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Biological characterization of hYVH1

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Biological Characterization of hYVH1: Subcellular Dynamics and Role in Cell Survival

by

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ABSTRACT

Human YVH1 is an atypical dual specificity phosphatase (DSP) that is widely conserved throughout evolution. Deletion studies in yeast have suggested a role for this phosphatase in regulating cell growth, sporulation, and glycogen accumulation. However, the functional role of the human orthologue is unknown. This study examines the interaction between hYVH1 and a recently identified binding partner Hsp70, in addition to deciphering domains and motifs important in hYVH1 localization. The results established that the zinc binding domain mediates the subcellular targeting of hYVH1. Moreover, a putative nuclear export sequence was identified to have some effect on the shuttling of hYVH1 between the nucleus and cytoplasm. Hsp70 and hYVH1 were found to be colocalized to the perinuclear region following heat stress. Furthermore, hYVH1 expression repressed heat shock induced cell death. The results suggest that hYVH1 cooperates with Hsp70 to positively affect cell viability by targeting the MAPK signaling pathway.

DEDICATION

I would like to dedicate this thesis to my family.

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My time as a graduate student at the University of Windsor has been nothing short of great and I would like to thank many people for making my experience an enjoyable one. Firstly, I would like to thank Dr.Vacratsis for giving me the opportunity to work in his lab. I appreciate all the guidance you have offered and I look forward to reading about what becomes of hYVH1. I would like to thank my committee members, Dr.Crawford and Dr.Pandey, for taking the time to read my thesis. Also, thank you again to Dr.Pandey for providing me with my first lab experience and always remaining helpful. I would also like to extend my gratitude towards the remaining faculty in the Biochemistry department. Thank you to Dr.Ananvoranich, Dr.Mutus, Dr.Gauld, and Dr.Lee for all the advice, help, letters of references and use of equipment.

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LIST OF ABBREVIATIONS

Apaf-1	Apoptotic Protease-Activating Factor-1
ATP	Adenosine triphosphate
Bax	Bcl-2 Associated X protein
Bcl-2	B-cell Lymphoma-2
BSA	Bovine Serum Albumin
CDC	Cell Division Cycle
DMEM	Dulbeco's Modified Eagle Medium
DTT	Dithiothreitol
DSP	Dual-Specificity Phosphatase
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ERK	Extracellular Signal-Regulated Kinase
FISH	Fluorescence in situ Hybridization
FITC	Fluorescein Isothiocyanate
HeLa	Henrietta Lacks (cervical cancer cell line)
JNK	Jun N-terminal Kinase
LMPTP	Low Molecular weight Protein Tyrosine Phosphatase
MAPK	Mitogen-Activated Protein Kinase
MKB	Mitogen-Activated Protein Kinase Binding domain
MKP	Mitogen-Activated Protein Kinase Phosphatase
mRNA	Messenger Ribonucleic acid
PCR	Polymerase Chain Reaction
PMSF	Phenylmethylsulphonyl fluoride
NES	Nuclear Export Sequence
NLS	Nuclear Localization Sequence
NPC	Nuclear Pore Complex
NRPTP	Non Receptor-like Protein Tyrosine Phosphatase
PBS	Phosphate Buffered Saline
PFA	Paraformaldehyde
PTP	Protein Tyrosine Phosphatase
RPTP	Receptor-like Protein Tyrosine Phosphatase
SAPK	Stress-Activated Protein Kinase
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
TBS	Tris Buffer Saline
TUNEL	Terminal deoxynucleotidyl transferase dUTP Nick End Labeling

CHAPTER 1

Introduction

1.1 Cellular Phosphorylation as a Post-translational Modification

Covalent alterations to amino acid residues of proteins are defined as post-translational modifications. It is because of this process that the number of unique forms of proteins is larger than anticipated by genome analysis [1]. It is estimated that about 5% of the higher eukaryotic genome codes for enzymes which specifically contribute to this process [1]. Examples of post-translational modifications include methylation, carboxylation, acetylation, glycosylation, ubiquitination, and phosphorylation [1].

Phosphorylation is an essential mechanism necessary for many aspects of cellular function including cell growth, signal transduction, cell cycle progression, cell metabolism, and gene expression [2]. The two enzyme superfamilies responsible for this reversible modification of the phosphorylation mechanism are protein kinases and protein phosphatases [3]. Protein kinases catalyze the transfer of a γ-phosphoryl group from a molecule of adenine triphosphate (ATP) onto a serine, threonine, or tyrosine residue of the target molecule [3, 4]. Phosphatases act reversibly and dephosphorylate target molecules by removing the phosphate group and returning the molecule back to its original conformation [3].

The conformational change accompanying the phosphorylation/ dephosphorylation process occurs through the negatively charged oxygen atoms on the phosphoryl group. This addition of negative charge causes alterations in the surrounding environment of the substrate molecule either by forming stabilizing electrostatic interactions with positively charged amino acid side chain groups or by disrupting

hydrophobic regions of the protein. It is through these interactions that a change in conformation of the target molecule occurs as well as an accompanied alteration in its biological activity.

The reversible process of phosphorylation, catalysed by protein kinases and phosphatases permits the control of a variety of proteins, lipids, nucleotides, and metabolic intermediates [5]. For instance, phosphorylation/dephosphorylation can increase or decrease a protein's biological activity, stabilize or indicate a protein for destruction, or mediate protein-protein interactions [6]. However, it would be false to presume that kinases and phosphatases act constitutively. Both protein families are under tight control by regulatory subunits, inhibitors, and post translational modifications [3]. When this control is disrupted or an error in the coding sequence or folding of a kinase or phosphatase occurs, a number of different types of disorders can arise including various forms of cancers, metabolic and immune disorders, and neurodegenerative diseases [7-9]. Therefore, both kinases and phosphatases are a vital class of enzymes to study in order to gain insight, understanding, and perhaps even an approach of treatment for a number of human diseases. However, in comparison to the study of kinases, research regarding the equally important phosphatase family is lagging.

1.2 The Phosphatase Family

Phosphatases can be classified into two main families; the Ser/Thr-specific phosphatases and the Tyr-specific phosphatases [2].

The Ser/Thr phosphatases dephosphorylate Ser and Thr residues exclusively. They are metalloenzymes that contain two divalent metal ions (eg. Fe^{2+} and Zn^{2+}/Mn^{2+}) within its catalytic site [10]. Mechanistically, a water molecule is bound to one of two

metal ions and is responsible for the nucleophilic attack of the phosphorous atom on the phosphate group [10]. The metal acts as a lewis acid and can enhance the nucleophilicity of the metal-bound water molecule [10]. A conserved histidine residue is responsible for donating a proton to the leaving group oxygen of the serine or threonine residue side chain and site-directed mutagenesis of this histidine indeed shows a loss of catalytic activity [10-12]. This dephosphorylation mechanism functions in a one-step SN2 reaction where no phosphoenzyme intermediates are detected.

In contrast, the protein tyrosine phosphatases (PTP), which are characterized by the presence of a conserved active site motif, CX_5R, follow an entirely unique mechanism that requires the formation of a phosphoenzyme intermediate complex [13]. PTPs catalyze a two step dephosphorylation mechanism. The first step involves the nucleophilic attack of the phosphorus atom on the substrate molecule by the active site cysteine, which forms a covalently bound phosphoenzyme intermediate [14, 15]. Simultaneously, a strategically placed general acid residue, usually an Asp residue, contributes a proton to the oxygen of the leaving group on the substrate molecule, which becomes free to leave the active site [13]. During the second step, the same Asp residue behaves as a catalytic base to remove a proton from a water molecule and activating it as a nucleophile. The nucleophilic attack of the phosphoenzyme intermediate causes the release of inorganic phosphate and the regeneration of the active site of the phosphatase (Fig.1) [13].

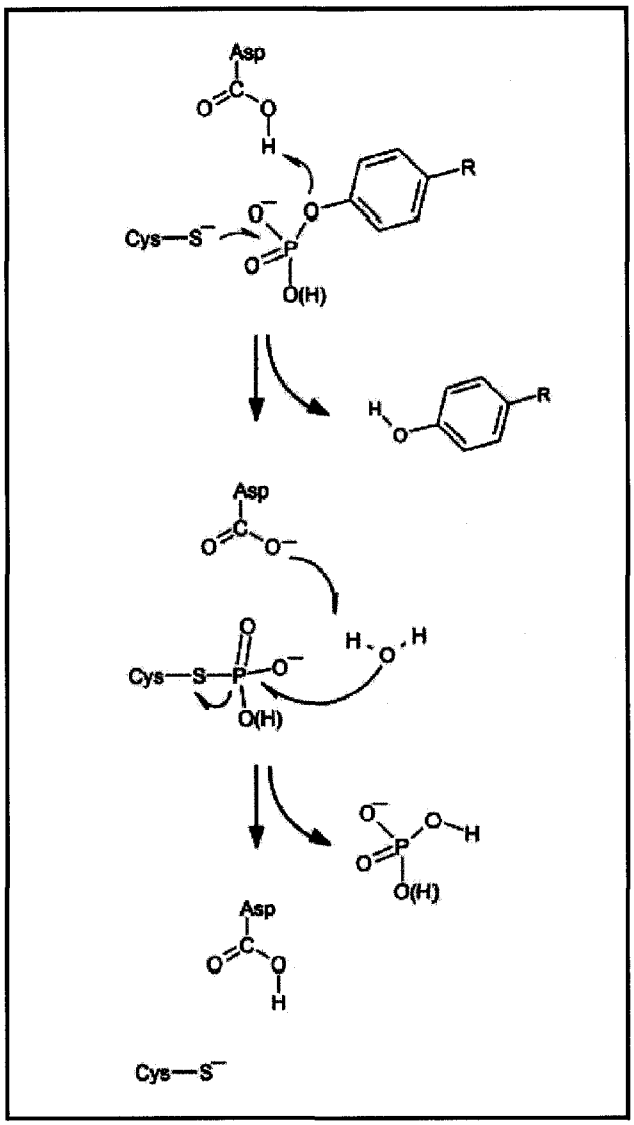


Figure 1 – Catalytic mechanism of protein tyrosine phosphatases [13]

The two step mechanism of dephosphorylation via a protein tyrosine phosphatase requires first the nucleophilic attack of the phosphate group by the catalytic cysteine and a proton donation from an acid to neutralize the negatively charged oxygen on the substrate molecule and second the base catalysis of the removal of a proton on a water molecule to form a nucleophile that readily attacks the phosphoenzyme, forming inorganic phosphate and regenerated phosphatase.

1.3 Classification of Protein Tyrosine Phosphatases

Further investigation of PTPs reveals the discovery of 107 genes within the human genome that encode for these proteins [5]. This number was much higher than anticipated, based on the 90 genes encoding protein tyrosine kinases [5, 16]. The apparent discrepancy can be reasoned through the realization that of the 107 genes, 13 dephosphorylate inositol phospholipids, 2 dephosphorylate mRNA, and 11 are considered catalytically inactive, which leaves 81 catalytically active PTPs [5]. This number is much more fitting when compared to the 85 known catalytically active protein tyrosine kinases [5].

Within the 107 members of the PTPs substantial variation exists and therefore they are further classified into three major groups; the classical PTPs, the lipid-specific PTPs, and the dual specific PTPs [5]. This classification is based on the amino acid sequence of their catalytic domains and their subsequent substrate specificity. However, despite the differences in primary sequence, there does exist high similarity of secondary and tertiary structure within the catalytic domains of each of the subgroups [17-19]. For instance, the environment surrounding the PTP signature motif, CX_5R, includes several important helices containing conserved amino acid residues [20]. Examples include the P-loop, containing the PTP signature motif and the WPD loop that carries the Asp residue involved in acid-base catalysis [9, 20]. The P-loop is conserved amongst all PTP members, while the WPD loop is specific to the classical PTPs [21].

The classical PTPs are a group of 38 tyrosine specific phosphatases that are further divided into receptor-like proteins (RPTPs), non-receptor-like proteins (NRPTPs), and low molecular weight phosphatases (LMPTP) [5, 22]. The RPTPs contain an

extracellular domain, a single transmembrane domain, and one or two intracellular phosphatase domains [23]. Members of this family include CD45 and PTPα, both of which are able to dephosphorylate members of the Src-family tyrosine kinases that have key functional roles in processes including cell differentiation and proliferation [23, 24]. The NRPTPs constitute most of the remaining tyrosine-specific phosphatases that do not contain a transmembrane domain and hence reside in the cytoplasm [5]. PTP1B is a member of the NRPTPs and has been demonstrated as a negative regulator of insulin signaling [25, 26]. The final group of classical PTPs are the 18 kDa, LMPTPs and while this class may have very low sequence similarity to the other classical PTPs, it does contain the conserved active site motif, implying a similar catalytic mechanism [27-29]. These phosphatases have been shown to dephosphorylate a number of growth factor receptors, thus influencing cell division [29].

The second group of PTPs are the lipid specific phosphatases, which are responsible for the dephosphorylation of the D3-phosphate of inositol phospholipids [30]. These phosphatases are allotted into two different subgroups, PTENs and myotubularins. PTEN members are responsible for the dephosphorylation of phosphatidyl-inositol-3,4,5-trisphosphate on the plasma membrane and is mutated in a high percentage of cancers, while myotubularins mainly dephosphorylate phosphatidylinositol-3-phosphate of internal cell membranes and loss of function mutations cause myotubular myopathy and Charcot-Marie-Tooth disease [5, 30-32].

The final and most diverse group of PTPs are termed dual-specific phosphatases (DSP); this name denotes the group's diverse substrate specificity for serine/ threonine and tyrosine phospho-residues [5]. Members of the DSP family can be further

subdivided into unique subgroups. The first, known as the mitogen-activated protein kinase phosphatases (MKPs), are characterized by their specificity for the pTXpY signature sequence of mitogen-activated protein kinases (MAPKs) [5, 33, 34]. Another well characterized group of DSPs are the CDCs, which participate in the regulation of the cell cycle [5]. Meanwhile, the last major subgroup, known as the atypical DSPs, are poorly characterized, but are not thought to target MAPK or cell cycle regulators [5].

1.4 The Saccharomyces cerevisiae Homologue of VH1

The first identified DSP, VH1, originates from the H1 open reading frame of the vaccinia virus and has been shown to dephosphorylate a viral histone-like protein required in the replication process of the virus life cycle [14, 26, 35]. The yeast homologue of VH1, commonly known as YVH1 or DUSP12, was the first eukaryotic DSP classified and falls under the atypical subclass of PTPs [36]. Functional studies have shown that under various stress conditions, such as nitrogen starvation or cold shock, YVH1 mRNA expression is significantly induced [36, 37]. Also, deletion of the *yvh1* gene results in defects relating to spore maturation, glycogen accumulation, and vegetative growth [38, 39]. In yeast cells expressing *yvh1* mutants, spore development is initiated, but occurs much slower and seldom compared with wildtype strains [38]. Through examination of dityrosine production, a late event of spore maturation, it was demonstrated that yeast strains containing *yvh1* mutants did not complete the sporulation process [38]. This phenotype was found to be genetically independent of the growth defect demonstrated by upstream suppression experiments [38]. Regarding glycogen accumulation, wildtype *yvh1* strains of yeast demonstrate increased glycogen accumulation during the S phase of the cell cycle, however mutant strains failed to

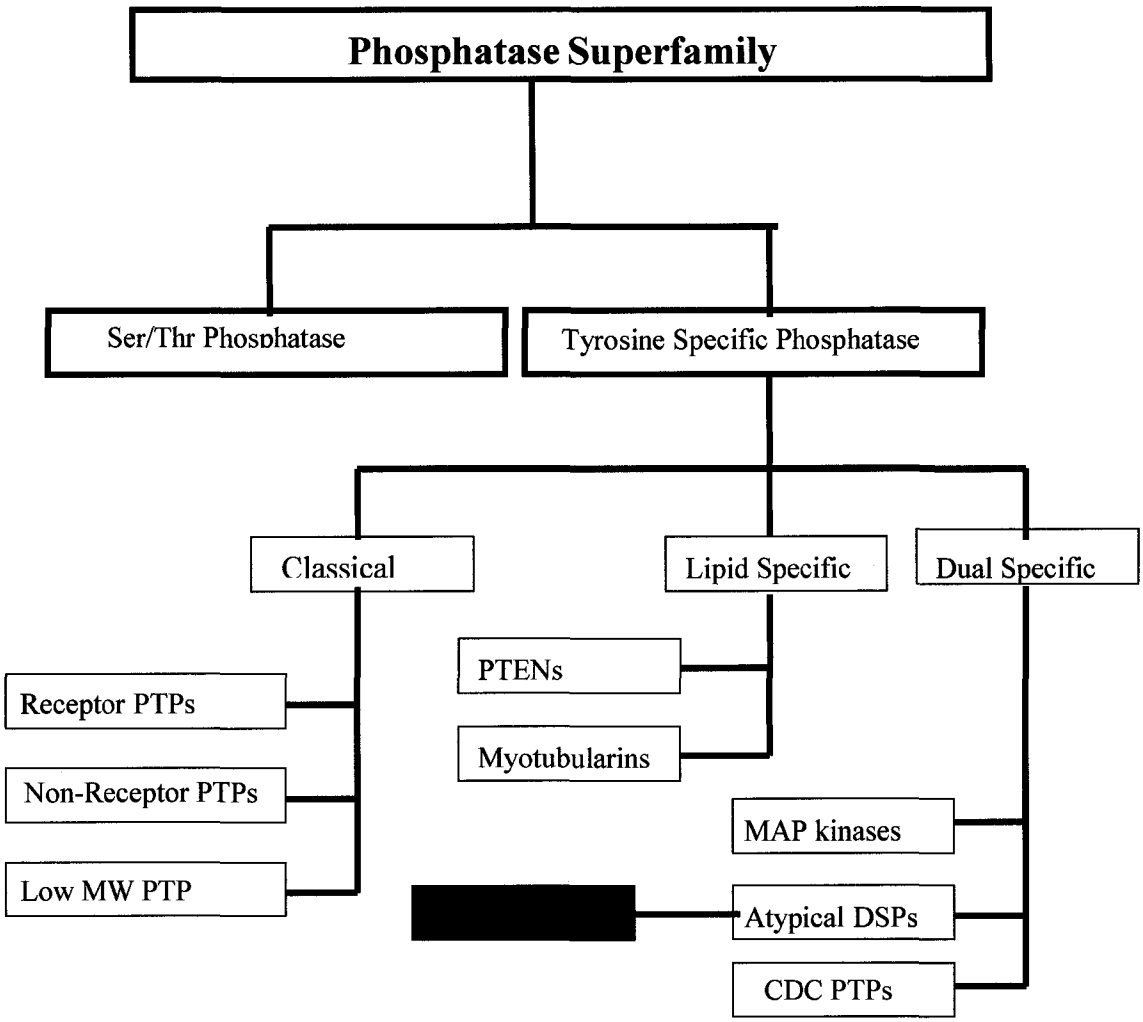


Figure 2 - Classification of the Protein Tyrosine Phosphatase Family

The protein tyrosine phosphatase family is further classified into subgroups based on amino acid sequence of the catalytic domain and substrate specificity, however, despite differences all members within this group share the signature catalytic motif, CX_5R, and the ability to dephosphorylate phospho-Tyr residues.

produce this event [39]. Surprisingly, these defects were recovered in yeast cells expressing a catalytically inactive YVH1 variant, where the conserved Cys residue was mutated to a Ser, as well as in cells expressing a YVH1 deletion mutant possessing only the C-terminus of YVH1 and completely lacking the phosphatase domain [39]. Oddly, this implies the YVH1 phosphatase activity is not required for the aforementioned phenotypes [39]. One possible explanation for this peculiar observation is that the C-terminus is the binding domain for the substrate, thus, instead of dephosphorylating its substrate, the variant forms of YVH1 are simply inactivating its substrate by direct binding [40].

Additionally, the *yvh1* gene and its mouse orthologue have both demonstrated modified mRNA expression in response to the cell cycle [41, 42]. The expression of the *yvh1* gene was down regulated in yeast cells synchronized to the G2 phase of the cell cycle [41]. Moreover, the mRNA expression of the mouse orthologue of YVH1 in NIH 3T3 cells was highest in cells synchronized to the G1/S interface and dropped during the G2/M phase [42]. Therefore, like the CDC DSPs, YVH1 may participate in a regulatory role within the cell cycle.

As for the exact identification of YVH1 regulators, yeast two-hybrid studies have revealed the yeast pescadillo homolog, YPH1 as a potential candidate [43]. The YPH1 protein has been shown to be involved in the cell cycle progression of yeast and found to bind directly to the catalytic domain of YVH1 [43, 44]. However, as of yet, this interaction has not been demonstrated with full length YVH1 nor in vivo and no functional significance has been assigned to the pair [43, 44]. Another more recent study in the malaria parasite, *Plasmodium falciparum*, have identified that the *P. falciparum*

orthologue of pescadillo interacts with the *P. falciparum* orthologue of YVH1, but again no functional significance of this interaction was determined [45].

1.5 The Homo sapien Orthologue of YVH1

The conservation of YVH1 orthologues throughout evolution was further established by Muda *et al.* which demonstrated that the human orthologue of YVH1 (hYVH1) was able to rescue the slow growth phenotype in yeast cells expressing the *yvh1* disruptant gene [40]. Sequence alignments also showed that hYVH1 shares an overall sequence identity of 31% with YVH1 [40].

Structurally, hYVH1 is 340 amino acids long with a molecular weight of 37,687 Da [40]. It consists of two domains, the first is the N-terminal, DSP catalytic domain, containing the conserved signature motif and the second is the unconventional C-terminal, zinc binding domain [40]. The zinc binding domain consists of two zinc fingers, each coordinating one zinc molecule through the amino acid residues C_2HC and C_4 [40, 45]. The seven cysteines and one histidine responsible for zinc coordination are very highly conserved in all YVH1 orthologues including *S.pombe, C.elegans,* and *P.falciparum* [40]. Yet, despite this preservation throughout evolution, little is known about its actual function. However, it is generally agreed that the zinc finger is crucial for the in vivo activity of hYVH1 [40].

Hoping to gain functional insight regarding hYVH1, *Muda et al.* probed for mRNA in various human tissues [40]. It was found that this phosphatase was ubiquitously detected in most tissues, with higher levels present in the spleen, testis, ovary, and peripheral blood leukocytes while low levels were found in the lung and liver tissue samples [40]. This result highlights that not only is hYVH1 highly conserved

throughout evolution, but it is also expressed in various tissue types, implying a basic yet crucial functional role.

Recently, through the use of fluorescence in situ hybridisation studies (FISH) combined with microarray analysis, it was determined a number of sarcomas and ependymomas showed amplification in the chromosomal region 1q21-1q23, where the *hyvh1* gene is located [46, 47]. Further analysis revealed that the *hyvh1* gene was indeed overexpressed in these forms of cancers [46].

More recently using mass spectrometry, our laboratory has discovered an interacting partner for hYVH1 identified as heat shock protein 70 (Butt, unpublished), which itself has been implicated in a number of cancers [48]. Characterization of this interaction will likely be crucial in the elucidation of the functional role of hYVH1.

1.6 Heat Shock Protein 70

The heat shock protein 70 (Hsp70) is thought by many to be the most conserved protein in evolution [48-51]. Found in all organisms ranging from archaebacteria to humans, Hsp70 remains conserved in amino acid sequence as well as functional role throughout all species [48, 52-55]. It was shown in various cell types that subjection to a mild heat shock stress prior to a severe heat shock provided a protective effect for the cells, thereby linking the induction of Hsp70 to cell survival [56-58]. In 1984, it was suggested by Hugh Pelham that the protective effect of Hsp70 could be attributed to its ability to aid in the catalysis of the refolding of damaged proteins, otherwise referred to as its chaperone effect [52]. However, today it has become clear that Hsp70 has roles beyond its chaperone activity and it has been linked to other functions including; transportation of proteins, degradation of unstable proteins, and apoptosis [48].

The human Hsp70 family consists of eight members that differ slightly from one another in amino acid sequence, expression levels, and localization [59]. Members of the human Hsp70 family consist of three main domains; a conserved ATPase domain, which binds and hydrolyzes ATP, a peptide binding domain, and the C-terminal domain, which contains an EEVD motif for binding co-chaperones and other Hsp molecules [48].

All members of the hsp70 family perform as molecular chaperones through the ability to recognize and bind to long stretches of hydrophobic amino acids exposed from incorrect peptide folding [48]. Hsp70 can bind to these regions using its peptide binding domain to help refold the molecule into its proper conformation [48, 60]. Another important function of hsp70 is its ability to regulate the shuttling of various proteins between the cytoplasm and nucleus [61]. Hsp70 has been shown to stabilize the complex responsible for importing proteins into the nucleus as well as itself containing a sequence known to cause nuclear localization [61].

1.7 Nuclear/Cytoplasmic Shuttling

The cellular machinery responsible for the active translocation of proteins between the nucleus and cytoplasm act through nuclear pore complexes (NPC) found within the nuclear envelope [62]. Proteins belonging to the karyopherin family, otherwise known as the importin/exportin family, associate with the NPC to transfer proteins along the nuclear membrane [63]. Proteins targeted to the nucleus usually contain a nuclear localization sequence (NLS) composed of several basic amino acids that are recognized by importin α [62]. The opposing procedure involves the recognition of a leucine-rich nuclear export sequence (NES) by an export carrier protein (eg. CRM1, exportin-t, and CAS) [62, 63].

Many proteins are transported via this cellular mechanism across the nuclear membrane [62, 63]. For instance, the human homologue of Hsp70 is able to enter the nucleus through its NLS sequence, ^{246}KRKHKKDISENKRAVRR^{262}, however a functional NES has yet to be identified [61]. In addition to self-import, Hsp70 can regulate the entry of other proteins into the nucleus, examples include; p53, NF-κ B, and nucleoplasmin [64-66]. Furthermore, Hsp70 demonstrates involvement in the formation and stabilization of the NLS/importin α complex, thereby aiding in the overall mechanism of cellular transport [61]. Lastly, during cellular stress conditions, like heat shock and oxidative stress, Hsp70 localization shifts to the nucleus and participates in the prevention of DNA damage, which is linked to apoptosis [61, 67].

1.8 The Role of Hsp70 in Apoptosis

The cellular function of heat shock proteins directly counters the apoptotic signaling pathway. While heat shock proteins help to regulate the protection and ultimate survival of cells, apoptosis is responsible for the removal of cells through programmed cell death [68]. Recent studies have demonstrated that the protective outcome of the heat shock proteins is partly due to their ability to suppress apoptosis within cells [69]. Overall, apoptosis signaling is an extensive process with various routes, eliciting a common response of controlled cell suicide. Despite the vast number of signals that result in this type of cell death, the morphological changes characterized by apoptosis remains the same. Apoptosis is recognized through specific physical phenotypes including membrane blebbing, chromatin condensation, and nuclear fragmentation [68].

The first implication of the involvement of Hsp70 in cell protection was the effect that mild heat shock protected cells from subsequent and more severe heat shock stress

[70]. The cells were shown to defend against apoptosis and this resistance was seemingly related to an induction of Hsp70 [71]. Through ample evidence it has been revealed that Hsp70's involvement in the obstruction of apoptosis modulates the mitochondrial mediated pathway (Fig.3) [68]. This pathway involves the disruption of the outer mitochondrial membrane, leading to the leakage of cytochrome c into the cytoplasm [72]. Cytochrome c binds to the caspase recruitment domain (CARD) of the Apaf-1 protein, which oligomerizes in the presence of dATP/ATP and recruits initiator caspase, procaspase-9 [68]. The association of procaspase-9 with Apaf-1 activates caspase-9, which is now able to recruit and cleave procaspase-3 [68]. Activated caspase-3 directly acts upon hundreds of substrates and it is responsible for many of the morphological traits as well as actual cell death that are associated with apoptosis [73]. The release of cytochrome c from the mitochondria is regulated by the Bcl-2 family of pro-apoptotic and anti-apoptotic proteins [74]. Bax is a pro-apoptotic member of the Bcl-2 family and when activated is able to integrate itself into the outer mitochondrial membrane causing permeabilization [74]. Currently, it is unclear as to how Bax gets activated in response to stress, but it is clear that its activation results in cytochrome c release and ultimately apoptosis.

The apoptotic inhibitory action of Hsp70 is not yet fully characterized, but it has been shown to be involved along multiple points within the mitochondrial mediated pathway of apoptosis. Hsp70 is thought to directly bind with the CARD domain of Apaf-1 and inhibit the formation of the apoptosome, which is the protein complex comprising of Apaf-1/procaspase-9/cytochrome c [72]. On the other hand, Hsp70 was also discovered to inhibit apoptosis upstream of mitochondrial permeabilization through the

prevention of Bax translocation [74]. However, Hsp70 does not directly bind to Bax, but instead represses signals responsible for its activation [74]. Such signals may include the ERK and JNK signaling pathways, which are able to regulate members of the Bcl-2 family [74]. ERK has the ability to phosporylate the Bcl-2 protein, Bim and upon phosphorylation Bim can no longer associate and activate Bax [74]. Additionally, JNK associates with Bim and furthermore inactivates the anti-apoptotic protein, Bcl-2 [74]. Also, JNK phosphorylates the 14-3-3 protein, which usually associates with Bax sequestering it in the cytoplasm, therefore, upon 14-3-3 phosphorylation, Bax is released and able to interact with the mitochondria [74].

The role of Hsp70 in apoptosis is not limited to the caspase-dependent pathway described above. For instance, Hsp70 can bind directly to the apoptosis inducing factor (AIF), which is a mitochondrial intermembrane flavoprotein, and prevent chromatin condensation and apoptosis that is usually associated with this protein [75]. Also, Hsp70 can control the activity of enzymes involved in apoptosis, like poly-ADP-ribose polymerase (PARP) and phospholipase A_2 (PLA_2) [76]. Hsp70 downregulates PARP, which is a substrate of caspase-3 involved in activating apoptosis, as well Hsp70 can prevent TNFα induced apoptosis by inhibiting the activation of PLA_2 [76, 77]. Overall, the protective effects of Hsp70 are decidedly beneficial in aiding stressed cells to resist apoptosis, however, the overexpression of Hsp70 is also, not suprisingly, linked to a number of cancers [48, 78]. The involvement of Hsp70 in apoptosis is undoubtedly complicated and further adding to this complexity is the pro-apoptotic functions of Hsp70 [78, 79]. One example of such function is the association of Hsp70 with IκB kinase (IKK)

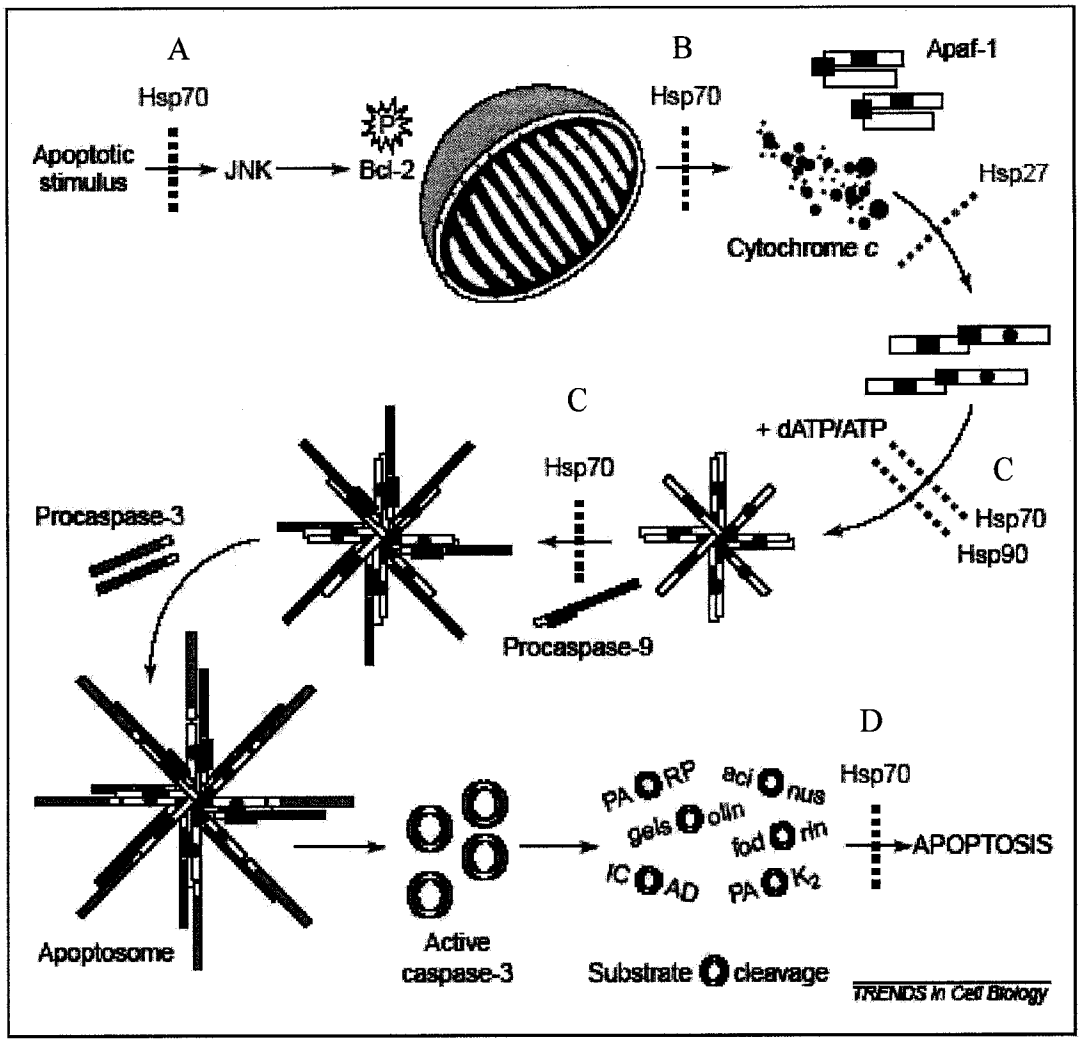


Figure 3 - Schematic of Hsp70 in the Mitochondrial Mediated Pathway of Apoptosis [68]

Upon the initiation of apoptotic signals *A,* Hsp70 can initially prevent the permeabilization of the mitochondria via the inactivation of the JNK signaling pathway through the stabilization of JNK inactivating phosphatases. However, once permeabilization of the mitochondria occurs apoptosis can still be prevented by Hsp70 through *B,* the blocking of cytochrome c release, *C,* the inhibition of the formation of the apoptosome, as well as even *D,* blocking apoptosis downstream of activated caspase-3 substrates.

to inhibit its activation of NF-κB anti-apoptotic gene induction [79]. The role of Hsp70 within apoptosis is still being unravelled, however, its importance is widely accepted.

1.9 The Mitogen-Activated Protein Kinase Signalling Pathway

The mitogen-activated protein kinase (MAPK) pathway is the mechanism used by cells to translate external stimuli, like hormones, growth factors, and environmental stresses into internal responses including cell proliferation, differentiation and apoptosis [34, 80]. There are three distinct subgroups defining this largely complex pathway; two are stress-induced – SAPK/JNK and p38 – while the third is the mitogen activated ERK pathway [81]. The regulation of the MAPK pathway is attributed to a cascade of phosphorylation, which leads to the eventual activation of the MAPKs, ERK, JNK, and p38. Upon activation, the phosphorylated forms of these proteins can relocate from the cytoplasm to the nucleus, where they are able to further activate transcription factors and alter gene expression [82]. Directly upstream from these MAPKs are a family of dual specificity kinases that phosphorylate the TXY motif, thereby activating the MAPKs, these enzymes are commonly referred to as the MAPKKs (MAP2K) [83]. Existing upstream from the MAP2Ks is a family of Ser/Thr kinases termed MAPKKK (MAP3K) that activate their MAP2K substrate by phosphorylating two residues of either serine or threonine found in its activation loop [83]. There is great diversity in the MAP3K family of kinases allowing for these pathways to be activated by and respond to diverse environmental stimuli [83].

The first subgroup of the MAPK pathway is the extracellular signal regulated kinase pathway (ERK), involved mainly in cell proliferation and differentiation [84]. An important stimulus of the ERK pathway are growth factors, and when these molecules

bind to receptor tyrosine kinases, the kinases are able to activate themselves through autophosphorylation [84]. The activated receptors can interact with adaptor proteins, which bind to Ras [84]. Ras-GTP can further activate the Ser/Thr kinase Raf, which phosphorylates the dual specificity MAP2K, MEKK1. MEKK1 directly interacts with ERK and phosphorylates Thr 183 and Tyr 185 [84]. While the activation of ERK is most commonly connected to the promotion of cell proliferation and differentiation, more recently, evidence has shown under some circumstances ERK can be linked to the promotion of apoptosis and cell cycle arrest [84]. Activated ERK has a number of substrate targets including transcription factors such as c-Jun, c-Fos, Elk-1, ATF-2, and c-Myc, as well as cytoplasmic targets such as phospholipase A2 and ribosomal S6 kinase [84].

The two remaining subgroups of the MAPK pathway are the c-Jun N-terminal Kinase / Stress Activated Protein Kinase (JNK / SAPK) and the p38 pathway. Unlike the ERK signalling pathway, these two branches require a stress stimulus for activation. Activating stresses include UV irradiation, oxidative stress, heat shock, inflammatory cytokines and osmotic shock, which in turn triggers a set of MAP4Ks, MAP3Ks, and MAP2Ks specific to JNK and p38 [85]. Targets for the JNK pathway include transcription factors c-Jun, ATF-2, Elk-1, p53, DPC4, Sap-1a, NFAT4, while p38 targets consist of PLA_2, ATF-2, Sap-1a, Pax6, CHOP, CREB, and GADD153 (Growth Arrest DNA Damaging transcription factor 153) [85]. The JNK pathway is most commonly associated with a pro-apoptotic role within mitochondrial dependent apoptosis as mentioned earlier. However, JNK has other functions such as encouraging cell proliferation. For instance, the JNK target transcription factor, c-jun, has been

demonstrated as essential for the transition of cells from G1 phase to S phase during the cell cycle [86]. Fibroblast cells exhibiting a JNK isoform knockdown show a decrease in cell density as well as a slowness of growth, which relates to an overall defect in cell proliferation [86]. Briefly, p38 is involved in an array of functions including apoptosis, adipocyte and neuron differentiation, proliferation, development and inflammation [86]. The p38 pathway is known to induce apoptosis through the activation of phospholipase A2, negatively regulate cell cycle progression through downregulation of cyclin D1 expression, and is required in the cdc-42 induced cell cycle arrest at the G1/S phase [86].

1.9.1 – The role of Hsp70 in the MAPK Pathway

The role of chaperone proteins within the MAPK pathway is of great interest and while some links have been made, there is still much to be discovered. Notably, Hsp70 interacts with co-chaperone Bag-1 and prevents it from binding to Raf-1 [87]. The Bag1/Raf-1 complex is an activator of ERK, hence Hsp70 can lead to a decrease cell proliferation [87]. A change in ERK expression levels is also linked to the cell cycle [88]. More specifically, ERK has shown to affect the cdc2/cyclin B complex, which is necessary for M phase progression [88]. The actual influence of ERK is contradictory, differing with experimental conditions [88]. In NIH 3T3 cells and Xenopus eggs, ERK activation was shown to aid in the progression of the cell cycle, specifically in the NIH 3T3 cells, blockage of ERK activation was associated with a delay in nuclear translocation of cyclin B [88]. However, in alternate study using Xenopus egg and fertilized egg, ERK was found to suppress cdc2 activation and block cell cycle progression [89]. Interestingly, a testis-specific Hsp70 molecule termed Hsp70-2 was found to interact with cdc2 to promote the formation of the cdc2/cyclin B complex and

promote cell cycle progression [84]. It has not been established whether Hsp70 has an effect on ERK within the cell cycle or vice versa, but it is exciting to note that both proteins target the same cell cycle regulator.

The p38 pathway has important functions within cell immunity. In response to heat shock or necrotic cell death, an increase in Hsp70 is present and binds to macrophage receptor proteins, such as CD40 [90, 91]. The interaction between Hsp70 and CD40 causes an upregulation of the p38 pathway, which is a known activator of phagocytosis [90, 91].

The main role of Hsp70 in the JNK pathway involves the regulation of phosphatases that deactivate JNK and therefore prevents apoptosis in a number of cellular contexts [92].

Overall, each segment of the MAPK pathway participates in a multitude of cellular processes and the regulation directing its action is dependent on a number of factors including, cell type, cell environment, stress conditions, and isoform specificity [84, 88]. It is apparent there is a great deal of overlap and crosstalk existing between the MAPK pathways and the equilibrium maintained in each regulatory step is crucial for normal cell homeostasis.

1.9.2 The Role of dual specificity phosphatases in the MAPK Pathway

As mentioned previously, a family of phosphatases associated with the dephosphorylation of MAPK members are termed the MAPK phosphatases or MKPs. These phosphatases recognize and dephosphorylate the pTXpY signature motif found on activated ERK, JNK, and p38 [34]. The MKPs can be further divided into three families based on their accessory domains and MAPK specificity [34]. The dephosphorylation of

simply one residue of the pTXpY motif is sufficient for the deactivation of MAPKs [93]. For instance, the Ser/Thr-specific phosphatase PP2A and the Tyr-specific phosphatase STEP were both shown to dephosphorylate and inactivate ERK in PC12 cells [93]. However, most of the literature regarding the dephosphorylation of MAPKs focuses on the dual-specific-MKPs.

It has been demonstrated that 13 DSPs can be classified into MKPs due to their association with the dephosphorylation of MAPKs, and can be further divided into four groups based on structure and function [34]. Type I MKPs only contain the DSP catalytic domain and consist of three members, VHR, DSP2, and MKP6, where VHR and MKP6 are ERK/JNK specific and DSP2 is p38/JNK specific [34, 94]. The remaining MKPs contain a MAPK binding domain (MKB), which acts as a docking site between MKPs and their corresponding substrate [34]. A substrate-induced activation model is suggested to describe the mode of binding between MKPs and their MAPK substrate [34]. It is believed in the absence of substrate the DSP catalytic domain is inactive, however, upon the binding of MKPs to its phosphorylated MAPK substrate through the MKB domain, a conformational change occurs [34]. This rearrangement allows the catalytic site to adjust to an active formation (Fig.4) [34]. Type II MKPs contain an MKB domain located at its N-terminal as well as a DSP catalytic domain [34]. Identified members of this group include the p38/JNK specific MKP1 and the ERK specific MKP3 and PAC1 [34]. Also belonging to this group are MKP2 and MKP4, which are able to dephosphorylate all three MAPK pathways and the poorly characterized VH3 and PYST2, which have both been shown to dephosphorylate ERK [34]. The type III MKPs have only one member, the JNK/p38 specific MKP5, which contain both MKB and DSP

domains in addition to an N-terminal domain of uncharacterized function [34]. This N-terminal domain is hypothesized to be involved in protein-protein interactions and could possibly be involved in cross-communication between the different MAPK pathways [34]. The type IV MKPs contains two JNK/p38 specific members, VH5 and MKP7 [34]. In addition to the MKB and DSP domains, type IV MKPs, contain proline, glutamate, serine, threonine rich domains (PEST), which is most commonly associated with ubiquitin-mediated proteolysis [34].

The MKPs play important regulatory roles by deactivating JNK, ERK, and p38 signaling and thus participate in the control of important cellular processes. Interestingly, the type I MKPs are able to dephosphorylate MAPK members despite the absence of the MAPK binding domain and it has been shown to have a preference for phosphorylated tyrosine over phosphorylated threonine [34]. This fact permits the possibility that other dual-specificity phosphatases with similar sequence or structure to VHR could also potentially dephosphorylate MAPKs.

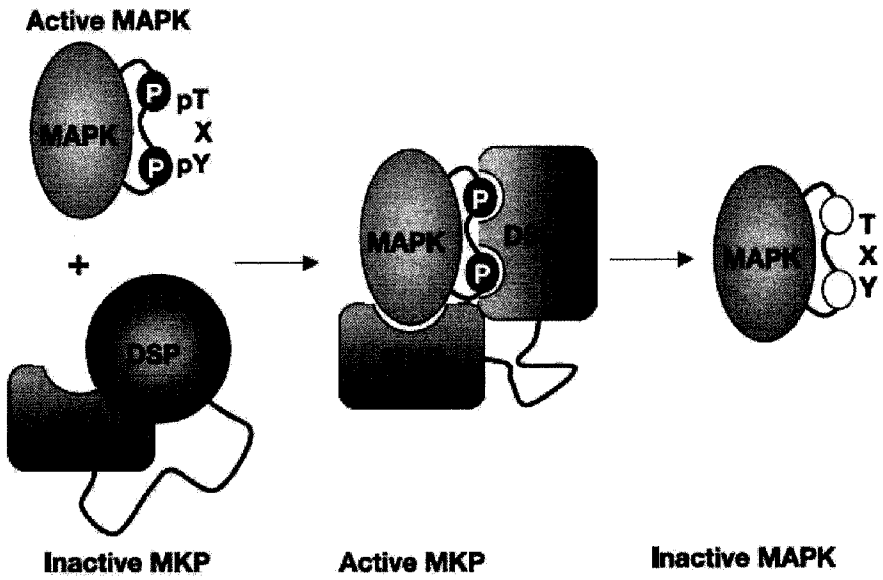


Figure 4 - Substrate Induced Activation of MKPs [34]

The MAPK substrate of the MKPs is able to allosterically initiate rearrangement of the catalytic domain of DSPs in order to instigate its own dephosphorylation.

1.10 Objectives

The atypical dual specificity phosphatase, hYVH1, is highly conserved and widely expressed in human tissues, yet remains poorly characterized. Studies reveal that YVH1 orthologues are able to complement one another in gene deletion experiments, implying the biological function of this protein is also well conserved. Specifically, the YVH1 gene was shown to be involved in cellular growth, sporulation, and glycogen accumulation in yeast, however precise biological details remain unknown. Also, a recent study found that the gene of the human orthologue of *yvh1* was amplified in a number of malignant sarcomas. Thus far, a well defined physiological role has yet to be elucidated regarding hYVH1. This is most likely due to the failure to identify a biological substrate, lack of in depth structural characterization, and poor definition of its regulation properties. Therefore, one objective of my project is to determine regions of hYVH1 important for subcellular localization. Additionally, the recent identification of Hsp70 as an interacting partner of hYVH1 in our lab has permitted examination of hYVH1 as functioning in cellular stress pathways.

The specific aims of this study are;

(i) To examine hYVH1 regulatory motifs mediating its localization

(ii) To confirm the interaction between hYVH1 and Hsp70 *in vivo*

(iii) To determine the functional significance of hYVH1 and its interaction with Hsp70

CHAPTER 2

Materials and Methods

2.1 Plasmid Construction

Truncated derivatives of hYVH1 were constructed by introducing each cDNA fragment into a pFLAG-CMV2 mammalian expression vector. The three hYVH1 Zn finger deletion constructs, ZnΔhYVH1, CT-2, and CT-3, were made using the same sense primer, 5'-CGGAATTCGATGTTGGAGGCTCCG-3', containing an initiation codon and an EcoRI restriction site and the antisense primers, 5'-CGTTAAGTCGACTC ATGGATACTTCTCTGT-3', 5'-CGTTAAGTCGACTCAATAAGATG TACATTG-3', and 5'-CGTTAAGTCGACTCATCCCAACAAAGCAGA-3', for ZnΔhYVH1, CT-2, and CT-3, respectively. The antisense primers all contained a stop codon and a SalI restriction site. The hYVH1 zinc finger domain (catΔ hYVH1) was constructed by PCR amplification using the sense primer 5'-CGGAATTCGGAATTGCAGATT-3' containing an EcoRI restriction site and antisense primer 5'-CGTTAAGTCGACTCATATTTTTCC TGTTTGTGA-3' containing a SalI restriction site. The construction of full length recombinant wildtype hYVH1 has been previously described [40].

For construction of variants within the potential nuclear export sequence (NES) of hYVH1, PCR-based site directed mutagenesis was carried out. The NES variant containing one Leu to Ser mutation used the following oligonucleotides: 5'-AAGTATCCAGAATCGCAGAATTTACCT-3' and 5'-AGGTAAATTCTGCGATTCT GGATACTT-3', while the doubly mutated Leu to Ser variant used the 5'-GAATCGCAG AATTCACCTCAAGAACTA-3' and 5'-GAGTTCTTGAGGTGAATTCTGCGATTC-3' oligonucleotides. Lastly, all Hsp70 constructs used were a gift from Dr.Frank Sharp at the

University of California. All above constructs were verified using automated DNA sequencing and prepared with either Sigma mini-prep kit or Qiagen maxi-prep kit.

2.2 Cell Culture, Transfection, and Treatments

HeLa cells originating from a human cervical carcinoma cell line were maintained in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% (v/v) fetal bovine serum at 37°C and 5% CO_2. Transient transfection of cells was carried out at 50-70% confluency using ExGen reagent (Fermentas) according to the manufacturer's protocol. For heat shock treatments, cells were grown at 70-80% confluency, placed in incubators at 42°C, 43°C, or 45.5°C and heat shocked and recovered for various time periods.

2.3 Cell Fractionation and Cell Lysis

Cell fractionation was performed by pelleting cells, washing with 1xTBS buffer (20 mM Tris base, 140 mM NaCl, pH=7.6) and resuspending cells in a cold hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.5 mM PMSF) with 0.5% Triton-X. Following a one minute high speed centrifugation, the supernatant was collected, labelled cytosolic fraction, and stored at -20°C while the pellet was resuspended in a second ice cold buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF) and vigorously rocked at 4°C for 15 minutes. After a five minute high speed centrifugation, the supernatant was collected, labelled nuclear fraction, and stored at -70°C.

Whole cell lysate samples were collected following treatment using a hypotonic lysis solution (50 mM Tris-HCl, pH=7.4, 1% Triton-X 100, 150 mM NaCl, 0.1% SDS, phenylmethylsulfonyl fluoride, and aprotinin). Cells were washed in PBS prior to being

resuspended in lysis buffer, incubated on ice for 15 minutes, and centrifuged at 15,000 rpm for 20 minutes. The supernatant was stored at -20°C.

2.4 Immunoprecipitation

Immunoprecipitation was performed using 20 μL of Protein A Agarose resin per sample (Invitrogen). The resin was washed twice with 500 μL of lysis buffer before being resuspended in 300 μL of lysis buffer and 1μg of anti-JNK antibody (Santa Cruz) and incubated at room temperature for 45 minutes. Next, the resin was centrifuged at 4000 rpm for 1 minute and the supernatant was removed. The resin attached to antibody was then incubated with fresh cell lysates, prepared as described above, for 2 hours at 4°C. Following incubation, samples were rinsed twice with wash buffer (50 mM Tris-HCl, pH 7.4, 0.1% Triton X-100 detergent, 150 mM NaCl, and 0.1% SDS), resuspended in 2 x SDS-PAGE loading dye buffer, heated at 95°C, and stored at -20°C.

2.5 Antibodies and Western Blotting

Protein concentration was estimated using BioRad Protein Assay reagent and Bovine Serum Albumin (BSA) standard. Equal amounts of protein were loaded and separated by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Millipore). Levels of hYVH1 were detected using a polyclonal anti-hYVH1 serum antibody at a 1:1000 dilution in 3% BSA. Recombinant protein expression was determined using a monoclonal flag antibody (Sigma) at a 1:1000 dilution in 5% milk in TBST buffer. Endogenous hsp70 levels were detected using a polyclonal anti-Hsp70 antibody (Santa Cruz) at 1:200 in a 5% milk TS solution (150 mM NaCl, 13 mM Tris base) and recombinant hsp70 protein expression was determined using a polyclonal anti-his antibody (Santa Cruz) at 1:200 in 5% TS milk solution. Active MAPK levels were

determined using anti-phospho-ERK and anti-phospho-JNK antibodies at a 1:2000 dilution. Antibodies were obtained from Cell Signalling and blots were incubated as per manufacturer's protocol. The corresponding inactive MAPK antibodies, anti-ERK and anti-JNK, were incubated in 5% TBST milk at a 1:5000 dilution (Sigma). Lastly, fractionation control antibodies, anti-actin (Sigma) and anti-laminB (Santa Cruz) were used at a 1:5000 dilution in 5% milk TBST solution and a 1:200 dilution in a 5% milk TS solution, respectively. Following overnight blocking in 5% milk, primary antibodies were incubated with blots for one hour, except in the case of the anti-phospho-MAPK antibodies and anti-laminB, where a 1 hour blocking and overnight primary incubation was used. Bound primary antibodies were detected with a horseradish peroxidase-coupled secondary antibody at a dilution of 1:5000 for all blots (Biorad, Santa Cruz, Vector Labs) and visualized using Super Signal West Femto (Pierce).

2.6 Immunofluorescence

For immunofluorescence analysis, cells were grown on coverslips in 6-well plates to 50-70% confluency. The cells were fixed in 3.7% paraformaldehyde for 12 minutes at room temperature, washed in PBS, and permeabilized with 0.15% Triton X-100 in PBS for 2 minutes. The fixed cells were then blocked for 1 hour in 5% rabbit serum in TBST and incubated with primary antibody at a dilution of 1:500 for anti-hYVH1 and anti-flag (Sigma) and a dilution of 1:50 was used for anti-Hsp70 (Santa Cruz). Fluorescein-conjugated secondary antibodies were used at a dilution of 1:500 in TBST for 1 hour (Vector Labs). For colocalization experiments, samples were incubated with anti-hYVH1 or anti-flag for 1 hour, rinsed with PBS, and incubated with anti-Hsp70 for an additional hour, as well, the corresponding fluorescein conjugated secondary antibodies were also

individually incubated at 1 hour intervals. Nuclei were stained with Hoechst 33342 dye (Molecular Probes) according to manufacturing protocol and coverslips were mounted on slides using SlowFade Antifade Kit (Molecular Probes). Cells were observed using fluorescence microscopy with either the Leica DM IRB microscope and Openlab software or with the Axiovert 200 microscope and Northern Eclipse software.

2.7 Annexin V Apoptosis Assay

Annexin V identifies apoptotic cells by binding to phosphatidylserine found on the outer surface of the plasma membrane in apoptotic cells. The FITC conjugated annexin V (Vybrant Apoptosis Assay Kit#3, Molecular Probes) differentiates between apoptotic cells and necrotic cells through a double stain procedure with propidium iodide. Following treatment, HeLa cells grown in 6-well plates were scraped and rinsed twice with 1x annexin binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$, pH = 7.4). After a 5 minute centrifugation at 2500 rpm, the remaining pellet was resuspended in Annexin binding buffer ($1x10^6$ cells/1mL binding buffer) and stained according to the manufacturer's protocol. These cells were also stained using Hoechst 33342 dye and viewed using fluorescence microscopy as described above. Approximately 500-1000 cells were counted per experiment and apoptotic cells were detected by a positive annexin V stain. Data is shown as a percentage of dead cells in the total sample population. The data is the culmination of three unique trials averaged together.

2.8 TUNEL Assay

A second method for apoptotic identification involved the use of APO-BrdU TUNEL Assay Kit (Molecular Probes) to measure DNA fragmentation. Another feature

of apoptosis is the activation of nucleases that eventually lead to the degradation of nuclear DNA. Once DNA fragmentation occurs as a result of the nucleases, the 3' hydroxyl end of DNA becomes exposed. This assay takes advantage of the exposed end by attaching a deoxythymidine analog 5-bromo-2'-deoxyuridine 5'-triphosphate (BrdUTP) to the 3' end using the terminal deoxynucleotide transferase enzyme (TdT). An antibody against BrdUTP accompanied with fluorescence microscopy is used to detect the binding. Samples were prepared following the manufacturer's protocol.

CHAPTER 3

Results

3.1 The Effect of the Zinc Finger Domain on hYVH1 Localization

Previously described by Muda *et al.,* the subcellular localization of GFP and endogenous hYVH1 determined by immunofluorescence in COS-7 cells was detected in both nuclear and cytoplasmic regions [40]. In order to further characterize the regulation of hYVH1 between the two cellular regions, a more in depth examination of hYVH1 motifs and their possible involvement in localization were conducted. Through the use of recombinant variants of the hYVH1 phosphatase in conjunction with indirect immunofluorescence in HeLa cells, localization patterns were detected (Fig.5 and Fig.6). The localization of transfected HeLa cells with full length recombinant hYVH1 demonstrated both nuclear and cytoplasmic localization, however, opposed to the Muda *et al.* findings, our localization pattern did not appear predominantly nuclear. Our results indicate the hYVH1 phosphatase localized with approximately 60% of the protein found in both the cytoplasm and nucleus and 40% remaining solely cytoplasmic (Fig. 7). However, Muda *et al.* described their hYVH1 localization as predominately nuclear with little diffusion in the cytoplasmic region. Therefore, our results put more emphasis on the cytoplasmic localization.

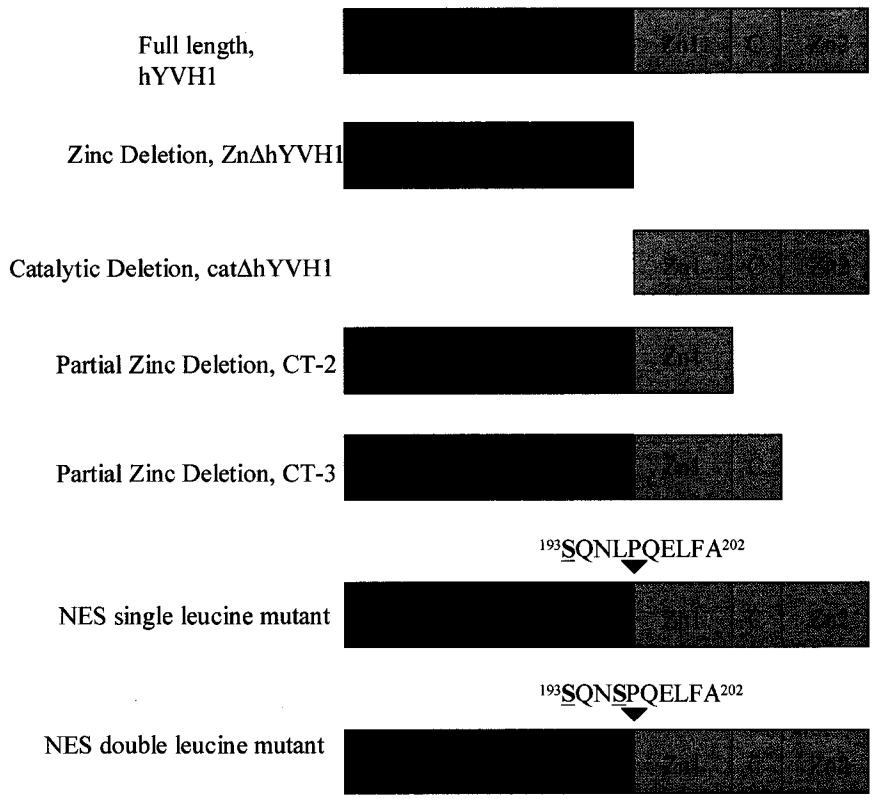


Figure 5 – Variants of hYVH1

Schematic diagram representing the various hYVH1 constructs used during transfection. Catalytic domain containing the signature DSP sequence is depicted in darker blue, while the zinc binding domain can be seen in pale blue. The zinc binding domain is composed of two separate coordinating zinc fingers, Zn1 and Zn2, which flank a conserved hydrophobic region (C). The partial zinc deletion, CT-2, contains only the first zinc coordinating site, while the CT-3 construct contains the first zinc coordinating site as well as a highly conserved hydrophobic motif. The NES mutants are derived from the potential NES site, LQNLPQELFA, located between the catalytic domain and the zinc binding domain.

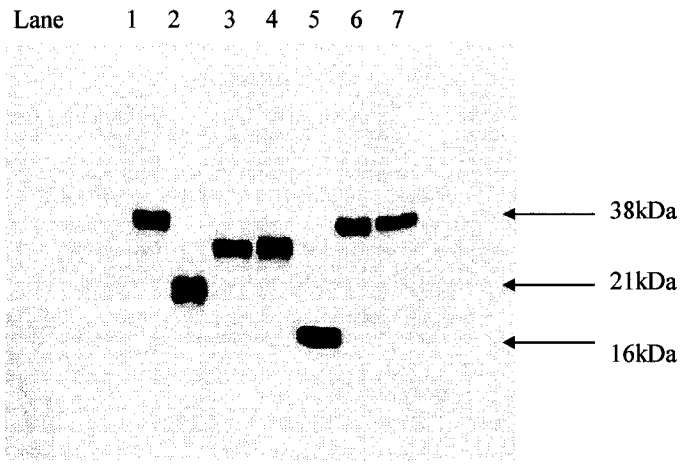


Figure 6 – Expression of hYVH1 Variants

Immunoblot showing expression of hYVH1 variants; Lane 1 full length hYVH1, Lane 2 Zn ΔhYVH1, Lane 3 CT-2, Lane 4 CT-3, Lane 5 catΔhYVH1, Lane 6 NES single leucine mutant, and Lane 7 NES double leucine mutant.

The ZnΔhYVH1 is the recombinant form of only the catalytic domain of hYVH1 (Fig.5). This construct transfected in HeLa cells demonstrated a dispersed localization where a cytoplasmic/nuclear ratio could not be concluded due to the fact the protein was diffused ubiquitously (Fig. 8A, 8B). On the other hand, catΔhYVH1, which is the catalytic deletion of hYVH1, shows a localization pattern that is increasingly focused in the nucleus. Approximately, 80% of recombinant catΔhYVH1 was found to be either exclusively nuclear or nuclear/cytoplasmic (Fig. 8C, 8D). This implies perhaps the zinc finger is responsible for the nuclear localization of hYVH1.

Further examination of the localization of the partial zinc finger deletion constructs demonstrated that with an incomplete zinc finger the nuclear localization remains strong. Transfection with CT-2, which contains the catalytic domain of hYVH1 and the coordination site of the first zinc finger, shows a strong nuclear localization of approximately 90% (Fig. 9A, 9B). Similarly, CT-3, which contains the catalytic hYVH1 domain, the coordination site of the first zinc finger as well as a hydrophobic motif that is highly conserved throughout evolution, also demonstrates an approximate 90% nuclear localization (Fig. 9C, 9D). A summarization of the cytoplasmic/nuclear percentages can be seen in Table 1. However, overall it appears that the zinc binding domain is responsible for mediating nuclear localization, while elements of the catalytic domain and zinc binding domain are necessary for cytoplasmic localization.

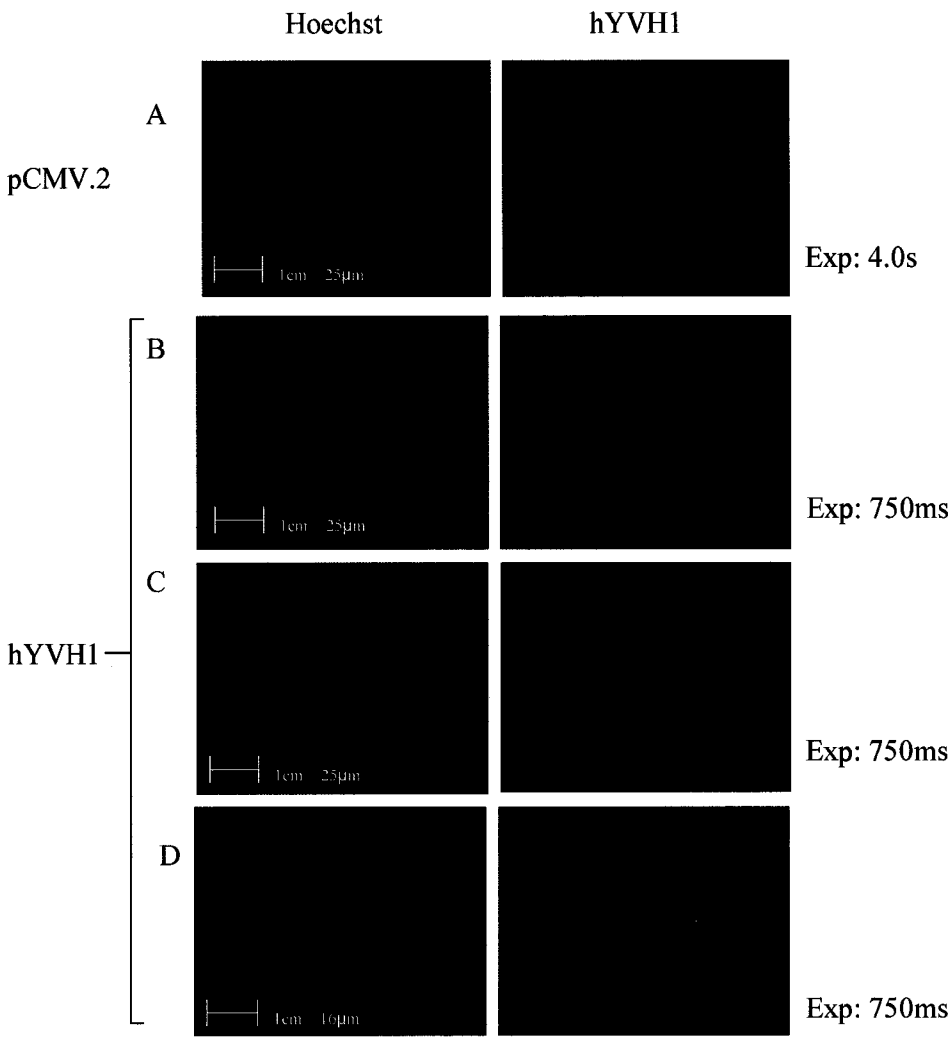


Figure 7 – Localization of Recombinant hYVH1

HeLa cells fixed with 3.7% PFA were examined using fluorescence microscopy. Nuclear staining is in blue, while hYVH1 is depicted in green. *A,* HeLa cells transfected with pCMV.2 empty vector DNA show very little non-specific binding of secondary anti-mouse FITC labeled antibody. *B,C,D,* HeLa cells transfected with full length wildtype hYVH1 DNA and visualized using anti-flag primary antibody followed by incubation with anti-mouse FITC labeled secondary antibody.

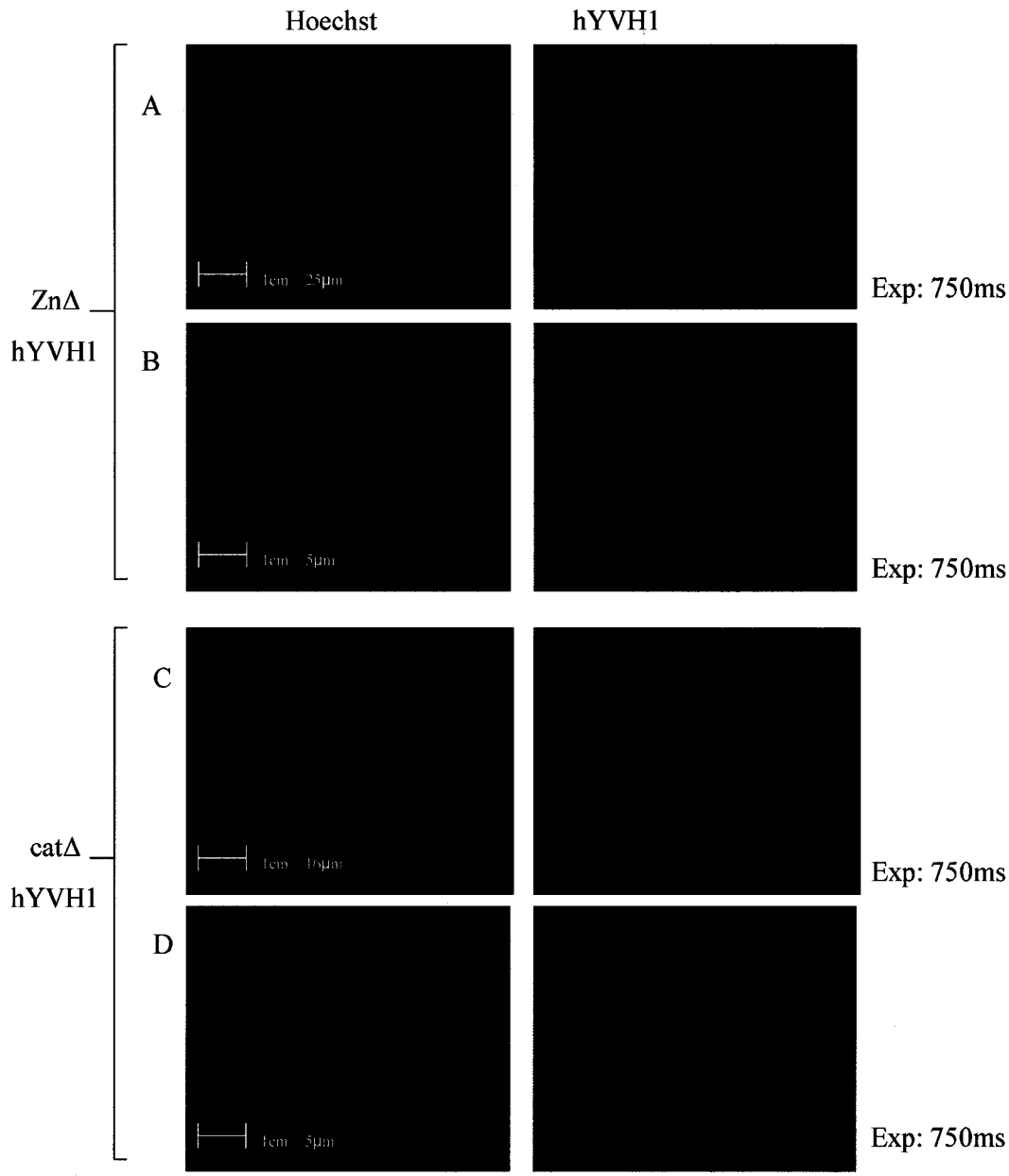


Figure 8 – Localization of the catalytic domain deletion and zinc finger domain deletion of hYVH1

A,B, Following transfection with ZnΔhYVH1, Hela cells were fixed and incubated with anti-flag primary antibody and FITC labelled anti-mouse secondary antibody. *C,D,* Cells were transfected with the catalytic catΔhYVH1 and treated as described previously.

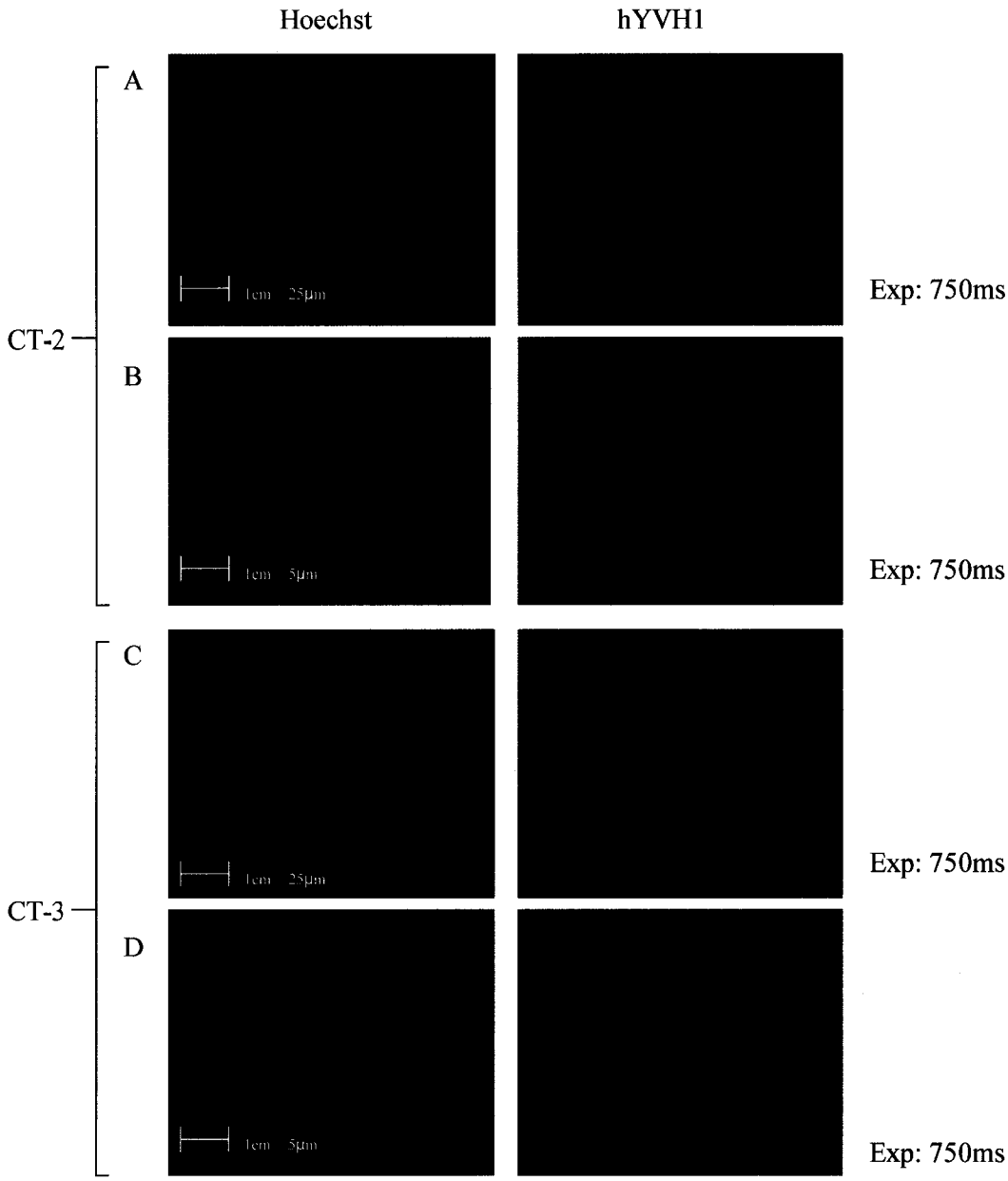


Figure 9 – Localization of partial zinc finger deletion variants of hYVH1

Hela cells were transfected with *A,B,* CT-2 variant of hYVH1 or *C,D,* CT-3 variant of hYVH1. Cells were fixed and visualized as described previously.

3.2 Determination of a possible NES site in hYVH1

One method of protein transportation across the nuclear membrane involves NES and NLS motifs. Characteristically, a NES motif is outlined as follows, $LX_{1-3}LX_{2-3}LXL$, where X is any amino acid and the last leucine may be replaced by any hydrophobic amino acid. Following this consensus sequence, a postulated NES of hYVH1 is ^{193}LQNLPQELFA^{202} located precisely at the beginning of the zinc binding domain (Fig.5). Using site directed mutagenesis, two hYVH1 variants were created, the first mutated one leucine into a serine to give the following, ^{193}SQNLPQELFA^{202}, and the second contained a double mutation of the first two leucines to create, ^{193}SQNSPQELFA^{202}. These constructs were transfected into HeLa cells and following immunofluorescence it was determined that both NES mutants were located in the cytoplasm and nucleus. More specifically, through cell counting it was established that approximately 75% of mutated hYVH1 was found in the nucleus, while 25% remained cytoplasmic (Fig. 10A-10D). Comparing these results to the full length recombinant hYVH1, there appears to be a significant increase in the nuclear retention of hYVH1. However, there still exists cytoplasmic localization despite the fact that the postulated NES was mutated, therefore, this suggests the putative NES sequence may not be the only factor contributing to hYVH1 nuclear/cytoplasmic shuttling.

3.3 Effect of Heat Shock on the Interaction and Localization of hYVH1 and Hsp70

In addition to NES/NLS motifs, another possible mechanism influencing subcellular localization is protein-protein interactions. Our lab has recently identified Hsp70 as a novel binding partner to hYVH1 using co-immunoprecipitation and mass

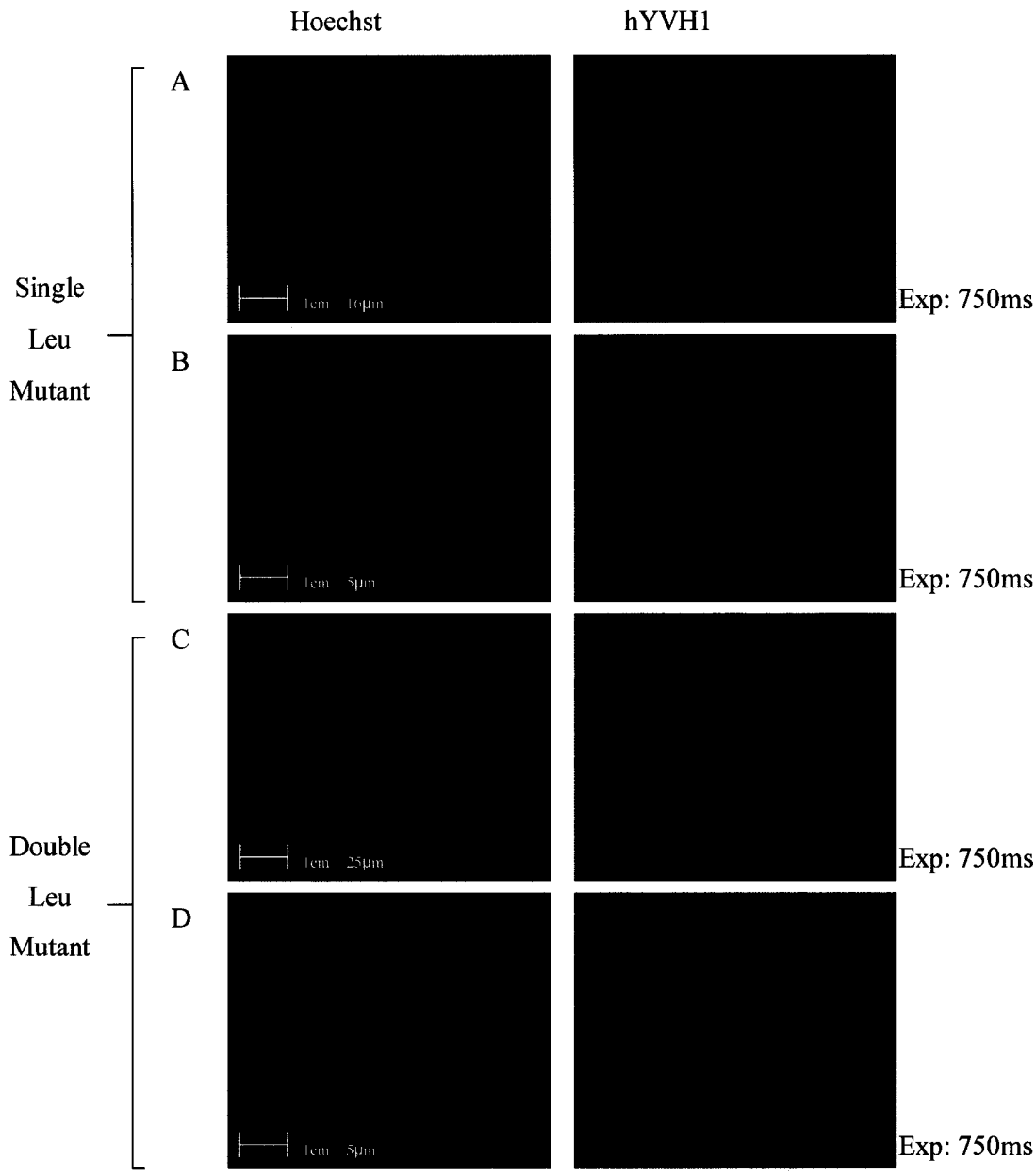


Figure 10 – Determination of potential NES

Hela cells were transfected with *A,B,* single leucine mutant NES construct of hYVH1 or *C,D,* double leucine mutant NES construct of hYVH1. Cells were fixed and visualized as described previously.

	Cytoplasmic (%)	Nuclear (%)
Recombinant hYVH1	40	60
ZnΔhYVH1	---	---
CatΔhYVH1	20	80
CT-2	10	90
CT-3	10	90
Single Leucine NES mutant	25	75
Double Leucine NES mutant	25	75

Table 1 Summarization of Cytoplasmic/Nuclear Localization

spectrometry. Therefore, to examine whether these proteins colocalize to a discrete cell compartment, fluorescence microscopy was employed. The colocalization between Hsp70 and hYVH1 was confirmed in HeLa cells using indirect immunofluorescence. The endogenous expression of both proteins was examined under untreated and heat shock conditions. Proper control pictures were taken to verify the absence of non-specific binding of secondary antibodies as well as false colocalization due to bleed through of microscope filters (images not shown). In agreement with the previous mass spectrometry results, a strong colocalization between hYVH1 and Hsp70 was demonstrated under untreated conditions (Fig. 11A-11C and 12A-12C). Furthermore, the colocalization of the two proteins was predominantly cytoplasmic with emphasis in the perinuclear region. However, upon mild heat shock of HeLa cells at 42°C applied for 2 hours and a 10 hour recovery period at 37°C (Fig. 11D-11F and 12D-12F) a significant shift in both proteins was detected. It appears the heat shock stress caused an accumulation of hYVH1 to the perinuclear region of the cell, while Hsp70 amassed around the perinuclear region as well as in the nucleus. Also, following an extreme heat shock stimulus at 45.5°C for 2 hours with a 3 hour recovery period at 37°C, the localization of both proteins appeared to be strongly perinuclear (Fig. 11G-11I, Fig.13). This severe heat shock treatment caused morphological changes prompting cells to round up, yet not undergo apoptosis (Fig. 13A-13E). Overall, despite the severity and duration of heat shock, the colocalization between Hsp70 and hYVH1 remained undisturbed. Interestingly, it has been previously shown that Hsp70 is capable of localizing at the perinuclear region and under heat shock conditions, the perinuclear localization is enhanced, but a purpose for this change in distribution has yet to be clearly elucidated

[95]. The strong colocalization between hYVH1 and Hsp70 combined with the mass spectrometry and co-immunoprecipitation findings that hYVH1 binds to Hsp70 suggests a functional possibility for the interaction within a cellular context.

3.4 Effect of Heat Shock on Protein Expression Levels of hYVH1

The change in distribution of hYVH1 and Hsp70 following heat shock stress led to further examination of hYVH1 endogenous protein expression levels using immunoblotting analysis. The level of hsp70 was shown to increase at the 10 hour interval following mild heat shock and remained unchanged after extreme heat shock and 12 and 24 hour periods of recovery. This coincided with another lab member's result showing Hsp70 levels increase and peak around 4 to 8 hours after the activating stimulus (Mucaki, unpublished). In response to a mild heat shock at 42°C for two hours followed by a recovery period at 37°C for 10 hours, a visible increase in the expression of hYVH1 occurred (Fig. 14A). A similar increase in hYVH1 protein expression was also seen after 12 and 24 hours of recovery post severe heat shock treatment of 1 hour at 45°C (Fig. 14B). Taking into account that nitrogen starvation and cold stress have been shown to increase the *yvh1* mRNA levels in yeast cells [36, 37], it is very possible that hYVH1 has some role in cellular stress pathways.

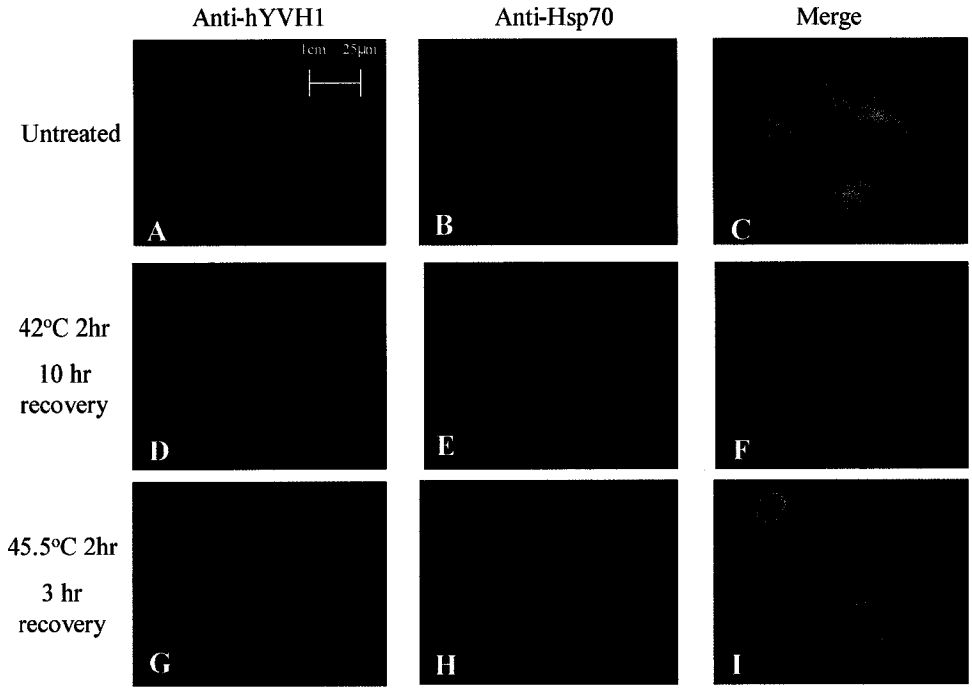
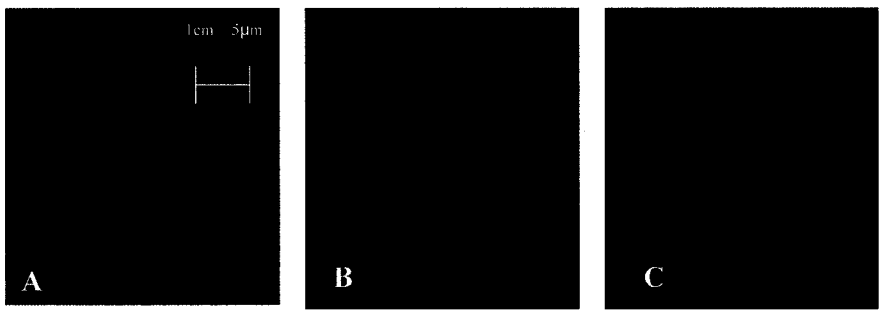


Figure 11 – Effect of heat shock on hYVH1 and Hsp70 in HeLa cells – Part I

Endogenous expression levels of hYVH1 (green) and Hsp70 (red) were detected using fluorescence microscopy. The hYVH1 phosphatase was stained with polyclonal anti-hYVH1 serum antibody and an anti-rabbit secondary antibody labeled with FITC *(A,D,G)*. Hsp70 was detected using a monoclonal anti-Hsp70 antibody and an anti-goat secondary antibody labeled with Texas Red *(B,E,H)*. By merging the two images, a suggested colocalization pattern between the two proteins can be seen in yellow *(C,F,I)*. HeLa cells were either untreated, *A,B,C,* heat shocked at 42°C for two hours and recovered at 37°C for 10 hours *D,E,F,* or heat shocked at 45.5°C for two hours and recovered for 3 hours *G,H,I,* before being fixed and permeabilized. HeLa cells are depicted at a magnification of 400x.

Untreated



42°C 2hr Heat Shock and 10 hr recovery

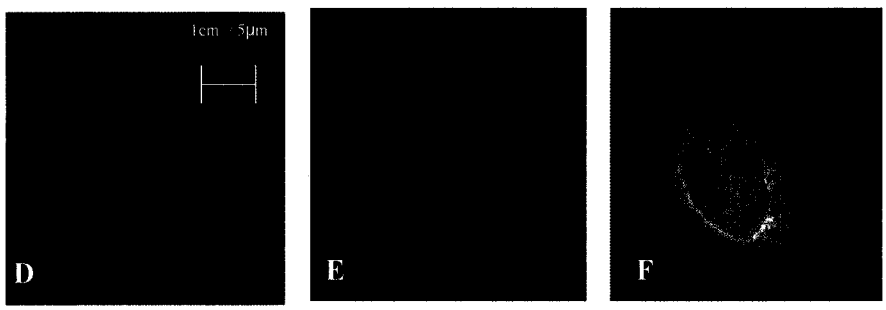


Figure 12 – Effect of heat shock on hYVH1 and Hsp70 in HeLa cells – Part II

Endogenous expression levels of hYVH1 (green) and Hsp70 (red) were detected using fluorescence microscopy. The hYVH1 phosphatase was stained with polyclonal anti-hYVH1 serum antibody and an anti-rabbit secondary antibody labeled with FITC *(A,D)*. Hsp70 was detected using a monoclonal anti-Hsp70 antibody and an anti-goat secondary antibody labeled with Texas Red *(B,E)*. By merging the two images, a suggested colocalization pattern between the two proteins can be seen in yellow *(C,F)*. HeLa cells were either untreated, *A,B,C,* or heat shocked at 42°C for two hours and recovered at 37°C for 10 hours *D,E,F* before being fixed and permeabilized. HeLa cells are depicted at a magnification of 630x.

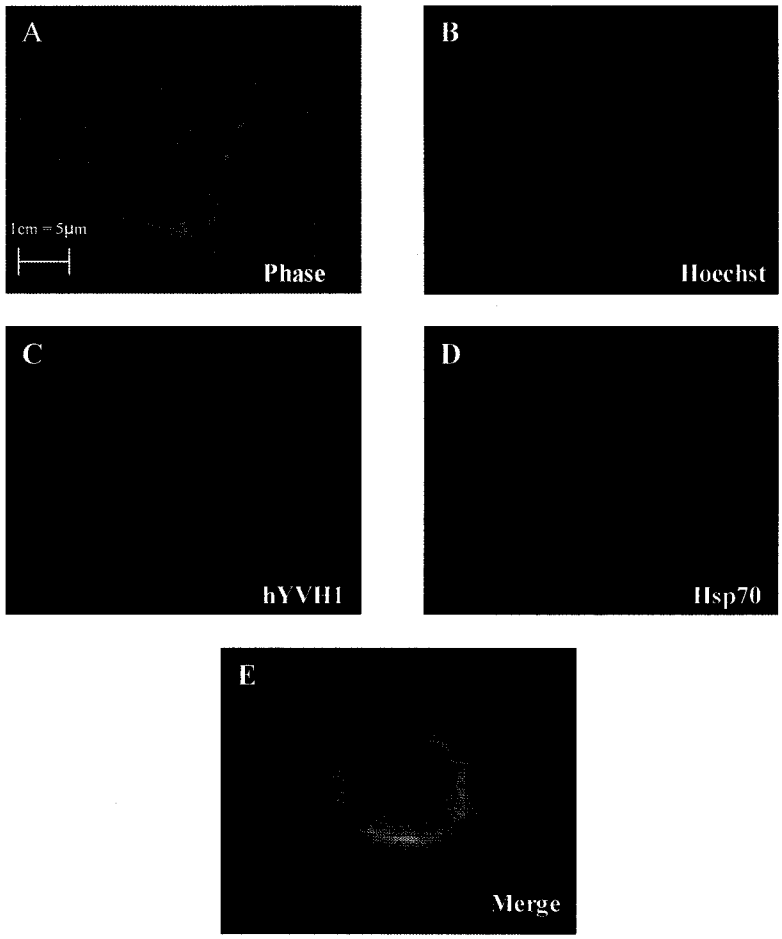


Figure 13 – Effect of Extreme Heat Shock on hYVH1 and Hsp70 in HeLa cells

HeLa cells were heat shocked at 45.5°C for two hours and allowed to recover at 37°C for 3 hours. Following heat shock cells were fixed and incubated with anti-serum hYVH1 primary antibody followed by incubation with a FITC labeled anti rabbit secondary antibody and a monoclonal anti-Hsp70 primary antibody followed by incubation with a Texas Red labeled secondary antibody. *A*, the phase contrast image of a HeLa cell following severe heat shock *B*, nuclear staining of the same cell using Hoechst 33342. *C,D,E,* the endogenous staining of hYVH1, Hsp70, and a merged image of hYVH1 and Hsp70, respectively.

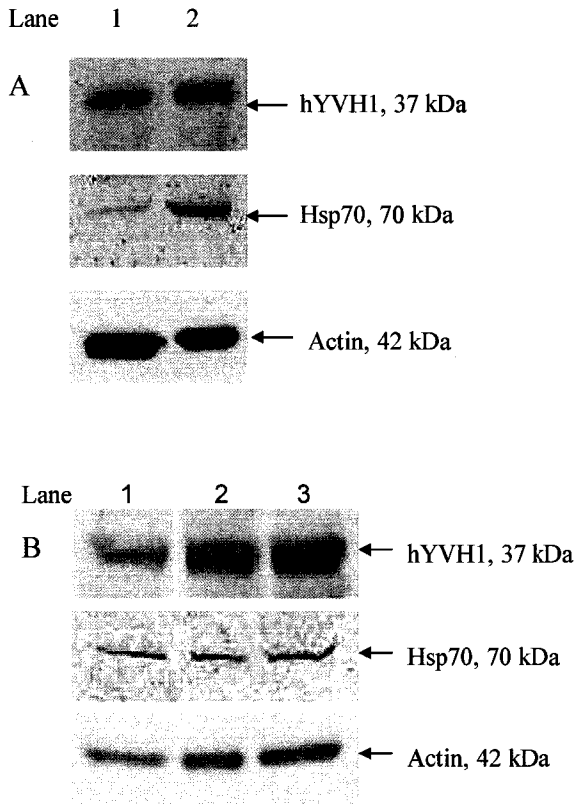


Figure 14 – Western blot analysis detecting expression level of hYVH1 during heat shock stimulus.

Endogenous expression of hYVH1, Hsp70, and actin were examined in HeLa cells. *A,* Cells remained untreated (Lane 1) or heat shocked for 2 hours at 42°C and recovered at 37°C for 10 hours (Lane 2). *B,* Cells either remained untreated (Lane 1), or were heat shocked for 1 hour at 45°C and allowed to recover at 37°C for 12 hours (Lane 2) or 24 hours (Lane 3).

3.5 Effect of Overexpression of hYVH1 on Cell Viability

In general, environmental stress can initiate various internal cellular responses. For instance, pro-apoptotic proteins can become activated to allow for controlled cell death when a stress becomes overwhelming, cell cycle arrest may occur to conserve cellular energy, and anti-apoptotic proteins, like Hsp70, can be activated as an attempt to protect cells. Therefore, the implication that hYVH1 participates in a stress related mechanism could imply a number of different functions. Interestingly, it was noticed that following transfection with recombinant full length hYVH1, cells that appeared apoptotic by Hoechst stain almost always remained negatively transfected (Fig. 7B). This led to the hypothesis that hYVH1 may participate as an anti-apoptotic protein with a positive effect on cell viability.

To test this hypothesis, HeLa cells were transfected with full length recombinant hYVH1 in the presence and absence of Hsp70, while control plates were normalized via transfection of pCMV.2 empty vector DNA. HeLa cells were treated to a heat shock stimulus for 1 hour at 43°C and allowed to recover at 37°C for 14 hours. Following recovery, cells were stained with FITC conjugated Annexin V and Hoechst 33342 dye to measure cell viability. Annexin V detects apoptotic cells by binding to phosphatidylserine that upon apoptosis flips from the inner leaflet of the plasma membrane to the outer plasma membrane surface. Hoechst 33342 dye is a nuclear stain that binds to DNA and can illustrate the nuclear blebbing phenotype associated with apoptosis. Consequently, upon heat shock an approximate 20% induction of apoptosis occurred in HeLa cells. Conversely, heat shocked cells transfected with either recombinant hYVH1 or hYVH1 accompanied by Hsp70 only implicated a 5% and 3%

increase in apoptotic cell death over control, respectively (Fig.15A, 15C). The percentages described above are the result of the number of dead cells, indicated by FITC conjugated Annexin V, divided by total cell number determined by Hoechst 33342 and is the average findings of three unique trials. Moreover, when the experiment was performed on coverslips, where lifted cells including those that underwent apoptosis were removed, a clear difference in cell number was observed. HeLa cells transfected with pCMV.2 empty vector DNA accompanied with a heat shock stress had much fewer cells present on its coverslip compared to those transfected with recombinant hYVH1 alone and hYVH1 with recombinant Hsp70 (Fig. 15B). Similarily, through the qualitative analysis of HeLa cells stained for DNA fragmentation using TUNEL assay, a reduced amount of apoptosis in response to hYVH1 overexpression was evident (Fig.16). Overall, these findings suggest the novel prospect that overexpression of hYVH1 alone can participate in the protection of cells from apoptotic death. This function would be consistent with previous studies suggesting the yeast orthologue functions in cell growth and is upregulated in response to cell stress [36-38].

3.6 Consequence of hYVH1 Overexpression on Phosphorylated ERK Levels

In response to heat shock, not only do the protein expression of hYVH1 and Hsp70 increase, but levels of phosphorylated ERK and JNK become elevated [81,92]. Interestingly, Hsp70 has been shown to prevent the activation of both ERK and JNK in response to heat shock [92, 96]. Considering hYVH1 is a DSP that has a strong affinity for Hsp70, a possibility that this phosphatase could have an affect on the MAPKs is fairly reasonable. In an attempt to examine the effect of hYVH1 overexpression on phosphorylated ERK (pERK), HeLa cells were fractionated and cytoplasmic and nuclear

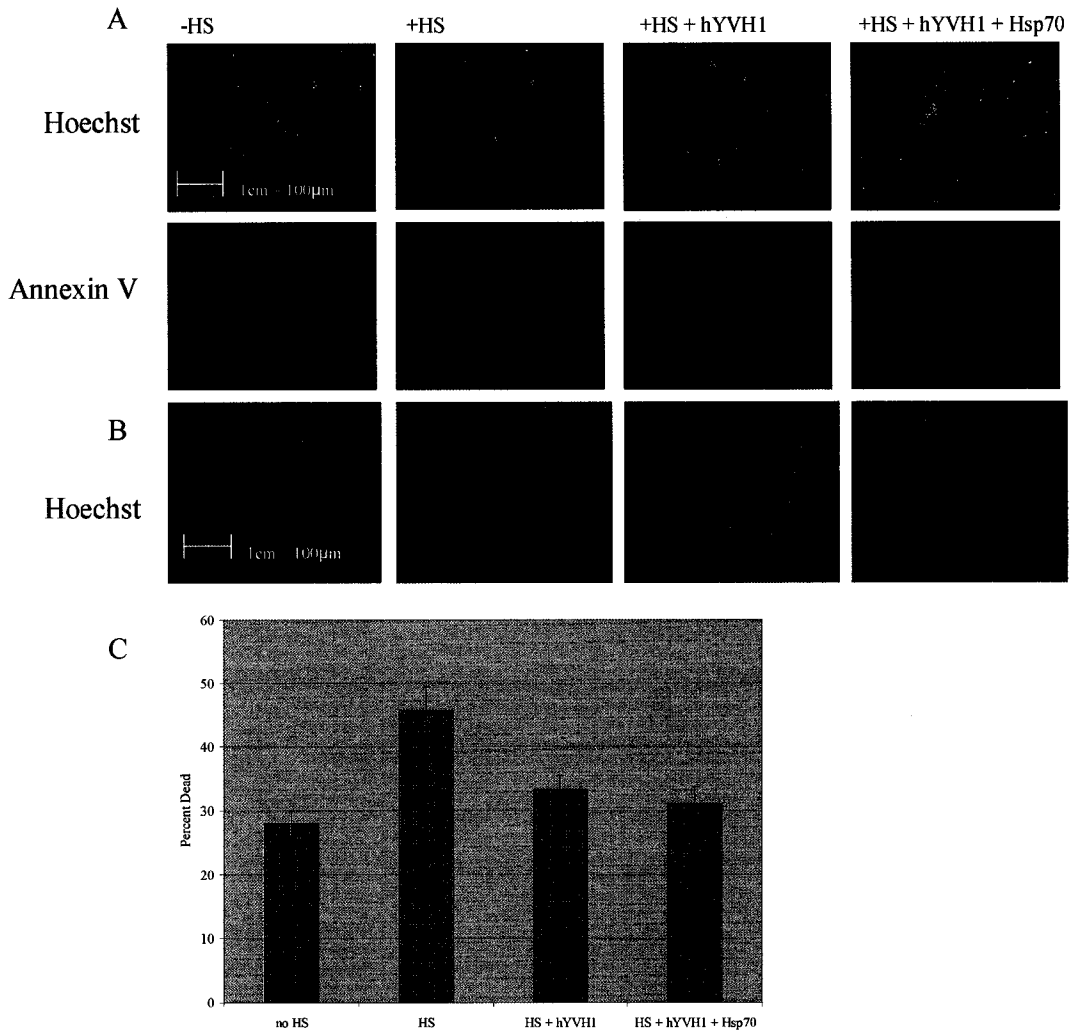


Figure 15 – Cell Viability determined using FITC conjugated Annexin V

Following 24 hours after transfection with either empty vector pCMV.2 DNA, recombinant full length hYVH1 or recombinant full length Hsp70 in conjunction with hYVH1 over expression, HeLa cells were left untreated *(-HS)* or heat shocked for 1 hour at 43°C and allowed to recover at 37°C for 14 hours *(+HS)*. *A,* Cell viability was detected using FITC conjugated annexin V (green) as per manufacturers protocol and total cell number was determined using Hoechst 33342 dye (grey). *B,* Total cell number was visually compared on slides stained with Hoechst 33342 dye (grey). *C,* Graphical representation expressing cell death as a percent of total cell number determined using FITC conjugated Annexin V and Hoechst 33342 nuclear stain.

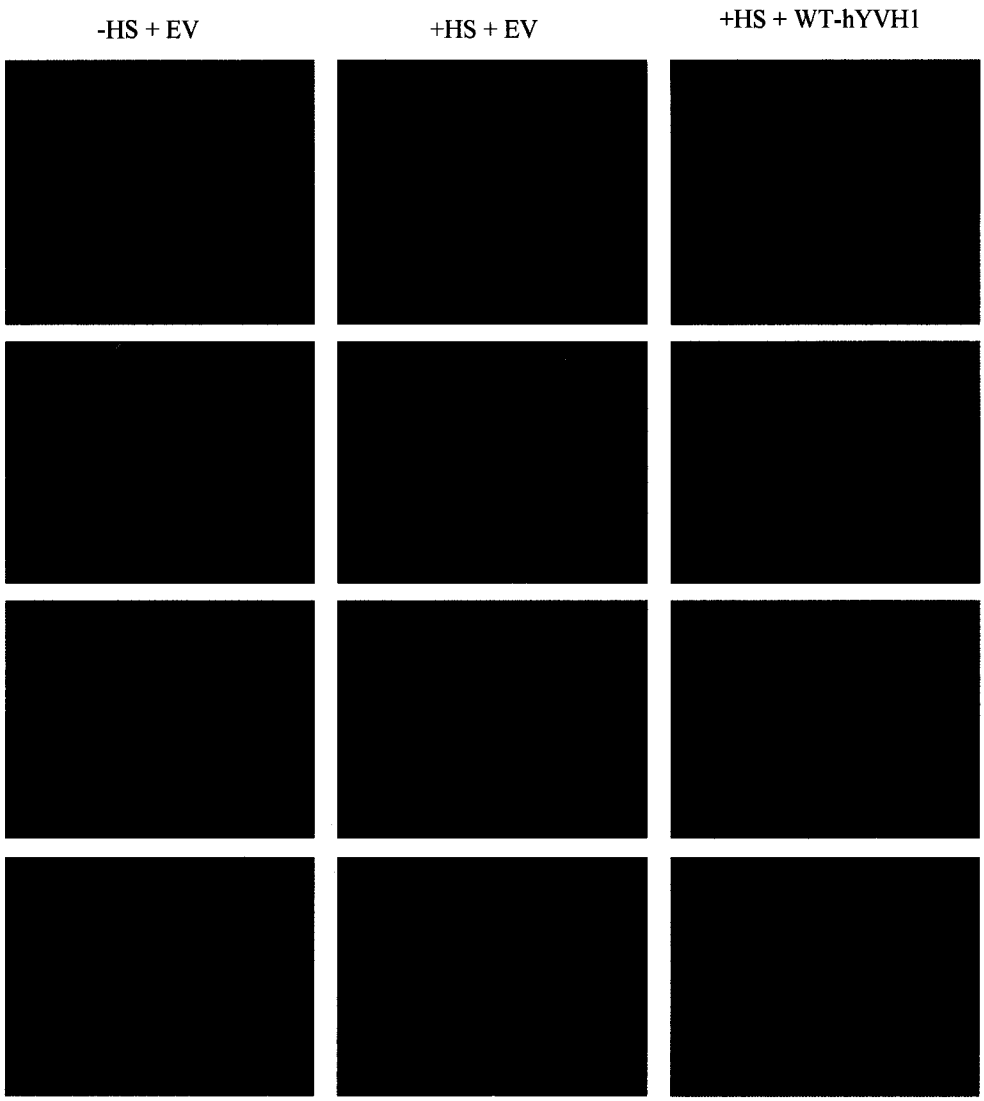


Figure 16 – Cell Viability determined using TUNEL assay

HeLa cells transfected with empty vector pCMV.2 (EV) or recombinant hYVH1 (WT-hYVH1) were subjected to a 2 hour heat shock at 45.5°C and recovered for 3 hours at 37°C. Cells were stained to indicate DNA fragmentation (green).

samples were collected. It appears the overexpression of hYVH1 in both cytoplasmic and nuclear fractions caused the dephosphorylation of pERK (Fig.17).

To further verify this trend, whole cell lysates from HeLa cells subjected to heat shock were examined. HeLa cells were incubated for 1 hour at 43°C and allowed to recover at 37°C for 3 hours before being lysed. The heat shock was successful in boosting Hsp70 levels and cells overexpressing hYVH1 under the heat shock stress demonstrated a notable decrease of pERK levels compared to those that were transfected with empty vector DNA (Fig.18). Therefore, it generally appears that hYVH1 is able to cause a significant, but not complete dephosphorylation of pERK. Alternatively, HeLa cells were examined after a heat shock of 1 hour at 43°C at the 0 hr and 6 hr recovery time points. Immediately following the heat shock stress, allowing for no recovery before cell lysis, the pERK levels demonstrated a supposed dephosphorylation in the overexpressed hYVH1 sample compared to cells transfected with pCMV.2 empty vector DNA (Fig.19). Alternately, after a 6 hour recovery period at 37°C, an increase in pERK levels was seen in cells transiently transfected with hYVH1 versus those transfected with pCMV.2 empty vector DNA (Fig.19). While these results seem contradictory, one possible explanation that provides some resolution is to note the trend in pERK levels in cells transfected with empty vector DNA versus hYVH1. More specifically, immediately following heat shock HeLa cells transfected with pCMV.2 vector DNA demonstrated increased pERK levels, but with time, these levels reduced to those of normal resting conditions (Fig.19). However, cells overexpressing hYVH1 deviated from this trend and instead demonstrate a prolonged increase in pERK levels and despite a 6 hour recovery period, pERK levels remained as high as immediately following heat shock (Fig.19).

Therefore, it is possible that instead of hYVH1 acting to dephosphorylate ERK, it may work on an upstream regulator of ERK to cause its prolonged activation.

3.7 Consequence of hYVH1 Overexpression on Phosphorylated JNK Levels

In addition to ERK, the phosphorylation and activation of JNK is also regulated by heat shock and Hsp70. The activated forms of ERK and JNK trigger a number of events, but most notably, phosphorylated JNK is a strong participant within apoptosis [92]. Therefore the possibility that hYVH1 can interact with Hsp70 to deactivate pJNK could result in the anti-apoptotic phenotype described earlier (Fig.15 and Fig.16). In the previous experiments examining pERK levels, the pJNK levels were also checked, however due to inadequacies of the pJNK antibody a signal was rarely detected. In an attempt to concentrate samples, a scaled-up fractionation experiment was conducted using HeLa cells. Only cytoplasmic fractions were examined owing to the fact that low concentration of nuclear samples that would remain undetected by the pJNK antibody. It was determined that cytoplasmic pJNK levels exhibited an increase in heat shocked cells overexpressing hYVH1 (Fig.20). One explanation can be that in the presence of hYVH1, pJNK gets sequestered in the cytoplasm. An increase in cytoplasmic pJNK, indicates less pJNK enters the nucleus to activate its pro-apoptotic substrates. Hence, the anti-apoptotic phenotype seen in HeLa cells overexpressed with hYVH1 may be due to the ability of hYVH1 to inhibit pJNK entry into the nucleus.

As mentioned earlier, examining hYVH1's affect on JNK using phospho-antibodies proved difficult. Therefore, to investigate whether hYVH1 could complex with JNK as an indication of its involvement in the JNK pathway, immunoprecipitation

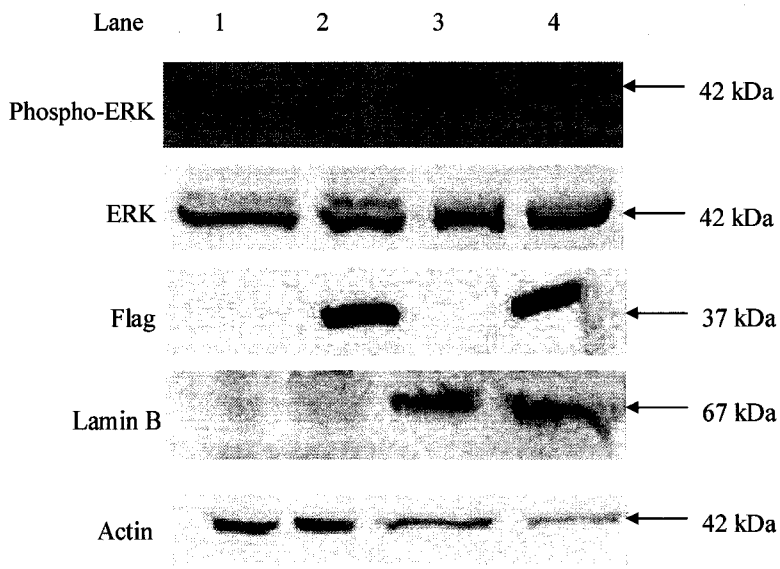


Figure 17 – Overexpression of hYVH1 related to pERK Levels in Fractionated HeLa Cells

HeLa cells either remaining untransfected or overexpressed with recombinant hYVH1 were fractionated. *A,* Samples of untransfected cytoplasmic fraction (Lane 1), overexpressed hYVH1 cytoplasmic fraction (Lane 2), untransfected nuclear fraction (Lane 3), and overexpressed hYVH1 nuclear fraction (Lane 4) were probed with pERK antibody to investigate the relationship between hYVH1 and pERK. Equal loading was determined using an anti-ERK antibody, while anti-lamin B and anti-actin acted as nuclear and cytoplasmic fractionation controls, respectively. Monoclonal anti-flag antibody was used to detect hYVH1 expression.

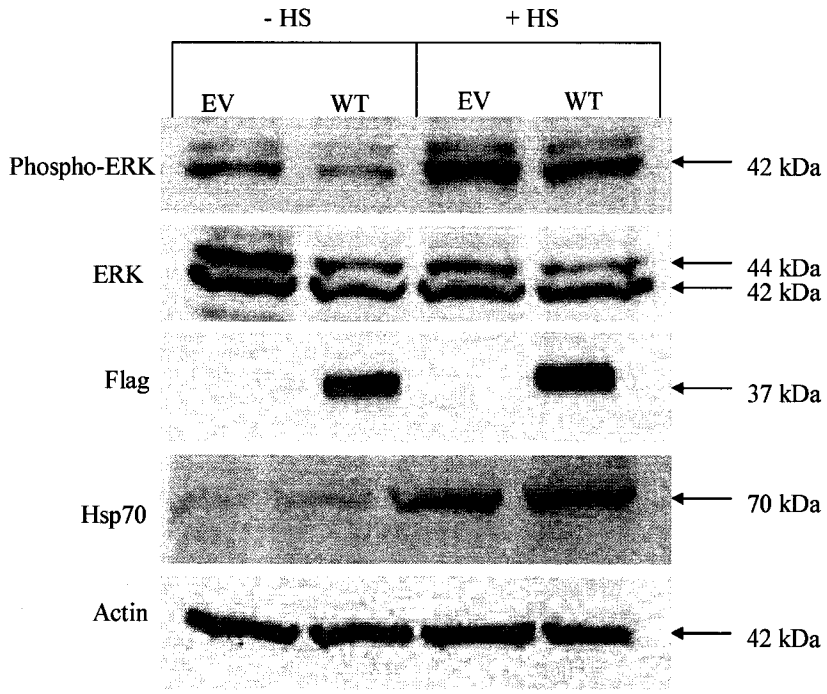


Figure 18 – Overexpression of hYVH1 related to pERK Levels in Whole Cells Lysates from HeLa Cells

HeLa cells were either left untreated (-HS) or subjected to 1 hour heat shock stimulus at 43°C and allowed to recover at 37°C for 3 hours (+HS) prior to lysing. *A,* Cell lysates from HeLa cells transfected with either empty vector pCMV.2 DNA (EV) or recominant wildtype hYVH1 (WT) were probed with pERK antibody. The overexpression of hYVH1 was verified using a flag antibody, while heat shock was deemed successful via anti-Hsp70 antibody. Anti-ERK and anti-actin both acted as loading controls.

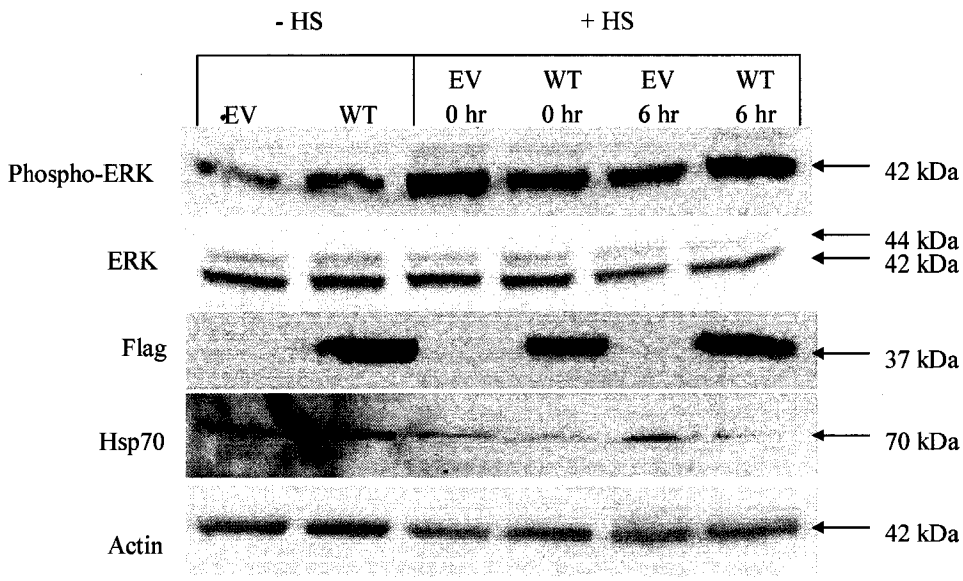


Figure 19 – Overexpression of hYVH1 related to pERK Levels following Heat Shock Stimulus

HeLa cells were either left untreated (-HS) or subjected to 1 hour heat shock stimulus at 43°C and allowed to recover at 37°C for 0 hours or 6 hours (+HS) prior to lysing. *A,* Cell lysates from HeLa cells transfected with either empty vector pCMV.2 DNA (EV) or recominant wildtype hYVH1 (WT) were probed with pERK antibody. The overexpression of hYVH1 was verified using a flag antibody, while degree of heat shock was measured via anti-Hsp70 antibody. Anti-ERK and anti-actin both acted as loading controls.

was employed. HeLa cells transfected with pCMV.2 empty vector DNA, recombinant full length hYVH1, or recombinant his-tagged Hsp70 were lysed and endogenous JNK was immunoprecipitated from these lysates using a polyclonal anti-JNK antibody. Interestingly, it was found that hYVH1 was in fact bound to JNK under both unstressed and heat shock conditions (Fig.21). Notably, it appears more hYVH1 was pulled down in HeLa cells that were lysed immediately succeeding 1 hour incubation at 43°C versus untreated cells. Overall, the determination of the co-immunoprecipitation of hYVH1 with JNK is an exciting prospect that with further investigation can lead to the deciphering of a significant role of hYVH1 in the MAPK signaling pathway.

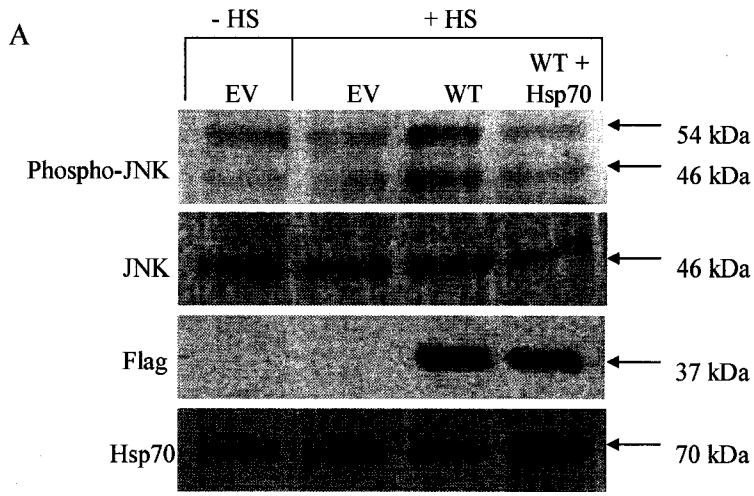


Figure 20 – Overexpression of hYVH1 related to pJNK Levels in Fractionated HeLa Cells

HeLa cells were heat shocked for 1 hour at 43°C before being fractionated. *A,* Cytoplasmic samples of HeLa cells transfected with either pCMV.2 (EV), hYVH1 (WT), or Hsp70 were probed with anti-pJNK to check for variation in pJNK levels. Antibody against JNK acted as a loading control and anti-flag and anti-Hsp70 were used in the detection of transfection efficiency.

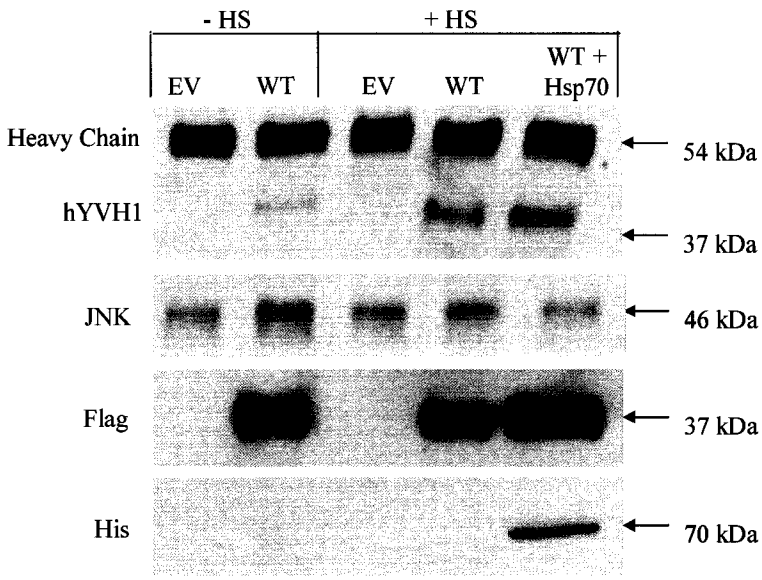


Figure 21 – Potential Interaction Between JNK and hYVH1

HeLa cells transfected with either pCMV.2 DNA (EV), recombinant hYVH1 (WT), or His-tagged Hsp70 (Hsp70) were lysed following 1 hour incubation at 43°C. Transfection efficiency was verified of hYVH1 and Hsp70 were verified in lysates using anti-flag and anti-his antibodies respectively. Lysates were then incubated with resin and anti-JNK antibody and JNK and hYVH1 levels were probed within the immunoprecipitated samples.

CHAPTER 4

Discussion

Identified in 1999, little is still known about the dual-specificity protein tyrosine phosphatase, hYVH1 [40]. The yeast orthologue of this phosphatase has been shown to play a role in spore maturation, glycogen accumulation, and vegetative growth, while the mouse orthologue has been implicated within the cell cycle [38, 39, 42]. However, despite its high conservation in amino acid sequence throughout evolution, the human YVH1 orthologue remains scarcely studied [40]. To date, only a single paper has been published that actively sought to characterize aspects of this phosphatase [40]. Muda *et al.* has confirmed the high conservation between hYVH1 and its yeast orthologue by demonstrating hYVH1 could suppress the characteristic phenotypes caused in yeast cells expressing YVH1 disruptant mutant [40]. Additionally, hYVH1 was established to localize in both the cytoplasmic and nuclear regions of the cell as well as shown to be nearly ubiquitously expressed in human tissues [40]. The hYVH1 phosphatase is mapped to the chromosomal region of 1q21-1q23, which has been shown to be amplified in various human sarcomas and ependymomas [46, 47].

This study attempts to continue in the characterization of the hYVH1 phosphatase by examining important motifs and domains that contribute to the subcellular localization of hYVH1 phosphatase. Also, recently our lab has discovered Hsp70 as a novel interacting partner for hYVH1. Therefore, this study sought to gain insights into the functional purpose of this interaction.

4.1 Subcellular Localization of hYVH1

Through the use of indirect immunofluorescence, the cytoplasmic/nuclear ratio of hYVH1's subcellular localization was determined. In agreement with the results published by Muda *et al.,* it was found that recombinant full length hYVH1 localized to both the cytoplasm and nucleus. However, where the Muda study suggests that the localization of hYVH1 was predominately nuclear, our results differ in that they suggest a more predominant cytoplasmic localization with particular focus in the perinuclear region. The reason behind this difference may be attributed to the use of a GFP-tagged recombinant hYVH1 construct and non-specific binding of secondary antibody in the Muda study to give a false nuclear localization. In addition to full length hYVH1, the subcellular localization of recombinant variants of hYVH1 were also examined. The ZnΔhYVH1 recombinant protein represents the catalytic domain/zinc binding domain deletion of the hYVH1 phosphatase. The distribution of ZnΔhYVH1 was found diffused throughout the cells and did not retain any of the perinuclear localization that is strongly visible with full length hYVH1. Due to the indiscriminate dispersion of the ZnΔhYVH1 construct throughout the cell, a cytoplasmic/nuclear ratio could not be determined. However, catΔhYVH1, which is the recombinant form of only the zinc binding domain of hYVH1, demonstrated a heavily favoured nuclear localization. Interestingly, this localization was not exclusively nuclear, but indeed showed a combined nuclear/cytoplasmic localization. In contrast, the partial zinc deletion recombinant forms of hYVH1, CT-2 and CT-3, demonstrated near exclusive nuclear localization with much less cytoplasmic localization as compared to catΔhYVH1.

Collectively, these results suggest a role of the zinc binding domain in the mediation of hYVH1 nuclear localization as well as the importance of both the catalytic domain and zinc binding domain in regard to cytoplasmic and perinuclear localization. It was found that the catalytic domain localized to both the cytoplasm and nucleus and by attaching on the first coordination site of the zinc finger and a highly conserved hydrophobic motif the subcellular localization of hYVH1 becomes strongly nuclear. Recombinant hYVH1 devoid of its catalytic domain also retains a strong nuclear localization, however some cytoplasmic localization is retained that was absent with the incomplete zinc finger constructs. This leads us to believe that both catalytic and zinc binding domains are necessary for localizing hYVH1 in the cytoplasm. One possible putative model of hYVH1 structure that supports these localization conclusions is the potential interaction between opposite ends of the hYVH1 protein. The hypothesized interaction of the N-terminal catalytic domain with the C-terminal zinc binding domain may support the proper conformation of hYVH1, which in turn allows it to interact with another protein that sequesters it in the cytoplasm. In order to test this hypothesis it would be necessary to construct a recombinant chimera protein from the catalytic domain and the second zinc coordinating site of hYVH1. It is assumed that the opposite ends of the hYVH1 protein will be able to interact and retain the normal localization pattern of full length hYVH1. Additional confirmation is currently underway in the form of structural identification of full-length hVYH1 through x-ray crystallography. Identifying the 3-dimensional structure of this phosphatase would confirm whether the opposite ends of hYVH1 do indeed interact. Agreeable to this hypothesis is the recent discovery in our lab that demonstrated through the use of gel filtration that the hYVH1 protein eluted at a

molecular weight corresponding to 30 kDa, rather than the expected 38 kDa. This is indicative of a compact structure of hYVH1, which supports the theory that the two opposite ends of the protein may interact with one another.

A second possibility rationalizing the subcellular localization results would be the presence of a NLS within the first zinc coordinating site of hYVH1. Through the examination of the hYVH1 sequence the following motif has been identified as a putative NLS, ^{219}KCRKCRR^{225}, due to its abundance in basic amino acid residues. An interesting feature of this sequence is the fact that the two cysteines embedded in the putative NLS motif also compose part of the first zinc finger. This may allow hYVH1 to adopt different conformations, regulated by zinc binding that can expose or hide a NLS motif. Therefore, it is possible that the recombinant zinc finger construct and the partial zinc finger deletion constructs change conformation in a manner that does not allow the coordination of a zinc molecule to the first zinc finger coordination site, which leads to the exposure of the putative NLS and causes the heavy nuclear localization. Our lab has recently shown that in response to oxidative stress one of the two zinc ions is ejected (Bonham, unpublished). This suggests that the zinc ejection is a plausible mechanism regulating hYVH1 conformations. Additionally, the protein SOD1 has also shown to change its conformation in response to oxidative stress accompanied by a loss of its coordinating zinc further providing evidence for this hypothesis [97].

On the other hand, the localization of ZnΔhYVH1 appears diffused to all parts of the cell, which can be reasoned by the loss of the binding domain that controls hYVH1 localization. This second hypothesis puts emphasis on the zinc binding domain as the key factor that controls hYVH1 subcellular localization. Again, one method of conformation

of this hypothesis can transpire through x-ray crystallography of the recombinant forms of hYVH1 and currently Dr. Brian Crane of Cornell University is solving the crystal structure of both full length hYVH1 and the catΔhYVH1.

4.2 The Putative Nuclear Export Sequence

The process of nucleocytoplasmic transport is central in allowing proteins to target their downstream nuclear substrates. The control of nuclear/cytoplasmic shuttling is often dictated by the presence of NLS and NES motifs. Therefore, in addition to the putative NLS sequence described above, a potential NES motif is also present in hYVH1. The following sequence, ^{193}LQNLPQELFA^{202}, situated between the end of the catalytic domain and the beginning of the zinc binding domain, fit the characteristics of an NES motif [62, 63]. In order to construct NES mutants, recombinant flag-tagged hYVH1 was mutated at ^{193}Leu and ^{196}Leu to two non-hydrophobic Ser residues using site-directed mutagenesis. As a result, the localization of hYVH1 appeared increasingly nuclear, however, significant cytoplasmic localization was still retained. It is plausible that the cytoplasmic localization present with overexpression of hYVH1 NES mutant is a result of the protein not entering the nucleus. Notably, the constitutive localization of wildtype hYVH1 is split between the nucleus and cytoplasm, therefore if the hYVH1 NES mutant does not initially enter the nucleus, its mutation has no effect on the nuclear export mechanism. To further this claim, a fusion protein between the putative NES and the NES mutants with the NLS of another protein, like the SV40 T-antigen, could be created [98]. Upon transfection the localization of the mutant fusion proteins would be expected to be strongly nuclear, while the intact NES should demonstrate cytoplasmic localization. Also, in an attempt to create a more severe NES mutant, Ser residues should replace

Leu^{200} and Ala^{202} because often the last two hydrophobic amino acid residues are key for NES function [99]. The successful completion of these additional approaches will strongly confirm our current data suggesting that hYVH1 possesses a NES motif that regulates the temporal localization of hYVH1 in the nucleus.

4.3 The Effect of Heat Shock on hYVH1

The functional data regarding hYVH1 remains scarce, however, studies on the yeast orthologue, YVH1, demonstrate that in response to nitrogen starvation or cold shock, YVH1 mRNA expression was significantly induced [36, 37]. Therefore, regarding the recent identification of Hsp70 as an interacting partner of hYVH1, it was reasonable to assume that hYVH1 may respond to a heat shock stimulus. HeLa cells were treated to a mild and severe heat shock stress and subcellular localization and protein expression levels of hYVH1 were analyzed.

Protein expression levels of hYVH1 in response to both mild and extreme heat shock conditions following a recovery period of 10-24 hours was increased. Previous studies demonstrated an induction of mRNA hYVH1 levels in response to stress [36, 37], however this is the first time an alteration in hYVH1 protein expression was demonstrated as a result of a stress stimulus. The increase in hYVH1 protein expression appears to occur later in recovery. Earlier time points were examined and recovery periods of up to 8 hours show no induction of hYVH1. This suggests that the function of hYVH1 is involved in a later event of the cell's stress response system. For instance, hYVH1 protein induction may respond to changes in the protein expression of an upstream or downstream regulator and therefore it must wait for the induction of another protein to signal a change in its own expression levels. Interestingly, HeLa cells are a

cancer cell line that demonstrates rapid growth, high resistance to stress, and altered protein expression levels, therefore the late effect of hYVH1 induction may be due to the cell line and therefore, other cell types should be further considered.

In regard to subcellular localization, under normal resting conditions endogenous hYVH1 and Hsp70 demonstrate a tight colocalization. This is the first evidence suggesting an interaction between hYVH1 and Hsp70 within a cellular context. Upon mild and extreme heat shock, both proteins displayed an increased enrichment in the perinuclear region of the cell, while in response to mild heat shock with a lengthy recovery period, only Hsp70 increased its nuclear localization. The alteration of the subcellular localization of hYVH1 in response to heat shock is extremely interesting. It implies that under stress hYVH1 has a purpose to congregate at the perinuclear region of the cell. Some functions associated with proteins that localize in the perinuclear region include membrane trafficking and cell signaling [99].

Previous studies have also shown an enhanced perinuclear localization of Hsp70, however a purpose has yet to be elucidated [95]. Notably, there is a study regarding the yeast orthologue of an Hsp70 co-chaperone, YDJ1, where the authors describe its localization as a "perinuclear ring" accompanied with cytoplasmic distribution [99]. This is on par with the hYVH1 localization detected in HeLa cells. Another interesting feature of YDJ1 is that it contains a cysteine rich motif similar to a zinc finger protein and it has been demonstrated that the *E.coli* orthologue of YDJ1, dnaJ, does in fact bind to Zn^{2+} [100]. Additionally, disruption of the YDJ1 gene results in a slow growth phenotype, which is also a characteristic of YVH1 disruption mutant [38, 100]. The similarities between the two proteins are striking and furthermore it was established that the

perinuclear localization of YDJ1 is most likely due to association with the endoplasmic reticulum (ER) [100]. In an attempt to analyze whether hYVH1 colocalized with the ER, immunofluorescence was conducted using ER-Tracker (Molecular Probes). Unfortunately, upon permeabilization of the cell to allow the binding of primary serum hYVH1 antibody, the ER-Tracker continuously leaked into the nucleus and lost all cytoplasmic localization. Interestingly, a number of functions can be assigned to ER localization including protein assembly, membrane trafficking, and cell signaling [100, 101].

Briefly, in a second study regarding another zinc finger containing dual-specificity phosphatase, FYVE-DSP-2, perinuclear localization was also revealed [101]. FYVE-DSP-2 was established to bind to phosphotidylinositol 3-phosphate (PI(3)P) located in cell membranes and furthermore reference a number of other zinc finger containing proteins that bind to PI(3)P [101]. The proteins that interact with PI(3)P on cell membranes have been shown to be involved in cellular functions including protein trafficking and cell signaling [101]. It is very interesting to consider the possibility that upon heat shock stress, hYVH1 accumulates in the perinuclear region of the cell, binding to the nuclear or ER membrane through the interaction of the zinc finger with PI(3)P and participate in a stress related mechanism. However, further research in the form of subcellular fractionation and lipid overlay assays needs to be conducted in order to validate this hypothesis.

4.4 The Involvement of hYVH1 in Apoptosis

The cellular stress response includes a number of events to cope and re-establish cell homeostasis. A protein implicated in a stress response role can participate in a

number of events, which include the sensing of DNA damage and other changes in cell homeostasis, cell cycle regulation, cellular repair mechanisms, and control of cell metabolism [102]. If a stress becomes overwhelming, a cell can respond through apoptosis, which refers to the mechanism of controlled cell suicide. The advantage of such a mechanism lies in the control feature, apoptosis can cause cell death within individual cells without disturbing the surrounding environment and causing adverse distress in the remaining cell population. Therefore, the perinuclear localization and presence of two zinc fingers, which is indicative of protein trafficking and cell signaling, could imply a plethora of roles for hYVH1 within the stress response mechanism.

Early evidence in the form of the absence of overexpression of hYVH1 in cells that appeared apoptotic via Hoechst stain, led to the belief that hYVH1 may participate in an anti-apoptotic role. Further evidence was provided through the lack of binding of FITC conjugated annexin V, a known binding partner of phosphatidylserine, in unlysed cells overexpressing hYVH1 under heat shock conditions. Upon apoptosis phosphatidylserine relocates to the outer surface of the cell membrane, therefore the binding of annexin V of unlysed cells indicates the presence of phosphatidylserine on the cell surface, which is indicative of apoptosis. Directly comparing cells transfected with empty vector pCMV.2 DNA with cells overexpressing hYVH1 following a heat shock stimulus, a significant decrease in the number of apoptotic cells in hYVH1 overexpressed cells was present. The extent of protection as a result of hYVH1 overexpression was near that of cells transfected with empty vector DNA, but did not undergo heat shock treatment. This result was further verified through the examination of a second apoptotic marker, DNA fragmentation. Apoptotic signaling can activate nucleases, which are

responsible for the nicking of DNA ends and causing DNA fragmentation. Qualitative analysis using TUNEL assay, which detects the DNA fragmentation phenotype of apoptosis, also shows a reduced amount of staining in response to hYVH1 overexprssion.

Additionally, the effect of dual overexpression of hYVH1 and Hsp70 regarding apoptosis measured by annexin V was also examined. It was found that the protection provided by the doubly transfected cells was near that of cells overexpressing in hYVH1 exclusively. According to these results it would appear that Hsp70 had minimal effect on cell survival, despite the fact it is well-known as an anti-apoptotic protein [68]. This anomaly can be reasoned in a number of ways. Firstly, low transfection efficiency of Hsp70 could render its affect as too mild to be detected. Another possibility is an inadequate heat shock could result in minimal apoptosis, which is seemingly reduced by overexpression of hYVH1 and its interaction with endogenous Hsp70. A third possibility is the regulatory mechanism or substrate hYVH1 and Hsp70 are involved with become saturated out and therefore overexpression of either proteins beyond a certain level would have no affect on cell viability.

Notably, different heat shock and recovery time periods were tested, however, at best only a 20% increase in apoptotic death was observed. It is believed that the lack of cell death can be attributed to the resilience of the cancer cell line used in this experiment. Therefore, the effect of hYVH1 overexpression related to apoptosis should be characterized in different cell types. In an attempt at testing this hypothesis NIH 3T3 cells (mouse embryonic fibroblasts) were transfected with hYVH1, however transfection efficiency was deemed poor as determined by immunoblotting. This addresses a second factor of concern with this experimental system. The use of transient transfection results

in a variable number of cells expressing the gene of interest. This can be standardized using flow cytometry and transfecting cells with GFP-tagged hYVH1. This would allow cells that stained positive for annexin V and did not overexpress hYVH1 to be eliminated from the data set and therefore a more pronounced relationship between hYVH1 and apoptosis could be established. An alternative solution would also be the use of a stable cell line overexpressing hYVH1. This provides 100% transfection efficiency and again eliminates the effect of untransfected cells in response to heat shock. However, despite the limitations of the system used, a clear reduction of heat shock induced apoptosis was demonstrated upon overexpression of hYVH1. In agreement with our findings is the large-scale RNAi screening study conducted by the Blenis group at Harvard [102]. In their study, they systematically knocked down and screened all kinase and phosphatase proteins in HeLa cells [103]. It was discovered that hYVH1 demonstrated one of the highest apoptosis scores and when HeLa cells were transfected with siRNA hYVH1 and then measured for the pro-apoptotic events of PARP and caspase-9 cleavage, both events were found to occur in cells expressing the knockdown of hYVH1.

It is tempting to consider that the anti-apoptotic effect of hYVH1 could result in the cancerous phenotype present in liposarcomas resulting from gene amplification of the chromosomal region 1q21-1q23 and subsequent overexpression of hYVH1 [46]. Furthermore, the involvement of Hsp70 in apoptosis presents a promising theory that the interaction between Hsp70 and hYVH1 are together involved in an anti-apoptotic function. As discussed earlier, Hsp70 has been shown to be involved with apoptosis in a number of situations [68-78], as well, its anti-apoptotic effect has demonstrated to be crucial in the survival of many human cancer cells of diverse origins [79, 104]. To

further validate the importance of the interaction between hYVH1 and Hsp70, our lab has determined which domains of the two proteins interact with one another (Mucaki, unpublished). Interestingly, it was established that hYVH1 did not interact with the C-terminal domain of Hsp70, which is associated with its chaperone activity, but instead with the N-terminal ATPase domain. This suggests that the interaction between hYVH1 and Hsp70 has a functional purpose other than maintaining proper protein folding. Considerable research needs to be conducted in order to establish the mechanism in which hYVH1 and Hsp70 function together in an anti-apoptotic manner.

4.5 Can hYVH1 act as a MAPK Phosphatase?

In an attempt to begin to elucidate the mechanism of the anti-apoptotic role of hYVH1, a closer examination of the MAPK signaling pathway was conducted. The choice was based on the fact that 13 dual specificity phosphatases have been previously characterized as MKPs [34], in addition to the involvement of Hsp70 within the signaling cascade in an anti-apoptotic context. In response to heat shock two main pathways of the MAPK signaling cascade become activated; the first is the cell death inducing JNK pathway and the second is the cell survival ERK pathway.

The effect of overexpression of hYVH1 on the activated ERK substrate, pERK, was investigated using phospho-antibodies and immunoblotting techniques. Initially, it appears that hYVH1 may lead to the dephosphorylation of pERK and consequently the inactivation of the ERK pathway. However, as the results of figure 19 illustrate, when comparing pERK expression levels in cells transfected with pCMV.2 empty vector DNA and recombinant hYVH1 in a temporal sense, the opposite conclusion is suggested. These results indicate that instead of hYVH1 causing the dephosphorylation of pERK, it

is actually prolonging the activation of pERK. The extended activation of pERK is in agreement with the apoptosis data suggesting that hYVH1 participates in a function that increases cell viability. The supposed prolonged activation of pERK subsequently lengthens the activation of its anti-apoptotic effects, which range from the phosphorylation and inactivation of the proapoptotic Bcl-2 family member Bad to its unknown anti-apoptotic roles downstream of cyochrome c or upstream of caspase 8 [96].

Another possibility to explain the initial dephosphorylation of pERK followed by its phosphorylation can be explained through a sequestration effect of hYVH1. Initially following heat shock, hYVH1 could actually bind and sequester pERK, protecting it from dephosphorylation until the cell restores normal homeostasis. After homeostasis is re-established, hYVH1 may release pERK, so it may return to its most well known function, which is the induction of cell proliferation. The collective release of pERK would appear as an increase in phosphorylation as detected by pERK specific antibodies. Also, the binding of hYVH1 to pERK could inhibit the phosphorylation of pERK by the upstream kinases, thereby indicating a false dephosphorylation at the early time points via western blot.

Lastly, owing to the complex nature of the MAPK signaling pathway, activated pERK has also been shown to participate in key roles regarding the cell cycle. Much evidence supports the claim that ERK activation is essential for cell cycle progression from the G1 phase to the S phase through the regulation of various cyclin-dependent kinases [105]. Therefore, the inactivation of pERK through its dephosphorylation can also result in cell cycle arrest, the temporary stop in cell cycle progression and cell proliferation would allow a population of cells to cope with a stress stimulus (ie. heat

shock) instead of initiating apoptosis [105]. Therefore, the deactivation of ERK is able to confer some anti-apoptotic function. Interestingly, it would appear that not all activated ERK is involved within the cell cycle progression function and therefore only a limited pool of the total ERK population would operate in this regard. This would be consistent with the partial dephosphorylation of ERK seemingly caused by hYVH1. Also, it would explain the inconsistent pattern of dephosphorylation and phosphorylation of pERK in regard to figure 19. It is possible that the pool of pERK supposedly being dephosphorylated by hYVH1 potentially within the cell cycle context is being masked by the effect of the other population of pERK within other functional circumstances, including cell proliferation. It would be quite interesting if hYVH1 could in some localization manner specifically target activated ERK involved within the cell cycle. Obviously, the fact that both hYVH1 and Hsp70 respond to changes in the cell cycle make this an interesting area of study and currently through immunofluorescence, the endogenous hYVH1 localization at different stages of the cell cycle is being investigated.

However, the possibility that the incomplete dephosphorylation of activated ERK is a result of cross-talk between the different MAPK signaling branches cannot be ruled out. Cross-talk is a result of many overlapping proteins that function in multiple branches of the MAPK pathway, therefore a protein that has a strong effect on the JNK pathway may also show minimal effect to the ERK and p38 pathways. Therefore, levels of activated JNK, which also have been shown to respond to heat shock stress, were evaluated through phospho-antibodies and immunoblotting. Unfortunately, the phospho-JNK antibody used to measure activated JNK levels was unable to detect a signal in most cases. Unlike ERK, JNK is stress-induced, therefore while basal levels of ERK and

activated ERK are high enough to be detected by western blot, it appears the same is not true of JNK. In an attempt to boost JNK levels, a scaled-up fractionation experiment was conducted and only cytoplasmic levels were examined. In this case, hYVH1 appears to be responsible for the cytoplasmic retention of pJNK. Previous studies have indicated that upon activation JNK is able to translocate into the nucleus to further activate various transcription factors including c-Jun, c-Fos, and ATF-2, all of which have shown to be involved in the execution of apoptosis through gene regulation [82, 106]. Therefore, sequestering activated JNK in the cytoplasm would prohibit interaction with its nuclear pro-apoptotic substrate, thereby conferring an overall anti-apoptotic effect on the cell. However, activated JNK also triggers apoptosis within the cytoplasm, but it should be noted that dual-specificity phosphatases play an important role in the deactivation of JNK and therefore, the overexpression of hYVH1 may also contribute to the dephosphorylation of activated JNK. The possibility that hYVH1 can directly dephosphorylate pJNK is very tempting due to the fact that Hsp70 has been directly related to the activation of another dual-specificity phosphatases, MKP-1 [106]. The direct interaction between MKP-1 and Hsp70 is able to cause increased phosphorylation of MKP-1, this activated phosphatase was found to be directly responsible for the deactivation of JNK [107].

Therefore, in order to determine whether hYVH1 and JNK could form a complex as well as establish an alternative method to examine the relationship between hYVH1 and JNK due to the poor detection of the phospho-JNK antibody, co-immunoprecipitation was conducted. Endogenous JNK was co-immunoprecipitated in lysates containing overexpressed amounts of hYVH1. It was established that hYVH1 was coupled to JNK,

which is an exciting surprise considering the fact that hYVH1 lacks a MKB domain. There are two possibilities that can reason this result. Firstly, JNK and hYVH1 could bind through an adapter protein or secondly, if determined that hYVH1 directly binds to JNK, it may do so in a manner analogous to VHR, which also lacks an MKB domain. The possibility that hYVH1 could deactivate JNK and prevent its pro-apoptotic effect fits nicely with the data presented earlier. However, much research still needs to be conducted in order to validate this theory. Firstly, to determine whether hYVH1 and JNK directly bind, various deletion mutants should be created and GST-pull-down assays can be conducted. Also, kinase assays can determine the direct effect of hYVH1 on JNK activity in vitro. In order to improve the detection levels of phospho-JNK antibody, recombinant JNK should be overexpressed via transfection. Lastly, JNK is also known to be activated under other stress conditions like UV irradiation, serum starvation, and oxidative stress. The oxidative stress is particularly interesting due to the results of in vitro studies conducted in our lab suggesting that H_2O_2 can alter hYVH1's ability to coordinate zinc (Bonham, unpublished).

Lastly, it should be noted that HeLa cells demonstrate a high endogenous expression of hYVH1. Therefore, the possibility endogenous hYVH1 protein expression could saturate out the substrate, scaffolds, and other regulators of hYVH1 could lead to a minimal effect of the overexpression of hYVH1. In this regard, an attempt at knocking down endogenous hYVH1 via RNAi and then overexpressing the protein may lead to a clearer effect of hYVH1 on the MAPK signaling pathway.

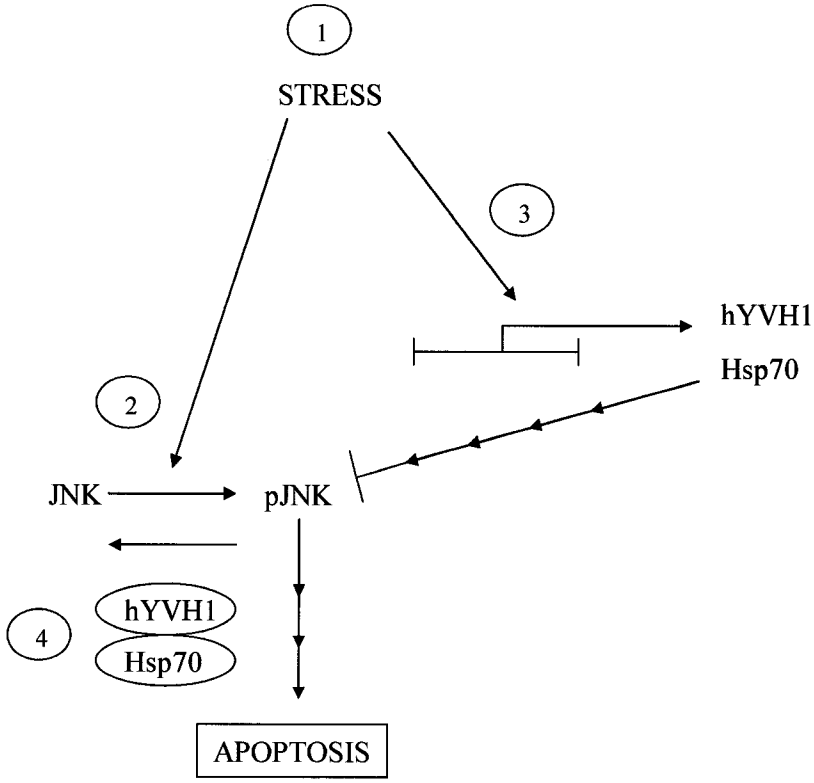


Figure 22 – Schematic of Proposed Anti-Apoptotic Mechanism via hYVH1

(1) An initial external stress (ie. heat shock) causes the (2) activation of JNK through phosphorylation and (3) the increase in protein expression of hYVH1 and Hsp70. (4) It is proposed that hYVH1 and Hsp70 interact together at the perinuclear region of the cell in order to cause the deactivation of JNK, which can lead to the prevention of apoptosis.

4.6 Final Remarks

The YVH1 DSP orthologues represent one of the most evolutionary conserved phosphatase members. Remarkably though, very little is known about its main role in regulating cellular processes. The current study has provided significant insight into YVH1 function and will act as a framework for future studies. Notably, our results point to a role for hYVH1 in cell survival. This function likely entails collaboration with Hsp70 and preliminary efforts suggests the mechanism of action involves targeting aspects of the MAPK signaling cascades.

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