Regulation of the Ribosome Biogenesis Factor hYVH1 by Src-mediated Phosphorylation

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Regulation of the Ribosome Biogenesis Factor hYVH1 by Src-mediated Phosphorylation

By

Ashley Anne DaDalt

A Dissertation
Submitted to the Faculty of Graduate Studies
through the Department of Chemistry and Biochemistry
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the Degree of Doctor of Philosophy
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Windsor, Ontario, Canada

2021

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DECLARATION OF ORIGINALITY

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ABSTRACT

The protein tyrosine phosphatase (PTP) superfamily is a major segment of the signal transduction landscape, responsible for regulating the biomolecular phosphorylation status of the cell. Diverse PTP subclasses exist, some of which are understudied and whose cellular functions are not yet fully elucidated. YVH1, an atypical PTP of the dual-specificity phosphatase (DUSP) subclass, is a pleiotropic enzyme with no known substrate. Human YVH1 (hYVH1) protects cells from cellular stressors, including heat shock and oxidative stress, regulates the cell cycle, disassembles stress granules, and acts as 60S ribosome biogenesis factor. Additionally, heat shock protein 70 (Hsp70) has been found to be a binding partner of hYVH1. The functional significance of this interaction includes improving the cell survival phenotype, but further details remain to be resolved, including their temporal and spatial regulation in vivo.

In this study, we investigate cellular effects of the recently discovered novel Src-mediated phosphorylation site at tyrosine 179 on hYVH1. First, this phosphorylation event negatively regulates the ability of hYVH1 to perform its stress granule disassembly function. Phosphorylated hYVH1 also displays enhanced shuttling to the nucleus, in contrast to its typical steady-state localization pattern that presents as a more cytoplasmic distribution. Biochemical evidence suggests that this phosphorylation event induces a higher level of cellular translational fitness, due to its increased binding to the 60S ribosome. Quantitative proteomics reveal that upon Src-mediated phosphorylation of hYVH1, formation
of ribosomal species that represent stalled intermediates are attenuated through the alteration of associating factors that promote translational repression. Furthermore, the robust interaction of hYVH1 and Hsp70 is disrupted upon Src phosphorylation. Using limited proteolysis, we propose a putative binding interface consisting of residues 68-77 on hYVH1 and 326-361 on Hsp70.

Collectively, we demonstrate the relevance of the Src-mediated phosphorylation event at tyrosine 179 on the subcellular localization of hYVH1, its disassembly function at stress granules, and the interaction between hYVH1 and Hsp70. Most notably, we have identified Src phosphorylation of hYVH1 as increasing the ability of hYVH1 to perform its 60S ribosome biogenesis role, thereby increasing cellular translational fitness and allowing for fine-tuning of protein synthesis. As the ribosome continues to emerge as a major scaffold structure for integrating various inputs regarding cellular homeostasis, insights into the mechanism of hYVH1 are essential.
DEDICATION

To my family, friends, and all who have supported me
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Figure 3.3 - hYVH1 subcellular localization (+/- Src). Immunofluorescence images with FLAG-hYVH1 as a marker of hYVH1 expression (green), nuclei stained with Hoechst (blue), and a merged depiction of these images. Scale bars = 25 µm. A) Top row panels display empty vector (EV) control. Second row panels display representative cells with overexpressed hYVH1 WT, with prominent cytoplasmic localization. Third row panels display hYVH1 co-expressed with Src, which displays higher nuclear localization. B) Top row panels display cells expressed with hYVH1 Y179F, showing largely cytoplasmic localization, while the bottom panel shows hYVH1 Y179E and its predominant nuclear localization. C) Bar graph illustrating the frequency of subcellular localization in % nuclear and % cytoplasmic for the various hYVH1 constructs, calculated via ImageJ software. Means ± SD of three independent experiments (n = 30 cells) are shown. Using Student’s t-test, p values were calculated and compared to hYVH1 WT, with differences considered statistically significant at p < 0.0001 (**).
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>A site</td>
<td>aminoacyl site</td>
</tr>
<tr>
<td>AB</td>
<td>ammonium bicarbonate</td>
</tr>
<tr>
<td>ACN</td>
<td>acetonitrile</td>
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<td>adenosine diphosphate</td>
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<td>diethyl pyrocarbonate</td>
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<td>DMEM</td>
<td>Dulbecco’s Modified Eagles Medium</td>
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<td>exit site</td>
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<tr>
<td>EF2</td>
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</tr>
<tr>
<td>EIF6</td>
<td>eukaryotic translation initiation factor 6</td>
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</tr>
<tr>
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<td>formic acid</td>
</tr>
<tr>
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<td>fetal bovine serum</td>
</tr>
<tr>
<td>FLAG-IP</td>
<td>FLAG-immunoprecipitation</td>
</tr>
<tr>
<td>HDMS$^E$</td>
<td>high definition mass spectrometry</td>
</tr>
<tr>
<td>HeLa</td>
<td>Henrietta Lacks (cervical cancer cell line)</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>Hsp70</td>
<td>heat shock protein 70</td>
</tr>
<tr>
<td>hYVH1</td>
<td>human ortholog of the yeast VH1-related phosphatase</td>
</tr>
<tr>
<td>IFRD1</td>
<td>Interferon-related developmental regulator 1</td>
</tr>
<tr>
<td>LC-MS</td>
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</tr>
<tr>
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<tr>
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</tr>
<tr>
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<tr>
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<td>phosphatidylinositol (3,5)-bisphosphate</td>
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<td>PIP₃</td>
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<td>protein phosphatase Mg²⁺- or Mn²⁺-dependent</td>
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<td>phosphoprotein phosphatase</td>
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</tr>
<tr>
<td>PSP</td>
<td>protein serine/threonine phosphatase</td>
</tr>
<tr>
<td>PTK</td>
<td>protein tyrosine kinase</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PTM</td>
<td>post-translational modification</td>
</tr>
<tr>
<td>PTP</td>
<td>protein tyrosine phosphatase</td>
</tr>
<tr>
<td>pTyr</td>
<td>phosphorylated tyrosine</td>
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<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
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<td>receptor of activated protein C kinase 1</td>
</tr>
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<td>substrate-binding domain</td>
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<td>sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
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<tr>
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<tr>
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<td>trifluoroacetic acid</td>
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<tr>
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<td>yeast VH1-related phosphatase</td>
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<td>ZBD</td>
<td>zinc-binding domain</td>
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CHAPTER 1:  
INTRODUCTION

1.1 Cellular phosphorylation background

1.1.1 Protein phosphorylation

Post-translational modifications (PTMs) are a well-known mechanism utilized by proteins to regulate their activity, localization, structure, stability, interactions, and more.\textsuperscript{1,2} One of the most prevalent PTMs, phosphorylation, was first reported in 1906 by Phoebus Levene.\textsuperscript{3} With regards to proteins, modification of hydroxyl amino acids with a phosphate moiety introduces negative charges on an otherwise neutral amino acid. This new chemistry induces conformational changes within a three-dimensional fold of a protein that mediates the various consequences on protein function. Phosphorylation modifications are achieved through the transfer of the terminal (\(\gamma\)) phosphate from adenosine triphosphate (ATP) onto a substrate. This modification most commonly occurs on tyrosine, threonine, and serine residues, although there is evidence of other amino acids as target substrates.\textsuperscript{4} Two superfamilies of enzymes, known as protein kinases and protein phosphatases, catalyze the addition and removal of a phosphate group to a protein, respectively (Figure 1.1). Because phosphorylation is a central mechanism of regulating protein function, it is imperative that homeostasis of these enzymes is maintained.
Figure 1.1 - Reversible protein phosphorylation. A protein kinase catalyzes the transfer of the γ-phosphate from an ATP molecule onto a serine, threonine, or tyrosine residue. The original neutral -OH group now contains two negative charges. This phosphate moiety can be removed as inorganic phosphate by a protein phosphatase, returning the amino acid substrate back to its neutral form. Figure created with BioRender.com.

The enzymatic addition of a phosphate group catalyzed by protein kinases was first discovered in the 1950s by Edmond Fischer and Edwin Krebs,\(^5\) and has since been a widely studied topic. Genes encoding for protein kinases constitute 2% of the human genome and kinases function in phosphorylating approximately 30% of known cellular proteins.\(^6\) There are currently 518 known protein kinases, which consist of 428 protein serine/threonine kinases (PSKs) and 90 protein tyrosine kinases (PTKs) in the human genome.\(^2\) In contrast, there are \(~140\) known phosphatase genes, with \(~30\) being protein serine/threonine phosphatase (PSP) catalytic subunits and 107 being members of the protein tyrosine phosphatase (PTP) superfamily.\(^7\) This disparity between the number of protein kinases and phosphatases can be partially explained by the large number of genes encoding PSP regulatory subunits that culminate in approximate equal numbers of phosphatase holoenzymes as the kinase superfamily. The large number of phosphatase
genes, along with the diverse array of regulatory motifs associated with these enzymes, highlights the staggering dynamic capabilities of phosphatases, as they are not merely housekeeping enzymes. In fact, the activities of phosphatases are as tightly regulated as kinase enzymes. This rather recent revelation has encouraged the scientific community to acquire greater understanding of phosphatases in cellular homeostasis and disease.

1.1.2 Protein phosphatases

The two main categories of protein phosphatases, PSPs and PTPs, can be further subdivided into various subclasses. The PSP catalytic subunits arise from two distinct gene families, the first being protein phosphatases that are Mg\(^{2+}\)- or Mn\(^{2+}\)-dependent (PPM), related structurally to PP2C, and the phosphoprotein phosphatase (PPP) family, typically subdivided into three subfamilies, related to PP1, PP2A, and PP2B (calcineurin).^8^

In terms of the PTPs, this superfamily is typically categorized into four main classes (I-IV) based primarily on domain architecture and homology between the catalytic domains (**Figure 1.2**). Class I is the largest, which contains both the classical PTPs and dual-specificity phosphatases (DUSPs). Proteins in the DUSP category include enzymes capable of removing a phosphate moiety from serine/threonine and tyrosine residues, along with members that dephosphorylate carbohydrates and specialized lipids.
1.1.3 PTP catalytic mechanism

Common between the PTPs is the presence of a highly conserved catalytic motif, \( \text{HCX}_5 \text{R(S/T)} \), in the active site of the phosphatase domain. This invariant sequence allows for a specific microenvironment which reduces the pKa of the catalytic cysteine, allowing it to perform a nucleophilic attack on the phosphorylated substrate. The subsequent contribution of a catalytic acid, which donates a proton to the leaving group of the target, releases the dephosphorylated product from the active site of the phosphatase and forms a thiol-phosphate enzyme intermediate. In the second half of the reaction, an ordered water molecule is deprotonated by a catalytic base, allowing the
hydroxide ion to perform a nucleophilic attack on the thiol-phosphate intermediate. This releases phosphoric acid as the second product and regenerates the PTP (Figure 1.3).

Substrate specificity of protein phosphatases is determined by the depth of the catalytic cleft, as well as other regulatory domains that often mediate substrate recognition.

![Figure 1.3 - PTP catalytic mechanism](image)

The phosphorylated substrate (“S”) is electrostatically stabilized in the hydrophobic cleft of the phosphatase by the arginine residue, while the catalytic cysteine performs a nucleophilic attack on the phosphate moiety. The dephosphorylated substrate is released, and a thiol-phosphate intermediate forms. The phosphate group is then liberated from the phosphatase through acid/base catalysis via an aspartic acid residue and water molecule as depicted, allowing for the phosphatase to return to its initial state for further enzymatic activity.

1.1.4 DUSP subclass

As stated above, Class I of the PTPs contains the subclass of DUSPs and can be further broken down into even more specific groups as depicted in Figure 1.2. Most notably are the myotubularins, which dephosphorylate the lipids phosphatidylinositol 3-phosphate (PI3P) and phosphatidylinositol (3,5)-bi-phosphate (PIP₂) which are enriched on endocytic structures, the PTENs, which act on the proximal signaling lipid phosphatidylinositol (3,4,5)-trisphosphate (PIP₃), and the map kinase phosphatases (MKPs), which remove phosphate groups from threonine and tyrosine residues within the activation loop of mitogen-activated protein kinases (MAPKs).
Of great intrigue in the DUSP subclass are atypical DUSPs, which share certain characteristics with the MKPs but lack the N-terminal CH2 domain. The proteins in this category are relatively less understood due to their lack of commonality with the other subclasses, but do share the conserved catalytic domain of DUSPs (Figure 1.4). Prevalent atypical DUSPs include Laforin (a polyglucose phosphatase which contains a carbohydrate-binding domain and causes Lafora disease when mutated), VHR/DUSP3 (specifically dephosphorylating and inactivating ERK1 and ERK2), and DUSP12, also known as the human ortholog of the yeast-VH1 related phosphatase, hYVH1.

**Figure 1.4 - Domain layout of the atypical DUSPs.** Of the 19 atypical DUSPs, all share a common catalytic domain (“PTP”). In addition, Laforin contains a carbohydrate-binding domain (“CBM-20”), HCE1 contains a guanylyltransferase domain (“GTase”), and DUSP12 (hYVH1) contains a zinc-binding domain (“Zn”). Figure from Bayón and Alonso, *Emerging Signaling Pathways in Tumor Biology*, 661:2 (2010) 185-208.
1.2 hYVH1 background

1.2.1 hYVH1 discovery & genetics

In 1992, Guan and Dixon cloned and characterized the yeast VH1-related phosphatase (YVH1), marking it as one of the earliest eukaryotic DUSPs identified. The mRNA levels of YVH1 were found to be dramatically induced by nitrogen starvation and low temperatures. Additionally, knockdown of the *yvh1* gene in yeast exhibited a slow growth phenotype, with defects in glycogen accumulation and spore maturation. Following these studies in yeast, the human ortholog (termed “hYVH1”) was identified and shown to share 31% sequence identity with YVH1. Interestingly, the *hyvhl* gene has now been shown to be widely conserved throughout evolution in eukaryotes from yeast to humans, with only one gene copy being present in each species. A further element of intrigue is that the *hyvhl* gene was found to be amplified in various cancers, including retinoblastoma, liposarcomas, and intracranial ependymoma. The genetic details of hYVH1 and initial documented phenotypes observed in yeast highlighted the importance of investigating its structure and biological functions.

1.2.2 hYVH1 structure

Wild-type hYVH1 contains 340 amino acids and consists of two domains: an N-terminal catalytic (phosphatase) domain, which possesses the characteristic CX5R motif, and a C-terminal zinc-binding domain (ZBD), as shown in Figure 1.5. These two domains are connected by an intrinsically disordered region (residues 176-219). It is important to note that, to date, hYVH1 is the only known PTP member with this unique ZBD, which is characteristic of all orthologs. The ZBD of hYVH1 is capable of binding 2
moles of zinc per mole of protein and has been established to play a critical role in the biological functions of hYVH1.

**Figure 1.5 - Domain architecture of hYVH1.** The N-terminal phosphatase domain (residues 1-175) contains the highly conserved CX$_5$R motif, as shown. The C-terminal zinc-binding domain (residues 220-340) indicates the 7 cysteines and 1 histidine residue, capable of binding 2 moles of zinc/mole of protein.

Currently, only the phosphatase domain of hYVH1 has had its crystal structure solved.\textsuperscript{25} As the ZBD is critical to the cellular functions of hYVH1, the pursuit of more structural information is necessary.

1.2.3 Biological roles of hYVH1

Initial studies on hYVH1 have shown its relevance in various cellular functions, including cell survival and the cell cycle. Overexpression of hYVH1 is able to repress cell death by cellular insults, specifically heat stress, H$_2$O$_2$, and Fas receptor activation.\textsuperscript{26} This ability of hYVH1 was shown to require the ZBD, as well as the catalytic activity of hYVH1. Notably, these are all redox-sensitive signaling pathways, and hYVH1 did not display this cytoprotective effect when subject to cisplatin, which triggers apoptosis via the DNA damage response pathway.\textsuperscript{26} This cell survival ability of hYVH1, coupled with the aforementioned hyvh1 gene amplification in various cancers, allows for speculation that hYVH1 can give tumour cells the capacity to survive unfavourable conditions and support uncontrolled cell growth.
In terms of the cell cycle effect, overexpression of hYVH1 showed increased multinucleation and G2/M phases of the cell cycle, whereas silencing of hYVH1 expression using siRNA arrested cells in the G0/G1 phases and caused a susceptibility to cellular senescence. For these observed phenotypes, solely the ZBD of hYVH1 was sufficient and the protein’s catalytic activity was not required. This data is intriguing, as other DUSPs shown to regulate cell cycle require the phosphatase activity of the enzyme. These studies were the first examples of hYVH1 as a novel cell survival factor and cell cycle regulator.

When investigating further into the capability of hYVH1 to protect cells from oxidative stress induced by H$_2$O$_2$, it was revealed that the ZBD acts as a redox sensor, capable of forming intramolecular disulfide bonds. This allows for hYVH1 to avoid irreversible inactivation during severe oxidative stress by protecting the catalytic cysteine residue, again highlighting the importance of this unique ZBD. Once the stress is removed and reducing conditions have been established, zinc ejection is readily reversible (and required) to recover hYVH1’s intrinsic phosphatase activity \textit{in vitro}.

A compelling aspect of the various hYVH1 studies in cells is that a direct physiological substrate of the phosphatase activity of hYVH1 has yet to be identified, despite various efforts in this regard. This is a common theme among atypical DUSPs.

1.2.4 hYVH1 & Hsp70 interaction

Heat shock protein 70 (Hsp70) is a ubiquitous molecular chaperone which functions primarily in response to stress for proper protein folding and for regulating the assembly/disassembly of various multiprotein structures. Hsp70 consists of three domains, which are the N-terminal ATPase domain, the substrate-binding domain (SBD),
and the C-terminal domain or “lid” (Figure 1.6). The ATPase domain binds ATP, and upon hydrolyzing it to adenosine diphosphate (ADP), confers conformational changes in the other two domains. Hsp70’s SBD has an affinity to neutral, hydrophobic residues, and will surround the substrate’s peptide backbone. Dependent on the ATP- or ADP-bound status of the ATPase domain, the C-terminal lid will be open or closed, respectively. In the closed (ADP-bound) state, the substrate will be tightly bound in the SBD.33,34

Figure 1.6 - Domain architecture of Hsp70. The N-terminal ATPase domain (residues 1-382) will bind ATP and hydrolyze it to ADP, influencing the conformation of the other domains. The substrate-binding domain (residues 398-537) will allow for tight or loose binding of a substrate, which can be “locked in” by the C-terminal lid (residues 538-641) when Hsp70 is in the ADP-bound state.

In relation to hYVH1, Hsp70 was discovered to be a novel binding partner of hYVH1, but not in the typical chaperone-substrate manner.26 Domain deletion studies of this interaction showed hYVH1 binds to the ATPase domain of Hsp70, whereas the domain of hYVH1 involved was not as clearly elucidated, although speculated to be the ZBD.26 The physiological relevance of this interaction is that Hsp70 was shown to enhance the cell survival phenotype of hYVH1 in response to the apoptotic-inducing stressors previously mentioned. To further understand these findings, it will be imperative to elucidate specifically how these two proteins bind, as well as discern the temporal and spatial regulation of this interaction.
1.3 hYVH1 stress granule influence

1.3.1 Stress granule background

When cells are exposed to stress, a mechanism to halt production of protein at the translation initiation step is often employed. This causes the formation of stress granules (SGs), which are conserved, cytoplasmic assemblies of translationally repressed mRNA. SGs can disassemble upon removal of the stress, and therefore allow for quick return of mRNAs for translation, thus protecting the mRNA from degradation while the cell is undergoing the cellular insult. However, the untranslated mRNA is not the only component of SGs; factors affecting translation initiation, and both RNA-binding and non-RNA binding proteins are also present. Examples of non-RNA binding components observed in SGs include enzymes for PTMs, metabolism, remodeling complexes for protein and/or RNA, as well as factors needed for signaling and apoptosis. The composition of SGs will vary, depending on the stress condition the cell is undergoing.

There are multiple key factors involved in both the assembly and disassembly of SGs that need to be tightly regulated in order to maintain this key stress-induced mechanism. Two of the relevant major players involved are discussed below.

1.3.2 Stress granule assembly & disassembly

A key RNA-binding protein that has a primary role in assembling SGs is T-intracellular antigen-1 (TIA-1). TIA proteins have two main domains: an RNA-binding domain and the glutamine-rich prion-related domain (PRD). The former is responsible for bringing RNA to the SG, while the latter creates cytoplasmic aggregates, which are required for recruiting the components of a SG. This illustrates the importance of the PRD of TIA-1, in that it is able to form an aggregate, acting as a scaffold for abortive
preinitiation complexes. The aggregation of the PRD will actually induce expression of Hsp70, and is also regulated by Hsp70. It is relevant to note that when TIA-1 is subject to an oxidizing agent, for example H$_2$O$_2$, SG assembly is suppressed. More specifically, H$_2$O$_2$ stress on TIA-1 will promote apoptosis. This is due to the fact that SGs will typically house, along with their other components, apoptotic regulatory factors. If SGs cannot be formed due to TIA-1 being oxidized, then these apoptotic factors will no longer be sequestered in the SG and will be available to the cell to proceed with apoptosis. These insights feature the major role TIA-1 plays in the assembly of SGs and allow for TIA-1 to be commonly used as a SG marker in biochemical experiments.

In regard to the disassembly of SGs, heat shock proteins play a crucial function. Hsp40 has a direct and important relationship with Hsp70, acting to trigger the ATPase activity of Hsp70 (intrinsically fairly weak), and also assist in providing substrate specificity for Hsp70 by acting as an adapter protein. It has been shown that different Hsp70 and Hsp40 family members co-localize with SGs, therefore implying their likelihood to play a role in SG dynamics. Studies performed in both yeast and mammals that looked into inhibiting the function of Hsp70 showed both a boost in SG assembly, as well as a hindrance in disassembly. Moreover, if there are mutations causing a defect in Hsp70 or Hsp40 function, there is a halt in the clearance of SGs when recovering from stress, further suggesting their valuable role in SG disassembly. Specifically, two Hsp40 family members, Ydj1 and Sis1, are crucial to clearance of SGs. These two proteins, interestingly, have differential effects on SGs and lead to two distinct fates. Firstly, the untranslated mRNAs being held in a SG have the potential to re-enter the translation process, and consequently be converted into proteins. Secondly, the SG can simply be
cleared by autophagy, and thus the mRNAs would be destroyed. Ydj1 was found to act on SGs as per the first process, returning the mRNA for translation, while Sis1 appears to promote SGs being targeted for autophagy. A schematic outlining these two fates is shown in Figure 1.7. Without Ydj1 functioning correctly, an accumulation of SGs is seen in both the cytosol and in the vacuole. Although this is not the full scope of factors which influence SG assembly and disassembly, understanding these central regulatory proteins are relevant to begin further elucidation of this mechanism.

Figure 1.7 - Two fates of stress granules dictated by Hsp70 adaptor proteins. Dependent on which adaptor protein interacts with Hsp70, the SG will be subject to two distinct fates. The left-hand side illustrates Ydj1, where the mRNA is being released from the SG, and therefore becoming available once again for translation. The right-hand side depicts the influence of Sis1 on the SG, targeting it for autophagy. Figure from Walters & Parker, Trends in Biochemical Sciences, 40:10 (2015) 552-559.
1.3.3 Role of hYVH1 in stress granule disassembly

Upon performing an hYVH1 interactome analysis, our laboratory discovered multiple ribonucleoprotein (RNP) complex proteins, having direct or indirect interactions with hYVH1. Specifically, signature SG proteins were identified and therefore led our lab to investigate if hYVH1 potentially played a role in SG dynamics. Upon further investigation, it was demonstrated that hYVH1 is involved in the disassembly of SGs. This was demonstrated by the observation that overexpression of hYVH1 in mammalian cells reduces the size of SGs induced by oxidative stress, as well as seeing more numerous and larger SGs when hYVH1 expression is knocked down. Importantly, the disassembly function of hYVH1 could be rescued using siRNA resistant cDNA clones of hYVH1 during stress granule recovery experiments. Of note, the assembly of SGs was unaffected in the knockdown experiments. Additionally, using domain deletion constructs of hYVH1, it was shown that only the ZBD of hYVH1 is required for this cellular phenotype; the catalytic domain, and therefore catalytic activity, was not necessary for the disassembly of SGs.

The finding that hYVH1 plays a role in the disassembly of SGs is compelling with the well-studied role of Hsp70 in this regard, as well as the knowledge of the strong protein-protein interaction between hYVH1 and Hsp70. Further details on the precise regulation of this newly discovered function are currently being investigated.

1.3.4 hYVH1 interactome - tyrosine kinase Src

During the aforementioned interactome analysis performed on hYVH1, it was reproducibly observed that Src kinase was present. Src is a non-receptor tyrosine kinase that regulates numerous cellular processes, most notably cell growth, proliferation, and
cell survival.\textsuperscript{43} Src is known to be activated by stress and mediates signal transduction in multiple pathways related to cell migration, invasion, and survival.\textsuperscript{44} Although there is not yet evidence of Src kinase being directly involved in SG dynamics, the protein tyrosine kinase Syk, which can be activated by Src family members, has been studied for its role in clearance of SGs via the autophagy pathway.\textsuperscript{45}

1.4 Ribosome biogenesis

1.4.1 Eukaryotic ribosome biogenesis

Eukaryotic ribosomes consist of the asymmetric 60S large subunit and the 40S small subunit, which initiate in the nucleolus separately and come together to form the full-functioning 80S ribosome in the cytoplasm, allowing for translation of mRNA into protein. The pathways for formation of each subunit are intricate and involve many trans-acting factors, allowing for a gradual assembly into a mature ribosome. The stages of subunit biogenesis are often referred to as early, intermediate, and late-stage maturation. The process of translation costs the cell significant energy, and therefore needs to be tightly regulated; it is also broadly halted when the cell is under stress.\textsuperscript{46}

Of note is a relatively new concept emerging about ribosomes known as ribosome heterogeneity, which refers to ribosomes with distinct compositions that have the ability to translate specific pools of mRNAs.\textsuperscript{47,48} The diversity is not limited to the ribosomal proteins, which can vary in their stoichiometry, composition, or PTMs, but additionally in the rRNA components through variants or modifications. Moreover, proteins associated with the ribosome, or even the subcellular location of the ribosome, can contribute to ribosome heterogeneity.
Specifically of interest is the ability of ribosomes to contribute to translational control, which is a critical factor in gene expression. It is imperative the cell can adjust its protein levels quickly and efficiently in response to internal or external factors. This is particularly important during scenarios such as embryonic development, where cell fate is being determined, as well as under conditions of stress. This notion highlights how ribosomes are more specialized than conventionally thought, and hence, the ribosome allows for another layer of gene regulation based on the ribosomal components.

1.4.2 Ribosomal subunit maturation

Both the 40S and 60S subunit maturation pathways initiate in the nucleolus. To begin, precursor rRNAs (pre-rRNA), including the 18S, 5.8S, and 28S, are transcribed here by RNA polymerase I as a single transcript. In eukaryotes, this forms the 47S precursor RNA which then undergoes required modifications and processing, while ribosomal proteins are simultaneously incorporated. The 18S rRNA will be associated with the small subunit and remains present as part of the mature 40S. For the mature 60S large subunit, there are three rRNAs present, which are the 5.8S, 28S, and the 5S that is transcribed independently in the nucleus by RNA polymerase III, and then transported into the nucleolus with the others.

RNA polymerase II is responsible for synthesizing the mRNA for ribosomal proteins and is active in the nucleus. As these mRNAs become translated in the cytoplasm by fully formed ribosomes, the ribosomal proteins will translocate back to the nucleus to associate with their respective subunits. The 40S and 60S subunits are exported to the cytoplasm separately before joining to form the mature 80S, capable now of translating mRNAs. As stated above, this process requires many trans-acting factors.
that must be coordinated in a specific manner to accurately move from the early stages of subunit biogenesis to the late, mature stage.

1.4.3 hYVH1 in 60S biogenesis

A central aspect of the 60S biogenesis pathway is the exchange of the nuclear protein Mrt4 for its cytoplasmic counterpart, P0. P0 is required in the mature ribosomal stalk, allowing for full function of the 80S ribosome. Particularly, the ribosomal stalk is required for recruitment of translation factors and is essential for ribosome activity. In yeast, this exchange has been shown to be mediated by YVH1, where YVH1 displaces Mrt4 on the pre-60S particle in the nucleus, translocates to the cytoplasm, and is subsequently replaced by P0 (Figure 1.8). YVH1 in this scenario is commonly referred to as a “recycling factor,” and is shown to be necessary for proper maturation of the 60S large subunit. These recycling factors associate and participate with pre-ribosomal particles at specific stages of maturation. Once their role is completed, these factors can be released from the pre-ribosome and then recycled to participate in the next round of ribosomal maturation. Although these studies were performed in yeast, hYVH1 has also been shown to complex with the 60S in human cells. Furthermore, these studies demonstrated the ZBD of YVH1 was sufficient to perform these ribosomal functions. This is in line with what was observed for the role of hYVH1 in SG disassembly previously discussed.
**Figure 1.8 - Model of YVH1’s role in ribosome stalk assembly.** Nuclear Mrt4 is present on the pre-60S subunit before Yvh1 releases it, allowing the pre-60S to export to the cytoplasm. At this stage, P0 is able to replace Yvh1 and therefore form the mature, translationally active 60S subunit. Figure from Lo, et al., *Journal of Cell Biology*, 186:6 (2009) 849-862.53

1.4.4 Hsp70 in 60S biogenesis

Biogenesis of both the 60S and 40S subunits are complex, intricate pathways which involve a plethora of different factors which are still being discovered. Pertinent to the study of hYVH1 and its established 60S biogenesis role, Hsp70 proteins also have an active function. Specifically, Ssa (an Hsp70-like molecular chaperone) acts as a co-chaperone for protein Jjj1, which is a pre-60S factor involved in the late cytoplasmic steps of the large ribosomal subunit biogenesis.55–57 It is also well-established that Hsp70 is one of the main scaffolding proteins, required for proper protein folding once the fully-functioning ribosome has translated mRNAs. Without Hsp70 at this stage, misfolding and aggregation of proteins occurs.32,34 As the interaction between hYVH1 and Hsp70 is explored in more comprehensive detail, these known functions of Hsp70 should be considered.
1.4.5 Stages of translation

Once the mature 40S and 60S ribosomal subunits are assembled, there are three main stages of the translation process. The first step begins with the initiation phase. During initiation, a first tRNA molecule will attach to the 40S small subunit, which then attaches to the 5’ end of the mRNA strand to be translated.\textsuperscript{58} Once a start codon is encountered, they will stop. This first tRNA molecule that attaches to the start codon on mRNA will correspond to the first amino acid in the protein sequence to be synthesized, and is almost always methionine.\textsuperscript{59,60} This then recruits the 60S large subunit to form around the mRNA strand as well and assemble with the 40S subunit, forming the full 80S ribosome around the mRNA. Once these pieces have come together, they form what is called the “initiation complex.”\textsuperscript{58} A simplified depiction of the initiation process is shown in Figure 1.9.\textsuperscript{61}
Figure 1.9 - Eukaryotic translation initiation schematic. During the first, initiation step of translation, the 40S small ribosomal subunit will attach to the first tRNA (corresponding to methionine - Met), which then binds to the 5’ end of an mRNA strand. Once the initiator tRNA detects and binds a start codon (AUG), the 60S large ribosomal subunit assembles onto the 40S-mRNA complex to form the full initiation complex. Also shown are the E, P, and A sites, relevant for the following translation step of elongation. Figure from Khan Academy, Stages of translation.⁶¹
The second step is known as elongation, in which tRNAs will bring amino acids to the ribosome, allowing for lengthening (“elongating”) of the polypeptide chain. It is important to recognize the three “sites” in the ribosome, known as the E (exit), P (peptidyl), and A (aminoacyl) sites. The initial methionine tRNA begins in the P site, and new incoming tRNAs will insert themselves into the A site, also sometimes referred to as the “landing site.” A tRNA molecule will bind when its associated anticodon is a perfect complement to the next exposed codon on the mRNA. At this point, a peptide bond forms between the first amino acid and the new one, and the first amino acid is transferred onto the second one (still attached to its tRNA). As the ribosome now begins to read the mRNA strand, the empty tRNA will enter the E site, and therefore exit the complex (Figure 1.10). The tRNA with the growing polypeptide will have moved to the P site, and the A site is now vacant again for a new tRNA to enter, complementary to the newly exposed codon. This cycle then repeats until a stop codon is encountered.
When elongation begins, the methionine tRNA will occupy the P site of the ribosome. The next amino acid, complementary to the next codon, will enter the A site via its tRNA, where a reaction will then take place to create a peptide bond between these two amino acids. This allows the initial, now empty, tRNA to release from the E site, and the newly forming polypeptide will enter the P site. The A site is then open for the next tRNA to enter and the process can continue. Figure from Khan Academy, *Stages of translation*.61
The third and final stage is aptly named as “termination.” When a stop codon (UAA, UAG, or UGA) enters the A site, they are recognized by proteins known as release factors. Although release factors are not tRNAs, they will fit into the P site of the ribosome and add a water molecule onto the final amino acid, rather than allowing another peptide bond to form. The peptide will now be liberated from the final tRNA and is released from the ribosome. At this stage, the protein is ready for any required processing or folding and will be ready to perform its cellular function. The complex steps of initiation, elongation, and termination are controlled by numerous regulatory proteins to ensure fidelity and tune translation in response to cellular needs. Alterations in the regulation of ribosome biogenesis and protein synthesis can have catastrophic results for the cell and are hallmarks of numerous human diseases. Since many of these regulatory systems are poorly characterized, advancing our understanding of the mechanisms regulating ribosome dynamics remains a critical area of research.

1.5 Objectives

hYVH1 has elucidated roles in modulating the cell cycle, acting as a redox sensor, protecting the cell from certain stressors, regulating stress granule dynamics, and as a trans-acting factor in 60S ribosome biogenesis. Hsp70 is a known binding partner of hYVH1, whose relationship, in terms of their combined cellular effects and their detailed physical interaction, still needs to be further investigated. The current hypothesis is that hYVH1 acts as a rheostat for cellular growth and cell survival through its regulation of mRNA dynamics, both at the level of ribosome biogenesis and various RNP structures, including stress granules.
The main aim of this thesis is to explore further intricacies of the dual-specificity phosphatase, hYVH1. The consequence of Src-mediated phosphorylation of hYVH1 on cellular processes will be examined, along with mechanisms regulating hYVH1 in mammalian cells.

Specifically, the objectives are:

1) Investigate the effects of Src-mediated phosphorylation of hYVH1 on stress granules, subcellular localization, and 60S ribosome association using immunofluorescence microscopy and ribosomal profiling

2) Development of a robust ribosomal profiling method to examine hYVH1-mediated protein alterations by label-free quantitative proteomics

3) Elucidate details of the binding interface between hYVH1 and Hsp70 using in-solution limited proteolysis
CHAPTER 2:
MATERIALS & METHODS

2.1 Plasmids

Wild-type (WT) hYVH1 FLAG-tagged DNA was described previously.\textsuperscript{21} Site-directed mutagenesis was used to create the Y179F and Y179E mutants of hYVH1 by Dr. Christopher Bonham and confirmed by automated DNA sequencing (ACGT Corp.). Human His\textsubscript{6}-tagged Hsp70 mammalian construct was generously provided by Dr. Frank Sharp (UC Davis). Human myc-Src Y530F mammalian construct was generously provided by Dr. Michel Tremblay (McGill University).

2.2 Cell culture

HeLa cells (ATCC\textsuperscript{®}, CCL-2\textsuperscript{TM}) were grown and maintained as a monolayer in Dulbecco’s Modified Eagles Medium Nutrient Mixture F12-HAM (DMEM; Sigma-Aldrich, #D8437), supplemented with 10\% (v/v) fetal bovine serum (FBS; Sigma-Aldrich, #F7942) and 1\% (v/v) penicillin-streptomycin (Thermo Fisher, #15140122) at 37 °C and 5\% CO\textsubscript{2}. For overexpression experiments, cells were split 24 h prior to transfection into antibiotic-free media. Respective cDNAs were introduced via cation-mediated transfection using linear polyethylenimine (PEI; Polysciences, #23966). Five hours post-transfection, cells were washed with phosphate buffered saline (PBS; Thermo Fisher, #SH3002802) and incubated overnight in fresh media with antibiotics. 24 h post-transfection, cells were washed with cold PBS, and lysed in buffer containing 50 mM Tris-HCl (pH 7.4), 1\% Triton X-100, 150 mM NaCl, and 0.1\% SDS, supplemented with protease inhibitors - 1mM phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich,
#78830) and 10 µg/mL Aprotinin (Sigma-Aldrich, #A3428). Soluble lysates were separated from cell debris by centrifugation at 24 000 x g for 10 min at 4 °C. Any alterations to the above protocol are noted below.

For ribosomal profiling experiments, 24 h post-transfection, cells were treated with 100 µg/µL cycloheximide (CHX; Sigma-Aldrich, #C1988) for 10 min at 37 °C. Subsequently, cells were washed two times with cold PBS supplemented with 100µg/µL CHX. Before cell lysis, transfected cells were pooled from three 15 cm cell culture plates by the addition of 3 mL of PBS with 100 µg/µL CHX per plate, followed by scraping cells and aliquoting the cell suspension into one 15 ml conical tube. Cells were harvested by centrifugation at 500 x g for 10 min at 4 °C. Cells were lysed in buffer containing 20 mM Tris-HCl (pH 7.4), 100 mM KCl, 5 mM MgCl₂, and 0.3% NP-40 in diethyl pyrocarbonate (DEPC; Sigma-Aldrich, #D5758) H₂O, supplemented with 1 mM PMSF, 10 µg/mL Aprotinin, 100 µg/µL CHX, 2 mM dithiothreitol (DTT; Sigma-Aldrich, #D9163), and 1% RNaseOUT (Invitrogen, #10777-019). Buffer solutions were made in Milli-Q H₂O treated with DEPC. Soluble lysates were separated from cell debris by centrifugation at 24 000 x g for 10 min at 4 °C. Fresh lysates were then loaded onto sucrose gradients for ribosomal profiling (section 2.5.2).

### 2.3 Affinity chromatography

FLAG-hYVH1 was isolated from cellular lysates using Anti-FLAG® M2 Affinity Gel resin (Sigma-Aldrich, #A2220) via a 3 h incubation on a nutator at 4 °C, while His₆-Hsp70 was isolated from cellular lysates using HIS-Select® Nickel Affinity Gel resin (Sigma-Aldrich, #P6611) via a 1.5 h incubation on a nutator at 4 °C. Samples were
washed three times in buffer containing 50 mM Tris-HCl (pH 7.4), 0.1% Triton X-100, 150 mM NaCl, and 0.1% SDS and subsequently resuspended in 6X SDS-PAGE loading dye for analysis by Western Blotting or Coomassie staining using Imperial™ Protein Stain (sections 2.4 and 2.6).

For limited proteolysis experiments, Anti-FLAG® M2 Magnetic Beads (Sigma Aldrich, #M8823) and His-Mag™ Agarose Beads (Novagen, #71002) were used in the same manner as described above.

2.4 Western blotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels were run at 125 V for 2 h and then transferred onto polyvinylidene difluoride (PVDF; Millipore, #IPVH00010) at 100 V for 1 h using the Mini-PROTEAN system by BioRad. PVDF membranes were blocked for 1 h at room temperature in either 5% skim milk or 5% bovine serum albumin (BSA; Sigma-Aldrich, #A9647) on a platform rocker. Membranes were then incubated on a rocker overnight at 4 °C in respective primary antibodies, including mouse anti-FLAG® M2 (Sigma-Aldrich, #F3165), mouse anti-His (Santa Cruz, #sc-8036), rabbit anti-Actin (Sigma Aldrich, #A2066), mouse anti-phospho-tirosine, clone 4G10 (Millipore, #05-1050), mouse anti-c-Myc (Santa Cruz, #sc-40), mouse anti-LP0 (Santa Cruz, #sc-293260), and rabbit anti-S3 (Cell Signaling, #2579S). The following day, membranes were washed three times in 1X Tris-buffered saline, 0.1% Tween (1X TBST), and then incubated on a rocker for 45 mins at room temperature in the respective secondary antibodies, including goat anti-mouse-HRP (Sigma-Aldrich, #A4416) and goat anti-rabbit-HRP (BioRad, #170-6515). After three more washes in 1X
TBST, chemiluminescent images were obtained using SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher, #34095) on BioRad ChemiDoc Imaging System (cat. #17001401).

2.5 Ribosomal profiling

2.5.1 Sucrose gradient preparation

Sucrose solutions were prepared using DEPC-treated Milli-Q H₂O at 5% and 40% sucrose (Sigma-Aldrich, #S7903) concentrations in a buffer of 20 mM HEPES (pH 7.4), 150 mM KCl, 15 mM MgCl₂, 100 µg/µL CHX, and 1 mM DTT. Sucrose gradients were prepared by layering 5% sucrose solution into 17 mL Open-Top Polyclear centrifuge tubes (Seton Scientific, #7042), followed by addition of 40% sucrose solution to the bottom of the tube. Gradients were formed using BioComp Gradient Maker (model #108) with tube holder SW28.1, on settings short, 5-40% sucrose (w/v) with long caps. Once formed, gradients were stored at 4 °C for 1 h.

2.5.2 Sucrose gradient fractionation

Equal amounts of lysate (equivalent OD₅₆₀ of 10) were carefully layered on top of the sucrose gradients and ultracentrifuged at 167 000 x g at 4 °C for 3 h (Thermo Scientific, Sorvall® SureSpin™ 630). Upon completion, gradients were loaded onto a piston gradient fractionator (BioComp Instruments, #157) and fractionated with the parameters of speed: 0.2 mm/sec, distance: 85.0 mm, and number of fractions: 30. Throughout fractionation, absorbance was continually monitored at a wavelength of 254 nm using a UV Monitor (Bio-Rad, #731-8160) and a Fraction Collector was used to
collect fractionated samples (Gilson, #FC203B). Fractions were then stored at -20 °C until analysis via Western Blotting (section 2.4) or in-gel digestion (section 2.6.1).

2.6 In-gel digestion

2.6.1 Digestion for ribosomal fractions

Ribosomal fractions were resuspended in 2X SDS-PAGE loading dye and loaded onto a 15% SDS-PAGE gel. Samples were run using a “short stack” method, where the gel running is stopped once all of the sample has entered the resolving layer of the gel (30 min). Gels were stained overnight with Imperial™ Protein Stain (Thermo Fisher, #24615) and de-stained the following day with Milli-Q H2O.

The entire sample area was excised from the gel with a scalpel blade and cut into small pieces. Gel pieces were then de-stained further in a 2:1 acetonitrile (ACN; Honeywell Burdick & Jackson®, #015-4):50 mM ammonium bicarbonate (AB; Thermo Fisher, #A643-500) solution, at 37 °C for 15 min, and repeated a second time. Gel pieces were subsequently dehydrated by addition of 100% ACN, vortexted, and dehydrated a second time. The shrunken, semi-dried gel pieces were then dried completely via vacuum centrifugation (Thermo Fisher) for 15 min. Gel pieces were rehydrated in trypsin digestion buffer, consisting of 15 ng/µL sequencing-grade modified trypsin (Promega, #V5111) diluted in 50 mM AB. Samples were incubated on ice for 30 min, before the addition of more AB to ensure gel pieces were fully immersed and placed in a shaking incubator overnight at 37 °C.

Peptides were extracted from the gel pieces by adding 150 µL of extraction buffer, consisting of 2:1 ACN:5% formic acid (FA; Thermo Fisher, #A117-50), incubated
at 37 °C for 15 min, and this step then repeated a second time. Peptides were concentrated via vacuum centrifugation for 1.5 h and reconstituted in 0.15% trifluoroacetic acid (TFA; Thermo Fisher, #28904).

Peptides were purified using Oasis® HLB 1cc Extraction Cartridge columns (Waters, #186000383). Initially, columns were activated with 100% ACN, followed by equilibration with 0.15% TFA, repeated five times total. On the fifth equilibration, 1% ACN was added to the 0.15% TFA solution. Peptide solutions were then loaded onto their respective columns, followed by three washes with 0.15% TFA. Elution of peptides was performed in a stepwise manner, with the first elution being 20:80 ACN:0.15% TFA, second elution 50:50 ACN:0.15% TFA, and the third and fourth elutions being 80:20 ACN:0.15% TFA. These purified peptides were then concentrated again via vacuum centrifugation for 2 h, reconstituted in 0.1% FA in mass spectrometry grade water (Honeywell Burdick & Jackson®, #365-4), and kept at -20 °C until mass spectrometry analysis.

2.6.2 Mapping in vivo tyrosine phosphorylation sites on Hsp70

HeLa cells were transfected with myc-Src and His6-Hsp70 as described above. Following nickel affinity chromatography, proteins were separated on 12% SDS-PAGE gels and stained with Imperial™ Protein Stain overnight. Gels were de-stained using Milli-Q H2O, and bands of interest corresponding to Hsp70 were excised using a scalpel blade. Tryptic peptides were generated following the in-gel digestion protocol detailed above. Desalted peptides were vacuum centrifuged and reconstituted in Optima LC grade water (ThermoFisher) containing 0.1% formic acid. These samples were kept at -20 °C until mass spectrometry analysis.
2.7 In-solution limited proteolysis

Cell culture and immunoprecipitation was performed as described above (sections 2.2 and 2.3). Samples with individually expressed FLAG-hYVH1 and His₆-Hsp70, as well as samples double-transfected with both plasmids, were subject to limited proteolysis. Following the immunoprecipitation washes, beads were washed three times with PBS, and then washed three times with 50 mM AB. Sequencing-grade trypsin or GluC (Promega, #V165A) digestion buffers were added containing 13 ng/µL of respective protease diluted in 50 mM AB. Samples were placed in a shaking incubator at 37 °C for varying time slots (5 min, 10 min, 15 min, 30 min, 1 h, 2 h, and 3 h), with 5 µL of solution being removed at each time point. 100 µL of mass spectrometry grade water was added to these samples and boiled for 10 min to inactivate the proteases, then vacuum centrifuged for 2 h to concentrate peptides. The peptides were then reconstituted in 0.1% FA in mass spectrometry grade water and kept at -20 °C until mass spectrometry analysis.

2.8 Mass spectrometry

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 (HDMS\textsuperscript{E}) operating mode was utilized consisting of data independent acquisition (DIA) with ion mobility separation activated using a wave speed of 650 m/s. HDMS\textsuperscript{E} 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 MassLynx (version 4.1).

For ribosome quantitative proteomics, raw data from three biological replicates and three technical replicates for each condition were processed using the Progenesis QI (Nonlinear Dynamics) software package for chromatogram alignments and normalization adjustments. Label-free quantitation was accomplished by the Hi-3 method\textsuperscript{65} which uses the intensity of the three most abundant peptides per protein to calculate relative abundance. Protein identification was accomplished utilizing the human UniProtKB/SwissProt database (26 465 proteins), acquired July 10, 2018. The following parameters were used to process the raw data: a low energy noise reduction of 135 counts, a high energy reduction noise reduction threshold of 30 counts, and an intensity threshold of 750 counts. Lock mass calibration correction occurred post-acquisition using Glu-fib as a standard. The following parameters were used for protein identification: a minimum of 3 fragment ions per peptide, a minimum of 7 fragment ions per protein, and a minimum of 1 unique peptide match per protein. The maximum false discovery rate used was 1% using a decoy reverse database. A maximum of one missed cleavage following trypsin digestion was permitted and the variable modification of methionine oxidation +15.9949 was included.
For limited proteolysis experiments, the mass spectrometry conditions were identical to the proteomic experiments except for the use of a shorter ACN gradient (3-30% B for 30 min, 30-55% B for 15 min, and 55-80% B for 10 min). Data was collected using MassLynx (version 4.1) and analyzed using ProteinLynx Global Server.

2.9 Immunofluorescence assay

U2OS cells (ATCC®, HTB-96™) were seeded at 50 000 cells/mL on eight-chamber slides with 500 µL chambers (BioBasic, #SP41219) using Opti-MEM® reduced serum media (Thermo Fisher, #31985-062). Transfection was achieved using Lipofectamine® 3000 (Invitrogen, #L3000) with 0.3 µg of FLAG-hYVH1 wild-type, Y179F, Y179E, FLAG-pCMV empty vector and myc-Src Y530F in antibiotic-free media. Five hours post-transfection, cells were washed with PBS and incubated overnight in fresh media with antibiotics. For stress granule analysis, sodium arsenite (NaAsO₂) treatment was carried out 23 h post-transfection by incubating cells with a final concentration of 0.5 mM NaAsO₂ at 37 °C for 1 h. At 24 h post-transfection, cells were washed once with PBS and then fixed with 3.7% paraformaldehyde (PFA; Thermo Fisher, #AAJ19943K2) for 15 min at room temperature. Cells were washed with PBS and then permeabilized using 0.15% Triton X-100 in PBS for 2 min at room temperature, followed by a final PBS wash. Cells were blocked with 5% BSA in PBS for 1 h at room temperature with gentle shaking. Primary antibody incubations were conducted for 1 h at room temperature in 1% BSA, using either mouse anti-FLAG® M2 (Sigma-Aldrich, #F3165) or goat anti-TIA-1 (Santa Cruz, #sc-1751) gently shaking at room temperature. The cells were then washed three times with PBS and subsequently incubated in
secondary antibody for 1 h at room temperature in 1% BSA, using either horse anti-
mouse-fluorescein (Vector Labs, #FI-2000) or rabbit anti-goat-Texas Red (Vector Labs,
#TI-5000), respective to the primary antibody used. Cells were washed another three
times with PBS and incubated for 2 min at room temperature with 0.5 mg/mL Hoechst
33342 stain (Invitrogen, #H3570) diluted in PBS. After a final PBS wash, the slides were
then allowed to dry for 15 min, followed by coverslip mounting with 50% glycerol and
sealed on all edges with clear nail polish (Super Dry®).

Fluorescence microscopy images were obtained with a Leica DMI6000 using a
40X oil objective. Images were quantitatively analyzed using ImageJ, with the JACoP
plugin used for determination of stress granule size and Pearson correlation coefficients
(PCCs), and the subcellular localization percentages determined by the Intensity Ratio
Nuclei Cytoplasm plugin. Measurements were obtained on 30 cells per sample, totaled
from three independent experiments. Statistical analysis was performed using the
Student’s t-test (Prism), with differences considered statistically significant at $p$ values <
0.005 (specific values shown with each relevant figure).
CHAPTER 3:
RESULTS & DISCUSSION

3.1 Phosphorylation of hYVH1 and its cellular effects

3.1.1 Phosphorylation site identification on hYVH1

As phosphorylation is a central mechanism employed to regulate protein function, it is beneficial to identify and investigate phosphorylation sites on a protein of interest. Because the tyrosine kinase Src was reproducibly observed in an interactome analysis with hYVH1, we investigated if there were phosphorylated tyrosine (pTyr) residues on hYVH1 induced by Src. To examine this, wild-type (WT) hYVH1 was overexpressed in cells along with Src. Following lysis and FLAG-immunoprecipitation (FLAG-IP) of hYVH1, western blot analysis revealed phosphorylated tyrosine residues on hYVH1 using an anti-pTyr antibody, clone 4G10 (Figure 3.1, A). Co-expression with Src results in a drastic increase of pTyr on hYVH1, thereby indicating Src is likely the mediator, as seen in the top panel. To confirm this and elucidate exact tyrosine residues being modified, in-gel digestion and mass spectrometry (MS) analysis was performed by Dr. Christopher Bonham using trypsin to prepare samples for MS. Upon analysis of the spectra obtained, phosphorylation of the tyrosine 179 (Tyr\textsuperscript{179}) residue was observed. A depiction of where this residue is located in the domain architecture of hYVH1 is shown in Figure 3.1, B. Tyr\textsuperscript{179} resides in a helix located at the N-terminal portion of the linker region between the phosphatase and zinc-binding domains. Interestingly, this tyrosine residue is conserved in all species of YVH1, including yeast, highlighting its importance to remain as a tyrosine evolutionarily. This was also the first evidence of tyrosine phosphorylation of hYVH1, mediated by Src.
Figure 3.1 - Phosphorylation of hYVH1 residue tyrosine 179. A) Western blot depicting an increase in tyrosine phosphorylation signal (using anti-pTyr antibody) of hYVH1 when co-expressed with Src, compared to hYVH1 expressed alone (top panel, center two lanes). The following four panels show controls, specifically the anti-FLAG FLAG-IP, and the lysates, blotted for anti-FLAG, anti-myc, and anti-Actin, respectively. B) Location of Tyr\textsuperscript{179} in the simplified domain structure depiction of hYVH1. This residue is in a conserved linker region of hYVH1, C-terminal to the phosphatase domain.

3.1.2 Phosphorylation of hYVH1 effect on stress granules

Discovery of the pTyr\textsuperscript{179} site prompted examination of its role as a regulatory modification. Due to the recent findings of hYVH1 as a stress granule (SG) disassembly factor,\textsuperscript{42} we chose to examine the impact of phosphorylated hYVH1 on this phenotype.
The subcellular localization of hYVH1 when solely expressed or co-expressed with Src was determined using immunofluorescence assays. As the protein TIA-1 is a well-known SG assembly factor, it is commonly used experimentally as a marker of SGs.\(^{39,66}\) Additionally, the FLAG-tag was exploited for visualization of overexpressed hYVH1. As seen in Figure 3.2, A, SGs were present after having been induced by oxidative stress using arsenite (see Materials & Methods, section 2.9), and detected by TIA-1 fluorescence, presented in red. In agreement with previously published data on the function of hYVH1 at SGs,\(^42\) hYVH1 is seen to co-localize with SGs, highlighted by the white arrows (Figure 3.2, A; 2\(^\text{nd}\) row). However, upon Src co-expression, the ability of hYVH1 to localize to SGs was reduced (Figure 3.2, A; 3\(^\text{rd}\) row). Additionally, the size of the SGs appeared larger when hYVH1 was phosphorylated. These findings suggest that the association of hYVH1 with SGs and its disassembly role is negatively regulated in a Src-dependent manner.

It is common in studying phosphorylation sites to mutate the known phosphorylated residue of interest to an amino acid that cannot be phosphorylated but is a conservative mutation of the original amino acid, as well as an amino acid that best mimics a phosphorylated residue. To do this, site-directed mutagenesis was used to mutate Tyr\(^{179}\) of hYVH1 to a phenylalanine (F) residue and a glutamic acid (E) residue, addressing each of the mentioned conditions respectively. The hYVH1 Y179F mutant represents a version of hYVH1 that cannot be phosphorylated at this site, and therefore acts as a similar construct to wild-type hYVH1 without Src co-expressed. The hYVH1 Y179E mutant attempts to mimic the negative charge of phosphorylated hYVH1.
To substantiate the above finding, analysis of hYVH1 at SGs was recapitulated using these two mutants. In concurrence with what was previously observed, U2OS cells expressing the Y179F mutant displayed fewer and smaller SGs, similar to that observed in cells expressing WT hYVH1 (Figure 3.2, B; 1st row). Interestingly, in contrast, the cells expressing the phosphor-mimetic mutant Y179E had a greater number of SGs, which were also larger in size, as we had seen with Src-phosphorylated hYVH1 (Figure 3.2, B; 2nd row).

To quantify the above qualitative observations for all four samples (hYVH1 WT, hYVH1 WT + Src, and the Y179F/Y179E mutants), image analysis was performed via ImageJ using the JACoP plugin. First, the localization of hYVH1 to SGs was analyzed on n = 30 cells from three independent experiments, and Pearson correlation coefficients (PCCs) were calculated and plotted in Figure 3.2, C. A PCC of 1.0 indicates complete positive correlation, 0 meaning no correlation, and a value of -1.0 indicating negative correlation. The discovered qualitative observations were confirmed statistically and show that the SGs present are highly co-localized with hYVH1 WT and the hYVH1 Y179F mutant when each of them was expressed, as indicated by relatively high PCCs, at 0.814 and 0.782, respectively. When hYVH1 was co-expressed with Src, and for mutant hYVH1 Y179E, much lower PCC values were obtained at 0.456 and 0.510 respectively, indicating loss of co-localization with SGs (Figure 3.2, C). Additionally, after measuring the size of the SGs for each set (n = 100 SGs), the SGs were statistically smaller when hYVH1 WT or hYVH1 Y179F were expressed (means of 25.16 pixels$^2$ and 23.73 pixels$^2$, respectively), and larger for hYVH1 + Src and hYVH1 Y179E (means of 67.32 pixels$^2$)
and 72.76 pixels$^2$, respectively). Taken together, these results suggest Src-mediated phosphorylation of hYVH1 impairs the SG disassembly role of hYVH1.
C

Colocalization between hYVH1-positive particles with TIA-1-positive stress granules

![Colocalization diagram](image)

- hYVH1 WT
- hYVH1 WT + Src
- hYVH1 Y179F
- hYVH1 Y179E

**** = p < 0.0001
ns = not significant

D

Size of TIA-1-positive stress granules

![Size of granules diagram](image)

- hYVH1 WT
- hYVH1 WT + Src
- hYVH1 Y179F
- hYVH1 Y179E

**** = p < 0.0001
ns = not significant
Figure 3.2 - hYVH1 co-localization with stress granules (+/- Src). Immunofluorescence images with TIA-1 as a marker of stress granules (red) induced by arsenite stress, FLAG-hYVH1 as a marker of hYVH1 expression (green), and a merged image of these images with Hoechst-stained nuclei (blue). White arrows are used to indicate granules of interest. Scale bars = 25 µm. A) Top row panels display empty vector (EV) control with visible SGs. Second row panels display representative cells with overexpressed hYVH1 WT, with hYVH1-positive granules co-localizing with stress granules. Third row panels contain hYVH1 WT co-expressed with Src, where co-localization between SGs and hYVH1 is no longer observed. B) Top row panels display cells expressed with hYVH1 Y179F and shows co-localization with SGs, while the bottom row panels shows hYVH1 Y179E and its lack of co-localization with SGs. C) Following statistical analysis via ImageJ, Pearson correlation coefficients were calculated to quantify the extent of co-localization between hYVH1 and SGs. Means ± SD of three independent experiments (n = 30 cells) are shown. Using Student’s t-test, p values were calculated and compared to hYVH1 WT, with differences considered statistically significant at p < 0.0001 (** *). D) Statistical analysis of SG size in pixels²/granule. Means ± SD of three independent experiments (n = 100 stress granules) are shown. As in C, Student’s t-test was used and p values were calculated and compared to hYVH1 WT, with differences considered statistically significant at p < 0.0001 (** *).

3.1.3 Src phosphorylation effect on subcellular localization of hYVH1

Phosphorylation can influence the subcellular localization of a protein. Interestingly, while analyzing the effect of Src phosphorylation of hYVH1 on SGs, it was also observed that Src appeared to influence the subcellular localization of hYVH1. When WT hYVH1 was expressed alone, the protein primarily localized to the cytoplasm (Figure 3.3, A; 2nd row). Conversely, upon co-expression with Src, hYVH1 localized primarily to the nuclear region (Figure 3.3, B; 3rd row). These data were again recapitulated using the phospho-site mutants. hYVH1 Y179F showed similar cytoplasmic localization patterns to WT hYVH1, whereas Y179E, mimicking Src phosphorylation of hYVH1, was primarily localized to the nucleus (Figure 3.3, B).

The proportions of cytoplasmic and nuclear localization were quantified to validate these findings, shown in Figure 3.3, C. For hYVH1 WT, the relative,
proportional subcellular localization was determined to be 21.64% nuclear and 78.36% cytoplasmic, while hYVH1 Y179F had 21.03% nuclear and 78.97% cytoplasmic localization. In contrast, quantification of hYVH1 co-expressed with Src displayed 52.15% nuclear and 47.85% cytoplasmic localization and hYVH1 Y179E showed 49.64% nuclear and 50.36% cytoplasmic. This was an intriguing observation, as it demonstrates Src-mediated phosphorylation of hYVH1 increases the amount of hYVH1 found in the nucleus versus the cytoplasm.
Figure 3.3 - hYVH1 subcellular localization (+/- Src). Immunofluorescence images with FLAG-hYVH1 as a marker of hYVH1 expression (green), nuclei stained with Hoechst (blue), and a merged depiction of these images. Scale bars = 25 µm. A) Top row panels display empty vector (EV) control. Second row panels display representative cells with overexpressed hYVH1 WT, with prominent cytoplasmic localization. Third row panels display hYVH1 co-expressed with Src, which displays higher nuclear localization. B) Top row panels display cells expressed with hYVH1 Y179F, showing largely cytoplasmic localization, while the bottom panel shows hYVH1 Y179E and its predominant nuclear localization. C) Bar graph illustrating the frequency of subcellular localization in % nuclear and % cytoplasmic for the various hYVH1 constructs, calculated via ImageJ software. Means ± SD of three independent experiments (n = 30 cells) are shown. Using Student’s t-test, p values were calculated and compared to hYVH1 WT, with differences considered statistically significant at $p < 0.0001$ (****).

Collectively, these results point to a mechanism by which Src-mediated phosphorylation of hYVH1 capable of shuttling hYVH1 into the nucleus. There is previous evidence of hYVH1’s subcellular localization being influenced by phosphorylation at its serine 335 residue,\(^\text{27}\) therefore suggesting the phosphorylation at the tyrosine 179 residue is a further layer of regulation. The observation that Src-
mediated phosphorylation affects the nuclear localization of hYVH1 is compelling when we consider its role in ribosome biogenesis. Trans-acting factors such as hYVH1 maintain a steady-state of ribosome biogenesis through recycling back into the nucleus from the cytoplasm. Therefore, our observation that Src-mediated phosphorylation results in higher nuclear localization may indicate that this phosphorylation event increases the rate of hYVH1 ribosome recycling.

3.1.4 Src phosphorylation of hYVH1 in ribosome biogenesis

There is evidence that nuclear/cytoplasmic shuttling is a key feature of the maturation role of hYVH1 during 60S ribosome biogenesis.\textsuperscript{53,54} The proposed function of hYVH1 is to act as a trans-acting/recycling factor to displace Mrt4 on the pre-60S subunit in the nucleus and then be replaced by the cytoplasmic analog of Mrt4, P0, once the pre-60S is exported from the nucleus. At this point, hYVH1 would be liberated and free to perform further roles. Because of the subcellular localization impact on hYVH1 we observed by Src phosphorylation, in addition to the impact seen on SG co-localization, this led to the pursuit of determining if phosphorylated hYVH1 affects this ribosome biogenesis function. To address this, ribosomal profiling using sucrose gradient fractionation was performed.

Ribosomal profiling experiments can differ significantly based on experimental objectives. For this reason, it was imperative to first develop a robust technique which allows for clear distinction between the various ribosomal subunits. Specifically, we needed to be able to discern between the 40S, 60S, and 80S particles, as well as visualize polysomes. Polysomes occur when multiple 80S mature ribosomes are present on a single mRNA transcript. The purpose of developing this workflow was not only to investigate if
Src phosphorylation was affecting the association of hYVH1 with the 60S subunit, but to also use this system for scaled-up experiments. This allowed us to explore protein alterations at the systems biology level in response to hYVH1 and Src co-expression using quantitative proteomics (addressed in Chapter 3.2).

The general optimized workflow is depicted in Figure 3.4 and described in detail in Materials & Methods, section 2.5. Essentially, a 5-40% sucrose gradient was formed using a mechanical gradient maker. Soluble cellular lysates were then carefully added to the top of the formed gradient. Cellular contents, particularly ribosomal particles, were separated along this gradient via ultracentrifugation and then mechanically fractionated using a top-down piston mechanism at an optimized flow rate and fraction volume of 500 µL. The least dense material, such as free ribonucleoproteins (RNPs), reside at the top of the gradient in lower sucrose conditions, and are therefore the first to be fractionated. More dense particles, such as the 40S, 60S, 80S, and polysomes, are distributed further within the gradient at regions of higher sucrose percentage, respectively. Thus, the denser the particle, the later it is fractionated. As the gradient is fractionated into small, equal volumes, the absorbance at 254 nm is simultaneously measured to view translational activity. The ribosomal profile is ultimately presented as a chromatogram of Abs254 vs. gradient position (related to elution volume/fraction number). Individual fractions can be further processed using additional biochemical methods, including western blotting and MS.
Figure 3.4 - Workflow of ribosomal fractionation and profile generation. Once a 5-40% sucrose gradient is formed, samples of interest are gently loaded and subsequently ultracentrifuged to separate the ribosomal subunits. This gradient, now containing the separated particles, is then subject to fractionation while simultaneously having its absorbance measured at 254 nm. A ribosomal profile corresponding to components throughout the entire gradient is produced. Figure created with BioRender.com.

3.1.5 Ribosomal profile analysis

To develop a standard ribosome profile, the above workflow was utilized on HeLa cells transfected with an empty vector (EV) plasmid. Lysate sample was loaded onto the sucrose gradient, ultracentrifuged, fractionated, and a profile was obtained by monitoring absorbance. A typical profile is shown in Figure 3.5, A. The areas of interest are labelled on the profile as 1-5, as the initial peak before the 10 mm gradient position would correspond to free RNPs and materials that were not fractionated through the gradient.

Ribosomal subunits were identified via western blot by analyzing the presence of known, unique associating proteins. Proteins specific to the small and large subunits were chosen as markers to indicate these peaks, specifically the 40S ribosomal protein S3 and 60S ribosomal protein P0. As shown in Figure 3.5, B, the sample corresponding to peak 1 on the profile displays a strong signal for the 40S ribosomal marker. In accordance with
this, peak 2 was confirmed to correspond to the 60S subunit. These proteins were each seen in the 80S fractions as well (peak area 3-5), which is expected as they are both present on the final mature ribosome. This confirmed the identities of the peaks obtained and validated the workflow designed to elucidate these various ribosomal particles. A general schematic illustrating these designations is shown in Figure 3.5, C.
A
Ribosomal profile of EV-transfected HeLa cells

B
Ribosomal peak fractions
IB: α-S3 (40S)

C
Ribosomal profile of EV-transfected HeLa cells
Figure 3.5 - Ribosomal profile peak identification from EV-transfected HeLa cells. 
A) This graph displays a standard ribosomal profile obtained when EV-transfected cells were subject to the designed ribosomal profiling workflow. Of interest are the peaks labelled 1-5. B) Western blot images of the 5 peaks of interest, as indicated in A. The top panel shows the 40S ribosomal marker, anti-S3, while the bottom panel shows the 60S ribosomal marker, anti-P0. C) As per the western blot analysis of samples pertaining to the ribosomal peaks of interest, peaks were designated as indicated. Peak 1 corresponds to the 40S small subunit, followed by the 60S large subunit (peak 2), and a split peak at 3-5 which corresponds to the mature 80S ribosome. Following this, small peaks corresponding to polysomes are present.

3.1.6 Examination of ribosomal peaks via mass spectrometry

Interestingly, we reproducibly observed peak splitting at the gradient position associated with the 80S particle. To our knowledge, this is indicative of a high-resolution ribosomal profile not yet described in literature. Specifically, we speculated the split peak labelled 3-5 as resolution of monosomes and disomes. Monosomes refer to a single 80S linked to an mRNA strand, while a disome contains two 80S ribosomes on mRNA. These two states occur respectively before multiple ribosomes attach, creating polysomes. This phenomenon is not commonly visible in a ribosomal profile, where there is typically one broad peak corresponding to the 80S. Therefore, the resolution obtained through this protocol allows for more accurate distinctions, as well as quantitative proteomics, to be executed on the mature ribosome. To confirm this theory and characterize the peaks of interest as their respective subunits, the fractionated samples corresponding to each of the four peaks were subject to an in-gel digest and subsequent MS analysis. The samples were analyzed by data independent acquisition mass spectrometry (DIA-MS) and data processing against the UniprotKB/SwissProt human database was accomplished using the Progenesis software program. Importantly, the measurements unambiguously confirmed that peaks 1 and 2 represent the 40S and 60S ribosomal subunits, respectively.
3.6). The proteomic profiling analysis for peaks 3 and 4, assumed to correspond to the 80S, confirmed that peak 3 contains an abundance of 40S and 60S core ribosomal proteins. This indicates that peak 3 corresponds to the 80S monosome (Figure 3.6). Peak 4 also contained every core ribosomal protein identified in peak 3, as well as additional core ribosomal proteins. Moreover, the relative abundance of all peptides/proteins were substantially higher in the peak 4 fractions, suggesting the additional core ribosomal proteins identified was due to higher concentration of ribosomal proteins in the sample. This is consistent with peak 4 representing the disome form of the mature ribosome and indicates that our chromatographic fractionation method was able to partially resolve monosome and disome ribosomal species. In addition to confidently characterizing the ribosomal subunit peaks, proteomic profiling of the fractions allowed for assessing the purity of the ribosomal subunits from other subcellular entities. Thus, sucrose gradient density centrifugation provides sufficient resolving power to isolate the ribosomal segment of the proteome that can be used to explore hYVH1-mediated regulation of RNP dynamics.
Figure 3.6 - Proteomic analysis of peaks of interest. Fractions specific to the four peaks of interest from a representative spectrum were subject to in-gel digestion and MS analysis. Peaks 1 and 2 were unambiguously confirmed as the 40S and 60S ribosomal subunits, respectively, as indicated by the core proteins identified. Regarding the 80S split peak (peaks 3 and 4), these were also confirmed as the mature 80S ribosome, containing 40S and 60S proteins and a notably higher abundance in peak 4, indicating the likelihood of two mature ribosomes (disome).

3.1.7 Ribosomal profiles of hYVH1

With an optimized ribosomal profiling workflow, we next sought to capture profiles corresponding to expression of hYVH1, as well as hYVH1 co-expressed with Src, with the goal of determining the effect of tyrosine phosphorylation on the role of hYVH1 in 60S biogenesis. The proteins were overexpressed in HeLa cells, and lysates were subject to the prepared sucrose gradients. A standard profile obtained using lysates from HeLa cells overexpressing hYVH1 is shown in Figure 3.7, A, and hYVH1 with Src in 3.7, C. As with previous western blotting efforts, P0 was used as a marker of the 60S
subunit, while overexpressed hYVH1 was tracked using the FLAG epitope (Figure 3.7, B and D). We observed hYVH1 present in highest amounts at the peak corresponding to the 60S (lane 2), an expected outcome based on its demonstrated role in 60S ribosome biogenesis.⁴³,⁵⁴
Figure 3.7 - Ribosomal profiles of hYVH1 and hYVH1 with Src. A 5-40% sucrose gradient with separated lysate samples was fractionated and the absorbance at 254 nm was recorded. These profile outlines are representative of the reproducible profiles obtained, n > 5. A) Profile obtained for hYVH1 expressed alone. B) Western blot analysis of indicated peaks 1-5, using anti-FLAG (top panel) and anti-P0 (bottom panel) antibodies, on the hYVH1 sample. C) Profile obtained for hYVH1 co-expressed with Src. D) Western blot analysis of indicated peaks 1-5, using anti-FLAG (top panel) and anti-P0 (bottom panel) antibodies, on the hYVH1 with Src sample.

Based on our previous results showing Src-mediated attenuation of the ability of hYVH1 to co-localize with SGs, we hypothesized that Src-mediated phosphorylation may diminish hYVH1 association with the 60S ribosomal subunit. However, analysis of the fractionated samples by western blotting revealed that when hYVH1 is phosphorylated by Src, the amount of hYVH1 in the 60S peak sample was substantially higher (lane 2 in Figure 3.7, B and D). This observation was reproducible in multiple experimental replicates, which suggests that Src-mediated phosphorylation of hYVH1 increases its ability to bind to the 60S ribosomal subunit and, therefore, potentially influences normal ribosomal production and function.

When comparing these profiles, other notable distinctions were detected. Firstly, the disome peak was observed to be significantly smaller when hYVH1 was co-expressed with Src compared to hYVH1 expressed alone, which is seen clearly when the two profiles are overlayed (Figure 3.8, A; black box). It was imperative here to implement controls to ensure the differences seen were caused by hYVH1 + Src and not Src alone. Using EV-transfected cells, along with EV + Src, profiles were obtained and are overlayed in Figure 3.8, B. After looking closely at the monosome and disome peaks specifically, it is clear this shift to a larger monosome peak and smaller disome peak only occurs in the hYVH1 + Src profile (Figure 3.8, C; blue), while EV, EV + Src, and
hYVH1 alone display the opposite phenotype. Secondly, increased polysome peaks are reproducibly observed when Src was co-expressed with hYVH1 indicative of higher translational rates (Figure 3.8, D). This further confirms that Src-mediated phosphorylation of hYVH1 may increase its targeting to the 60S ribosomal subunit that results in a state of higher translational fitness. To confirm these findings, we were interested in exploring potential proteomic differences caused by Src-phosphorylated hYVH1, specifically at the monosome and disome.
A

hYVH1 & hYVH1 + Src Overlay

Absorbance @ 254 nm

Gradient position (mm)

B

EV & EV + Src Overlay

Absorbance @ 254 nm

Gradient position (mm)
Figure 3.8 - Ribosomal profiles comparison with EV controls. The 5-40% sucrose gradient with separated lysate samples was fractionated and the absorbance at 254 nm was recorded. These profile outlines are representative of the reproducible profiles obtained, n > 5. A) Overlay of hYVH1 (pink) and hYVH1 + Src (blue) ribosomal profiles. Highlighted by the black box is the difference in intensities between the monosome and disome peaks. B) Overlay of EV (purple) and EV + Src (green) ribosomal profiles. C) Overlay of all four profiles, zoomed into the monosome/disome peak area. Notable is the hYVH1 + Src line (blue) displaying a larger monosome peak and smaller disome peak. D) Overlay of all four profiles, zoomed into the polysomes. Evident is the highest peak intensities in the hYVH1 + Src sample (blue), and low peak intensities of both EV (purple) and EV + Src (green).
3.1.8 Working model of Src phosphorylation cellular effect on hYVH1

With the experimental results attained thus far, we propose that Src-mediated phosphorylation of hYVH1, particularly at Tyr\(^{179}\), allows for a significant increase of hYVH1 steady-state levels in the nucleus. This is in contrast with its common, predominantly cytoplasmic localization when un-phosphorylated. It is relevant to relate this to the differences in ribosomal profiles obtained. The three major contrasting features when hYVH1 was phosphorylated in cells by Src were: 1) the disome peak intensity decrease relative to the monosome peak, 2) an apparent increase of polysomes based on chromatographic peak intensity in contrast to the profiles obtained for cells where Src was not co-expressed, and 3) increased binding of hYVH1 to the 60S subunit. Taking these effects into account with the nuclear localization of hYVH1 caused by Src, it is hypothesized that this phosphorylation event increases the nuclear/cytoplasmic shuttling properties of hYVH1, which is directly related to its role in 60S ribosome biogenesis. We propose that this data collectively indicates more efficient ribosome biogenesis occurs when hYVH1 is phosphorylated by Src, and therefore higher translational fitness. A simplified schematic of this concept is shown in Figure 3.9. In both cases, a mature 80S ribosome is formed and translation can occur, but it is occurring at a higher rate when the ability of hYVH1 to translocate to the nucleus is increased by its phosphorylation (Figure 3.9, B). This raised the inquiries of potential proteomic differences at the ribosome caused by Src phosphorylation of hYVH1, as well as if there are other mechanisms regulating this shuttling feature.
Figure 3.9 - Schematic of Src-mediated phosphorylation effect on hYVH1. A) Under basal conditions, the steady-state localization pattern of hYVH1 is primarily observed in the cytoplasm (1). In this case, hYVH1 can translocate into the nucleus to perform its 60S ribosome biogenesis role, replacing Mrt4 on the 60S (2), and then export to the cytoplasm for replacement by protein P0 (3). The full 80S mature ribosome then forms (4). B) When hYVH1 is phosphorylated at its Tyr179 residue, it shuttles into the nucleus at a higher rate, altering its steady-state localization pattern to be higher in the nucleus. hYVH1 is still capable of performing its regular 60S ribosome biogenesis role, potentially at a higher rate due to increased nucleus recycling, and therefore leading to increased efficiency of forming mature, translationally fit ribosomes. Figure created with BioRender.com.
3.2 Proteomic analysis on ribosomal subunits

3.2.1 Rationale for quantitative proteomic efforts and workflow

With the ribosomal profiles exhibiting qualitative differences that were reproducible and significant between hYVH1 and hYVH1 that has been phosphorylated by Src, the next step was to search for quantitative proteomic differences. Particularly, the purpose of pursuing this was to give further insight into the consequences of Src phosphorylation at a proteomic level, specifically where qualitative changes were observed on the profiles. The first samples of interest were those corresponding to the monosome and disome peaks, as the shift between samples was so distinctive. Using in-gel digestion as the sample preparation technique, a modification to standard SDS-PAGE protocol was made to ensure the entire fractionated sample was digested. The approach of running an SDS-PAGE in a “short stack” method allows for the entire sample to migrate into the resolving layer, but instead of allowing the sample to fully resolve, gel running is stopped prematurely. This effectively concentrates the sample to allow for excision of the sample area from the gel, and then subject it to the standard in-gel digestion procedure. For these experiments, trypsin was chosen as the protease to create peptide fragments. A general workflow schematic is shown in Figure 3.10.
3.2.2 Characterization of monosome and disome protein alterations

To assess the proteomic alterations occurring during the distinct change in the monosome/disome chromatographic peak pattern upon co-expression of hYVH1 and Src, we compared this phenotype at the protein level with the empty vector control sample using label-free quantitative proteomics. Following sucrose gradient fractionation, samples corresponding to the monosome and disome were prepared for MS analysis using the short stack SDS-PAGE approach described above (Figure 3.10). Generated tryptic peptides were separated by nano liquid chromatography using an 85 minute acetonitrile gradient and electrosprayed directly into the mass spectrometer. Mass analysis was performed using data independent acquisition (DIA) and ion mobility separation was used to increase resolution and improve protein identification yields. Samples were generated from 6 biological replicates (3 from empty vector transfected control and 3 from hYVH1 + Src co-transfected HeLa cells). Altogether, 154 proteins in the monosome samples and 244 proteins in the disome samples were confidently
identified. Gene ontology (www.pantherdb.org) of the proteomic data confirmed the vast majority of proteins identified were ribosomal proteins and regulators of translation (Figure 3.11).

Figure 3.11 - Gene ontology designations for monosome and disome proteins. Proteomic analysis was performed on six biological replicates of ribosomal samples and separated into their respective protein classes. A) Gene ontology breakdown for the monosome peak. B) Gene ontology breakdown for the disome peak. Gene ontology was obtained using the Panther classification system. In both samples, there is a significantly higher number of proteins related to translation compared to all other categories.
For quantitation, only proteins that were identified with ≥ 3 unique peptides were considered. A Student's \( t \)-test was used to determine protein alterations that were statistically significant \( (p < 0.05) \). Application of the Student's \( t \)-test identified 23 and 32 statistically significant protein alterations in the monosome samples and disome samples, respectively. Additional criteria were employed to provide enhanced statistical rigor; ANOVA analysis of all technical replicates with a \( p \) value cut-off of < 0.001 and considered alterations significant if the fold change difference was >1.5. Collectively, statistical analysis of the proteomic data yielded 10 proteins in the monosome sample and 9 proteins in the disome sample that displayed altered protein levels in response to hYVH1 and Src co-expression. For the disome sample, all 9 of the statistically significant protein alterations were the result of protein levels being reduced in response to hYVH1-Src co-expression. This result is consistent with the observed decrease in the 254 nm chromatographic peak when hYVH1 and Src are co-expressed. For the monosome sample, the levels of 6 proteins were reduced and 4 proteins elevated in response to hYVH1-Src co-expression. Importantly, the protein levels of the core ribosomal proteins were statistically similar between both conditions, providing additional confidence that the observed protein alterations are valid.

To visually depict the protein alteration data, a volcano plot was constructed by comparing the \( -\log_{10} p \) value to the \( \log_2 \) fold change (Figure 3.12) for the monosome and disome samples. As expected, most of the identified proteins populate the base of the volcano plot indicating that the majority of ribosomal proteins identified in the monosome/disome samples were not significantly affected by co-expression of hYVH1.
and Src. However, there are several significant protein alterations that are consistent with
the working model that hYVH1 and Src co-expression enhances translational fitness.
Figure 3.12 - Volcano plots indicating significant protein alterations for monosome and disome. Comparison between control sample (purple) and hYVH1 + Src (blue). Significant proteins are indicated by the coloured circles, while proteins deemed insignificant are coloured in gray. **A)** Monosome peak samples. **B)** Disome peak samples. Only proteins identified with ≥ 3 peptides in all technical and biological replicates are represented.
3.2.3 Analysis of significant protein alterations

The most statistically significant protein alteration was a reduction in the levels of Nucleolin in both the monosome (3.4-fold higher in control) and disome samples (3.8-fold higher in control). Nucleolin is a multifunctional protein that has been shown to regulate chromosome condensation, DNA replication, and ribosomal RNA biogenesis.\textsuperscript{68–70} Moreover, a distinct pool of Nucleolin has also been shown to associate with 3’ and 5’ regions of select mRNA molecules at the fully mature ribosome, leading to enhanced translation or, more commonly observed, repressed translation depending on the cellular context.\textsuperscript{69,70} Another intriguing protein alteration was the reduction in Nuclease-sensitive element-binding protein 1, better known as Y-box binding protein 1 (YB-1). YB-1 was found to be 2.1-fold higher in the control monosome sample and 2.9-fold higher in the control disome sample. YB-1 has been well characterized as a translational repressor in a wide variety of organisms.\textsuperscript{71–74} Finally, reduced levels of the protein Interferon-related developmental regulator 1 (IFRD1) in the disome sample was reproducibly detected in response to hYVH1-Src co-expression (3.8-fold higher in control). The role of IFRD1 in translational regulation is currently unclear; however, a close homologue, IFRD2, has been shown to induce translationally inactive ribosomes by associating with ribosomes that have a tRNA bound to a non-canonical site, suggesting that IFRD family members participate in repressing translation at the elongation stage.\textsuperscript{75,76} These proteomic findings suggest that overexpression of hYVH1 and Src may attenuate the formation of disome ribosomal species that represent stalled intermediates through the regulation of associating factors that promote translational repression or ribosomal pausing.
In addition to the reduction of select translational repressive factors at the ribosome, co-expression of hYVH1 and Src increased levels of key proteins at the monosome that have been shown to enhance translational fitness. One critical factor that was found at elevated amounts was the GTPase, elongation factor 2 (EF2) (Figure 3.12, A). EF2 mediates the transfer of aminoacyl-tRNAs and mRNAs through the ribosome during translational elongation.\(^7^7\) Moreover, levels of EF2 at the 80S monosome is indicative of active elongation due to the fact that ribosome bound EF2 is inaccessible to its negative regulator EF2 kinase.\(^7^8\) Therefore, higher monosome levels of EF2 found in the hYVH1-Src co-expression sample is consistent with the model that hYVH1-Src co-expression induces ribosomal structures capable of increased translational fitness. Along the same lines, a notable 2-fold increase in eukaryotic translation initiation factor 6 (EIF6) in the hYVH1-Src co-expression sample is further evidence of an increased translational fitness response. EIF6 is a potent regulator of translational initiation through its ability to function as an anti-association factor.\(^7^9\) EIF6 docks onto the 60S subunit at the 60S-40S binding interface and is postulated to prevent translationally inactive 80S intermediates (e.g. 80S devoid of mRNA and stalled disomes).\(^8^0\) This apparent checkpoint role can be alleviated through phosphorylation by protein kinase C (PKC) to activate 80S formation in response to sufficient growth conditions.\(^7^9\) This phosphorylation event is facilitated by the scaffold protein receptor of activated protein C kinase 1 (RACK1) that associates with the 40S subunit.\(^7^9,^8^1\) Interestingly, we observed RACK1 to also be upregulated in the monosome in response to hYVH1-Src co-expression (Figure 3.12, A), suggesting hYVH1-Src may positively affect the EIF6-RACK1 signaling pathway that is crucial for proper translational fidelity. Consistent with
this finding is a previous study reporting that Src associates with and phosphorylates RACK1, indicating there is precedence for Src being in close proximity to the RACK1 scaffold.

It is unclear if the proteome alterations observed with hYVH1-Src co-expression is the result of a moonlighting function of hYVH1 unrelated to its 60S ribosome biogenesis role or if the alterations are the result of increased recycling of hYVH1 due to Src-mediated tyrosine phosphorylation. The immunofluorescent microscopy data showing increased nuclear localization in response to Src phosphorylation of hYVH1 supports the hypothesis of a recycling effect. However, it is also possible that once hYVH1 is released from the 60S ribosome following 60S ribosomal stalk association, it performs additional modulating activities that affect the monosome proteome and that Src-mediated tyrosine phosphorylation of Tyr179 facilitates shuttling hYVH1 back to the nucleus for another round of 60S ribosome maturation. While a substantial amount of work will be required to elucidate the mechanistic details of hYVH1-Src regulation of translational fitness, the quantitative mass spectrometry efforts have discovered valuable proteome alterations that will serve as useful biomarkers for future studies.
3.3 Investigating the structural mechanism of the hYVH1-Hsp70 complex

3.3.1 Rationale and protocol development for limited proteolysis

In addition to its role in 60S ribosome biogenesis and stress granule disassembly, we have previously shown that hYVH1 is a potent cell survival factor, protecting cells from thermal and oxidative stress. Importantly, this cell survival phenotype required both the zinc-binding domain and phosphatase activity. Furthermore, this study identified the well-characterized cell survival protein Hsp70 as a novel binding partner of hYVH1. Although the functional significance of the hYVH1-Hsp70 complex remains unclear, investigation into the binding interface of hYVH1-Hsp70 was previously performed using domain deletions of both proteins. Based on the constructs used for this analysis, it was determined the ATPase domain of Hsp70 is required and sufficient for binding to hYVH1. This indicates that Hsp70 is not binding to hYVH1 through its chaperone domain, suggesting that the proteins complex together to perform a critical cell survival function.

In contrast, the hYVH1 domain involved in complex formation was not as clearly elucidated. It was hypothesized that the ZBD of hYVH1 is required for the interaction due to loss of complex formation upon ZBD deletion. However, this could not be fully validated as the ZBD itself was expressed at low levels and was not experimentally shown to be sufficient for complex formation. These results stress the importance of examining the protein-protein interaction using full length proteins. For this purpose, limited proteolysis was chosen to map the regions on both proteins that represent the hYVH1-Hsp70 binding interface.
Limited proteolysis coupled with mass spectrometry (LP-MS) is a powerful footprinting technique to elucidate structural information of proteins including conformational changes and binding interfaces. With regards to the latter, the premise of the technique is that limited incubation with proteases will preferentially target the solvent exposed regions of native proteins (such as binding interfaces). Therefore, the digestion of the protein complex is performed at short time periods and compared to individual proteins digested alone. Peptides whose levels are significantly altered between the two conditions (single protein vs complex) will represent putative binding interface regions. A schematic of this workflow is shown in Figure 3.13.

![Figure 3.13](image)

**Figure 3.13 - Workflow for in-solution limited proteolysis analysis of protein-protein interaction.** Full-length hYVH1 and Hsp70 were overexpressed in HeLa cells and subsequently subject to affinity chromatography to isolate the complex. This sample was then exposed to trypsin for 5 min, quenched, and analyzed by mass spectrometry. Figure created with BioRender.com.

3.3.2 Mapping the hYVH1-Hsp70 binding interface using limited trypsin digestion

Due to sensitivity issues, LP-MS is most commonly performed on purified proteins. However, the hYVH1-Hsp70 complex can be isolated in sufficient amounts when co-expressed in mammalian cells and would be a more biologically relevant source to obtain the complex. Therefore, epitope-tagged constructs of hYVH1 and Hsp70 were expressed in HeLa cells and isolated by magnetic bead affinity chromatography. A
variety of critical factors were optimized, including protease selection, incubation time(s), and chromatography conditions to resolve and detect maximum protein sequence coverage. The results of our optimization efforts concluded that trypsin digestion of 5 min yielded sufficient sequence coverage in a limited time interval, while other proteases tested required longer periods of time to yield detectable peptides, compromising the goal of avoiding denatured protein derived peptides for the footprinting analysis. The optimized method reproducibly yielded sequence coverages of 39% and 34% for hYVH1 and Hsp70 respectively (Figure 3.14). Importantly, the identified peptides were derived from portions of the primary sequence that cover the different domain and linker regions of the two native proteins.

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134/340 = 39.4% hYVH1 sequence coverage
Figure 3.14 - Sequence coverage of hYVH1 and Hsp70 tryptic peptides via limited proteolysis. Sequence coverage maps outlining the regions where peptides were identified after 5 min of trypsin digestion on full-length A) hYVH1 and C) Hsp70. Red and blue colouring is used to distinguish between exact peptide boundaries. Full protein sequences with respective peptides highlighted are shown in B) hYVH1, with 39.4% sequence coverage and D) Hsp70, with 33.7% sequence coverage.
In order to elucidate putative binding interfaces between hYVH1 and Hsp70, the relative intensities of the tryptic peptides were compared between the individual transfected samples and the co-transfected samples. To account for variation between the conditions, peptide intensities were normalized to internal control peptides whose intensities minimally varied between conditions. This process resolved several peptide regions that reproducibly displayed significant intensity loss in the co-transfected sample.

For Hsp70, several peptides in the ATPase domain displayed the most significant alterations between the Hsp70 and hYVH1-Hsp70 samples (Figure 3.15). This was expected since previous work has demonstrated that the ATPase domain is sufficient to co-immunoprecipitate hYVH1 from mammalian cells.\textsuperscript{26} Interestingly, there were multiple overlapping peptides spanning amino acid residues 326-361 that showed the greatest loss of intensity in the hYVH1-Hsp70 sample (Figure 3.15, C and D). This peptide region resides in the C-terminal portion of the ATPase domain of Hsp70, close to the linker region that separates the ATPase domain and the substrate-binding domain.\textsuperscript{84} Specifically, these residues are within a section of subdomain IIA that does not mediate nucleotide binding or co-chaperone association.\textsuperscript{85} Therefore, the location of this putative binding interface suggests that hYVH1 may associate with a region of the ATPase domain of Hsp70 that would not affect or depend on nucleotide status or competition with co-chaperones.
Figure 3.15 - Hsp70 peptides of interest identified via limited proteolysis. High resolution MS spectra corresponding to A and C) Hsp70 alone and B and D) Hsp70 bound to hYVH1. In A and B, the control peptide used for normalization is indicated. Also shown is the peak of a peptide of interest (residues 57-71, green). In C and D, two other peptides of interest are highlighted (residues 349-361, blue, and residues 326-342, yellow). E) Fold change differences between control and three peptide regions, with differences considered statistically significant at $p < 0.05$ (*). F) Domain structure representation of the ATPase domain of Hsp70, with aforementioned regions highlighted (PDB: 3GDQ), as well as the four subdomains (IA, IIA, IB, and IIB).

With regards to hYVH1, we anticipated that the C-terminal ZBD would possess the Hsp70 binding region, as deletion of the ZBD attenuates Hsp70 co-immunoprecipitation from cells and the fact that the ZBD is required for most YVH1 biological activities. To our surprise, the LP-MS data revealed two regions in the N-terminal phosphatase domain that exhibited the most significant protection from trypsin digestion in the hYVH1-Hsp70 sample (Figure 3.16, A-D). The most reproducible and prominent alteration was observed in a peptide that corresponds to amino acids 68-77 (Figure 3.16, A and B). This stretch of residues reside in a large random coil between beta strand 3 and 4 of the phosphatase domain protruding away from the active site cleft.
Another region of interest was observed in three overlapping peptides corresponding to amino acids 141-163. This stretch of amino acids reside in a helix-loop region at the end of the phosphatase domain and the start of the linker region separating the phosphatase domain from the ZBD. Although protection from trypsin digestion in the hYVH1-Hsp70 sample was reproducible in these peptides, the protection was less prominent and exhibited greater variation compared to the 68-77 peptide. We therefore hypothesize that residues 68-77 represent the putative Hsp70 binding interface while residues 141-163 undergo conformational changes in response to binding to Hsp70.
Figure 3.16 - hYVH1 peptides of interest identified via limited proteolysis. High resolution MS spectra corresponding to A and C) hYVH1 alone and B and D) hYVH1 bound to Hsp70. In A and B, the control peptide used for normalization is indicated. Also shown are the peaks of a peptides of interest (residues 68-77, blue, and residues 154-163, green). In C and D, another peptide of interest is highlighted (residues 141-153, yellow). E) Fold change differences between control and three peptide regions, with differences considered statistically significant at $p < 0.05$ (*). F) Domain structure representation of the phosphatase domain of hYVH1, with aforementioned regions highlighted (PDB: 4JNB).

In order to definitively determine if the amino acid regions measured by LP-MS represent bona fide binding interfaces, additional supporting experimental evidence will be required. Current work in our laboratory is ongoing to employ complementary techniques to validate the LP-MS findings. This includes site-directed mutagenesis of amino acid residues within these regions followed by comparative co-immunoprecipitation experiments, as well as differential biotin labeling mass spectrometry studies. Collectively, these efforts hold great promise to identify the structural determinants of the hYVH1-Hsp70 interaction which will contribute valuable mechanistic details to understanding the role of this complex in cellular regulation.
3.3.3 Attenuation of the hYVH1-Hsp70 interaction by Src phosphorylation

Knowing that hYVH1 and Hsp70 have a strong, robust interaction both in vitro and in cells, as well as the cellular effects observed imparted by Src phosphorylation of hYVH1, we wanted to assess if Src would have an impact on this interaction. Furthermore, the region of interest containing residues 141-163 on hYVH1 is in close proximity to the Src phospho-site, suggesting phosphorylation of Tyr\textsuperscript{179} may induce a conformation change in hYVH1 that may promote or attenuate association with Hsp70. Therefore, we performed co-purification on each of the epitope-tagged constructs (FLAG-hYVH1 or His\textsubscript{6}-Hsp70), and measured association by chemiluminescent visualization of the other protein. This would then be compared to the same pulldown with Src also overexpressed in the system. Initially, using FLAG-immunoprecipitation (FLAG-IP), FLAG-hYVH1 was immobilized and acted as the bait, and the levels of His\textsubscript{6}-Hsp70 would be assessed via western blot, with and without Src co-expressed.

The strong interaction between hYVH1-Hsp70 in cells was observed after the FLAG-hYVH1 pulldown (Figure 3.17, A; lane hYVH1 + Hsp70, top panel). Upon Src co-expression, the amount of co-purified Hsp70 was drastically reduced, as seen in the adjacent lane (hYVH1 + Hsp70 + Src, top panel). After observing this reproducibly, we performed statistical analysis on the western blot images to quantify the difference. As seen in the bar graph in Figure 3.17, A, the interaction was decreased between the two proteins by ~50%.

To strengthen this finding, a reciprocal pulldown was performed. In this case, His\textsubscript{6}-Hsp70 was immobilized by nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography and therefore acted as the bait, and FLAG-hYVH1 was evaluated by
western blotting. The same trend was observed, in that phosphorylation by Src kinase reduced the interaction between hYVH1-Hsp70 significantly (Figure 3.17, B). Again, there was ~50% less protein bound (Figure 3.17, B; bar graph). These two sets of data strongly suggest that Src-mediated phosphorylation of the hYVH1-Hsp70 complex attenuates their interaction in cells. This result complements the previous results showing increased nuclear localization of hYVH1 in response to Src phosphorylation. We have previously shown that hYVH1 and Hsp70 do not localize in the nucleus but are co-localized to perinuclear structures in the cytoplasm.26 Therefore, the Src-mediated attenuation of the hYVH1-Hsp70 complex may be an important mechanism to target or recycle hYVH1 into the nucleus during ribosome biogenesis or during a recovery response following a stress stimulus.
Figure 3.17 - Analysis of hYVH1-Hsp70 interaction with and without Src. A) The top two western blot panels contain samples that were subject to FLAG-IP isolation of FLAG-hYVH1, with anti-His and anti-FLAG detection, respectively. The bottom three panels are the lysates, blotted for anti-His, anti-FLAG, and anti-Actin, respectively. Of note is the amount of Hsp70 interacting with hYVH1 in the top panel, which was quantified using three independent experiments in the bar graph (right). B) The top two western blot panels contain samples that were subject to nickel affinity isolation of His6-Hsp70, with anti-FLAG and anti-His detection, respectively. The bottom three panels are the lysates, blotted for anti-His, anti-FLAG, and anti-Actin, respectively. Of note is the amount of hYVH1 interacting with Hsp70 in the top panel, which was quantified using three independent experiments in the bar graph (right). Statistical significance calculated using Student’s t-test (respective p values indicated below graphs).
3.3.4 Comparing tyrosine phosphorylation between hYVH1 and Hsp70

The finding that Src is capable of targeting the hYVH1-Hsp70 complex leaves open the possibility that Src is able to phosphorylate Hsp70 in addition to targeting hYVH1. Therefore, we decided to examine the Src-mediated phosphorylation status of hYVH1 and Hsp70 expressed alone or co-expressed. This was achieved by using the phospho-tyrosine specific antibody (clone 4G10) via western blot. As seen in Figure 3.18 (lane 6, top panel), hYVH1 co-expressed with Src induces a strong band on the pTyr western blot, indicating the tyrosine phosphorylation occurring on hYVH1. This can be compared to when hYVH1 is expressed alone, where it becomes clear the tyrosine phosphorylation seen is mediated by the co-expression of Src (lane 7). To determine if Hsp70 can be tyrosine phosphorylated, we compared the difference in tyrosine phosphorylation seen on Hsp70 when expressed alone (lane 2), with just hYVH1 (lane 3), and when co-expressed with Src and hYVH1 (lane 4).
Figure 3.18 - Western blot analysis of phosphorylated proteins. The top two panels contain samples after FLAG-IP isolation of FLAG-hYVH1, blotted for anti-pTyr and anti-FLAG, respectively. The bottom four panels are lysates blotted for anti-FLAG, anti-His, anti-Src, and anti-Actin, respectively. Of note is the top panel which corresponds to phosphorylated tyrosine residues, induced by Src.

Interestingly, we observed distinct changes when hYVH1 and Hsp70 are both expressed with Src. When comparing the two proteins co-expressed alone (lane 3) versus upon addition of the kinase (lane 4), a strong band corresponding to Hsp70 tyrosine phosphorylation was observed. Additionally, when Hsp70 is present, tyrosine phosphorylation of hYVH1 was dramatically reduced (when compared to YVH1 + Src, lane 6). Taken together, these results represent the first evidence that Hsp70 is also tyrosine phosphorylated by Src. Secondly, it appears Hsp70 either acts as a better substrate for Src or is able to “protect” hYVH1 from being phosphorylated by Src. *In vitro* kinase assays and binding assays using recombinant purified proteins will be
required in the future to determine the order and competitive details of this phosphorylation mechanism.

3.3.5 Identification of a Src-mediated tyrosine phosphorylation site on Hsp70

The finding that Src phosphorylates Hsp70 is an opportunity to deepen our understanding of how Src disrupts the hYVH1-Hsp70 interaction. In order to understand the consequence of Src-mediated Hsp70 phosphorylation, mapping of the residue modified is required. An analogous mass spectrometry-based approach that was used to map the Tyr<sup>179</sup> site on hYVH1 (outlined in 3.1.1), was employed to identify the modified Tyr residue on Hsp70. Hsp70 was co-expressed with Src kinase in cells and isolated from lysates via nickel affinity chromatography. Following in-gel digestion and desalting steps, Hsp70 tryptic peptides were analyzed by tandem mass spectrometry using the data independent acquisition mode (DIA). Shown in Figure 3.19, A is a high-resolution mass spectrum of a tryptic peptide corresponding to amino acids 37-49 with the addition of a phosphate moiety. DIA-MS/MS analysis confirmed that residue Tyr<sup>41</sup> was phosphorylated within this peptide. This residue lies in the ATPase domain of Hsp70, near the N-terminus (Figure 3.19, B).
Figure 3.19 - Phosphorylation of Hsp70 residue tyrosine 41. A) High resolution MS spectrum focused on the peak corresponding to the phosphorylated Hsp70 peptide, TTPS(pY)VAFTDTER. B) Location of Tyr\textsuperscript{41} in the domain structure depiction of Hsp70. This residue is in the ATPase domain of Hsp70, near the N-terminus of the protein sequence.

The identification of a Src-mediated phosphorylation site on the ATPase domain of Hsp70 is intriguing considering the fact that Hsp70 associates with hYVH1 via its ATPase domain and that Src expression disrupts the hYVH1-Hsp70 protein-protein interaction. This suggests that the phosphorylation of Tyr\textsuperscript{41} may induce conformational changes in the ATPase domain which then affects the ability of Hsp70 to bind hYVH1. Interestingly, a well characterized cyclin dependent protein kinase site is located in close proximity to the Src-mediated Tyr\textsuperscript{41} of Hsp70 and has profound effects on cell cycle progression.\textsuperscript{87} Phosphorylation of Thr\textsuperscript{36} disrupts interaction with co-chaperones making
Hsp70 available to associate with client proteins including cyclins necessary for G1/S and G2/M checkpoint release. Hsp70-cyclin association leads to cyclin degradation and is induced in response to cellular insults in a mechanism that is conserved from yeast to humans. Similarly, Tyr oxidation may release hYVH1 in order to be available for targeting specific client proteins at the ribosome or elsewhere in response to stimuli that signal through Src kinase. Further studies into the effect of this phospho-site are underway as well as determination of other potential Src-mediated tyrosine phosphorylation sites on Hsp70 that may be relevant for the hYVH1-Hsp70 biological function.

3.3.6 Hypothesized model of hYVH1-Hsp70 interaction affected by Src phosphorylation

Hsp70 has been shown to enhance the cell survival capabilities of hYVH1; however, investigation into regulation of this interaction and its functional significance is ongoing. Upon discovery of Src-mediated phosphorylation of Tyr on hYVH1 and Tyr on Hsp70, it is evident this interaction is attenuated. This finding, taken together with the increased translational fitness and increased nuclear localization phenotypes observed with Src phosphorylation of hYVH1, suggests that the interaction with Hsp70 may impact the temporal and spatial steps of the hYVH1-mediated ribosome biogenesis function. We propose that when these proteins are in their dephosphorylated state, they exist as a complex in the cytoplasm, and function at steady-state levels to perform their respective cellular roles. However, upon Src phosphorylation of each protein at Tyr on hYVH1 and Tyr on Hsp70, this interaction is disrupted and allows for increased shuttling of hYVH1 into the nucleus, resulting in higher amounts of hYVH1 available to perform its 60S ribosome biogenesis role, leading to higher translational fitness of the
cell. This would also cause higher amounts of free Hsp70 to be available to perform various chaperone roles, although there is not enough data in this current study to speculate where the liberated Hsp70 may be targeted. Building on the initial model suggested in Figure 3.9, Figure 3.20 also takes Hsp70 into account. In a steady-state condition where Src is not present, the proteins are bound and hYVH1 will shuttle into the nucleus as needed during ribosome formation (Figure 3.20, A). Src-mediated phosphorylation of this complex appears important to maintain translational efficiency during cell growth conditions by attenuating their interaction to ensure translational efficiency. Another situation where this mechanism may be critical is during recovery from cellular insults. (Figure 3.20, B). Once phosphorylated, hYVH1 is released and contributes to restoring protein synthesis to levels needed for cell survival and proper function.
Figure 3.20 - Schematic of Src-mediated phosphorylation effect on hYVH1 and Hsp70. A) In the absence of Src, hYVH1 and Hsp70 are bound in the cytoplasm (1). In this case, hYVH1 can translocate into the nucleus to perform its 60S ribosome biogenesis role at a basal level, replacing Mrt4 on the 60S (2), and then exported to the cytoplasm with the maturing 60S before being replaced by protein P0 (3). The full 80S mature ribosome then forms, as we deem steady-state translation rates (4). B) When hYVH1 is phosphorylated at its Tyr179 residue and Hsp70 at Tyr41, their interaction is disrupted. Phosphorylated hYVH1 shuttles into the nucleus at a higher rate, increasing its amount in the nucleus and causing increased translational efficiency, including higher levels of polysomes. Figure created with BioRender.com.
4.1 Conclusions

Src has been identified as a novel regulator of hYVH1. Its phosphorylation of the Tyr\(^{179}\) residue on hYVH1 was demonstrated to have implications in the stress granule disassembly function of hYVH1, specifically in its ability to localize and therefore aid in their disassembly. Src-mediated phosphorylation of hYVH1 also caused increased nuclear shuttling of hYVH1, which is predominantly cytoplasmic when unphosphorylated. These findings led to the exploration of the impact on the role of hYVH1 in ribosome biogenesis, where we found increased binding of hYVH1 to the 60S ribosome, increased amounts of polysomes, and decreased disome formation (stalled ribosomes) upon Src phosphorylation. Quantitative proteomic efforts corroborated the theory of hYVH1-\(p\text{Tyr}^{179}\) increasing cellular translational fitness due to the noted differences in translational repressors and markers of translational efficiency, seen at both the monosome and disome peaks of the ribosomal profiles.

The interaction of hYVH1 and Hsp70 was further detailed via limited proteolysis, which elucidated a putative binding region on each protein. Specific residues of interest identified were 68-77 on hYVH1 and 326-361 on Hsp70. Their attenuated interaction upon Src phosphorylation, at both Tyr\(^{179}\) on hYVH1 and Tyr\(^{41}\) on Hsp70, suggests their binding can also be regulated by the kinase and is perhaps relevant for the modulation of each of these proteins. As this interaction is disrupted, we hypothesize that hYVH1 is liberated to translocate into the nucleus and perform its ribosome biogenesis function in response to cellular needs.
The previously published roles of hYVH1 include its capacity to act as a cell survival phosphatase, modulate the cell cycle, redox sensing, disassemble stress granules, and its critical role in 60S ribosome biogenesis. These roles could all be attributed to its regulation of mRNA dynamics, both at the level of ribosome biogenesis and RNP particle dynamics, and are influenced by the phosphorylation status of hYVH1.

Ultimately, these studies have allowed for further insight into the regulation of the dual-specificity phosphatase, hYVH1, suggesting Src-mediated phosphorylation of this protein increases cellular translational efficiency. Furthermore, the large-scale ribosomal profiling technique designed will allow for further exploration into the mechanistic details present at the various ribosomal particles, which can be elucidated using quantitative proteomics.

### 4.2 Future Directions

With this developed ribosomal profiling technique, it is imperative to examine if there are any differences in monosome, disome, and/or polysome formation with Hsp70 co-expressed with hYVH1, as well as with and without Src. Performing this analysis with the hYVH1 Y179 F/E mutants will also be useful to corroborate the trends seen with hYVH1 WT and hYVH1-Src, as well as domain deletions of hYVH1 to discern exact regions responsible for the observed phenotypes. Overexpression experiments will benefit greatly from complementary knock-down studies of hYVH1, Src, and Hsp70 to document the ribosomal response to the absence of these factors in the context of various extracellular stimuli and stressors. In addition to the aforementioned mutants, it is imperative to also include a kinase-dead mutant of Src (K298M), the inactivated SH2
domain mutant (R178A), and the inactivated SH3 domain mutant (W121A). The effect of
these Src mutations in our model would be relevant in terms of their different protein
binding capabilities. The Src family member, Fyn, has also been shown to activate
mRNA transport proteins, and would be useful to assess in the stress granule effect seen
via Src-mediated phosphorylation.

With the findings in this study, we have gained insight into the role of hYVH1 at
the 60S ribosome, but the detailed mechanism still needs to be elucidated and the search
for a physiological substrate continues. In this regard, a substrate trap mutant of hYVH1
will also be employed in search for a potential substrate at the ribosome. Moreover,
studying this system under differing cellular conditions, along with various cell types,
will be key to fully discerning all elements involved.

In terms of the hYVH1-Hsp70 interaction, it will be valuable to determine the
precise amino acids participating, as the regions of interest have now been identified. To
do this, site-directed mutagenesis will be employed for both proteins, and co-
immunoprecipitation experiments repeated to test their binding. We will also implement
differential biotin labelling mass spectrometry for this effort which will be able to discern
between proteolytic protection due to conformational change or physical binding to
Hsp70. The functional significance of this interaction is likely related to cellular
functions of hYVH1, as it is regulated by Src phosphorylation and both proteins are
involved in overlapping cellular processes, and therefore imperative to fully characterize.

The search for other novel tyrosine phosphorylation sites on hYVH1 is ongoing.
Although it has become clear through our studies that Tyr179 is the most prominently
phosphorylated residue, this does not rule out other potential layers of regulation
occurring at other sites. Once other residues of interest are identified, their roles will be examined in terms of subcellular localization, stress granule dynamics, and potential effect on the relationship of hYVH1 with Hsp70. Additionally, other tyrosine kinases need to be tested to determine if the phosphorylation sites we have identified thus far are specific to Src, or if there are other tyrosine kinases that are capable of acting on these residues. The Src family of tyrosine kinases have nine members, and testing with the other Src family kinases is currently underway.

The overarching purpose of studying hYVH1 is to determine its primary cellular function and potentially evaluate the protein as a drug target, if appropriate. In recent years, important functions of hYVH1 have been revealed, but many questions remain. With the absence of a known physiological substrate, it is difficult to explicitly state the main role of hYVH1, although evident to play a role in multiple cellular processes (cell cycle, cell survival, stress granule dynamics, and ribosome biogenesis). Further determining structural information, its regulation, and its relationship with Hsp70 will be imperative as studies of hYVH1 continue.

Perhaps the most exciting and intriguing role of hYVH1 thus far is its ribosome biogenesis role. The ribosome is continuing to emerge as a major scaffold structure for integrating various inputs regarding cellular homeostasis, allowing for the fine-tuning of protein synthesis and maintenance of cellular homeostasis. Considering the influence of this macromolecular complex, it is vital to discern the inner workings of its intricate biogenesis. As hYVH1 is revealed to play a meaningful role in precise 60S formation, the mechanistic details are of great interest, along with the potential of revealing the physiological substrate of hYVH1.
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