DISSECTING FUNCTIONAL DIFFERENCES IN HSP70 ISOFORMS IN NEMATOSTELLA VECTENSIS

by

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ABSTRACT

SHAWN J. WALLER. Dissecting functional differences in Hsp70 isoforms in *Nematostella vectensis*. (Under the direction of DR. ANDREW TRUMAN)

Heat shock protein 70s (Hsp70s) are a highly conserved class of molecular chaperones that are involved in cellular processes in all organisms such as stress response, homeostatic maintenance, and cell cycle progression. The organism *Nematostella vectensis*, or the starlet sea anemone, experiences a multitude of physiological stressors through abiotic factors such as temperature, UV radiation, salinity, and pollutants like toxic metals. Due to the dynamic estuary environments that *N. vectensis* resides, these stressors are experienced with both daily and seasonal fluctuations. In marine cnidarians, Hsp70 proteins can be effective biomarkers in order to determine adaptations and evolutionary responses to environmental stress: a pressing issue as concerns about climate changes grow. Within *N. vectensis*, 3 isoforms of cytosolic Hsp70 (NvHsp70) exist: A, B, and D. Transcriptional data has shown dramatic differences among expression profiles of these NvHsp70 isoforms under stress. To bypass the lack of *in vivo* protein technologies available for marine invertebrates, the 3 NvHsp70 isoforms were expressed in *Saccharomyces cerevisiae* budding yeast lacking native Hsp70. These cells grow at different rates and display altered tolerance to stressors such as heat shock, DNA damage, and salinity. After functional differences at the organismal level were investigated, each isoform and their respective complex of bound proteins (“interactomes”) were characterized using mass spectrometry. From these characterizations, differences in binding behaviors were observed. Each isoform expressed a unique interactome upon heat stress, and bound to different co-chaperones.

Of the 3 isoforms, NvHsp70B showed a substantially large number of increased interactions in response to heat shock as compared to NvHsp70A and NvHsp70D. Using the data gathered throughout the course of this thesis, each of the NvHsp70 isoforms' roles were further characterized.

ACKNOWLEDGMENTS

This thesis was made possible with the help of many individuals, and I would like to extend my sincere thanks to all of them. First and foremost, I would like to thank my advisor Dr. Andrew Truman at the University of North Carolina, Charlotte, for being a great mentor during the entirety of my graduate experience. I can't imagine completing my Master's without his enthusiasm and support. I would also like to thank Dr. Adam Reitzel for extending to me the collaborative opportunity to work with *N. vectensis*. Dr. Richard Chi rounded out my fantastic committee, and provided insightful feedback and assistance throughout the process.

I would also like the acknowledge my colleagues in the Truman lab, who helped me learn and optimize techniques and assisted me in experimentation. From letting me know where things were stored the many times I forgot, to helping me practice important presentations, my labmates were a crucial element to my success.

Finally, I would also like to thank my friends and family for their support throughout my time in graduate school. They made my time in Charlotte an enriching and enjoyable experience that extended beyond just academic work.

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DEDICATION

I would like to dedicate this to my parents, Tom and Leslie Waller, and my fiancé, Chadd McGurn, for their support and encouragement for the duration of my time in graduate school.

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LIST OF ABBREVIATIONS

HSP- Heat shock protein

HSP70- Heat shock protein 70

NBD- Nucleotide binding domain

SBD- Substrate binding domain

ATP- Adenosine triphosphate

ER- Endoplasmic reticulum

NvHSP70 – *Nematostella vectensis* heat shock protein 70

NvHsp70A: Isoform A

NvHsp70B: Isoform B

NvHsp70D: Isoform D

YPD- Yeast peptone dextrose (yeast media)

MS- Mass spectrometry

PTM- Post-translational modification

PMF- Protein mass fingerprinting

SILAC- Stable isotope labeling of amino acids in culture

HSF- Heat Shock Factor

HU- Hydroxyurea

RNR- Ribonucleotide Reductase

NJ- Neighbor joining

GO- Gene ontology

Ln- Natural log

sHSP- Small heat shock protein

TCA- Tricarboxylic acid cycle (Krebs cycle)

HU- Hydroxyurea

DDR- DNA damage response

ME- Maine

MA- Massachusetts

NC- North Carolina

HSR- Heat shock response

CHAPTER 1: INTRODUCTION

1.1 BACKGROUND

1.1.1. HEAT SHOCK PROTEIN 70

Heat shock proteins are molecular chaperones that engage in various cellular processes, ranging from the facilitation of cell repair and reproduction to cellular homeostasis. They are characterized by their molecular weight and categorized into different classes. Of these, the Hsp70 and Hsp90 families have been the most commonly utilized classes for studies of environmental stress both in laboratory conditions and in field research. Large Hsp size classes are ancient, meaning that they have broad conservation among most eukaryotes. Although retaining conserved properties throughout speciation events, large Hsp classes are also diverse. The Hsp70 family has multiple genes within its class with distinct cellular localization and inducibility.

The Hsp70 family is present in every cell of all living organisms. The Hsp70 protein is comprised of 2 main components: a 44-kD nucleotide binding domain (NBD) on the N-terminal, and an 18-kD substrate binding domain (SBD) near the C-terminal. The NBD and SBD are joined together by a linker region that is conserved among the Hsp70 family (Flaherty, DeLuca-Flaherty, & McKay, 1990). When Hsp70 binds to ATP in the NBD, a 10-kD variable C-terminal “lid” ensures that the substrates properly attach to the SBD, utilizing an EEVD amino acid motif (Scheufler et al., 2000). Hsp70 acts as a clamp through this conformational change that occurs upon the action of ATP hydrolysis, thereby trapping substrate proteins in the SBD. Regulation of protein folding is allosteric, mediated by the hydrolysis performed in the NBD region of the molecular chaperone

(Sharma & Masison, 2009). Therefore, modifications upon the protein at either the N-terminal or C-terminal domain can affect the regulation of Hsp70 function (Chirico, Markey, & Fink, 1998; Truman et al., 2012).

This protein family has been studied as a biological marker in ecology and stress evolution (Feder & Hofmann, 1999). When under stressors such as heat shock, anoxia, changes in salinity, or chemical stress, the Hsp70 family can be upregulated or activated. This is an essential component of proteostasis, otherwise known as the homeostasis of proteins. It serves to assist in the folding of newly synthesized proteins and denatured proteins, protein transport, and the degradation of aggregated proteins (Hartl, Bracher, & Hayer-Hartl, 2011).

Different isoforms of Hsp70 localize to different regions in the cell. In different organisms, there may be different patterns of Hsp70 localization (Evans, Chang, & Gestwicki, 2010). In the budding yeast *Saccharomyces cerevisiae*, 9 isoforms are cytosolic, 3 are mitochondrial, and 2 are restricted to the ER (Walsh, Bursac, Law, Cyr, & Lithgow, 2004). Determining isoform localization using a molecular basis involves examining the conserved C termini of HSP70: cytosolic HSP70 contains an EEVD motif, mitochondrial HSP70 contains a PEAEYEEAKK motif, and the plastid motif is PEGDVIDADFTDSK (Guy & Li, 1998).

1.1.2. HSP70 AS A BIOMARKER

Heat shock proteins have been broadly studied as biological markers in the ecology and evolution of organismal physiology and stress (Feder & Hofmann, 1999), including cnidarians such as *Nematostella vectensis*, or the starlet sea anemone (Tarrant, Reitzel, Kwok, & Jenny, 2014). Of all the class sizes of Hsps, Hsp70 has been one of the

most often utilized classes for studies of environmental stress both in laboratory settings and in field research. Because Hsp70 is an ancient class of the heat shock family, it has extensive conservation across most eukaryotes while still maintaining diverse genes with distinctive cellular localization patterns and inducibility (Georgopoulos & Welch, 1993).

Over the past few decades, Hsp70 consequently developed as an early response biomarker for determination of organismal stress in a variety of animals (Kultz, 2005). Prior studies of Hsp70 in reef building corals has determined that different abiotic stressors elicit upregulation of various isoforms of the chaperone (Louis, Bhagooli, Kenkel, Baker, & Dyall, 2017; Nakamura, Morita, Kurihara, & Mitarai, 2012). Similar results can be found when studying the Hsp70 isoforms of sea anemones and hydrozoans (Gellner, Praetzel, & Bosch, 1992; Meyer & Weis, 2012). The Hsp70 protein has also been a target of interest for understanding cnidarian evolutionary ecology. Populations of particular species living in different locales exhibit distinct expression patterns for specific Hsp70s that are consistent with adaptation to surviving in more physiologically challenging conditions (Barshis et al., 2013; Bellantuono, Granados-Cifuentes, Miller, Hoegh-Guldberg, & Rodriguez-Lanetty, 2012).

1.1.3. NEMATOSTELLA ORGANISM: A MODEL CNIDARIAN

Nematostella vectensis, or the starlet sea anemone, is a marine cnidarian that can be used as a model organism (Darling et al., 2005). They are some of the simplest animals at the tissue level of organization, and date back approximately 700 million years as a sister group to the Bilateria (Putnam et al., 2007). Cnidarians such as starlet sea anemone, and others like jellyfish, coral, and hydra, are important players in marine ecosystems like coral reefs and pelagic environments (Pandolfi et al., 2003).

Starlet sea anemones are found in coastal marine waters on the Atlantic and the Pacific coasts of the United States and Canada, from as far north as Nova Scotia and as far south as Florida. Small populations of the starlet sea anemone have also been introduced to the coast of the United Kingdom (Hand & Uhlinger, 1994; Reitzel, Darling, Sullivan, & Finnerty, 2008) and likely Brazil (Silva, Lima, Perez, & Gomes, 2010). In these locations, *N. vectensis* usually inhabits estuaries near land, such as salt marshes and saline lagoons. According to climatic models, these habitats will undergo considerable change in temperature in the coming decades, leading to higher average annual temperatures and more dramatic seasonal fluctuations (Lima & Wethey, 2012; Solomon et al., 2007). This shows a capability for tolerance of broad environmental conditions, such as varied temperatures, salinity, and sunlight exposure. Specifically, *N. vectensis* has been observed in environments of temperatures from 1.5°C to 32.5°C, and salinities of 2 to 52 parts per thousand (Kneib, 1988; Sheader, Suwailem, & Rowe, 1997). Starlet sea anemone habitats may also have high or low levels of pollution (Elran et al., 2014). Acute and chronic changes to the estuary habitat of *N. vectensis* are influenced by rainfall patterns, biological productivity, and nutrient and food availability. Sea anemones are especially affected due to their limited migratory ability, and must therefore rely on existing physiological plasticity or genetic adaptations to the often-changing environment (Parmesan, 2006; Visser, 2008). Studying these organisms gleans insight into mechanisms by which individuals adapt and acclimate to variation, providing information about population health in both current conditions and those that will arise due to future climate change.

N. vectensis routinely spawns under laboratory conditions. Depending on abiotic environmental factors, it may form mature eggs every 7-8 days while spawning continuously during the year (Genikhovich & Technau, 2009). Despite the ease of reproducing and caring for *N. vectensis*, previous work on this organism has had molecular limitations. Although taxonomic sampling and environmental ranges for work on sea anemone has increased, there exists a lack of *in vivo* protein technologies for these and other emerging model organisms. Previous studies on NvHsp70 have relied solely on transcriptional analysis, resulting in a lack of existing knowledge regarding isoform-specific functions of NvHsp70 and its interactions at the protein level. Assessments of HSP isoform diversity in *N. vectensis* remain largely uncharacterized. Early genomic surveys suggested perhaps a dozen members of Hsp20 family, 5 Hsp70s, and 3 Hsp90s (Goldstone, 2008; Reitzel et al., 2008). Transcriptome-wide surveys from adult *N. vectensis* cultured under various stressors have shown particular HSPs in each size class to have significant changes in expression (Elran et al., 2014; Oren et al., 2015). The ability to perform studies on specific isoforms and protein interactions in these emerging model organisms will further increase the value of Hsps as informative or predictive biomarkers in understanding a cnidarian ecology and comparative biology.

1.1.4. MASS SPECTROMETRY BACKGROUND

Proteomics, or the large-scale study of proteins in molecular biology, is a field of study facilitated by the development of mass spectrometry technology (MS). Its goal is to completely characterize features of proteins, such as localization, structure, expression, activity, and interactions with other proteins and molecules. Traditionally, individual proteins were studied. However, as complete genome sequences have become more

available, comprehensive analyses with multiple treatments/modifications and proteins of interest have been performed (Xuemei, Aslanian, & Yates, 2008).

The mass-to-charge ratio (*m/z*) of gas-phase ions is measured by mass spectrometry in order to gain information on these features. Using this proteomic data, there are 3 primary applications of MS: uncovering protein expression, defining protein interactions, and defining and elucidating sites of protein modification. Tandem mass spectrometry is a technique that is often used for post-translational modification (PTM) analysis. This is a technique that involves performing more than one step of MS, and often some sort of fragmentation of the sample occurs between the steps (IUPAC, 1997). The multiple steps can involve different types of separation strategies: reverse phase, size-exclusion, isoelectric focusing, and ion exchange. This can be performed at the protein level or peptide level (Fournier, Gilmore, Martin-Brown, & Washburn, 2007; Issaq, Chan, Janini, Conrads, & Veenstra, 2005).

1.1.5. PROTEOMIC ANALYSIS

There are two main categories of proteomic analysis using MS: “top-down” proteomics and “bottom-up” proteomics. Top-down proteomics analyze entire proteins, whereas bottom-up proteomics analyze the peptides that have been produced chemically or enzymatically. The original strategies for mass spectrometry involve bottom-up methods to infer protein presences through peptide identification. This can be used to identify and analyze highly complex samples. Within the bottom-up approach, there are 2 types of workflows: “sort-then-break” and “shotgun proteomics”. “Sort-then-break” approaches, as their name suggests, utilize protein separation prior to protein digestion (Henzel et al., 1993; Ogorzalek Loo et al., 2005). After the separation and digestion is

complete, peptide analysis is performed by peptide mass fingerprinting (PMF). A common analytical technique, PMF is the comparison of peptide masses to a database or other resource of known protein components. If no database exists, these peptides can even be compared to other proteins in the genome of the studied organism (Clauser, Baker, & Burlingame, 1999). The other bottom-up technique is the inverse of "sort-then-break", and is called "shotgun proteomics." This method first digests proteins to create peptides, and then separates these products using liquid chromatography. Therefore, the proteins are broken first and then sorted (Alves et al., 2007). Once sorted, tandem mass spectrometry is used to identify the peptides. Shotgun method can allow for the profiling of dynamic proteomes, as well as protein identification at a global scale (C. C. Wu & MacCoss, 2002). However, this method can be prone to under-sampling due to dynamic exclusion filtering procedures before performing tandem MS. This can cause peptides occurring at low abundances to go unaccounted for, even if they are crucial in a protein's identification (B. Zhang et al., 2006). Overall, bottom-up proteomics methods as a whole are advantageous in front-end separation of peptides, and provide high sensitivity (Yates, Ruse, & Nakorchevsky, 2009). The most obvious limitation of bottom-up proteomic methods are the reliance on identified peptides, loss of PTM identification, and redundant peptide sequences (Y. Zhang, Fonslow, Shan, Baek, & Yates, 2014).

In top-down proteomics, the analyzed proteins remain intact without any prior digestion until fragmentation in the mass spectrometer. Theoretically, this is a more complete characterization of PTMs and protein isoforms than the bottom-up approach, because without the prior digestion the entire protein sequence can be investigated at once- including proteins up to 229 kDa in size. Consequently, this method directly

examines mass discrepancy differences between the measured mass and predicted values of the DNA sequence in any modifications that are tested. Typically, this approach is performed using only simple protein mixtures or singular proteins. Top-down proteomics is advantageous when studying the distinct characteristics of individual proteoforms without risk of degradation and loss of components within the sample. It is also more successful at the characterization of small proteins that cannot be broken into significant peptide chains, and it is the approach of choice when quantifying protein expression and PTM mechanics due to its higher tolerance of impurities when keeping proteins intact (Lorenzatto et al., 2015). However, the disadvantages of top-down proteomics are their limitations on sample complexities scale (Durbin et al., 2016). Additionally, top-down proteomics has a lower sensitivity than the bottom-up methods (Yates et al., 2009). In order to avoid common disadvantages of both top-down and bottom-up proteomic methods, newer studies have attempted work with "middle-down" proteomics, in which size-dependent fractionation of protein is combined with restricted proteolysis (C. Wu et al., 2012). This allows for front-end separation of protein mixtures, but does not allow the level of digestion that occurs in bottom-up proteomics. It produces peptides that are larger than 5kDa, and has been shown to be useful in new experimental techniques for solving problems such as deciphering the histone code (Jenuwein & Allis, 2001). Middle-down proteomics can also further elucidate characterizations of different PTMs working in combination with each other.

Mass spectrometry technology can also be used to quantify protein abundance within a sample. Stable isotopes can be used to label samples, causing a shift in mass that distinguishes the labeled individuals from identical peptides in the sample. ^{2}H, ^{13}C, ^{15}N,

and ^{18}O are the stable isotopes often used in these labeling quantitative procedures (Xuemei et al., 2008). These isotopes are combined with the samples during protein synthesis, and are uniform across the proteome. SILAC, or the use of stable isotope labeling of amino acids in cell culture, is a labeling technique in which select amino acids are labeled- most often arginine and lysine (Ong et al., 2002). The amino acids are cleaved with the use of trypsin, and all peptides are labeled. This technique has been successful in measuring signaling pathways and characterizing protein interactions (Guo et al., 2008; Vermeulen et al., 2007). It has also been shown to characterize the relationship of Hsp70 with Hsc82 in previous yeast experiments (Truman et al., 2015). SILAC can achieve absolute measurement of protein abundance, whereas other MS quantification is relative to the sample provided (Ross et al., 2004).

1.3. RATIONALE

In this project, a multidisciplinary approach combining analyses from novel molecular approaches will be used to identify the unique and shared functions of Hsp70 isoforms from the starlet sea anemone *N. vectensis* in response to environmental variation common to their estuarine habitats. Performing gene expression experiments as well as protein-protein interaction studies allows for a more complete understanding of the role of Hsp70 as a marine cnidarian biomarker.

CHAPTER 2: HYPOTHESIS AND SPECIFIC AIMS

2.1. HYPOTHESIS

Functional differences among NvHsp70 isoforms A, B, and D in *N. vectensis* are due to specific protein binding interaction differences.

2.2. RESEARCH FOCUS

To observe the effect of differential protein binding patterns among the NvHsp70 A, B, and D isoforms on their functional differences.

2.3. SPECIFIC AIMS

2.3.1. Specific aim 1: To determine whether the isoforms of Hsp70 in *N. vectensis* have distinct cellular functions.

2.3.2. Specific aim 2: To dissect observed functional differences by understanding their specific client interactions using mass spectrometry.

CHAPTER 3: CHARACTERIZING FUNCTIONAL DIFFERENCES IN SEA ANEMONE HSP70 ISOFORMS USING BUDDING YEAST

3.1. MATERIALS AND METHODS

3.1.1. ANIMAL CULTURES AND ABIOTIC EXPOSURES

Adult *Nematostella vectensis* from three populations (Saco, ME; Sippewissett, MA; Wilmington, NC), which represent a large part of the species' latitudinal distribution along the Atlantic coast of the United States (Reitzel et al., 2008), were collected and transported to UNC Charlotte. Individuals from each location were cultured under standard conditions (20°C, 13‰ artificial seawater, fed *Artemia* three times weekly) for approximately one year prior to beginning experiments.

3.1.2. IDENTIFICATION OF HSP70 GENES IN THE *NEMATOSTELLA* GENOME

Candidate HSP genes for *N. vestensis* have been previously identified through similarity searches using BLAST searches of the reference genome and transcriptome (see Introduction). In an earlier study (Reitzel et al., 2014), identified three HSP70 isoforms, named NvHSP70A, B, and D, from *N. vectensis* that likely grouped with other cytosolic forms from other animals that also showed inducible expression under acute temperature stress (e.g. 40°C). We focused on these three HSP70s for this study to: 1) determine transcriptional dynamics under temperature changes that mirror natural oscillations and to then compare these changes for individuals from each of three locations and 2) determine similar and unique properties for each HSP70 isoform when heterologously expressed in yeast.

3.1.3. PHYLOGENETIC ANALYSIS OF NVHSP70 ISOFORMS

Yeast and human Hsp70 sequences were looked up on Saccharomyces Genome Database (SGD, http://www.yeastgenome.org/) and Universal Protein Resource (UniProt, http://www.uniprot.org/) respectively. Sequences were aligned using Clustal Omega Multiple Sequence Alignment (http://www.ebi.ac.uk/Tools/msa/clustalo/). Using these aligned sequences and the software program Mega 7, a phylogenetic tree was constructed using the Neighbor-Joining method with 1000 bootstrap replications.

3.1.4. EXPRESSION OF HSP70 ISOFORMS IN *NEMATOSTELLA* POPULATIONS

Adult *Nematostella* from each location were exposed to two temperature regimes ("acute" and "chronic") in order to determine the impacts of thermal environment on HSP70 transcription. In the acute experiment, adults from each population were divided into four replicate bowls per population (12 bowls total, 200 mL volume) at 20°C overnight. At the beginning of the experiments, 2-3 adults cultured at 20°C were sampled and immediately preserved in RNAlater. The temperature was increased 2°C per hour for the next 8 hours to a final temperature of 36°C. Adults were sampled from each bowl at 28°C (4 hours) and 36°C and preserved. For the chronic temperature treatment, half of the remaining adults in each bowl were cultured for an additional 14 hours at 36°C, while the other half were cultured at 20°C. Adults sampled from each replicate and preserved in RNAlater. RNA was extracted from all samples using the RNAqueous® Total RNA Kit (Ambion), DNase treated, and then quantified with a NanoDrop. cDNA was synthesized from 200ng of total RNA in a 20 μL reaction using the iScript cDNA synthesis kit (Bio-

Rad). Expression of each HSP70 was determined following methods by Reitzel and Tarrant (2009) using an Applied Biosystems® 7500 Real-Time system. Briefly, a 20 μL reaction consisted of 10 μL Power SYBR® Green Master Mix (Thermo), 1 μL of cDNA and 400 nM of gene-specific primers. Expression was calculated by comparing the threshold cycle of amplification against a standard curve constructed from a serially diluted plasmid standard containing the amplicon of interest. The PCR conditions were 95°C for 2 minutes followed by 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds. After 40 cycles, a melt curve analysis was used to verify single amplicons during the amplification steps. Due to the uneven loss of samples during quality control of the data (poor RNA yields or quality), we were unable to complete a formed statistical analysis of the expression data. Instead, we present the expression data as a more qualitative comparison to characterize the dynamics of expression between HSP70 isoforms and populations in these temperature treatments.

3.1.5. CLONING OF NVHSP70 ISOFORMS INTO YEAST EXPRESSION PLASMIDS

We assembled the open reading frame from each HSP70 (A, B, D) using sequence resources available through *Nematostella* JGI genome portal. We then designed primers to amplify the full open reading frame. Each primer set also included the nucleotides for directional cloning using the In-Fusion® HD Cloning Kit (Clontech). Each full length Hsp70 was amplified from cDNA synthesized from RNA isolated from *Nematostella* originally collected from Sippewissett and cloned into the pAG415GPD-ccdB vector purchased from Addgene for expression in yeast. All inserts were sequenced and confirmed with Sanger sequencing.

3.1.6. EXPRESSION OF NVHSP70 INTO *SACCHAROMYCES CEREVISIAE*

MH272 yeast cultures were grown in YPD (1% yeast extract, 2% peptone, 2% glucose) supplemented with the appropriate nutrients to select for plasmids and gene replacements. pNvHsp70 was transformed into this yeast. An empty vector of pAG415GPD-ccdB was also transformed into the MH272 yeast as a control. Through plasmid selecting on amino acid dropout yeast media, we confirmed that the NvHsp70 sequences were properly transformed into yeast. The transformed yeast was grown to mid-log phase and serially diluted on YPD plates to be incubated at both control and heat shock (37°C) temperatures.

3.1.7 SERIAL DILUTION PLATES TO TEST FUNCTIONALITY

Transformed Ssa1 and NvHsp70 yeast samples were diluted in factors of 10 (full concentration, 1/10 concentration, 1/100 concentration, and 1/1000 concentration) in a 96-well plate. From the plate, a multi-pronged tool is used to stamp the diluted samples of yeast onto plates containing chemicals or exposed to elements such as heat shock or UV radiation. After 3 days of growth, the phenotypes of the experimental plates are compared to the control plate in order to determine functional differences between isoforms. Environmental conditions for acute and chronic stress were determined from preliminary experiments and included: temperatures up to 36°C for 6 hours and 24 hours, which mimics natural thermal stress in *N. vectensis* habitats and elicits a molecular response for heat shock (Reitzel et al., 2013), salinity stress of 5 and 45 ppt for 6 and 24 hours (Sombatsaphay & Reitzel, 2016), and UV stress of 0.66 W/m^{-2} UV-B (Tarrant et al., 2014). We used these same conditions for the exposure of yeast transformed with the individual HSP70 isoforms.

3.2. RESULTS

3.2.1. PHYLOGENETIC ANALYSIS AND SEQUENCING OF HSP70 ISOFORMS

CLUSTAL analysis reveals similar sequences between the *N. vectensis* NvHsp70A, B, and D isoforms (Fig 3.1). Amino acid differences among the isoforms exist sporadically throughout the aligned sequences, but most of the differences between NvHsp70 isoforms occur near the C terminal in the protein binding region (amino acid positions 555-640). In an effort to place the NvHsp70 isoforms in a broader evolutionary context, we constructed a phylogenetic tree containing NvHsp70 isoforms of different organisms. MEGA software was used to create a neighbor-joining phylogenetic tree based on aligned amino acid sequences. From this tree, two distinctly clustered groups are formed (Fig 3.2). One of these groups contains all of the cytosolic yeast isoforms (Ssa1, Ssa2, Ssa3, and Ssa4). The other major group in the tree contains the cytosolic sea anemone isoforms (NvHsp70A, NvHsp70B, and NvHsp70D) and the human isoforms (Hsp70 and Hsc70). Although Hsp70 and Hsc70 are most closely related to each other, both being the human isoforms, they share a common ancestor with the sea anemone isoform in the Hsp70 family NvHsp70A. NvHsp70B and NvHsp70D are most closely related to each other of the sea anemone isoforms, and share a common ancestor with the branch containing human Hsp70 and Hsc70 as well as sea anemone NvHsp70A. This phylogenetic tree was supported with 1000 bootstrap replications.

Figure 3.1: Amino acid alignment of *Nematostella vectensis* Hsp70 isoforms. NvHsp70A, NvHsp70B, and NvHsp70D were aligned using Clutal Omega. Red lettering indicates the area of most variance in the C-terminal of the Hsp70.

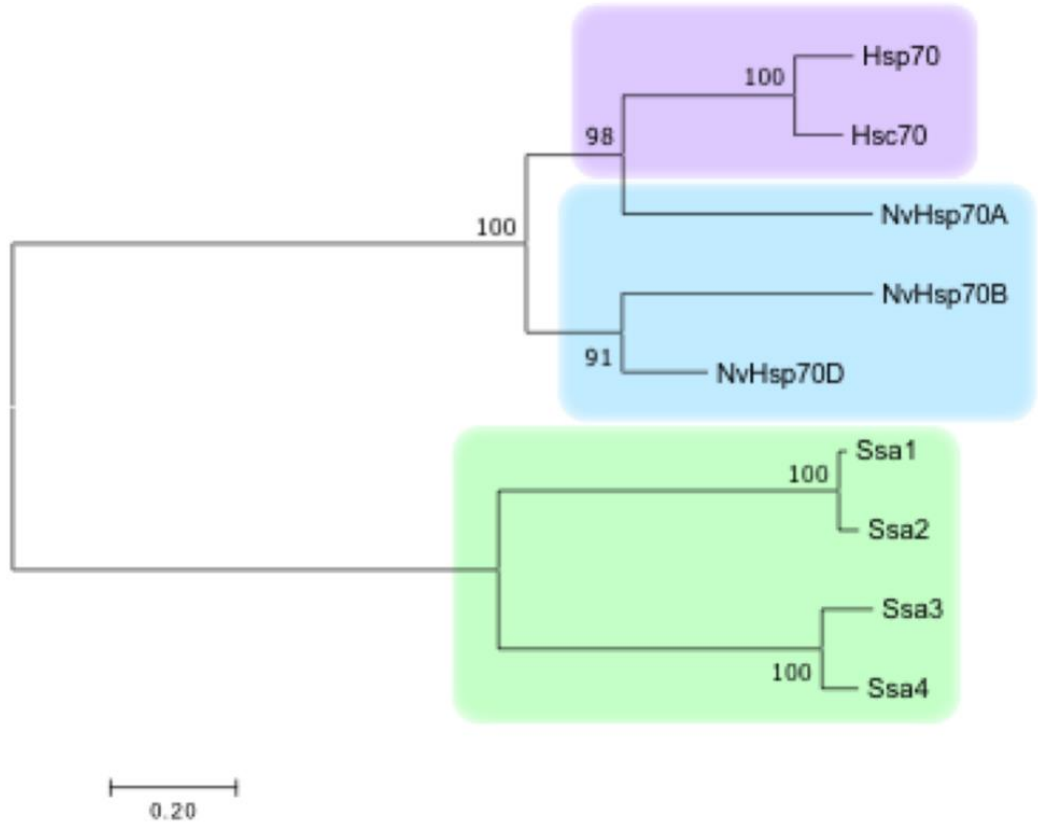


Figure 3.2: Phylogenetic analysis of NvHsp70 isoforms. Phylogenetic analysis of the Hsp70 isoforms in humans, yeast, and *N. vectensis*. Sequences of the isoforms were aligned using Clustal Omega and arranged into a NJ phylogenetic tree using the software Mega 7. The results show 1000 bootstrap replications. The numbers in the interior nodes represent in what percentage of replicates the associated taxa were joined together. The branch lengths are drawn to scale in the same units of evolutionary distances as determined by phylogenetic analysis. Lengths were computed using the Poisson correction method in the units of number of amino acid substitutions per site analysis. There were a total of 639 amino acid positions in the final dataset.

3.2.2. EXPRESSION OF NVHSP70 ISOFORMS UNDER ACUTE AND CHRONIC HEAT STRESS

The habitats of *N. vectensis* experience relatively large daily (>20°C) and seasonal (>25°C) changes in temperature. The acclimation and adaptation to diverse environmental regimes may include adjustments in both threshold and magnitude for induction of an Hsp70 response if the quantity of particular isoforms is specific for particular stressors.

Additionally, because different populations of *N. vectensis* show divergent organismal-level responses to abiotic variation based on their own environmental baselines (Reitzel et al., 2013), the induction of each NvHsp70 isoform was tested. The aim was to determine if each isoform's induction correlates with particular thresholds for each population. The selected testing temperatures followed the daily oscillations in temperature for a typical late spring or summer day, where temperatures recorded by field-deployed loggers have a low of 20°C and can increase to 36°C or more by the hottest point of mid-day. Experimentally, this temperature increase follows a trend of 2°C increased per hour (Fraune, Foret, & Reitzel, 2016; Reitzel et al., 2013). *N. vectensis* specimens from 3 different geographic populations (ME, MA, and NC) were exposed to "acute" and "chronic" high temperatures. The "acute" temperatures mimicked a temperature gradient these organisms experience in nature, and the "chronic" temperatures represented a more extreme shift in temperature that would active the heat shock response. All 3 geographic populations of *N. vectensis* displayed similar trends in transcriptional upregulation for each NvHsp70 isoform, however, each isoform responded differently from each other to the treatments (Fig 3.3). NvHsp70A transcription gradually increased during acute heat stress at both 28°C and 36°C measurements. When exposed to the chronic 36°C heat stress, NvHsp70A showed a similar increase in transcription to the acute stress of the same temperature. NvHsp70B transcription did not respond differently to acute temperature exposure at 28°C in comparison to the 20°C treatment, but there was a robust increase in transcription during the 36°C acute stress. There was a similar increase in transcription in NvHsp70B during exposure to chronic heat stress at 36°C, indicting a potential temperature threshold to activate the heat stress response that is higher than that of NvHsp70A.

NvHsp70D showed constitutive transcription when challenged by acute heat stress, with no discernable difference in transcription when measured at 20°C, 28°C, and 36°C. However, when exposed to the chronic heat stress at 36°C, NvHsp70D showed an induced increase in transcription compared to the specimens held at 20°C.

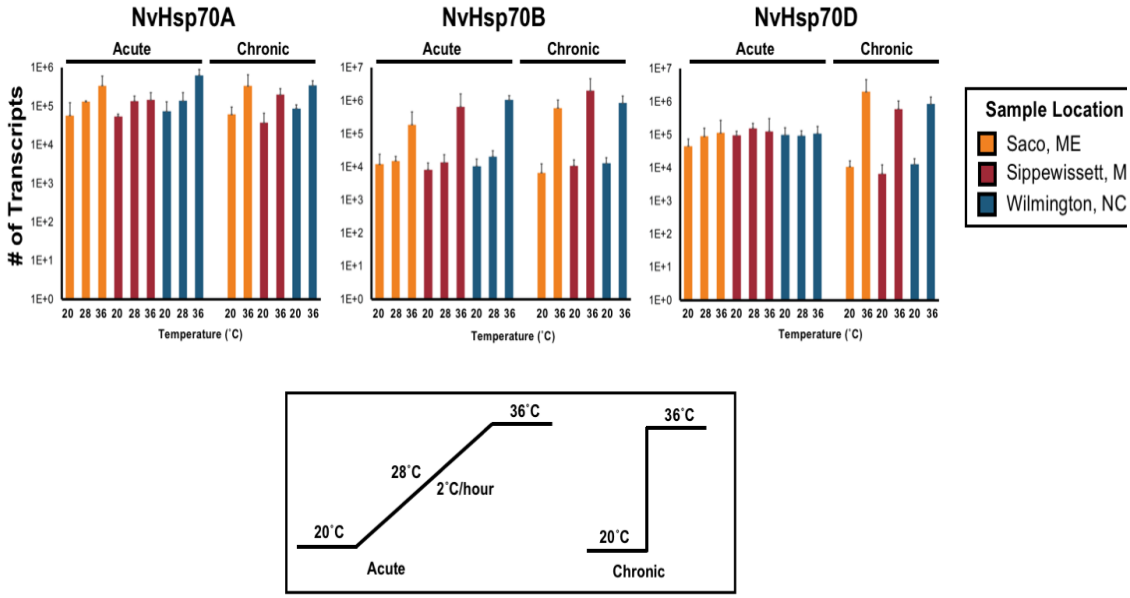


Figure 3.3: NvHsp70 isoforms display differential expression patterns under chronic and acute heat stress. RNA extracted from NvHsp70A, B, and D genes were converted into cDNA and analyzed using quantitative RT-PCR. Exposure to acute (28°C and 36°C) and chronic (24 hours at 36°C) temperature stresses elicits upregulation of all 3 NvHsp70 genes from each population. Results shown are the average of data obtained from 3 animals.

3.2.3. NVHSP70 ISOFORMS PROVIDE ESSENTIAL HSP70 FUNCTION WHEN EXPRESSED IN YEAST

The lack of both efficient gene editing and *N. vectensis* specific antibody reagents makes the direct study of proteins in this organism technically challenging. Therefore, to examine potential functional differences among NvHsp70 isoforms, they were expressed

in a yeast model system. NvHsp70 isoforms were transformed into an MH272 strain of the *Saccharomyces cerevisiae* budding yeast. The MH272 yeast Ssa1-4 genes were knocked out, and relied on a URA3 plasmid containing Ssa1 to survive the transformation (Jaiswal et al., 2011). After transformation of cells with plasmids expressing NvHsp70 isoforms from a LEU2-based constitutive GPD promoter, the original Ssa1 plasmid was removed using 5-FOA curing. In contrast to cells expressing the empty pAG415GPD-ccdB control, which did not grow and showed a lack of a second site suppressor effect, cells expressing the NvHsp70 isoforms were able to confer functionality in the yeast to keep it alive. While these cells were slower growing than cells expressing the Ssa1 plasmid, the data suggests that NvHsp70A, B, and D at least minimally complement essential Hsp70/Ssa1 function when expressed in budding yeast.

3.2.4. NVHSP70 ISOFORMS ARE FUNCTIONALLY DIFFERENT WHEN EXPRESSED IN YEAST

Hsp70 mediates the cellular response to a wide variety of abiotic stressors such as high temperature, DNA damage, pollution, and heavy metal exposure (Truman et al., 2015; Verghese, Abrams, Wang, & Morano, 2012; Wang, Gibney, West, & Morano, 2012). Having established that the NvHsp70-expressing yeast was capable of survival and growth by complementing essential Hsp70 function, the next step was to examine differences between NvHsp70 isoforms by performing various screens. There was a control plate containing YPD media that was incubated at 30°C, which is the optimal environment for yeast growth. The NvHsp70-expressing yeast was screened for resistance to NaCl (osmotic stress), UV radiation, H_2O_2 and HU (DNA damaging agents), $CdCl_2$ and $CuCl_2$ (heavy metal exposure), and high temperature (37°C). Experimental and control plates were

incubated for 3 days in a 30°C incubator (with the exception of the heat stress plates, which incubated for 3 days in a 37°C incubator). Cells that expressed NvHsp70A were the slowest growing, even in control settings, and were the most sensitive to all stressors tested (Fig 3.5). Cells expressing NvHsp70B conferred the most similar results to the cells containing Ssa1, with cells displaying minimal sensitivity to all agents examined (Fig 3.5). Interestingly, cells expressing NvHsp70D conferred an intermediate phenotype, falling somewhere between NvHsp70A and B for stress resistance. NvHsp70D cells were sensitive to NaCl and hydroxyurea relative to WT Ssa1- and NvHsp70B- expressing strains (Fig 3.5). Taken together, this suggests that even when transcriptional changes are compensated for (by constitutive expression in yeast), NvHsp70A, B, and D display differing functionality in vivo.

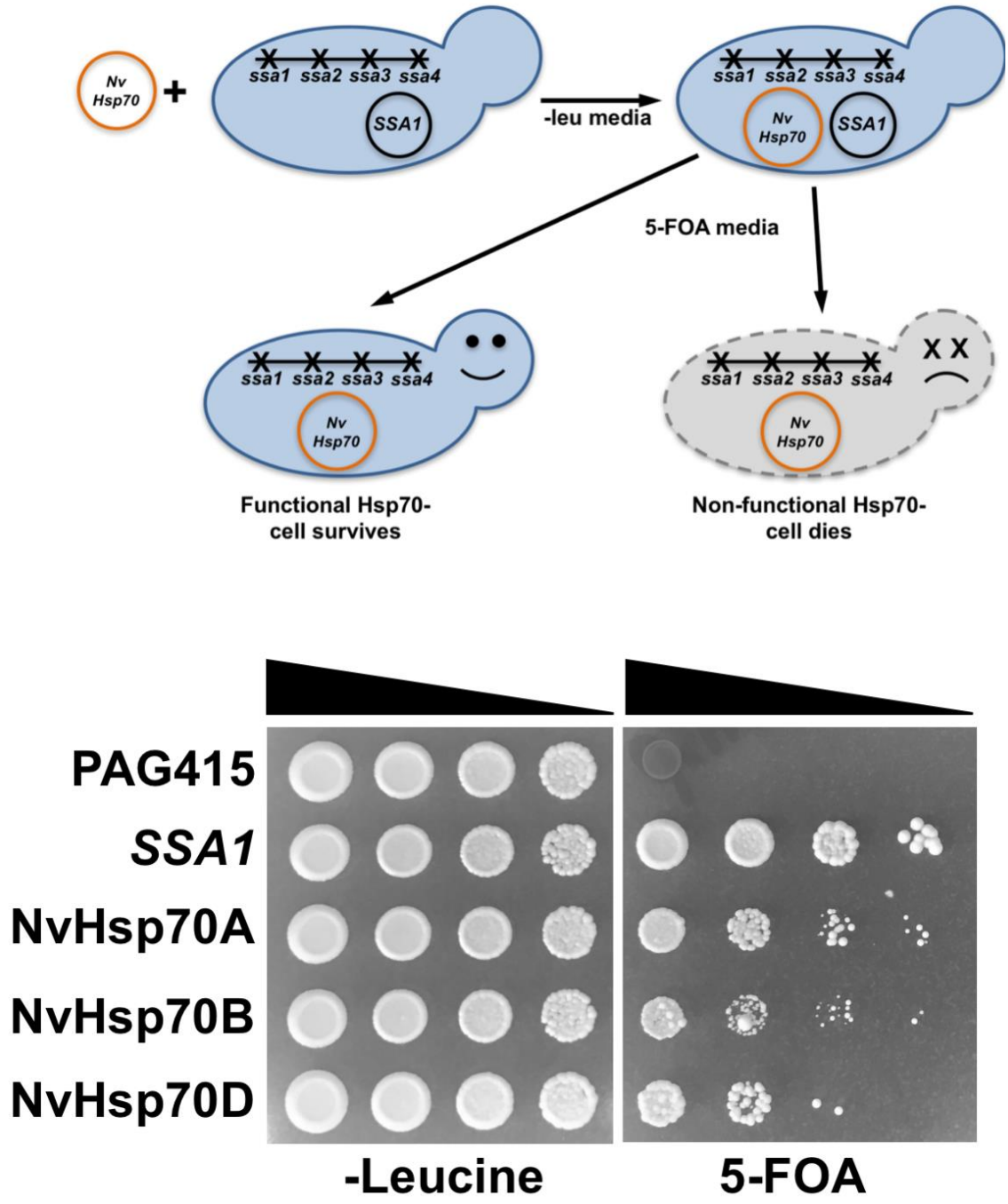


Figure 3.4: NvHsp70 isoforms provide essential Hsp70 function in yeast. 10-fold serial dilutions were performed with either control plasmid pAG415-ccDB (top row) or plasmids expressing SSA1/NvHsp70 isoforms. Dilutions were plated onto both media lacking leucine or containing 5-FOA before being incubated for 3 days at 30°C. Results were then photographed.

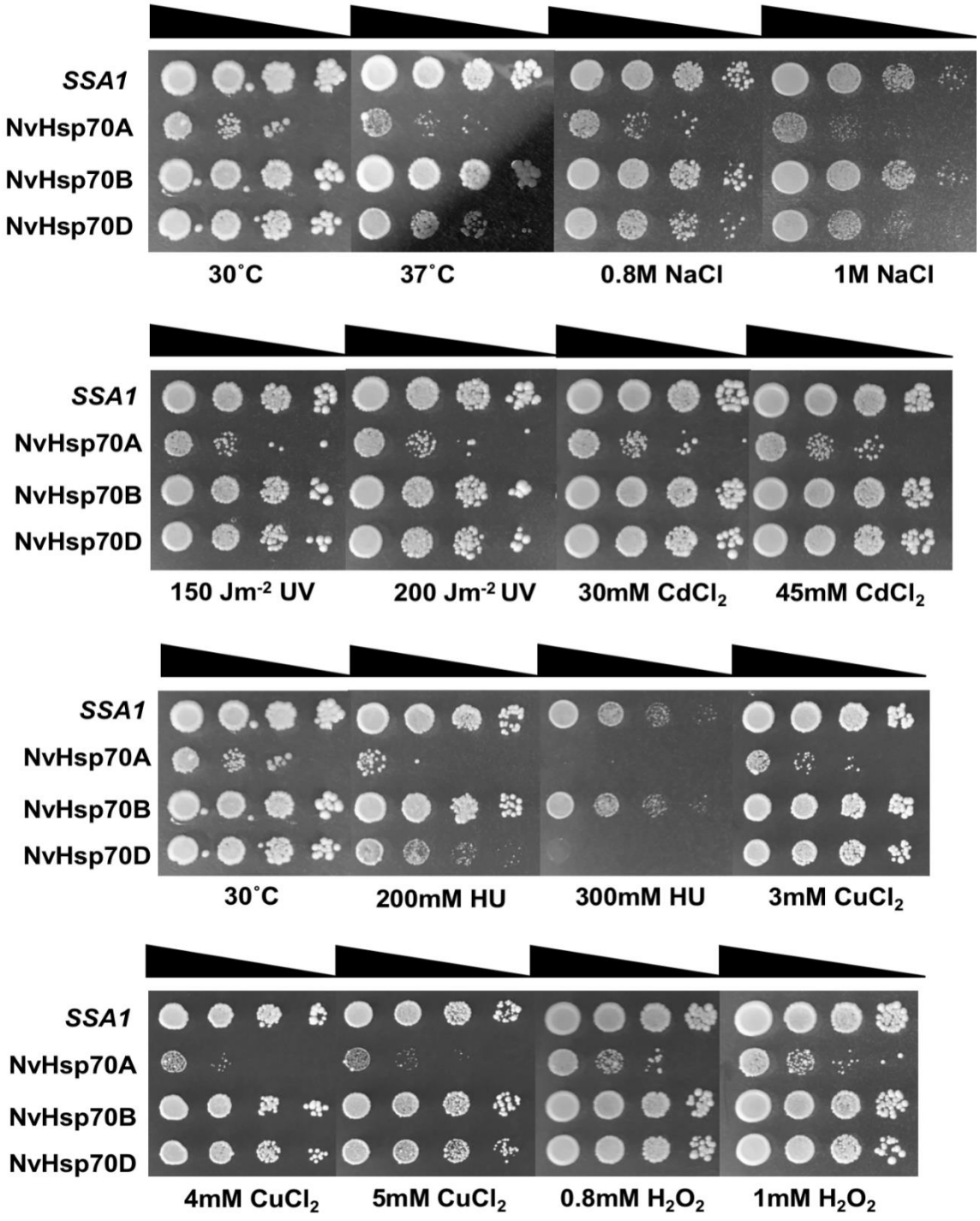


Figure 3.5: Yeast expressing NvHsp70 isoforms have altered stress resistance. Cells expressing either Ssa1 or NvHsp70A, B, or D as the sole cytosolic Hsp70 were grown to mid-log phase. 10-fold dilutions of the yeast were plated onto plates containing stress agents. Plates were incubated for 3 days at 30°C (except for the plate conferring heat stress, which was incubated for 3 days at 37°C) and photographed.

3.3. DISCUSSION

The differential transcription of HSPs by various organisms that have been exposed to stressful conditions has resulted in the general conclusions that these are general stress response proteins. NvHsp70 has been shown to be dynamically expressed under a variety of conditions in prior studies, including diel light exposure, metal stress, and UV exposure (Elran et al., 2014; Oren et al., 2015; Tarrant et al., 2014). In this study, *N. vectensis* Hsp70 isoforms vary both in their *in vivo* functionality and in the way they are induced under stress. *N. vectensis* adults were exposed to acute stress by incorporating a gradual temperature change to their environment that would mimic the temperature changes they experience in their natural habitats. Under these conditions, NvHsp70A was induced the earliest at 28°C, NvHsp70B was induced after exposure to 36°C, and NvHsp70D was expressed constitutively across all acute temperatures. The more nuanced expression profile for isoforms NvHps70B and NvHsp70D may indicate tightly orchestrated regulation of transcription that is dependent on an accumulation of cellular stress over a prolonged environmental exposure. Dramatic differences in expression profiles of any NvHsp70 isoform between populations were not observed. As a shallow water estuarine species, *N. vectensis* populations in all locations experience dramatic daily temperature fluctuations that have varying means depending on latitude. Future experiments that study even higher temperatures or lower temperatures may reveal more dramatic differences in transcription between populations that were not captured in this study.

Although molecular chaperones have been studied for over 50 years, the evolutionary reason for maintaining multiple isoforms of the Hsp70 gene has not been concluded. Previously, it was thought that most isoforms of a chaperone gene were

functionally identical, even though there were known unique transcriptional separators. Studies in both model organisms and mammalian cells have uncovered functional differences in chaperone isoforms (Hasin, Cusack, Ali, Fitzpatrick, & Jones, 2014; Millson et al., 2007; Prince et al., 2015). The sequence variations in client-binding domains of each NvHsp70 isoform may dictate the variety and number of client proteins that can be bound. Hsp70 isoforms may be induced to bind and stabilize specific client proteins in response to specific stressors, and these sequence differences may affect the regulatory capabilities of potential clients.

NvHsp70 isoforms were expressed from the same plasmid on a constitutive promoter in yeast in order to negate the possibility that the only difference in NvHsp70 isoforms was the pattern of expression upon stress. While all NvHsp70 isoforms could provide enough basal Hsp70 function to keep cells viable in the absence of Ssa1, there were basal differences in yeast growth with each isoform. The NvHsp70A-expressing yeast had the slowest basal growth, while NvHsp70B-expressing yeast showed the most similar basal growth to Ssa1-containing yeast. NvHsp70D-expressing yeast had an intermediate growth pattern. It is clear that NvHsp70A and NvHsp70D-expressing cells were compromised for several Hsp70-mediated stress responses, including high temperature growth and exposure to hydroxyurea. It is interesting to note that NvHsp70A aligns more closely with human, rather than yeast, Hsp70 isoforms. This may be one explanation for the poor growth and stress-resistance phenotype when NvHsp70A was expressed in yeast cells, even at basal levels, as the sole Hsp70.

Temperature resistance involves a complex coordination of the heat shock transcription factor HSF1 and associated regulating proteins. In both yeast and human cells,

Hsp70 directly regulates the activity of HSF1 through its binding patterns (Verghese et al., 2012). It may be that the HSF1-transcriptional program is controlled by specific NvHsp70 isoforms, or that NvHsp70 isoforms have intrinsically different levels of stability. If the isoforms have different intrinsic levels of stability, a high temperature environment may promote loss of NvHsp70 isoform activity. Hydroxyurea (HU) is a replicative stress agent that activates the DNA damage response (DDR) pathway in cells. Interestingly, HU directly targets the enzyme RNR, a known client protein of both heat shock proteins Hsp70 and Hsp90 (Truman et al., 2015). Because yeast cells expressing the NvHsp70A and NvHsp70D isoforms showed a reduced capability to grow on media containing HU, these isoforms may confer a loss of chaperone-RNR interaction and possible RNR destabilization. While beyond the scope of this study, we intend to examine this further by characterizing the global binding partners of each NvHsp70 isoform in *in vitro* and *in vivo* conditions using mass spectrometry technology.

CHAPTER 4: ANALYSIS OF NVHSP70 ISOFORM-CLIENT SPECIFICITY USING COMPARATIVE INTERACTOMICS

4.1. MATERIALS AND METHODS

4.1.1. YEAST CULTURE, STRAINS, AND HEAT TREATMENT

Yeast cultures were grown in YPD (1% yeast extract, 2% peptone, 2% glucose) supplemented with the appropriate nutrients to select for the NvHsp70 plasmid. The NvHsp70A, B, and D His-tagged sequences were transformed into *S. cerevisiae* yeast using the pAG415GPD-ccdB vector purchased from Addgene.

Untreated yeast was not manipulated in any way and incubated at the optimal temperature of 30°C. Heat shock treatment yeast were incubated in an environment of 39°C for 2 hours in order to elicit the heat shock response. Protein was extracted, run on SDS-PAGE gels, and Western blot analysis was performed using a His primary antibody.

4.1.2. PURIFICATION OF NVHSP70 INTERACTOMES FROM YEAST

100mL of yeast containing the NvHsp70 inserts was grown to an OD_{600} of 0.5 in YPD media. Cells were split into 2 flasks per isoforms: one flask per isoform was left untreated and incubated at 30°C, and the other flask was exposed to heat shock by incubation at 39°C for 2 hours. HIS-tagged NvHsp70A, B, and D along with their associated interactomes were isolated as follows: Protein was extracted via bead beating in 500uL of Binding/Wash Buffer (50mM Na-phosphate pH 8.0, 300mM NaCl, 0.01% Tween-20). 200ug of protein extract was incubated with 50uL His-Tag Dynabeads (Invitrogen) at 4°C for 15 minutes. Dynabeads were collected by magnet then washed 5 times with 500uL Binding/Wash buffer. After final wash, buffer was aspirated and the Dynabeads were incubated with 100uL elution buffer (300mM imidazole, 50mM Na-

phosphate pH 8.0, 300mM NaCl, 0.01% Tween-20) for 20 minutes, and then the beads were collected via magnet. The supernatant containing purified His-NvHsp70 isoforms was transferred to a fresh tube, 25uL of 5x SDS-PAGE sample buffer was added, and the sample was denatured for 5 minutes at 95°C. 10uL of sample was analyzed by SDS-PAGE and analyzed in a Western blot using a HIS primary antibody.

4.1.3. MASS SPECTROMETRY

Mass spectrometry was performed at the University of Chicago Proteomics Core Lab by Don Wolfgeher.

4.1.4. DATA ANALYSIS, STATISTICAL ANALYSIS, AND VISUALIZATION

Venn diagram showing unique interactors was created using curated lists of interactors for each NvHsp70 isoform and the online program Venny 2.1.0 (Oliveros, 2015). Gene Ontology analysis was performed using GO Slim Mapper on the Saccharomyces Genome Database (http://www.yeastgenome.org/cgi-bin/GO/goSlimMapper.pl). Interactome visualization was performed using Prism 7 graphing software for individual interactomes and comparative interactomes. Data points indicating an interaction of Ln >1 or Ln < -1 were colored according to various parameters.

4.2. RESULTS

4.2.1. QUANTITATIVE MASS SPECTROMETRY ANALYSIS OF NVHSP70 INTERACTOMES DURING HEAT STRESS RESPONSE

The interactomes of the NvHsp70 isoforms were characterized using mass spectrometry in order to determine their potential roles in the cell. Quantitative data regarding the number of interacting proteins each isoform can identify which interactors

are unique to each isoform. Yeast cells containing the respective *N. vectensis* isoforms were grown to mid-log phase and then either left untreated or exposed to heat shock conditions of 39°C for 2 hours. These conditions were chosen due to the ecological and evolutionary concerns of heat stress upon marine invertebrates. His-epitope-tagged NvHsp70A, B, and D proteins were pulled down along with any associated proteins with and without heat stress exposure. Quantitative proteomics was performed on these pulled down proteins. Using the software program Venny 2.1.0, a Venn diagram was produced giving percentages of unique and overlapping interactors (Fig 4.1). This diagram shows that in total, there are 1,171 interactors associated with the NvHsp70 isoforms. 549 of the interactors (46.9%) are shown to have interacted with all 3 of the isoforms. NvHsp70B has the most unique interactors at 211 (18%), and NvHsp70D has the fewest unique interactors at 38 (3.2%). NvHsp70A has 81 (6.9%) unique interactors. NvHsp70A and NvHsp70B share 240 interactors (20.5%), whereas NvHsp70A and NvHsp70D share 21 interactors (1.8%) and NvHsp70B and NvHsp70D share 31 interactors (2.6%). Overall, NvHsp70B has the most interactors, both unique and in total, whereas NvHsp70D has the fewest cumulative interactors. Of the 1,171 interactors present, NvHsp70A interacts with 891 (76.1%), NvHsp70B interacts with 1,031 (88.0%), and NvHsp70D interacts with 639 (54.6%). A unique interactor of note in NvHsp70 is the heat shock transcription factor Hsf1. Even in heat stress conditions, there is an interaction between NvHsp70A and Hsf1, which is counterintuitive to a heat stress response. Hsp70 and Hsf1 negatively regulate each other's function in wild-type conditions due to their interacting bond. In times of heat stress, Hsp70 and Hsf1 dissociate from each other. This allows Hsp70 to tend to the repair and refolding of proteins that are damaged due to this environmental condition,

while Hsf1's mechanism of action as a transcription factor transcribes more Hsp70 genes (Morimoto, 2011; Shamovsky & Nudler, 2008).

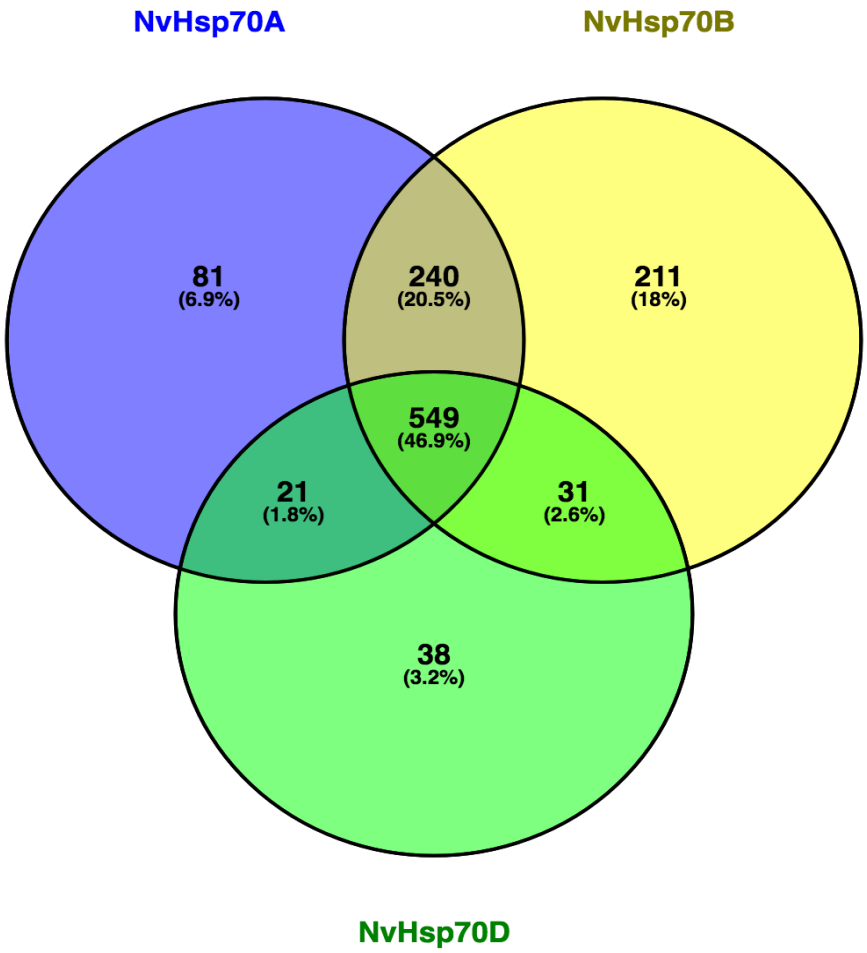


Figure 4.1: Venn diagram of candidate yeast NvHsp70A, B, and D interactors remaining after applying statistical filters. Quantitative display of unique interactors for each NvHsp70 isoform, as well as a display of how many interactors are shared between the isoforms.

4.2.2. GENE ONTOLOGY (GO) ANALYSIS OF NvHSP70 INTERACTOMES

Gene ontology (GO) analysis of the candidate interactors of each NvHsp70 isoform revealed significant enhancement of multiple cellular functions. Cytoplasmic

translation was the most enriched GO term for NvHsp70A and D isoforms, which underlines the role Hsp70 isoforms have in folding newly created proteins (Albanese, Reissmann, & Frydman, 2010; Craig, Eisenman, & Hundley, 2003). All of the NvHsp70 isoforms interact with 33 out of the 56 (61%) ribosomal 40S subunits. NvHsp70A interacts with 54 out of the 76 (71%) ribosomal 60S subunit proteins. NvHsp70D interacts with 49 out of the 76 (64%) ribosomal 60S subunit proteins. Although cytoplasmic translation is only the second-most enriched GO term for NvHsp70B interactions behind lipid metabolic processes, 52 out of the 76 (68%) 60S ribosomal subunit proteins are considered its interactors (Fig 4.2).

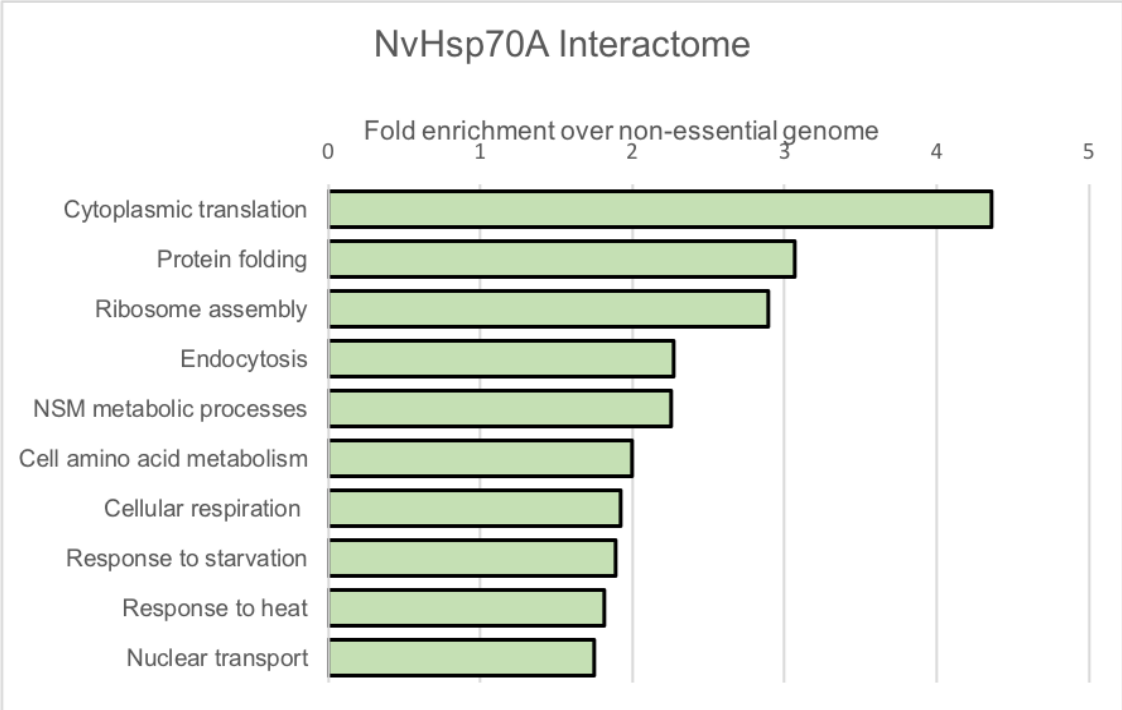


Figure 4.2: Gene ontology (GO) term analysis of NvHsp70A, B, and D interactors. Interactors of each isoform were characterized by cellular function using GO Slim analysis. Relative enrichment was calculated as compared to its occurrence in the non-essential genome. Continued on the next page.

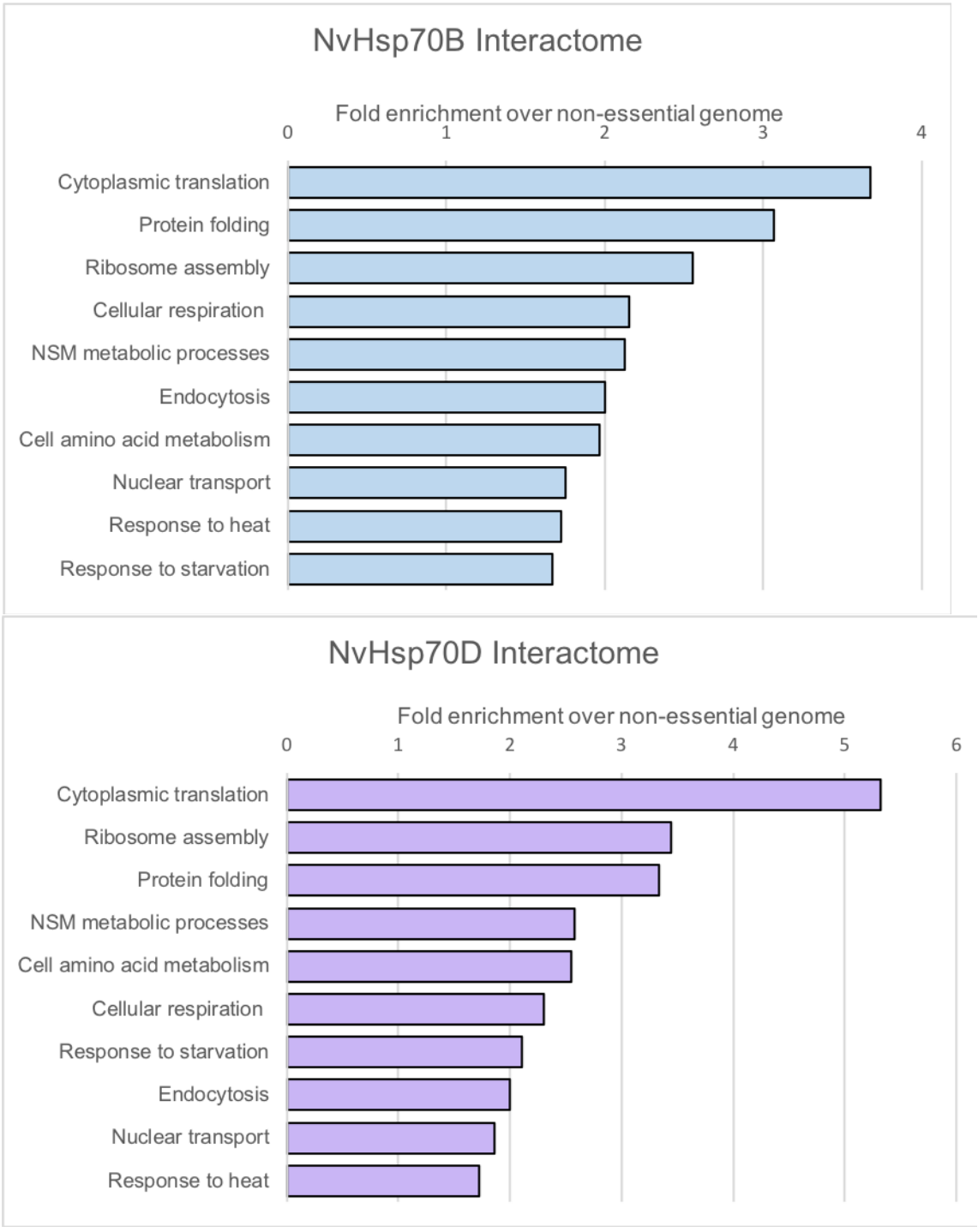


Figure 4.2 continued: Gene ontology (GO) term analysis of NvHsp70A, B, and D interactors.

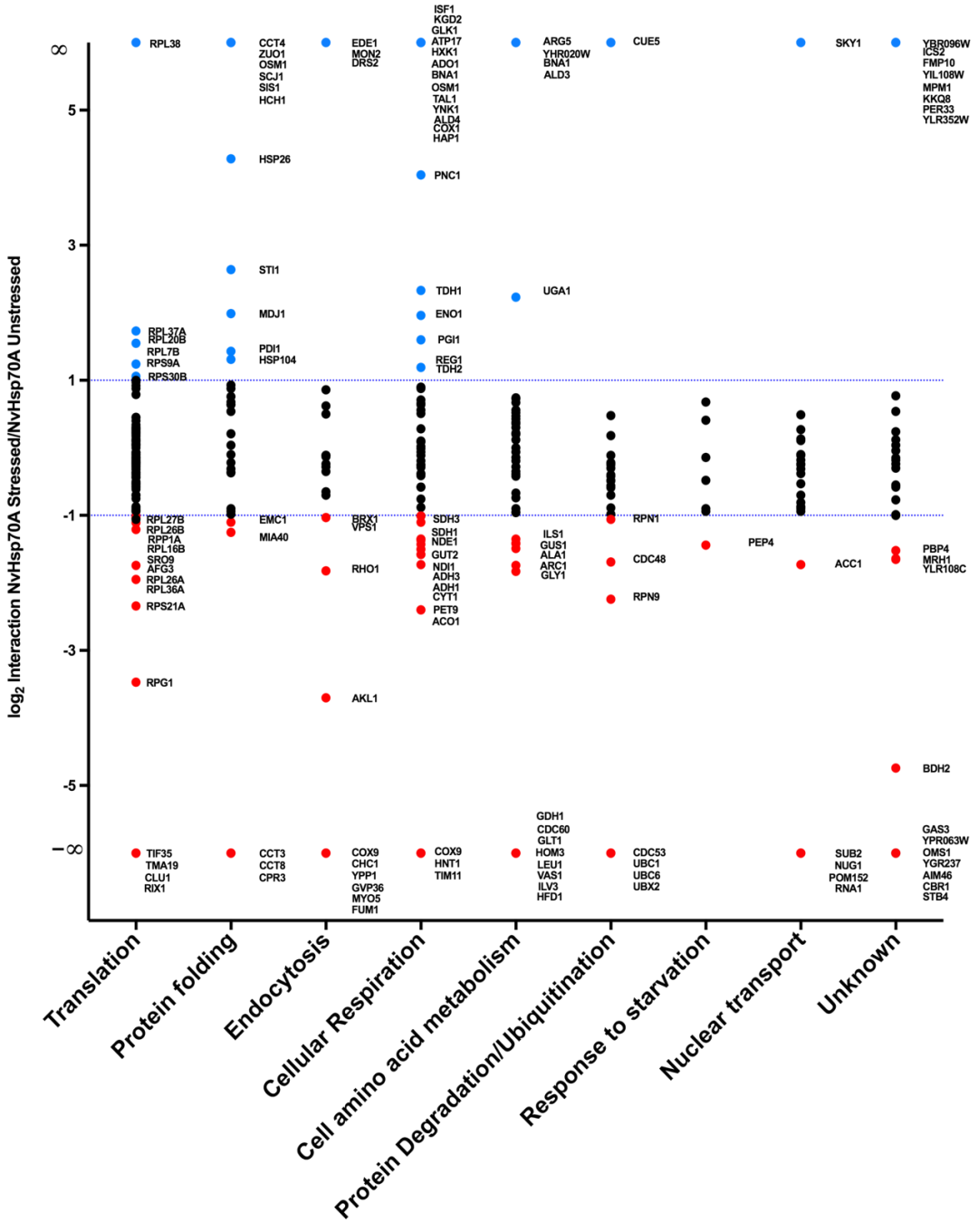
Similarly, the GO term “protein folding” was also enriched in all of the NvHsp70 isoforms’ interactomes. This reflects the wide range of chaperones and co-chaperones that bind to the NvHsp70 isoforms. Although NvHsp70A, B, and D were the only Hsp70/Ssa1 proteins localized to the cytosol, all of the *N. vectensis* isoforms interacted with other heat shock co-chaperones such as the ribosome-associated Ssb1 and the mitochondrial-associated Ssc1. All NvHsp70 isoforms also interacted with the Hsp90-encoding gene Hsp82.

4.2.3. ANALYSIS OF THE DYNAMIC NVHSP70 INTERACTOMES

To represent chaperone dynamics, the average natural log (Ln) ratio change in interaction upon heat stress treatment for each quantitated interacting protein was examined (Fig 4.3A-C). Any change in interaction that was a Ln of >1 or <-1 was considered significant. Values in between those parameters were classified as remaining unchanged. Interactors were sorted by non-redundant GO terms and graphed by average change in interaction with the NvHsp70A, B, or D isoform upon heat stress. Among NvHsp70A clients, 331 out of 644 (51%) interactions remained unchanged with 109 (17%) increasing and 204 (32%) decreasing after heat stress exposure. Among NvHsp70B clients, 311 out of 735 (42%) interactions remained unchanged with 378 (51%) increasing and 46 (6%) decreasing after heat stress exposure. Among NvHsp70D clients, 309 out of 492 (63%) interactions remained unchanged with 100 (20%) increasing and 83 (17%) deceasing after heat stress exposure (Fig 4.3).

Among the GO terms observed, “protein folding” and “cellular respiration” contained the most individual interactors that were significantly enriched. Between isoforms, there were different enrichment patterns with interactors: NvHsp70A had the

most interactors with increased interaction in the protein folding, cellular respiration, and unknown function categories. However, its most decreased interactors also occurred in the cellular respiration and unknown function terms. NvHsp70B, in comparison, has significantly enhanced interactions with proteins across all GO terms. Similar to NvHsp70A, the protein folding and cellular respiration terms contained the most increases. NvHsp70D had the fewest significant changes among protein interactions. Protein folding, cellular respiration, and cell amino acid metabolism had the most increases in individual interactions, but the cellular respiration category also had the most decreased interactions. This followed a similar pattern to the NvHsp70A isoform, but with fewer individual proteins as a whole.



A.

Figure 4.3: The dynamic NvHsp70A, B, and D interactomes during the heat shock response. Interactors were organized into functional categories following GO terms and plotted against interaction change (Ln ratio) with either (A) NvHsp70A, (B) NvHsp70B, or (C) NvHsp70D following heat stress treatment. The dotted lines represent an interaction chage of Ln > 1 or Ln < -1. Interactors are colored according to change in interaction as follows: blue (significant increase), red (significant decrease), or black (no significant change). Continued on the next 2 pages.

B.

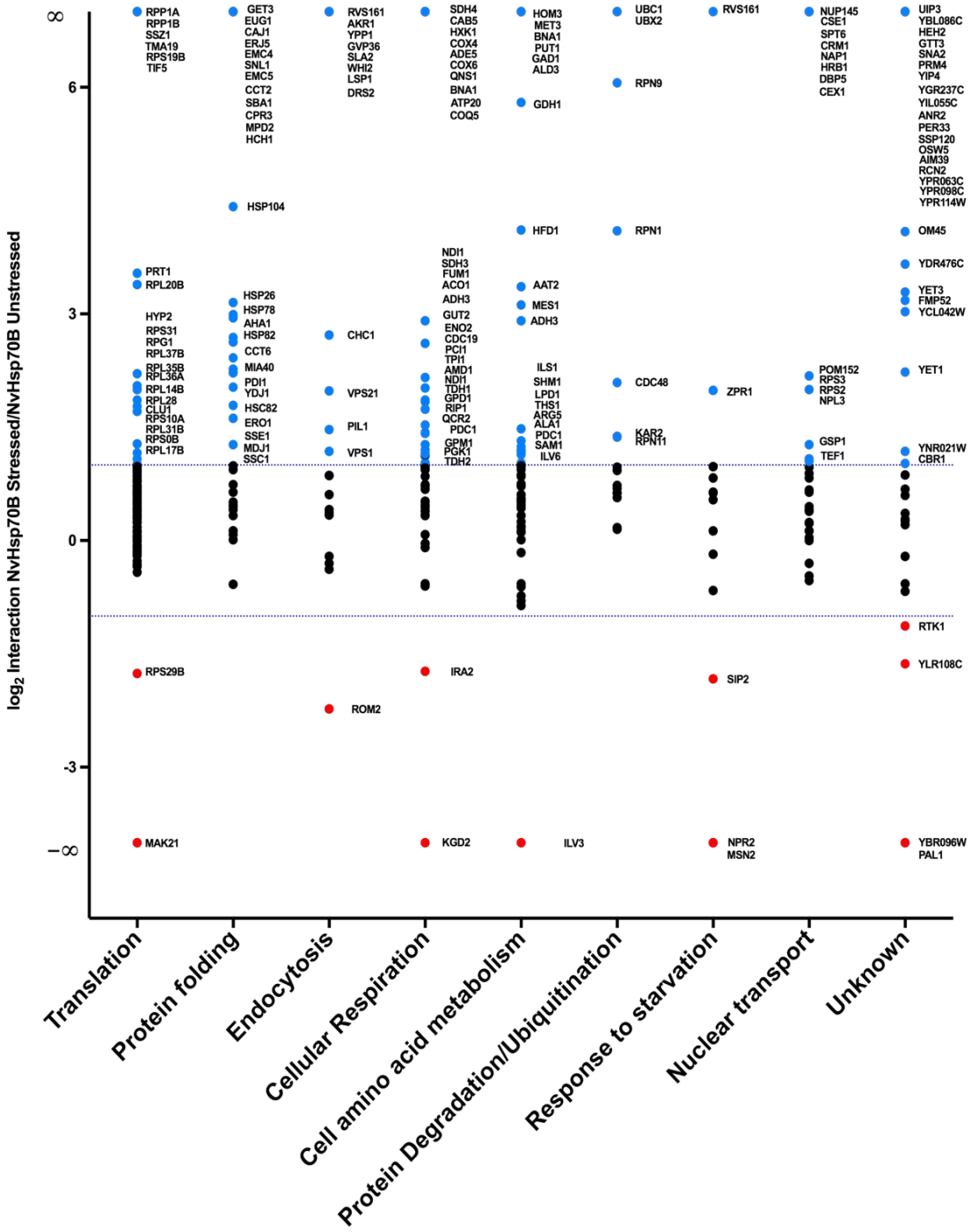


Figure 4.3 (continued): The dynamic NvHsp70A, B, and D interactomes during the heat shock response. Continued on the next page.

C.

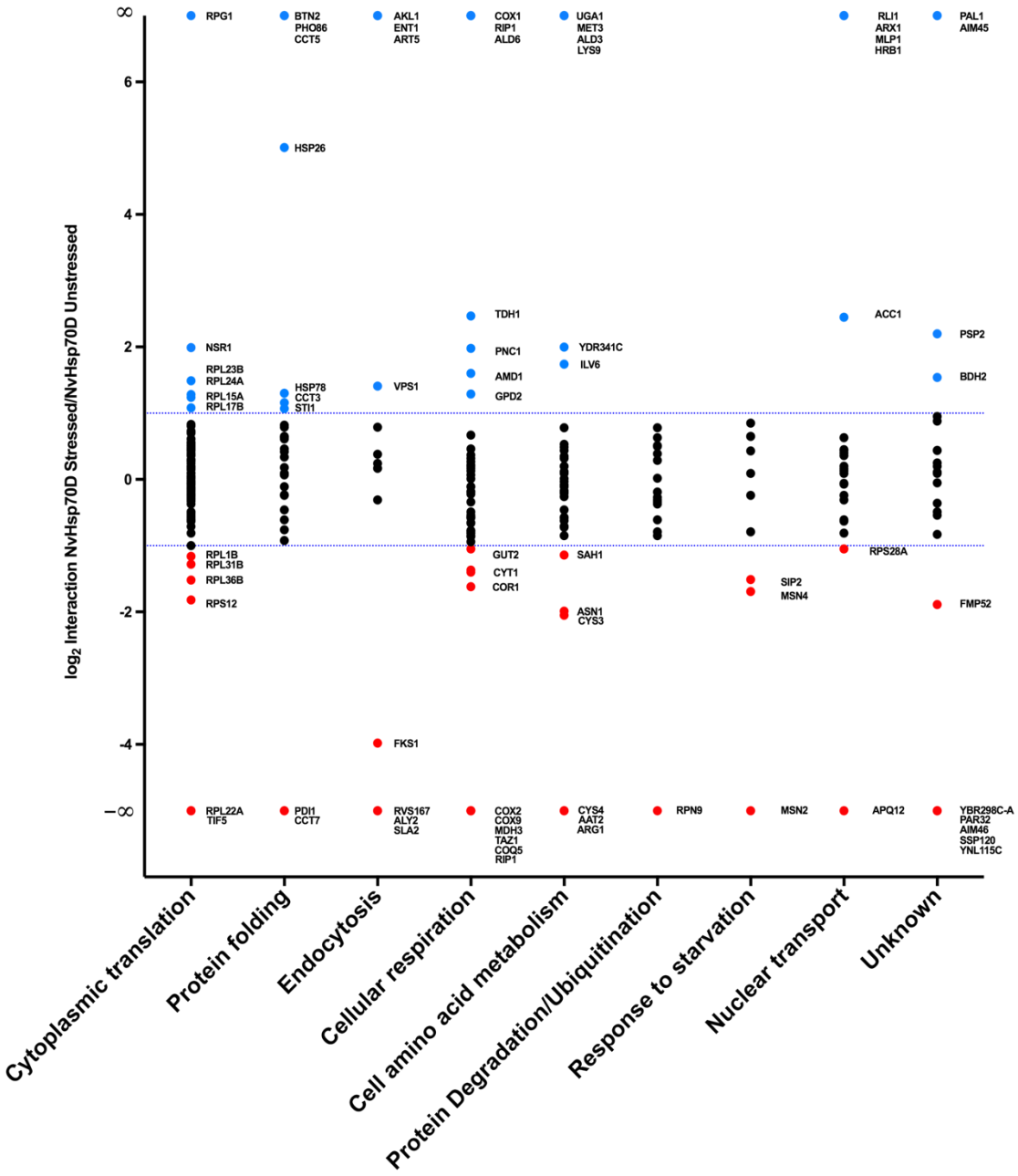


Figure 4.3 (continued): The dynamic NvHsp70A, B, and D interactomes during the heat shock response.

Molecular chaperones work in conjunction with other chaperones and regulators in order to facilitate the integrity and stability of folded and repaired proteins (Kim, Hipp, Bracher, Hayer-Hartl, & Hartl, 2013). As expected, these chaperones can be seen under

the GO term “protein folding” when exposed to heat shock stress, and their relationships with NvHsp70 differ by isoform. In NvHsp70A, the yeast co-chaperones Scj1, Sis1, Mdj1, Sti1, Hsp26, and Hsp104 have increased interactions after stress (Fig 4.3A). NvHsp70B had increased interactions with the co-chaperones Sba1, Hsp78, Hsp82, Sse1, Mdj1, Hsp26, and Hsp104 (Fig 4.3B). Interestingly, although NvHsp70B showed the most increased interactions across all GO terms, including protein folding, it did not show increased interactions with the co-chaperones Scj1, Sis1, or Sti1. NvHsp70D only showed increased protein folding interactions with the co-chaperones Hsp26, Hsp76, and Sti1 (Fig 4.3C). Across the 3 isoforms, the only co-chaperone with an increased interaction observed among all NvHsp70’s was Hsp26 (Fig 4.3A-C). This co-chaperone is a small heat shock protein (sHSP) that suppresses the aggregation of unfolded proteins and has mRNA binding activity (Petko & Lindquist, 1986; Susek & Lindquist, 1990).

4.2.4. ANALYSIS OF THE COMPARATIVE NVHSP70 INTERACTOMES

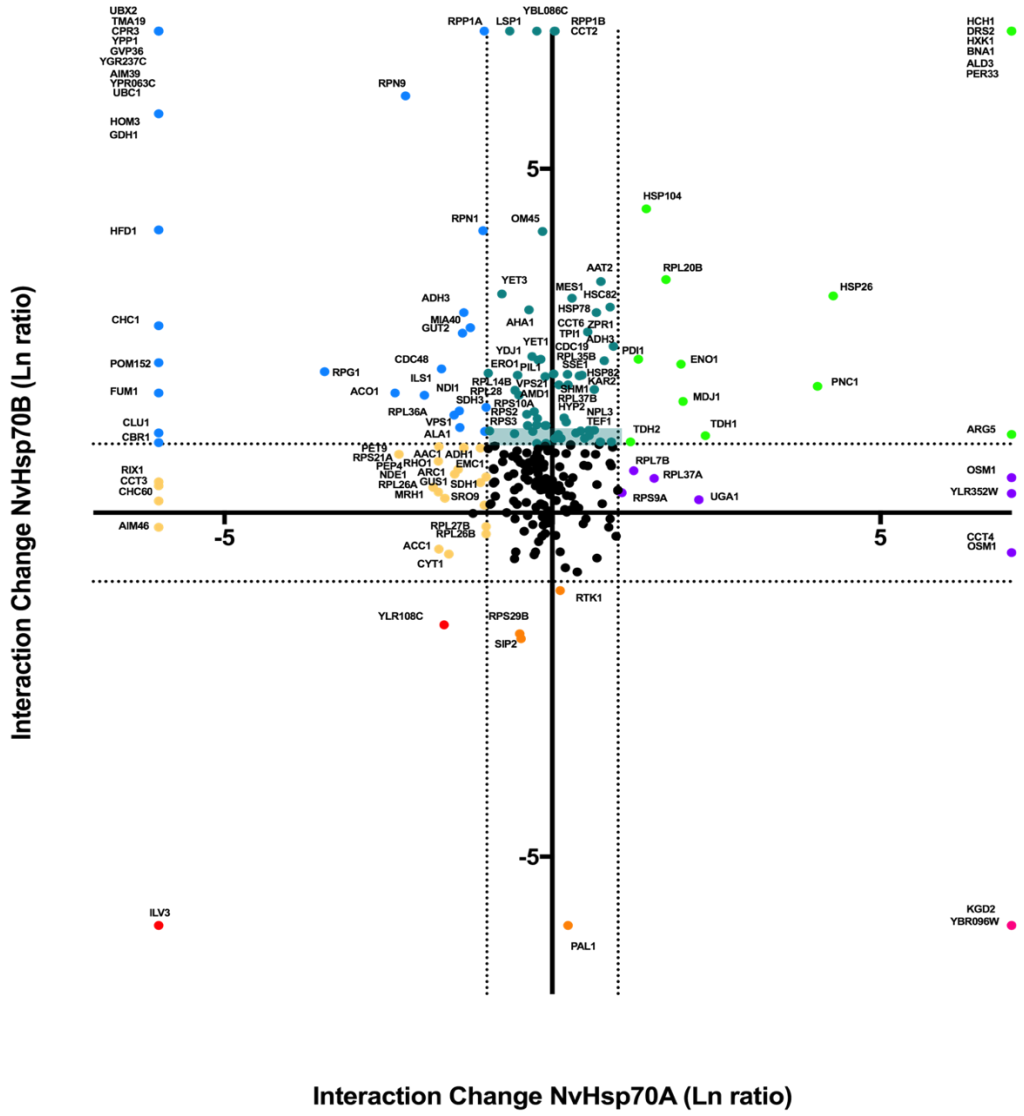
In order to determine common dynamic interactors among the NvHsp70 isoforms, comparative interactomes were plotted to demonstrate binding activity of interactors between the isoforms. Each isoform was plotted against each other in a separate comparative interactome in order to determine similar and opposing binding patterns. The interaction changes for each isoform exist as the Ln ratio seen in the interactomes shown in Fig. 4.3A-C. The comparative interactome of NvHsp70A and NvHsp70B has the most data points because of the large number of interactors both isoforms have. The majority of significant data points are either positive for NvHsp70B only (teal points in Fig. 4.4A) or positive for NvHsp70B yet negative for NvHsp70A (blue points). The least common data points were decreased interactions in both isoforms (red points) and those that had

an increased interaction with NvHsp70A but a decreased interaction with NvHsp70B (pink points). Each of these groups contained 2 proteins: YLR108C and ILV3 among the red points, and KGD2 and YBR096W among the pink points. ILV3 plays a role in the biosynthesis of branched-chain amino acids, and would therefore be found under the GO term for cell amino acid metabolism (Velasco et al., 1993). KGD2 is a component of the TCA cycle, and therefore has a role in cellular metabolism (Reinders et al., 2007; Repetto & Tzagoloff, 1990). Interestingly, both of the proteins YLR108C and YBR096W have unknown functions at the time of writing (Huh et al., 2003).

The NvHsp70A and NvHsp70D comparative interactome has the fewest number of plotted points. The majority of points were unchanged and towards the center of the plot (black points in Fig. 4.4B). Behind that, the next largest category of data points were interactors that decreased in NvHsp70A, but remained unchanged in NvHsp70D (yellow points). In general, the trend of this comparative interactome was for interactors that contained an increase or decrease in just one isoform but elicited no change in the other isoform were more common than interactors that affected both isoforms. This includes interactors that showed increased binding with NvHsp70A yet no change in NvHsp70D (purple points) and interactors that showed an increase or decrease in NvHsp70D yet no change in NvHsp70A (teal and orange, respectively). There was only one interactor that increased in NvHsp70A but decreased in NvHsp70D (the pink point): this protein was Pdi1. This protein is essential for disulfide bond formation in secretory and cell-surface proteins, and it falls under the GO term “protein folding” (Noiva & Lennarz, 1992).

Similar to the NvHsp70A vs. NvHsp70B comparative interactome seen in Fig. 4.4A, when NvHsp70B and NvHsp70D are compared, the majority of plotted points that

indicate change occur in the domain of an increased NvHsp70B interaction. Although there are many interactors that have not encountered an interaction change in either isoform (black points in Fig. 4.4.C), there is also a large number of interactors that only increase in NvHsp70B and are unaffected in NvHsp70D (purple points). Due to the large number of interactors with increased interaction in NvHsp70B, there are also many plotted points noting increased interaction in both isoforms (green points) and increased interaction in NvHsp70B yet decreased interaction in NvHsp70D (pink points). Although there are some interactors that increase or decrease in NvHsp70D with no change to NvHsp70B (teal points and orange points, respectively), there are very few points in which in interaction change is seen in NvHsp70D while NvHsp70B decreases. There is only one interactor that increases in NvHsp70D while decreasing in NvHsp70B (Pal1), and there are only two interactors that decrease in both NvHsp70B and D (Sip2 and Msn2). Sip2 is involved in the starvation response of the cell, and Msn2 is a transcriptional activator that is induced in times of stress (Ashrafi, Lin, Manchester, & Gordon, 2000; Martinez-Pastor et al., 1996). Pal1, although unique in its interaction pattern with NvHsp70B and NvHsp70D, has a yet-unknown function at the time of writing (Carroll et al., 2012; Huh et al., 2003).



A.

Figure 4.4: Analysis of comparative NvHsp70 interactomes in order to determine protein binding patterns common among isoforms. Interactomes were plotted comparing NvHsp70A to NvHsp70B (A), NvHsp70A to NvHsp70D (B), and NvHsp70B to NvHsp70D (C). The dotted lines represent an interaction change of Ln > 1 or Ln < -1. Because there are so many data points, interactors are colored according to change in interaction as follows: green (significant increase in both isoforms), purple (significant increase only on x-axis), teal (significant increase only on y-axis), pink (significant increase on x-axis, but significant decrease on y-axis), blue (significant increase on y-axis, but significant decrease on x-axis), yellow (significant decrease only on x-axis), orange (significant decrease only on y-axis), red (significant decrease in both isoforms), and black (no significant change). Continued on the next 2 pages.

B.

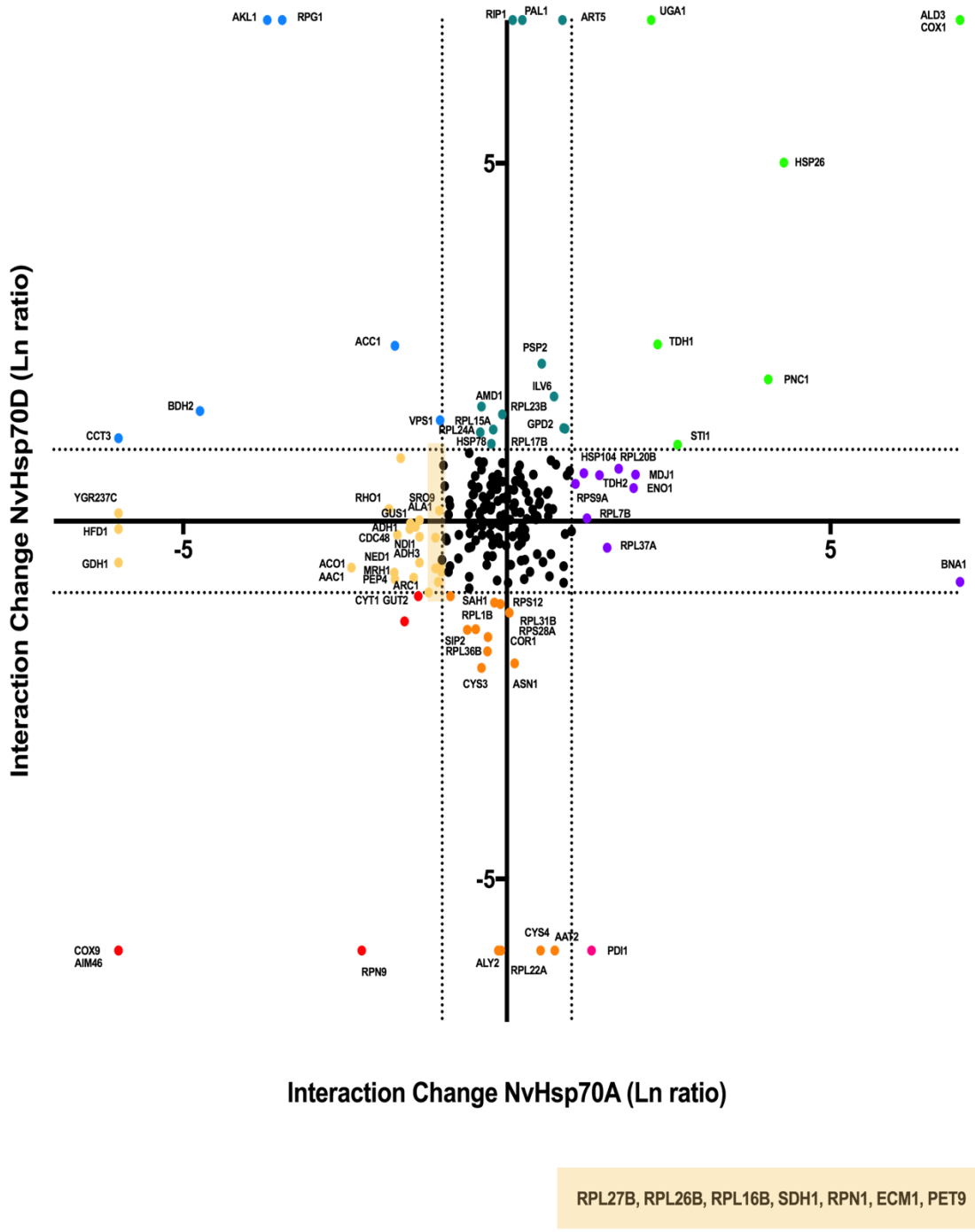


Figure 4.4 (continued): Analysis of comparative NvHsp70 interactomes in order to determine protein binding patterns common among isoforms. Continued on the next page.

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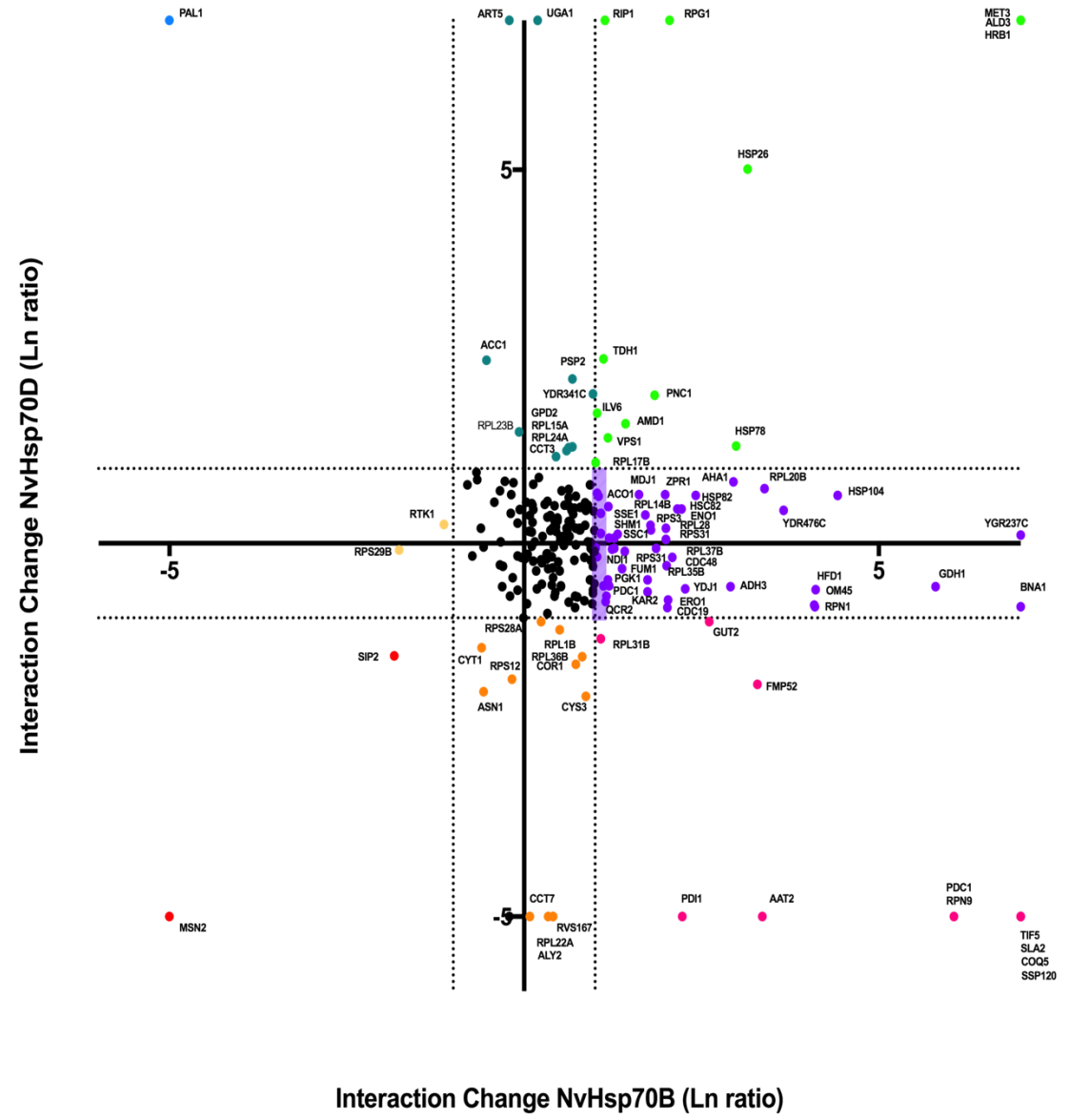

Figure 4.4 (continued): Analysis of comparative NvHsp70 interactomes in order to determine protein binding patterns common among isoforms.

4.3. DISCUSSION

Specific signaling pathways are activated by cells in response to stress. A crucial element of stress response, in this experiment heat shock, is the interaction of clients with molecular chaperones such as Hsp70. Clients and chaperones associate and disassociate with each other in order to maintain stability, proper activity, and correct cellular localization. These changing associations can be affected by heat stress, and are shown to have the potential to differ from interactions that occur in situations lacking a stressor. In this study, it was found that the change in interaction between control and heat-stressed NvHsp70 proteins and their clients differs among the cytosolic isoforms NvHsp70A, B, and D. In both of the isoforms NvHsp70A and NvHsp70D, the majority of interactions remained unchanged between the treatment conditions, however, more disassociations occurred than new associations in the presence of heat stress. In the isoform NvHsp70B, the majority of interactions again remained unchanged between treatments, however, there were more new chaperone/client interactions than disassociations in the presence of the heat stress treatment. This trend is especially highlighted in Fig 4.4A and Fig 4.4C, where the comparative interactomics favor whichever axis is representing NvHsp70B.

The chaperone-client interactions decreasing during heat stress implies that there is an important regulatory effect in the dissociation process. Even though Hsp70 binds and folds denatured proteins to help them regain lost activities, there are instances in which dissociation is important for proper function of the chaperone-client system. In some cases, proteins have been too damaged for the chaperone to repair. In these instances, Hsp70 targets the damaged proteins for degradation via the ubiquitin proteasomal system. Therefore, loss of Hsp70-client interaction can influence overall

cellular stability or destruction depending on the circumstances and conditions (Cyr, Hohfeld, & Pattereson, 2002; Wegele, Muller, & Buchner, 2004). Although a chaperone-client interaction may be important to cellular function under normal conditions, it may be preventing full client activity or more beneficial chaperone interactions under times of stress. In fact, stressing yeast cells with high temperature causes a heat shock response cascade to occur. This response is called the heat shock response (HSR), and it is an ancient and conserved transcriptional regulatory program that results in immediate induction of HSP's to protect and repair the cellular proteome (Wang et al., 2012). In all eukaryotes, including *S. cerevisiae* and presumably *N. vectensis*, the HSR is mediated primarily by HSF1, a heat shock transcription factor family. The HSF1 then binds to heat shock elements (HSE's) that exist in the promoters of target genes such as Hsp70 and Hsp90, another heat shock protein that has significant roles in stress response (Morimoto, 2008). This process is regulated by the Hsp70 chaperones themselves, because Hsp90/Hsp70 complexes repress HSF1 activation through its binding patterns under normal conditions. Upon encountering stressors, the Hsp70/Hsp90 complex dissociates from the HSF1 in order to promote its full transcriptional capabilities (Duina, Kalton, & Gaber, 1998; Harris, MacLean, Hatzianthis, Panaretou, & Piper, 2001; Zou, Guo, Guettouche, Smith, & Voellmy, 1998). The interactome of *N. vectensis* isoform NvHsp70B shows large increases in interaction upon heat stress exposure that may be due in some part to an effect in interaction with components of the HSR such as HSF1. Further studies of the NvHsp70 isoforms in order to investigate their regulatory capabilities of the HSR may show more dissociation between the NvHsp70B isoform and Hsf1 than in the other isoforms.

Chaperone-client interaction increases reveal additional information regarding the regulatory interactions of the NvHsp70. For example, NvHsp70B was the only isoform that conferred an increase of Ln ratio < 1 with the yeast Hsp40 homolog YDJ1. The Hsp40 co-chaperone family, also known as J domain-containing proteins, can assist in modulation and function of Hsp70 family proteins (Kampinga & Craig, 2010). In the NvHsp70B isoform, this increased interaction with YDJ1 may assist in the stability and growth of NvHsp70B-expressing yeast seen in prior work compared to the other *N. vectensis*-expressing cells (Waller et al., 2018). Interestingly, the yeast heat shock co-chaperone STI1 was only found to increase in NvHsp70A and NvHsp70D isoforms. Increased interaction with STI1 leads to increased activation of Ssa1 ATPase activity, eliciting a faster rate of heat shock response (Nicolet & Craig, 1989; Wegele, Haslbeck, Reinstein, & Buchner, 2003). All of the NvHsp70 isoforms showed an increased interaction with the sHSP Hsp26, which is a chaperone produced in the event of heat shock that assists in the recovery of misfolded proteins (Cashikar, Duennwald, & Lindquist, 2005). Thus, this increased interaction across all isoforms in the heat stress treatments exemplifies a well-conserved heat shock response between Hsp70 and Hsp26.

With only 46.9% of total interactors discovered using proteomics being common to all 3 NvHsp70 isoforms, unique interactors may explain differences seen functionally in NvHsp70-expressing yeast (Waller et al., 2018). Observing the dynamics of NvHsp70 interactomes adds a level of information to isoform difference studies that cannot be examined from other methods such as functional assays. Given that these chaperones have critical roles in *N. vectensis* protein function upon exposure to environmental stress, further study of each's isoform's dynamic interactomes under other abiotic stressors may

reveal further information about isoform differences in the study organism. Using yeast as a model, shared interactions with proteins between isoforms after heat stress reveal candidates for proteins that most effectively confer cell viability in stressful conditions.

4.4 CONCLUSION

After conducting experiments to explore both the phenotypic functionality of NvHsp70 isoforms in budding yeast, as well as to investigate the interactomes of each NvHsp70 isoform using mass spectrometry, clear differences between the isoforms emerge. The isoform with the fewest unique interactors, NvHsp70D, may act as a "housekeeping" gene that is constitutively expressed in order to maintain normal cell function. Evidence for this includes the transcriptional analysis of the isoforms, in which the NvHsp70D isoform across all 3 populations of *N. vectensis* retained constant transcription levels even when faced with acute stress. Although transcription of NvHsp70D increased in the face of chronic stress, immediate response did not occur in the wake of an environmental change. Yeast cells expressing NvHsp70D in control conditions were capable of growth that was similar to the control of Ssa1, showing that this isoform contains an interaction behavior that is conducive to cell viability. NvHsp70D maintained the fewest interacting proteins of 639, and 38 of these were unique to this isoform. Compared to the other 2 isoforms, NvHsp70D has the fewest unique interactors. This further supports the argument that NvHsp70D is interacting mainly with necessary entities for viability and growth. Its phenotype when expressed in yeast was hardy in the presence of most environmental stressors compared to NvHsp70A-expressing specimens, but did show more loss than the NvHsp70B-expressing results.

In contrast, there is evidence that the NvHsp70B isoform is an inducible response to immediate environmental stress. Transcriptionally, it showed a drastic increase in transcription levels when exposed to the acute and chronic temperatures of 36°C. At this level, there is an immediate response to the environmental heat stress. When tested in yeast, NvHsp70B-expressing cells not only grew similarly to Ssa1 in control conditions, but also was able to confer stability in the face of multiple stressors. Even when exposed to 300mM HU, on which NvHsp70D-expressing yeast could not grow efficiently, NvHsp70B-expressing yeast was still viable. In the proteomic results seen in Chapter 4, NvHsp70B has the most interactors at 1,031, and 211 of these are unique. When exposed to heat stress, there is a trend for the entire NvHsp70B interactome to shift upward, as even more proteins increase in interaction than even remain unchanged. As described in the discussion, this may be due to the dissociation of Hsf1 and NvHsp70B occurring easily in the face of environmental heat stress.

NvHsp70A has an interesting heat shock pathway, according to its interactor profile. One of its unique interactors is Hsf1, which interacts with NvHsp70A even under the condition of heat stress. This is unusual, because the dissociation of Hsf1 and Hsp70 is crucial for the protein repair and refolding mechanisms associated with heat shock proteins. Additionally, this dissociation of Hsf1 from Hsp70 allows for the Hsf1 to act as a transcription factor in the production of more Hsp70 genes. With NvHsp70A interacting with Hsf1 even under heat stress, it is unlikely that this isoform provides much stress-response protection and repair to the cell. This can be seen in NvHsp70A yeast, which even faces difficulties growing in control conditions. When exposed to environmental stress, this isoform consistently underperforms when compared to the

NvHsp70B and D isoform-expressing yeast. Considering neither of the other isoforms conferred Hsf1 interaction under stress, it can be concluded that the Hsf1-mediated heat shock response pathway is responsible for these differences.

In conclusion, from the work presented in this thesis, clear functional differences are seen in the transcriptional patterns of *N. vectensis*, as well as NvHsp70-expressing yeast phenotypes. Interaction patterns uncovered through the use of proteomic technology and analysis has identified NvHsp70D as constitutive heat shock protein that aids in maintenance of baseline activity. NvHsp70B is an immediate response that becomes more upregulated and active in the face of environmental stress, acting as a "rescue" protein. NvHsp70A is a suppressing force in the heat shock response pathway, and has a markedly different relationship with its own transcription factor than the other isoforms.

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