipitation was much lower than in the absence of stress suggesting a clear reduction in the ability of Src to attenuate the complex. This observation was
supported by the normalized data (Fig. 3.13, B) which outlined that in the presence of the low-level stressor, the Hsp70 levels were similar to the levels observed in their Src-absent companion samples (only an ~18% decrease in the average when exposed to stress compared to an ~75% decrease in the absence of stress). Interestingly, we also noticed oxidative stress moderately attenuated the hYVH1-Hsp70 interaction in the absence of Src. As Hsp70 is a well-known stress response protein that functions in a wide variety of other stress-related mechanisms, there may be less Hsp70 available to bind with hYVH1 due to its involvement in other cellular pathways under these conditions. Collectively, these results support our previous hypothesis that the presence of Src leads to the dissociation of the hYVH1-Hsp70 complex. However, this data also suggests a novel function of low-level hydrogen peroxide induced oxidative stress in protecting the complex from Src’s attenuating effect. This finding suggests that under stress, the interaction between hYVH1 and Hsp70 is resistant to the impact of Src kinase which may point to a temporal-spatial aspect to this regulatory mechanism.

We hypothesized that the Src-mediated dissociation of the hYVH1-Hsp70 complex was being caused by a phosphorylation event. Therefore, we investigated how oxidative stress alters the level of Src-mediated phosphorylation of either hYVH1 or Hsp70 using phosphotyrosine Western blot analysis. Under non-stress conditions, we saw that when Src was present, both Hsp70 and hYVH1 exhibited reactivity to the α-pTyr antibody implying that Src was phosphorylating both proteins. Interestingly, the band that corresponded to Hsp70 reproducibly appeared to be more intense suggesting that Src-mediated phosphorylation of Hsp70 was more readily occurring than Src-directed phosphorylation of hYVH1 (Fig. 3.12 and Fig. 3.13, A). We suggest that this difference in phospho-status is due to Hsp70 requiring phosphorylation for dissociation of the complex and subsequent phosphorylation of hYVH1 to occur. This would
imply that Hsp70, while possibly required for proper hYVH1 localization to the perinuclear region as previously discussed, may prevent hYVH1 from being targeted by Src until the complex is disassembled.

When we analyzed the samples exposed to oxidative stress, we observed that the phosphotyrosine levels for Hsp70 was significantly reduced but was not significantly different for hYVH1 following normalization of the immunoblot band intensities to protein levels present in the FLAG-IP. Since under oxidative stress conditions Src’s ability to disrupt the hYVH1-Hsp70 complex is attenuated, more Hsp70 is co-immunoprecipitated under these conditions. Therefore, the increase in α-pTyr band intensity visualized was due to more protein present for the antibody detection. Statistical processing of the collected data showed that all three replicates showed a reproducible decrease in phosphorylated Hsp70 when normalized and statistical testing using the T-test indicated that the reduction observed was statistically significant (Fig. 3.13, D). This supports our hypothesis that Hsp70 phosphorylation is critical for the disassembly of the hYVH1-Hsp70 complex as the stress positive samples now show that less Src-mediated phosphorylation of Hsp70 is occurring which is resulting likely resulting in the increased level of protein present following immunoprecipitation. Further examination into the impact of oxidative stress on hYVH1’s phospho-state elucidated a similar but significantly less pronounced effect. Comparative analysis between hYVH1’s level of phosphorylation both with and without stress, illustrated a reproducible decrease in phosphorylation as well. Of note, we typically observed a relatively variable change in phosphorylation with two of our replicates showing a relatively small decrease. Due to this variability in fold change, the recorded decrease in phosphorylation does not fall within a 95% confidence interval. Despite this, the replicability of the decrease does suggest that a decrease in hYVH1 phosphorylation is occurring in response to oxidative stress.
A possible explanation for the observed results may be attributed to cellular localization effects. We have previously discussed the prospect that the hYVH1-Hsp70 complex plays a role in localization of hYVH1 to the perinuclear region. Once here, Src-directed phosphorylation of hYVH1 may trigger it to enter the nucleus where it participates in ribosome biogenesis which has an indirect impact on protein translation in the cell. Based on the ability of Src to trigger the disassembly of the hYVH1-Hsp70 complex and the lack of previous research regarding the presence of Hsp70 at the 60S ribosomal subunit, we have suggested that hYVH1 is in its unbound form during phosphorylation and nuclear transport. The data collected here adds the additional observation that under low-level hydrogen peroxide induced oxidative stress, Src-mediated dissociation of the complex is hampered. This reduction is likely the result of a decrease in the ability of Src to phosphorylate Hsp70 which also suggests that Hsp70 must be in its phospho-state for dissociation to occur. The observed decrease in hYVH1 phosphorylation occurring when more complex is present also suggests that Hsp70 may be partially shielding hYVH1 making Src-directed phosphorylation of the protein more difficult. Less phosphorylation of hYVH1 under stress would mean less hYVH1 in the nucleus which may contribute to decreasing ribosome biogenesis rates during cellular insults. Furthermore, the hYVH1-Hsp70 complex has been strongly suggested to function in cell survival under oxidative stress and as such would support the theory that hYVH1’s primary function may shift from participating in ribosome biogenesis under basal conditions to cell survival when a stress is present.\(^5\)

Our observations regarding a reduction complex dissociation under stress resulting in a possible decrease in phosphorylated hYVH1 available for ribosome biogenesis is somewhat paradoxical when compared to the apparent increase in translation in cells co-overexpressing hYVH1 and Src when exposed to oxidative stress (Fig. 3.9 and 3.10) described earlier. We
suggest that the observed increase in translation despite the decrease in free hYVH1 may be due to 1) more efficient nuclear translocation during oxidative stress or 2) more efficient translation restoration during cellular stress recovery when hYVH1 and Src are co-expressed.

While less hYVH1 is in its free phospho-form under the stress, which would imply a reduction in its capacity to participate in ribosome biogenesis, previous research has suggested that the presence of free radical oxygen species can influence the transport of macromolecules, such as hYVH1, to cross the nuclear envelope. This could result in a higher efficiency of nuclear import resulting in less phospho-hYVH1 having a greater effect. This would also fit with the previously discussed possibility that oxidative stress may alter hYVH1’s conformation which simultaneously improves its nuclear transport capability. While significantly more research would be required to explore this possibility, especially localization studies of hYVH1 under oxidative stress, it does provide a plausible explanation to both the reduction in phosphorylated hYVH1, and increased translation observed assuming the boost in puromycin integration occurs during the stress.

The second explanation suggests that the increase in translation was the result of an increased demand for nuclear hYVH1 due to a stress recovery-induced boost in ribosome biogenesis. Our findings regarding Src-mediated dissociation of the complex supports this explanation. Assuming that the previously described increase in translation is due to the cell stress recovery response, these results would suggest that during oxidative stress hYVH1-Hsp70 complexes form to participate in stress response and cell survival. Once basal conditions have been restored, there would be a need for the cell to restore ribosome biogenesis and protein translation. This environment would be characterized by increased Src-mediated phosphorylation and dissociation of the complex. Liberated phosphorylated hYVH1 would then be available to
recycle back into the nucleus to return to its role as a regulator of 60S ribosome biogenesis that is critical for efficient protein translation. Further investigation, including the replication of this experiment with a recovery period similar to our puromycin experiment, will be necessary to support the role of hYVH1 as a key mediator of the translational stress response.
CHAPTER 4:
CONCLUSIONS AND FUTURE WORK

4.1 Conclusion

4.1.1 Confirmation of Src-Directed Phosphorylation of hYVH1

Co-overexpression of hYVH1 and Src Y350F in HeLa cells, showed that hYVH1 experiences significantly higher levels of tyrosine phosphorylation suggesting that Src kinase can target hYVH1 but did not specifically confirm it as a direct event. Moreover, the SFK Hck also resulted in phosphorylation of hYVH1, which due to the similarity of SFK catalytic domains, provides initial evidence that the increase phosphorylation of hYVH1 is a direct effect as different kinase family members, which are typically involved in different pathways, both result in a similar outcome. Using two independent in-vitro kinase assay setups we showed that Src was directly phosphorylating hYVH1. Subsequent mass spectrometric analysis of phosphorylated hYVH1 confirmed that Tyr179 on hYVH1 is a potent phosphorylation site of Src indicating the same phosphorylation site identified from cellular samples was phosphorylated in-vitro, and provided stronger evidence that hYVH1 is a novel substrate of Src.

4.1.2 A Src-directed Decline in Phosphatase Activity

Due to the low-level of in-vitro enzymatic activity exhibited by hYVH1 against artificial phosphosubstrates, and due to Tyr179’s proximity to the catalytic domain, it was reasonable to assume that this event could also influence enzyme activity. Utilizing a DiFMUP assay, we observed a reproducible and statistically significant decrease in phosphatase activity of hYVH1 following overexpression with Src. This finding led us to the conclusion that Src-directed phosphorylation of hYVH1 causes a reduction in its phosphatase activity. While the exact reason
for this decline is unclear, we suggest that the reduction is due to a conformational change induced by the addition of one or more phosphate moieties. While other Src phosphorylation sites appear to exist on hYVH1, Tyr179’s proximity to the catalytic domain and the abundance of charged polar amino acid residue nearby leads us to predict phosphorylation of this site as the likely cause of the presumed conformational change and reduced intrinsic phosphatase activity.

4.1.3 The Impact of hYVH1 on Translation Due to Phosphorylation

Utilizing the SUnSET method, we provided evidence that while overexpression of hYVH1 alone results in a reproducible increase in puromycin integration, which implies an increase in translation, the co-overexpression of hYVH1 and Src leads to a more pronounced increase. These findings corresponded with the previous findings regarding the increased number of monosome and polysome complexes present in HeLa cells following co-overexpression. The complementary nature of this data led us to make the conclusion that the Src-mediated phosphorylation of hYVH1 results in an increased ability of hYVH1 to function in late stage pre-60S ribosomal subunit maturation. While the exact mechanism hYVH1 plays in ribosome biogenesis is still being elucidated, we suggest, based on previous work, that the phosphorylation event allows for more efficient recycling of hYVH1 into the nucleus, increasing the amount of hYVH1 available for biogenesis.

Due to hYVH1’s known role as a cytoprotective factor and studies that show oxidative stress activates Src, we examine the possibility that oxidative stress may influence hYVH1’s role in biogenesis. We observed that in the presence of mild oxidative stress an increased level of puromycin integration was present when hYVH1 was expressed alone and with Src. We concluded that, due to the puromycin treatment occurring in the absence of the stress, the cells
were in a state of stress recovery and the increase translational rate was most likely occurring due to an increased recycling of hYVH1 into the nucleus to restore ribosome biogenesis during cellular recovery. While we believe this is the more likely explanation, it is also possible that the increased level of translation observed could be the result of the stress itself with prior research suggesting that reactive oxygen species, through unknown mechanisms, can increase the ability of cancerous cells to proliferate requiring higher levels of ribosome biogenesis and translation.

Further investigation of hYVH1’s impact on cellular translation through ribosome biogenesis was conducted by exploring the impact of Hsp70, a known hYVH1 binding partner. Overexpression studies of both proteins showed that individually both proteins increased translation, however, co-overexpression resulted in a more pronounced translational increase. While the trends we observed were replicable, the reason behind the observed increases is not fully understood. Due to Hsp70’s classical lack of characterization in ribosome biogenesis, but known function in translation, it is possible that the increase observed in co-overexpressed samples is purely an additive effect from each protein’s independent function. While this possibility prevents us from making a definitive conclusion regarding the cause, we also suggest the possibility that Hsp70 is important in the localization of hYVH1 to the perinuclear region for phosphorylation and subsequent nuclear shuttling.

4.1.4 Low-Level Oxidative Stress Influences the Ability of Src to Dissociate the hYVH1-Hsp70 Protein Complex

Having seen that the presence of Src, Hsp70, and stress appear to affect hYVH1’s ribosome biogenesis function, we sought to explore the interplay between all three factors. Previous research concluded that Src-mediated phosphorylation results in the attenuation of the
hYVH1-Hsp70 complex and this was further supported here. Interestingly, following the application of a low-level hydrogen peroxide induced stress, we observed that Src was less able to disrupt the hYVH1-Hsp70 complex. Also, the level of phosphorylation was significantly lower for Hsp70 and slightly lower for hYVH1 under the oxidative stress conditions employed. These findings led us to conclude that Hsp70 is less readily phosphorylatable in the presence of oxidative stress, and that the phosphorylation is required to trigger dissociation of the hYVH1-Hsp70 complex. We also suggest that Hsp70 may shield hYVH1 from Src-directed phosphorylation.

Based on the observations made in this study we propose the following working model. Under basal cell conditions, hYVH1 co-localizes to the perinuclear region with Hsp70 as a protein-protein complex. In response to growth signals, Src-mediated phosphorylation of Hsp70 results in dissociation of the complex and subsequent Src-directed phosphorylation of hYVH1 at Tyr179 occurs which results in a possible conformational change of the catalytic domain reducing phosphatase activity. This phosphorylation event also initiates shuttling of hYVH1 into the nucleus where it participates in late stage pre-60S ribosomal subunit maturation. Following release of hYVH1 from the 60S ribosomal subunit, hYVH1 is complexed again by Hsp70 to facilitate another round of hYVH1-mediated 60S ribosome biogenesis. This has an indirect effect on protein translation by increasing the amount of translationally fit 60S ribosomal subunits available. Under low-level oxidative stress conditions, Src-mediated phosphorylation of Hsp70 decreases resulting in an increase of the number of available hYVH1-Hsp70 complexes. These complexes are thus available to function in stress response activities (e.g. stress granule disassembly) which has been shown to be critical for cell viability. Following the termination of the stress, Src can once again attenuate the formation of the protein complex and target hYVH1
for phosphorylation at Tyr179 triggering nuclear localization and contributing to the restoration of ribosome biogenesis and translation during cellular stress recovery.

**Figure 4.1 – Current working model of hYVH1’s function cellular function.** A) Under basal cell conditions, the hYVH1-Hsp70 complex forms and localizes to the perinuclear region. Upon
arrival, Src-mediated phosphorylation of Hsp70 leads to dissociation of the complex and Src-directed phosphorylation of hYVH1 at Tyr179 triggers it to shuttle into the nucleus. Once in the nucleus, hYVH1 binds the pre-60S ribosomal subunit aiding in nuclear export. Following export, hYVH1 induces the ejection of Mrt4 from the subunit allowing Rpp0 to replace it and leading to ejection of hYVH1. The hYVH1-Hsp70 complex then reforms allowing hYVH1 to be recycled back to the perinuclear region for another round of ribosome biogenesis. B) Under mild oxidative stress, some of the hYVH1-Hsp70 complex dissociates allowing free Hsp70 and free hYVH1 to participate in independent stress response pathways. The remaining complexes localize to the perinuclear region where Src-mediated dissociation of the complex still occurs, but to a much lesser extent. The un-dissociated complexes are then able to function in stress response. Figure created with BioRender.com.

4.2 Future Work

While we have classified Tyr179 as a Src targeted phospho-site, mutational studies have suggested that this is not the only site Src targets on hYVH1. Having seen the impact modification of this site has on hYVH1’s localization and consequently its function, it is important to further examine hYVH1 for additional phosphorylation sites. Our MS analysis involved a relatively rapid but crude sample preparation procedure that used trypsin-based digestion and lacked alkylation. While this was sufficient for identifying Tyr179, an already suspected site, it makes the discovery of novel sites more difficult. Additional more comprehensive MS investigation needs to be conducted which utilizes cysteine alkylation and an alternative protease. Cysteine alkylation prevents the loss of novel phospho-site containing peptide fragments that also contain cysteine residues capable of reforming disulfide bonds. Moreover, alternative peptide bond cleavage using a protease with a different recognition sequence relative to trypsin would help minimize the loss of site containing fragments that are exceptionally large or small and get filtered out during MS. Optimization of our MS preparation
will likely aid in the identification of additional target sites which can then be characterized using point mutational studies.

Moreover, upon the development of our \textit{in vitro} phosphatase activity assay, we observed that Src-directed phosphorylation results in a decrease in hYVH1’s enzymatic activity. This is a crucial finding as any mechanism by which a phosphatase activity can be modified is important to understand. While we suggested that this is possibly due to the phosphorylation of Tyr179 causing a conformational change, further research is needed to definitively identify the reason behind this decline. One possible approach to confirming this theory, is through the utilization of X-ray crystallography. While the crystal structure of hYVH1’s catalytic domain has been obtained, the crystal structure of phosphorylated hYVH1 could provide invaluable insights into the impact Src-directed phosphorylation has on the protein structure of hYVH1 and would allow us to make more concrete conclusions regarding the impact of phosphorylation on hYVH1’s catalytic domain conformation. Moreover, while we have seen that Src can induce a reduction in activity, we have still yet to elucidate any conditions that increase hYVH1’s activity. Human YVH1 is classified as a phosphatase yet it demonstrates very poor \textit{in vitro} activity. This has led us to hypothesize that hYVH1 requires a specific set of conditions or cofactors to improve its phosphatase ability. For example, it is possible that association with the 60S ribosomal subunit induces a more catalytically active conformation. Also, with more and more evidence supporting hYVH1 as a stress response protein, it is crucial that we examine its activity under stress. The replication of our activity assay under conditions reconstituting the ribosomal environment and oxidative stress conditions will be important to test to elucidate novel activating factors and even the substrate of hYVH1.
While we have provided sufficient evidence to conclude that the increase in translation observed by the overexpression of hYVH1 and Src is due to the increased ability of hYVH1 to participate in ribosome biogenesis, it is unclear if hYVH1 must be phosphorylated to interact with the ribosome or if it is only required for nuclear localization and requires dephosphorylation prior to association with the 60S subunit. Utilization of phosphomimetic hYVH1 mutants and ribosomal fractionation would provide possible means to determining the importance of the phosphate moiety for 60S ribosomal subunit association. Additionally, determination of the cause behind the translational increase following oxidative stress is necessary. Repetition of that experiment with the puromycin treatment conducted concurrently with the hydrogen peroxide induced stress would provide an efficient means of determining if the increase was caused directly by ROS or in-directly due to the recovery process causing an initial surge in ribosome biogenesis. In a similar fashion, the cause for the increase depicted due to the presence of Hsp70 and hYVH1 needs to be further investigated. Immunofluorescent investigation of the localization of both proteins could help support our suggestion that Hsp70 localizes hYVH1 to the perinuclear regions to interact with Src.

Finally, and of critical importance, is the determination of these observed trends under endogenous conditions to support the physiological relevance. All experiments utilized here involve the overexpression of the relevant proteins which could influence the cellular readouts tested. Replication of these experiments utilizing siRNAs to knockdown hYVH1, Src, and/or Hsp70 should be conducted to complement the findings made here and confirm that the observations we have made are physiologically relevant and not due to abnormal interactions caused by excess protein. Establishing that the findings made here are applicable to conventional
cells is crucial in establishing the importance of this project and furthering our understanding of hYVH1 and its role in ribosome biogenesis.
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