The Atypical Dual Specificity Phosphatase hYVH1 Associates with Multiple Ribonucleoprotein Particles*

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Human YVH1 (hYVH1), also known as dual specificity phosphatase 12 (DUSP12), is a poorly characterized atypical dual specificity phosphatase widely conserved throughout evolution. Recent findings have demonstrated that hYVH1 expression affects cellular DNA content and is a novel cell survival phosphatase preventing both thermal and oxidative stress-induced cell death, whereas studies in yeast have established YVH1 as a novel 60S ribosome biogenesis factor. In this study, we have isolated novel hYVH1-associating proteins from human U2OS osteosarcoma cells using affinity chromatography coupled to mass spectrometry employing ion mobility separation. Numerous ribosomal proteins were identified, confirming the work done in yeast. Furthermore, proteins known to be present on additional RNP particles were identified, including Y box-binding protein 1 (YB-1) and fragile X mental retardation protein, proteins that function in translational repression and stress granule regulation. Follow-up studies demonstrated that hYVH1 co-localizes with YB-1 and fragile X mental retardation protein on stress granules in response to arsenic treatment. Interestingly, hYVH1-positive stress granules were significantly smaller, whereas knocking down hYVH1 expression attenuated stress granule breakdown during recovery from arsenite stress, indicating a possible role for hYVH1 in stress granule disassembly. These results propagate a role for dual specificity phosphatases at RNP particles and suggest that hYVH1 may affect a variety of fundamental cellular processes by regulating messenger ribonucleoprotein (mRNP) dynamics.

The protein tyrosine phosphatase (PTP)* superfamily catalyzes phosphate hydrolysis by way of a thiol phosphate enzyme intermediate. The PTP superfamily can be subdivided into subgroups that include receptor PTPs, intracellular PTPs, phosphoinositol lipid phosphatases, and dual specificity phosphatases (DSPs). The DSPs represent the most diverse group of PTPs. Their name denotes the extended substrate specificity of the group for serine/threonine and tyrosine phosphoresidues (2). Analogous to tyrosine phosphatases, DSPs contain the invariant catalytic sequence C(\(\text{X}^5\)R) and use a thiol phosphate intermediate as a catalytic mechanism (3). The broader, more shallow active site pocket of DSPs compared with tyrosine-specific phosphatases results in stabilization of phosphoserine and phosphothreonine residues in addition to phosphotyrosine (4). Members of the DSP family can be further subdivided into unique subgroups. The best characterized, known as the mitogen-activated protein kinase phosphatases, are characterized by their specificity for the pT\(\text{XpY}\) signature sequence of MAPKs (5). Another well-characterized group of DSPs are the cell division cycle phosphatases (Cdc14 and Cdc25), which participate in regulation of the cell cycle by dephosphorylating cell cycle regulators, including cell cycle-dependent kinases (6). Meanwhile, a subgroup known as the atypical DSPs is the least characterized subgroup of PTPs. These phosphatases lack the MAPK recognition motifs and the characteristic Cdc25 rhodanese fold and, hence, are predicted to dephosphorylate substrates other than the MAPKs and cell cycle-dependent kinases (7).

The atypical dual specificity phosphatase hYVH1 (also known as DUSP12) is an evolutionary conserved enzyme ranging from yeast to humans (8–10). Although the precise physiological role of hYVH1 remains uncharacterized, recent evidence indicates that YVH1 orthologs may be critical mediators of ribosome biogenesis (11–13), cell cycle regulation (14), and cell survival (15, 16). Furthermore, the \(\text{hyvh1}\) gene has been found to be significantly amplified in various cancers that have progressed to advanced stages (17–19), highlighting the importance of examining the biological activities of hYVH1.

In addition to the C-terminal phosphatase domain, YVH1 family members contain a highly conserved cysteine-rich C-terminal domain that is more highly conserved than the phosphatase domain. The C-terminal domain of hYVH1 has been shown to coordinate 2 mol of zinc per 1 mol of protein, defining it as a novel zinc-binding domain (ZBD) and the only phosphatase known to contain such a domain. However, the lack of known hYVH1 targets has hindered our understanding of the biological significance of this unique enzyme. In this study, we employed affinity chromatography methods coupled with mass spectrometry to identify novel hYVH1-containing macromolecular structures. Members of various ribonucleo-
protein (RNP) complexes were identified, and follow-up microscopy studies demonstrated for the first time that hYVH1 is a stress granule-associating protein. Moreover, we provide evidence that, through its unique ZBD, hYVH1 represents a novel factor regulating stress granule disassembly.

**Results and Discussion**

**Human YVH1 Co-sediments with the 60S ribosomal subunits**—Recently, YVH1 in yeast has been identified as a novel trans-acting factor important for 60S ribosomal biogenesis (14, 15). Studies have suggested that YVH1 is not present on the mature translationally active ribosome but required for exchanging the protein Mrt4 for the ribosome stalk complex (P0, P1, and P2) during late-stage 60S maturation (12, 13). Because hYVH1 shares ~30% sequence identity with YVH1, we were interested in determining whether the human ortholog also localizes to the 60S ribosomal subunit. To examine the relationship between hYVH1 and ribosomal complexes, HEK 293 cells expressing endogenous hYVH1 were lysed and applied to a sucrose gradient (10−50%). Absorbance (254 nm) was monitored (left panel), and fractions were collected by peak as shown (F1–F8), resolved by SDS-PAGE, and subjected to immunoblot (IB) analysis (right panel). Shown are the immunoblots for each of the samples adjacent to its corresponding ribosome profile trace. Each trace and corresponding immunoblot is representative of at least three independent experiments. B, ribosome profiling was repeated for FLAG-hYVH1 WT, with smaller-volume fractions to better resolve 60S and 80S complexes. Fractions corresponding to the 60S and 80S peaks (red box, indicated in A) were analyzed by immunoblotting for RPL26 and FLAG-hYVH1. C, FLAG-hYVH1 co-immunoprecipitation with endogenous RPL26. HEK 293 cells expressing empty FLAG vector (Control) or FLAG-hYVH1 were subjected to FLAG immunoprecipitation (IP) and analyzed by immunoblotting. The image is representative of three independent experiments. D, schematic of the hYVH1 deletion constructs analyzed in A. Amino acid boundaries for the hYVH1 variants are shown. E, 60S fractionation levels of hYVH1 variants were quantitated by densitometry and normalized to total input levels. Means ± S.D. from three independent experiments are shown. The p values were calculated using Student’s t test and compared with wild-type hYVH1 (**, p < 0.05).
somes (80S + mRNA) and half-mers (40S + mRNA). Immunoblot analysis was then performed on the fractions to determine the presence of hYVH1 and the 60S ribosomal subunit protein RPL26 as a fractionation control for the 60S and 80S subunits. As shown in Fig. 1B, endogenous hYVH1 (37 kDa) co-elutes within the 60S and 80S peak fractions (Fig. 1A, right panel, top blot). These fractions were confirmed by the presence of the large ribosomal subunit protein RPL26 (17 kDa) in Fig. 1A, right panel, fourth blot, fifth and sixth lanes, corresponding to the 60S and 80S ribosomal complexes, respectively.

To characterize the structural features mediating the interaction between hYVH1 and ribosomal subunits, it was important to determine whether ectopically expressed hYVH1 can also associate with the 60S ribosomal subunit. As shown in Fig. 1A (right panel), no appreciable change occurs in the ribosome profiles in the presence of overexpressed FLAG-hYVH1. Furthermore, FLAG-hYVH1 displays a similar pattern of co-fractionation as endogenous hYVH1.

The presence of hYVH1 in the 80S fractions was unexpected and could be due to an association with the 80S complex or due to low resolution between the 60S and 80S peaks, as these two complexes elute very close to one another. Thus, to increase resolution, smaller-volume fractions were collected for FLAG-hYVH1 samples and subjected to immunoblotting. This analysis revealed that although the ribosomal core protein RPL26 co-eluted with both the 60S and 80S fractions as expected (Fig. 1B, bottom panel), FLAG-hYVH1 elution was observed predominantly in the 60S fraction, with only trace levels of hYVH1 detected in the later fractions (Fig. 1B, top panel). To complement the fractionation data, a co-immunoprecipitation experiment was performed between hYVH1 and RPL26 (Fig. 1C). Immunoblot analysis shows FLAG-hYVH1 in the lysates of transfected cells but not in the negative control lysates (Fig. 1C, top row). Furthermore, although equal levels of RPL26 are observed in the lysates of both the negative control and FLAG-hYVH1 samples, only FLAG-hYVH1 was able to pull down RPL26 from cellular lysates (Fig. 1C, bottom row). Collectively, these results support the notion that hYVH1 associates with the 60S ribosomal complex in human cells, suggesting an analogous role as its yeast ortholog.

Additional RNP complexes, we conducted an interactome analysis in U2OS cells using FLAG affinity chromatography followed by tandem mass spectrometry. We sought a cell line that was responsive to hYVH1 expression for our interactome analysis. U2OS cells were selected because of our previously finding that hYVH1 expression in U2OS cells affects cell cycle progression (14). Following isolation using FLAG-hYVH1 affinity chromatography, isolated proteins were eluted from the resin using a “soft elution” SDS buffer adapted from Antrobus and Borner (21). Eluted proteins from control cells or FLAG-hYVH1-expressing cells were then acetone-precipitated and processed for trypsin digestion. The tryptic peptides were analyzed by LC/MS-MS in a SYNAPT G2Si Q-TOF mass spectrometer (see “Experimental Procedures” for details) for protein identification. To increase the number of proteins identified, we employed the ion mobility chamber positioned between the quadrupole and time of flight tube within the SYNAPT instrument. The ion mobility step adds an additional separation feature to the experiment, as peptides that co-elute off the reverse phase column will often have different mobility (drift time) through the ion mobility chamber. This additional chromatography step significantly increased the number of proteins identified (Fig. 2A). Proteins of interest were those that were confidently identified in the FLAG-hYVH1 sample but not in the FLAG-control sample (Fig. 2B, supplemental Fig. S1). Identified proteins consisted of numerous 60S ribosomal unit proteins, including the ribosome stalk proteins P0 and P2 (Fig. 2B and supplemental Fig. S1). The high number of core 60S ribosomal proteins identified was expected given the fact that YVH1 in yeast has been shown to associate with the 60S RNP particle (11–13), validating our affinity chromatography approach. Surprisingly, however, large numbers of 40S subunit proteins were also identified. This was unexpected because, in yeast, YVH1 has been characterized exclusively as a 60S biogenesis factor (12, 13). In contrast, our interactome data provide suggestive evidence that, in human cells, hYVH1 may be present on additional RNPs that possess 40S subunits. Along these lines, proteins characterized for their role in translational repression via storage mRNPs containing 40S ribosomal subunits were also present in the interactome analysis, along with proteins that function in pre-mRNA splicing (Fig. 2B and supplemental Fig. S1). Collectively, this dataset implies that, in addition to 60S ribosome biogenesis, hYVH1 may localize to multiple RNP particles important in regulating mRNA processing.

**Human YVH1 Localizes to Stress Granules**

**Human YVH1 Co-localizes with FMRP- and YB-1-containing Granules**—Our hYVH1 interactome findings showed a large number of proteins known to function in RNP dynamics. We therefore were interested in testing whether hYVH1 indeed localizes to RNPs other than the 60S ribosome. Of notable interest was the presence of the fragile X mental retardation protein (FMRP) and Y box-containing protein 1 (YB-1), both regulators of translation repression on mRNP granules (Fig. 2B and supplemental Fig. S1). FMRP-containing mRNP granules...
have been shown to mediate transport of specific mRNAs for localized protein expression (20, 22). Furthermore, FMRP and YB-1 are necessary factors for repressing translation during transport (20, 23). To confirm that hYVH1 associates with FMRP/YB-1-containing foci, we analyzed their co-localization using fluorescence microscopy (Fig. 3). To better visualize FLAG-hYVH1 on RNP particles, we treated the cells with a low concentration of the detergent saponin (Fig. 3A). Gentle treatment of cells with saponin prior to fixation and immunostaining has been shown to permeabilize plasma membranes, liberating soluble cytoplasmic proteins while retaining the localization of proteins found on suborganellar structures (24). In the absence of saponin, the hYVH1 localization pattern is quite diffuse, making it difficult to resolve hYVH1 association with subcellular structures (Fig. 3A). Gentle treatment of cells with saponin prior to fixation and immunostaining has been shown to permeabilize plasma membranes, liberating soluble cytoplasmic proteins while retaining the localization of proteins found on suborganellar structures (24).

Co-immunoprecipitation experiments were performed to complement the immunofluorescence studies. U2OS cells expressing FLAG-hYVH1 variants were immunoprecipitated with anti-FLAG-agarose resin and probed for endogenous YB-1 and FMRP (Fig. 4). We observed that both wild-type hYVH1 and hYVH1 C115S (a phosphatase-inactive mutant) co-immunoprecipitated YB-1 and FMRP to a similar extent, suggesting that phosphatase activity is likely not required to associate with YB-1 and FMRP particles. Regarding the modular domains of hYVH1, the ZBD of hYVH1 has been shown to be required, and in some instances sufficient, for the observed hYVH1-mediated cellular functions, such as ribosome biogenesis (11–13), cell cycle regulation (14), and cell survival (15). As shown in Fig. 4, deletion of the ZBD (hYVH1 ΔZBD) reduced co-immunoprecipitation with both FMRP and YB-1 by ~2-fold results suggest that hYVH1 associates with selective YB-1-containing particles. Co-localization was also observed between FMRP and hYVH1 (Fig. 3C). Similar to YB-1, co-localization between hYVH1 and FMRP was also observed on punctate structures in the cytoplasm, with no detectable co-localization of hYVH1 with the nuclear pool of FMRP. It has been proposed that FMRP associates with its mRNA target in the nucleus and assists in the transport of mRNA into the cytoplasm for targeted mRNP transport (28). Thus, our co-localization pattern suggests that hYVH1 association with FMRP-containing granules may occur after the particles are shuttled from the nucleus to the cytoplasm.

Co-immunoprecipitation experiments were performed to complement the immunofluorescence studies. U2OS cells expressing FLAG-hYVH1 variants were immunoprecipitated with anti-FLAG-agarose resin and probed for endogenous YB-1 and FMRP (Fig. 4). We observed that both wild-type hYVH1 and hYVH1 C115S (a phosphatase-inactive mutant) co-immunoprecipitated YB-1 and FMRP to a similar extent, suggesting that phosphatase activity is likely not required to associate with YB-1 and FMRP particles. Regarding the modular domains of hYVH1, the ZBD of hYVH1 has been shown to be required, and in some instances sufficient, for the observed hYVH1-mediated cellular functions, such as ribosome biogenesis (11–13), cell cycle regulation (14), and cell survival (15). As shown in Fig. 4, deletion of the ZBD (hYVH1 ΔZBD) reduced co-immunoprecipitation with both FMRP and YB-1 by ~2-fold.
Although immunoprecipitating equal levels of hYVH1 ΔDSP compared with the other hYVH1 variants was problematic, densitometry analysis of data normalized to FLAG-hYVH1 levels suggests that the ZBD associates with FMRP/YB-1 granules to a similar extent as wild-type hYVH1. It is important to note that, in our hands and reported by others (10), the catalytic domain alone (ΔZBD) is substantially more stable in vitro and expresses at much higher levels during transient transfection of mammalian cell lines compared with the ZBD (ΔDSP) and full-length hYVH1. Thus, the N-terminal DSP domain may help stabilize the ZBD. Although it remains inconclusive whether the ZBD is sufficient, we can conclude that the DSP domain alone maintains some ability to associate with the FMRP/YB-1 granules. However, the ZBD appears to be necessary for optimal hYVH1 association with FMRP/YB-1 complexes.

We also examined whether hYVH1 binds directly to FMRP or YB-1 in vitro using purified recombinant proteins and co-immunoprecipitation experiments. Although a variety of conditions were tested, there was no detectable evidence that hYVH1 could directly associate with FMRP or YB-1 (data not shown). As mRNP granules are multiprotein complexes, these results suggest that hYVH1 likely associates with YB-1- and FMRP-containing granules through a linking protein rather than directly through YB-1 or FMRP. Future work utilizing cross-linking tools and proximity-based mass spectrometry techniques will be needed to delineate the direct binding partner linking hYVH1 to FMRP/YB-1 containing granules.

**Human YVH1 Associates with Stress Granules under Arsenic Stress**—FMRP and YB-1 have been shown to play multiple roles in mRNP dynamics and regulation of translation. One common example is their presence on stress granules. Stress granules are critical RNP intermediates that temporarily store mRNAs and sequester proteins during cellular insults (29). FMRP overexpression is sufficient to form stress granules in the absence of...
cell stress, suggesting that its prion-like folding trait is an important component in stress granule architecture (30). YB-1 has also been described as a stress granule marker (31), whereas studies have implied that YB-1 levels affect stress granule dynamics. For example, a recent report demonstrated that knockdown of YB-1 expression reduces stress granule formation because of translational regulation of factors involved in stress granule nucleation (32). Given that hYVH1 has been implicated as a cell survival factor (15, 33, 34), we were interested in testing whether hYVH1 co-localizes with YB-1 and FMRP on stress granules during cellular insults. Thus, stress granules were induced by arsenic treatment, and immunofluorescence microscopy was performed to visualize co-localization (Figs. 5 and 6). Furthermore, stress granule formation was confirmed by staining for the well characterized stress granule marker T cell-restricted intracellular antigen 1 (TIA-1) (Figs. 5D and 6C). TIA-1 is a translational repressor with 3’ UTR RNA binding activity and is a core stress granule component because of its self-aggregation property (29). To resolve hYVH1 localization to YB-1/FMRP foci, we again treated cells with saponin. This treatment had to be carefully optimized because mild treatment poorly resolves hYVH1 at subcellular foci, whereas prolonged treatment overly permeabilizes cells, resulting in release of stress granules. Under these conditions, we observed significant co-localization of wild-type hYVH1 with YB-1- and FMRP-containing stress granules (Fig. 5, A–C, and 6, A and B).

To rule out stress granule localization because of general protein overexpression, we examined co-localization between TIA-1 and ectopically expressed FLAG-MTMR2, a lipid phosphatase (35, 36). Although FLAG-MTMR2 displays a punctate localization pattern, we detected no significant co-localization with TIA-1 stress granules (Fig. 5C). Moreover, we analyzed the domain deletion variants to determine the regions important for association. Deletion of the ZBD (hYVH1 ΔZBD) had the most significant effect on association with YB-1/FMRP-containing granules (Figs. 5A and 6A, center panels). Interestingly, although association was significantly abrogated, there were still detectable YB-1/FMRP-containing granules that were positive for the ΔZBD deletion variant. Therefore, these results again suggest a contributing role of the N-terminal DSP domain for hYVH1 localization to the YB-1/FMRP granules. With regard to the C-terminal ΔDSP variant, we did observe fewer positive cells overall because of either low transfection efficiency or instability, as noted above. However, the cells that did express hYVH1 ΔDSP displayed significant co-localization to YB-1/FMRP granules, indicating that the ZBD is likely the principal facilitator of hYVH1 association to these particles (Figs. 5A and 6A, bottom panels).

Human YVH1 Expression Levels Modulate Stress Granule Size—Stress granules are transient structures (29). The temporal regulation of stress granule assembly/disassembly is an inherent property, allowing strategically controlled repression and restoration of translation in response to cellular insults (29). The above results implicate hYVH1 as a novel stress granule-associating protein. We were interested in confirming this finding more directly with the core marker TIA-1 and investigating the relationship between hYVH1 expression levels and alterations in stress granule properties. To this end, we analyzed the ability of hYVH1 variants to co-localize with TIA-1 in response to arsenic treatment and measured stress granule size as a readout for hYVH1-mediated modulation of stress granule

![Image](https://www.jbc.org/content/292/2/544)
Wild-type hYVH1, as expected, localized to TIA-1 foci. Interestingly, we observed that the size of the stress granules was significantly smaller in cells expressing hYVH1 compared with control cells (Fig. 7). The hYVH1 ΔDSP variant was also able to localize to TIA-1 stress granules and, analogous to wild-type hYVH1, significantly reduced stress granule size. The hYVH1 ΔZBD variant also co-localized with TIA-1, although at a statistically significant lower level. Moreover, hYVH1 ΔZBD failed to significantly reduce the size of TIA-1-positive stress granules compared with wild-type hYVH1 and the hYVH1 ΔDSP variant. Taken together, these results establish hYVH1 to be a novel stress granule factor whose overexpression alters stress granule size/architecture. Moreover, deletion analysis revealed that the DSP domain possesses moderate stress granule binding ability, with the ZBD domain likely the primary mediator, as this region is sufficient for the hYVH1-mediated effects on stress granule size.

To rule out that localization to stress granules is unique to overexpressed hYVH1, we confirmed that endogenous hYVH1 localizes to TIA-1-positive stress granules under arsenite stress conditions.
We then tested whether hYVH1 is required for stress granule formation by reducing hYVH1 expression with a validated hyvh1 siRNA (14) and quantitating the percentage of cells containing stress granules. Because saponin can potentially affect stress granule solubilization during treatment, and considering that localization of hYVH1 was not the experimental objective, we did not treat cells with saponin for these experiments to more accurately quantitate stress granule-positive cells (Figs. 8 and 9). As shown in Fig. 8, knocking down the expression of hYVH1 did not significantly affect the percentage of cells exhibiting stress granules, suggesting that hYVH1 is not required for stress granule formation. The findings that knockdown of hYVH1 expression does not affect stress granule formation, whereas overexpression of hYVH1 reduces stress granule size, points to a possible role in promotion of stress granule disassembly.

To further explore this hypothesis, we examined the effect of reducing hYVH1 expression on the stress granule disassembly process (Fig. 9). Cells exposed to arsenite for 1 h were washed and provided fresh medium to initiate stress granule disassembly. After 1- or 2-h recovery periods, the percentage of cells possessing stress granules was determined. Cells transfected with control siRNA possessed less stress granules at the 1-h (37% ± 4%) and 2-h (13% ± 3%) recovery time points compared with cells transfected with hyvh1 siRNA (73% ± 7% and 35% ± 5%, respectively), consistent with the idea that hYVH1 is a contributing factor for stress granule disassembly.

These results were confirmed using siRNA rescue experiments. Site-directed mutagenesis was employed to produce siRNA-resistant hyvh1 constructs that could be exogenously expressed following siRNA treatment. As shown in Fig. 9B, expression of the wild-type hYVH1 siRNA-resistant variant was able to effectively rescue the disassembly phenotype induced by knocking down the expression of endogenous hYVH1. Moreover, we examined functional features of this mechanism by repeating these experiments with siRNA-
resistant hYVH1 variants that were catalytically inactive (C115S) or expressed the domain deletion variants (Fig. 9, B and C). The results show that the hYVH1 C115S mutant is as effective at rescuing the disassembly phenotype as wild-type hYVH1, indicating that phosphatase activity of hYVH1 is not required. This conclusion was also supported by the DSP sample, which also was able to rescue the phenotype (Fig. 9, B and C). In contrast, deletion of the ZBD clearly resulted in significant failure to rescue the disassembly phenotype, suggesting that the C-terminal ZBD is the primary region mediating the hYVH1-induced acceleration of stress granule disassembly. Collectively, these results suggest that depletion of hYVH1 expression affects the ability of cells to disassemble stress granules during recovery from cellular insults. Furthermore, analogous to past studies evaluating YVH1 cellular effects (11–15), the presence of the ZBD rather than the phosphatase activity is the key determinant for the observed effects on stress granule dynamics.

It is interesting to note that the heat shock protein HSP70 has also been implicated as an important disassembly factor because of its intrinsic chaperone function and ability to disassociate proteins from macromolecular complexes, including stress granules (37). HSP70 was identified in our current interactome screen but was not reported as a protein hit because of its low-level identification in the control sample (data not shown). However, we have previously reported that endogenous hYVH1 associates with HSP70 through a direct interaction between the ZBD of hYVH1 and the ATPase domain of HSP70, suggesting that the proteins exist in a functional complex (15). Thus, it is tempting to speculate that hYVH1 and HSP70 may collaborate to promote stress granule disassembly during recovery from cell stress exposure. We are currently investigating the potential for hYVH1 and HSP70 to form a functional complex to mediate stress granule disassembly.
examining this hypothesis along with elucidating mechanisms regulating hYVH1 stress granule activities.

In conclusion, interactome analysis provided an unbiased approach toward discovering novel subcellular compartments containing hYVH1. Although future detailed analysis will be required to fully understand the mechanism of action, our results point to an expanded role for hYVH1 in mRNP and stress granule regulation. We have evidence that recombinant hYVH1 has no detectable affinity for RNA when analyzed by the RNAcompete microarray technique (38, 39). This implies that hYVH1 likely targets to mRNPs through protein-protein interactions. The maturation and functionality of mRNPs is regulated through various trans-acting factors and a wide range of mechanisms, including reversible phosphorylation and allosteric protein-protein interactions (40). Thus, we are interested in investigating the contribution of hYVH1 toward regulating the protein complement of the maturing mRNPs in response to a variety of extracellular stimuli. Moreover, FMRP/YB-1 expression elicits cytoprotective and cell cycle effects (41, 42). These cellular phenotypes are postulated to be a result of temporal translational regulation of specific mRNAs involved in these cellular processes. Because hYVH1 expression exhibits increased translational control in response to a variety of extracellular stimuli, understanding the mechanisms underlying this regulation may provide insights into the cellular functions of hYVH1.

**Experimental Procedures**

**Plasmid Constructs**—The plasmids encoding FLAG-hYVH1 variants have been described previously (15). Site-directed mutagenesis was performed to generate hYVH1 siRNA-resistant variants. The forward and reverse primers used were as follows: 5′-GCTCTTGTGTTAGTGGATACACACC-TGCTTTTC-3′ and 5′-GAAAAAGCAGGTGTGATCC-ACCTACCAAGAGC-3′ for I313I and 5′-GAAAAAGC-GCGGTGTGATCCACCTACCAAGAGC-3′ and 5′-GCTCTTGTGTTAGTGGATACACCCGCTTTTC-3′ for I313I P315P.

**Ribosome Profiling**—All solutions used for ribosome profiling were made using diethylpyrocarbonate (DEPC)-treated water (Sigma-Aldrich, Inc.). All sucrose solutions had a buffer composition of 80 mM NaCl, 5 mM MgCl₂, 0.2 mM Tris-HCl (pH 7.4), and 1 mM DTT. Sucrose gradients were made by carefully layering 2 ml of a 5% sucrose buffer on top of 2 ml of a 60% sucrose solution containing hYVH1. Although future detailed analysis will be required to fully understand the mechanism of action, our results point to an expanded role for hYVH1 in mRNP and stress granule regulation. We have evidence that recombinant hYVH1 has no detectable affinity for RNA when analyzed by the RNAcompete microarray technique (38, 39). This implies that hYVH1 likely targets to mRNPs through protein-protein interactions. The maturation and functionality of mRNPs is regulated through various trans-acting factors and a wide range of mechanisms, including reversible phosphorylation and allosteric protein-protein interactions (40). Thus, we are interested in investigating the contribution of hYVH1 toward regulating the protein complement of the maturing mRNPs in response to a variety of extracellular stimuli. Moreover, FMRP/YB-1 expression elicits cytoprotective and cell cycle effects (41, 42). These cellular phenotypes are postulated to be a result of temporal translational regulation of specific mRNAs involved in these cellular processes. Because hYVH1 expression exhibits increased translational control in response to a variety of extracellular stimuli, understanding the mechanisms underlying this regulation may provide insights into the cellular functions of hYVH1.

**Affinity Chromatography and Mass Spectrometry**—Human U2OS osteosarcoma cells (ATCC) were grown in DMEM/F12 with 10% FBS and 2 mM L-glutamine and supplemented with 1% penicillin/streptomycin antibiotics at 37 °C and 5% CO₂. Cells (1 × 10⁷) were seeded in 20-cm dishes 24 h before transient transfection with 10 μg of DNA (FLAG-hYVH1 or empty vector) and FuGENE HD (Promega) following the protocol of the manufacturer. Cells were lysed and subjected to FLAG-affinity chromatography as described previously (15). Proteins were liberated from the FLAG resin using an elution buffer containing 0.25% SDS with 0.2% Tween 20 adapted from Antrobus and Bornor (21). The eluant was then precipitated with acetone and prepared for trypsin digestion as described previously (15, 16). The pools of tryptic peptides were analyzed by tandem MS with a SYNAPT G2Si Q-TOF mass spectrometer using the ion mobility feature of the instrument for increased ion separation. MS-MS was performed by data-independent acquisition methods for increased peptide identification. MS data of three technical replicates from three biological replicates were analyzed using ProteinLynx Global Server software (PLGS version 3.01). Data were further annotated using Excel and the software program Venny.

**Immunofluorescence Analysis**—U2OS cells were seeded on four-chamber slides (BD Biosciences) 24 h prior to transient transfection with 0.5 μg of DNA using FuGENE HD. For siRNA experiments, cells were transfected for 48 h with control siRNA or hyvh1 siRNA according to the protocol of the manufacturer using Lipofectamine Raiman. For siRNA rescue experiments, siRNA-resistant hYVH1 variants were transfected for 24 h following siRNA treatment. Prior to fixation, cells were treated with or without saponin (0.1%) for 45 s at room temperature as described previously (40, 41). Cells were then fixed for 15 min with 3.7% paraformaldehyde at room temperature. Thereafter, cells were permeabilized with 0.15% Triton X-100 for 2 min and blocked with 5% BSA for 1 h at room temperature. Primary antibodies, including mouse anti-FLAG (Sigma, F1804), rabbit anti-FMRP (Abcam, ab17722), rabbit anti-YB-1 (Sigma, Y0271), rabbit anti-hYVH1 (10), and goat anti-TIA-1 (Santa
noprecipitates were washed three times with 50 mM Tris-HCl and FLAG-protein A-agarose beads (Sigma) for 1.5 h at 4 °C. Immunoprecipitation—U2OS cells were transfected with FLAG-hYVH1 variants for 24 h, and cells were lysed as described above. Cellular lysates were incubated with 20 µl of FLAG-protein A-agarose beads (Sigma) for 1.5 h at 4 °C. Immunoprecipitates were washed three times with 50 mM Tris-HCl (pH 7.4), 0.1% Triton X-100, 150 mM NaCl, and 0.1% SDS and analyzed by immunoblotting using mouse anti-FLAG (Sigma, F3165), rabbit anti-L26 (Sigma, R0655), anti-FMRP, and anti-RNA polymerase II antibodies (Life Technologies, A11011) for 1 h at room temperature. Cells were washed and stained with Hoechst stain (0.5 mg/ml) (Invitrogen) for 2 min and mounted on the slide. Fluorescence microscopy was utilized to capture the images using a ×40 objective. Pearson’s correlation coefficients and stress granule size were determined using ImageJ (National Institutes of Health) using the JACoP plugin and analyze particle features, respectively. Calculations were determined by analyzing at least 30 cells from three independent experiments. Statistical analysis was performed using Student’s t test (Excel), with differences considered statistically significant at p < 0.01.

Co-Immunoprecipitation—U2OS cells were transfected with FLAG-hYVH1 variants for 24 h, and cells were lysed as described above. Cellular lysates were incubated with 20 µl of FLAG-protein A-agarose beads (Sigma) for 1.5 h at 4 °C. Immunoprecipitates were washed three times with 50 mM Tris-HCl (pH 7.4), 0.1% Triton X-100, 150 mM NaCl, and 0.1% SDS and analyzed by immunoblotting using mouse anti-FLAG (Sigma, F3165), rabbit anti-L26 (Sigma, R0655), anti-FMRP, and anti-YB-1 as described previously (15). Densitometry analysis was performed using ImageJ. Data were normalized to levels of the corresponding FLAG-hYVH1 variant and compared with wild-type FLAG-hYVH1. p < 0.05 was considered statistically significant. For direct protein-protein interaction analysis, recombinant hYVH1 was purified as described previously (15). The His-FMRP construct was a kind gift from Dr. Utz Fischer, and purification of recombinant FMRP was conducted as described above. GFP-YB-1 was purchased from Abnova. Recombinant proteins (10 ng) were incubated for 2 h at 4 °C. Protein complexes were isolated via immunoprecipitation and analyzed by immunoblotting as described above.

Author Contributions—Q. G. and P. O. V. designed and conceived the study. Q. G., B. X., C. K., and C. A. B. performed all experimental work. P. O. V. coordinated the study. Q. G. and P. O. V. wrote and edited the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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