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Characterizing the Role of HspB2 in Cardiac Metabolism and Muscle Structure Using Yeast and Mammalian Systems

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ABSTRACT

Characterizing the Role of HspB2 in Cardiac Metabolism and Muscle Structure
Using Yeast and Mammalian Systems

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HspB2 is a small heat shock protein encoded on human chromosome 11. Less than 1000 base pairs away from HSPB2 and situated in a head-to-head orientation lies the gene encoding another small heat shock protein, CRYAB. Because they are uncommonly close to one another they share regulatory elements. In addition, they share protein homology as sHSPs, suggesting that they perhaps perform similar functions. SHSPs such as HspB2 and CryAB are traditionally thought to provide protective effects to cells in response to a variety of stress inducers. In response to stress they form complexes around misfolded proteins or proteins in danger of denaturation. HspB2 has been shown to exhibit protective effects during cellular stress and to localize to the Z-line of skeletal muscle. It has also been implicated in cardiac energetics, specifically in the production of ATP, however little is known about its molecular targets. Here I report the use of yeast two-hybrid screening to uncover the molecular targets of HspB2. I also detail the process by which the screens are performed as well as the verification steps, including co-precipitation experiments in mammalian cells. Through these studies we identify many novel binding partners of HspB2, including CryAB as well as multiple muscle and mitochondrial proteins. Proteins discovered to bind to HspB2 include such proteins as actin and myosin, enzymes catalyzing various steps of glycolysis and the electron transport chain, as well as redox-, small heat shock protein-, kinase-, and electrolyte-related proteins, among others. Studies of the binding partners of HspB2 in cardiac tissue will provide important information clarifying the involvement of HspB2 in cardiac muscle maintenance and metabolism.

Keywords: small heat shock proteins, HSPB2, CRYAB, yeast two-hybrid, coimmunoprecipitation

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CHAPTER ONE: Introduction, Background, Specific Aims Summary

Introduction: sHSPs

Researchers have identified HspB2 as one of eleven presently-known small heat shock proteins (sHSPs) encoded in the human genome.[2, 3] Typically functioning as protein chaperones, heat shock proteins are primarily involved in protecting cells from a variety of stresses including shock related to extreme heat or cold, oxidative/reductive stress, and heavy metal-induced stress.[4] Cellular stress often causes proteins to misfold or to unfold. Proteins in these undesirable states bind to each other and form protein aggregates—nonfunctional protein complexes. The occurrence of protein aggregation causes organ dysfunction such as in cataract formation and amyloid fibril development leading to neurodegenerative diseases.[5] In an ATP-independent manner, sHSPs form complexes around misfolded or unfolded proteins in order to prevent protein aggregation.[6-9] Members of the small heat shock protein family, while similar in their general form and function, often exist in varying levels throughout the body, suggesting a specific and unique function from one another. Since the discovery of HSPB2 in 1997 researchers have tried to deduce its functional role, leaving us with a presently incomplete understanding of this small heat shock protein. In this chapter I will continue to provide an introduction of the family of sHSPs and will then specifically focus on our current understanding of HspB2, briefly reviewing various papers and focusing on its relationship with the heart. Following an adequate introduction to sHSPs and necessary background about HspB2, I will emphasize both the significance of HspB2 and that of our study.

Morphologically, sHSPs share many similarities, yet present knowledge of their structure remains incomplete. One reason for the lack of understanding is that none of the human sHSPs have yet been crystallized, however we can gain understanding from crystal structure studies of sHSPs found in *Methanococcus jannaschii* (HSP16.5), wheat (HSP16.2), and *Taenia saginata* (TSP36).[10-12] These studies shed light into heat shock protein fundamentals such as how heat shock proteins oligomerize and function as chaperones. However, Basha et al. note that there is no universal model to describe the structure, function, and mechanism of action of the sHSPs.[13] On a general level, sHSPs are structurally characterized by low monomeric molecular mass (15-43 kDa).[14-16] While each of the sHSPs are of differing lengths they share a conserved alpha-crystallin domain in their C-terminal regions.[17-19] Hsp27 (HspB1) and HspB5 (CryAB or αβ-crystallin) are among the most researched sHSPs. In a study of Hsp27, Lambert et al. suggest that the alpha-crystallin domain of Hsp27 is functionally involved in Hsp27-Hsp27 interactions, suggesting that this domain is involved in oligomerization.[20] The N-terminal region contains nonconserved sequences and commonly one or two regions rich in proline and phenylalanine called WD/EPF motifs.[21-23] Lambert et al. also show in the aforementioned study that the WD/EPF region is involved in oligomerization in response to phosphorylation of certain amino acid residues.[20] The C-terminal region of the alpha crystallin domain contains a flexible tail conferring solubility to the sHSPs and has been shown to regulate oligomerization .[24, 25]

From a molecular perspective HspB2 is the most divergent of the sHSP family, containing only 30% sequence identity to the other sHSPs (excluding HSPB11).[26] Although the sequence of HSPB2 is quite different than other sHSPs, it localizes to similar areas as several members of the small heat shock protein family (see .1 1).[27]

Protein	Alternate Name	Localization
HspB1	HSP27	Ubiquitous
HspB2	MKBP	Cardiac and Skeletal Muscle
HspB3	HspL27	Cardiac and Skeletal Muscle
HspB4	αA-crystallin	Eye Lens
HspB5	αB-crystallin	Ubiquitous
HspB6	Hsp20	Ubiquitous
HspB7	cvHSP	Cardiac and Skeletal Muscle
HspB8	Hsp22	Ubiquitous
HspB9	--	Testis
HspB10	ODF	Testis
HspB11	Hsp16.2	Ubiquitous

Table 1.1 SHSPs, their alternate names, and localizations in the human body. Adapted from Laskowska et al. 2010

sHSPs are generally expressed ubiquitously in the human body, although some are specific to certain organs. Many, while expressed in various tissues, are found at higher levels in some tissues than in others. HspB5 (αB-crystallin), for instance, is found throughout the body but it has higher abundance in the heart and eye, making up approximately 50% of the protein mass in the human lens.[28] SHSPs that localize to specific parts of the body include HspB2, HspB3, HspB4, HspB7, HspB9, and HspB10. While HspB2, HspB3, and HspB7 are expressed ubiquitously, they are especially localized to cardiac and skeletal muscle.[27] HspB9 and HspB10 are found solely in the testis.[29, 30] Understanding localization to different areas of the body and, more appropriately, the respective tissues and even the intercellular locations, will perhaps help us better understand the function of each of the sHSPs.

Background: HspB2

In the following section I provide background information specific to HspB2 in a more-or-less chronological manner, outlining key papers, experiments, and results that compose the current knowledge in the field, and especially include information that is pertinent to deducing the role of HspB2. HspB2 was first referred to as myotonic dystrophy binding protein (MKBP) since it was discovered bound to myotonic dystrophy protein kinase (DMPK).[31] It is more properly known as HspB2, thus I will refer to it as HspB2 regardless of what it was termed in the original literature.[15, 32, 33]

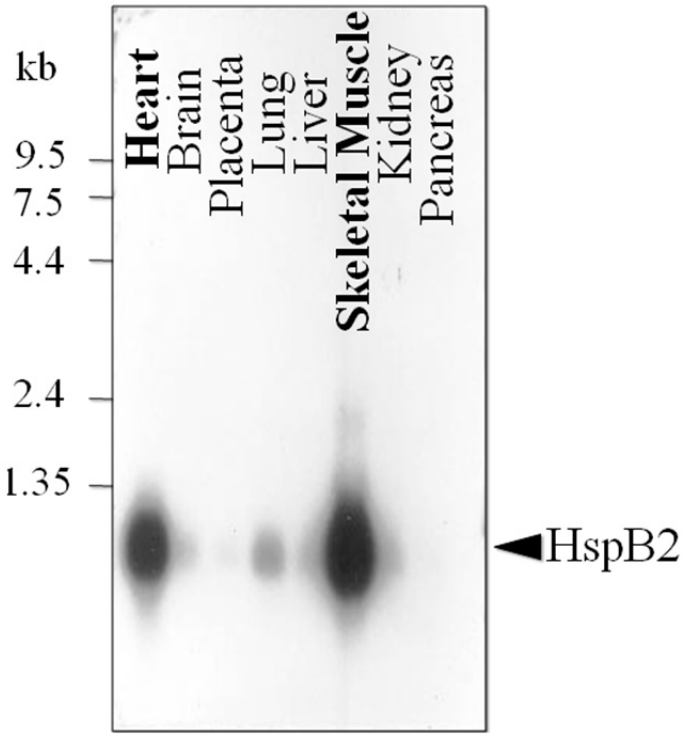

Figure 1.1 Northern blot showing that HSPB2 localizes to the heart and to skeletal muscle. Adapted from Suzuki et al. 1998.

As stated previously, HspB2 is one of eleven known sHSPs and is primarily expressed in the heart and skeletal muscle (see Table 1.1).[31] Using the yeast two-hybrid system—the same system we are using in our study—Suzuki et al. looked for binding partners of DMPK. DMPK was of particular interest because certain DMPK mutations cause myotonic dystrophy, an autosomal dominant disease manifesting various symptoms including myotonia and muscle weakness. Patients also often show cardiac conduction defects, cataracts, atrophy of the testicles/ovaries, diabetes, and mental dysfunction as a result of the disease.[34] The mutation responsible for the disease was located within DMPK, however it appeared to be in an

untranslated region of the gene, meaning that DMPK should function normally when expressed.[35-37] In hopes of understanding the role of DMPK, Suzuki et al. searched for binding partners using a cDNA library as the prey protein and DMPK as the bait in a traditionally yeast two-hybrid screen (Chapter 2). From this screen they uncovered HspB2 bound to DMPK. They also noted that HspB2 formed a complex with itself but not with several of the other sHSPs, including CryAB. Additionally they also noted that HspB2 localized to sarcomeric Z-lines and hypothesized that it may provide protection to the proteins found in this region. Unpublished data by Suzuki et al. reports that HSPB2 was upregulated in stretched neonatal mouse cardiomyocytes, also suggesting an involvement in muscle structure maintenance. This early look at HspB2 was surprisingly not the first discovery of the protein. Another lab a year earlier discovered a novel heat shock protein-encoding gene while investigating the region upstream of CRYAB.[32] The protein they found was identical to HspB2.[31]

Upstream of CRYAB, Iwaki et al. discovered another sHSP encoded by the chromosome.[32] They found that HSPB2 and CRYAB are situated in a head-to-head fashion on human chromosome 11, only 958bp from each other.[32] In this orientation they are transcribed in opposite directions. Iwaki et al. noted that, traditionally, genes transcribed in this manner share physiological and structural similarities and share regulatory elements.[38-41] Particularly interesting to their study is the fact that northern blot analysis of various rat tissues showed HspB2 localization to heart and femoral muscle tissues (see Figure 1.2). In the same northern blot experiment they found that CryAB also localizes to heart and femoral muscle, but is additionally expressed at a high level in lens tissue. They also noted that levels of HSPB2 expression were far less than that of CRYAB in all tissues where the proteins were both present. If, indeed, CRYAB and HSPB2 share regulatory elements, this finding begs the question of how

and why they are differentially expressed in various tissues. In contrast to differential expression, similarities may also exist between them; they noted that HSPB2 and CRYAB may share similar pathologic roles such as in mitochondrial myopathies of the muscle.[42, 43]

Yoshida et al. furthered the investigation of the physiologic and perhaps pathogenic role of HspB2 in a study identifying the intracellular locations of HspB2 in the ischemic heart.[44] They measured HspB2 quantity for three fractions of the cell: the nuclear-myofibril, membrane, and cytosolic fractions and found that, the longer the heart was under ischemic conditions, an increased amount of HspB2 was found in the nuclear-myofibril and a decreased amount found in the cytosolic fractions. The membrane fraction did not yield any HspB2 detection. In the same study they also noted that Hsp27 translocates in a similar manner to the nuclear-myofibril fraction, perhaps suggesting similar roles for the two sHSPs. Further immunohistochemical observation showed that both HspB2 and Hsp27 translocate to the myofibrillar Z-line. Yoshida et al. also noted that there was no increase in phosphorylation of HspB2 in the ischemic heart and that translocation occurs regardless of

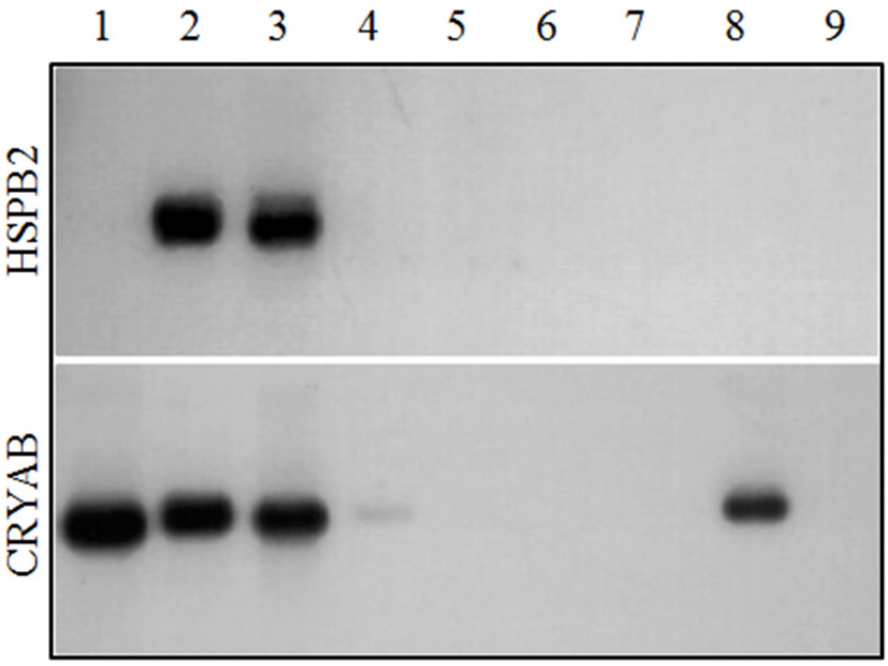

Figure 1.2. Northern blot of various rat tissue. 1, lens; 2, femoral muscle; 3, heart; 4, kidney; 5, brain; 6, thymus; 7, liver; 8, U-373MG cells; 9, HeLa cells. U-373MG is a glioblastoma/astrocytoma cell line.[1] Note that although CRYAB and HSPB2 share regulatory elements, CRYAB localizes to the eye lens while HSPB2 does not. Adapted from Yoshida et al., 1997.

whether cells were exposed to inhibitors of MAP kinase and protein kinase C. This suggests that perhaps the translocation of HspB2 is driven by an alternate phosphorylation-independent pathway. This study by Yoshida et al. is important because it points out similar roles for HspB2 and other sHSPs. CryAB, for instance, was also shown to translocate to myofibrillar Z-lines in response to ischemia.[45, 46]

In a collaborative work with Yoshida and others, Shama et al. showed increased levels of HspB2 and Hsp27 in neonatal rat heart myocardium.[47] These levels peaked one week after birth then decrease over the following weeks. As a comparison they also note that human levels of HspB2 in the heart follow a similar pattern, peaking between two and 11 months and remaining low in the following months and years. Also significant to the study by Shama et al. is the discovery that HspB2 and desmin colocalize to the myofibrillar Z-line. Desmin is an intermediate filament present in cardiac, skeletal, and smooth muscle and mutations in desmin have been shown to cause multiple muscle problems, including cardiomyopathy.[48-50] Additionally, Shama et al. showed that HspB2 also localizes to cardiac intercalated discs by immunofluorescent microscopy. In their discussion as to why levels of HspB2 are found to be increased in neonatal heart they note that during birth the heart goes through a period of ischemia followed by reperfusion as the rat begins to breathe on its own. This process introduces the heart to oxidative stress and could identify the need for increased sHSP expression.[51, 52] They also suggest that since in this study they observed high cytosolic levels of HspB2, perhaps the protein is involved in protecting actin or other cytosolic proteins that proliferate during the initial stages of life.

Aside from providing an overview of the five sHSPs known at the time, Sugiyama et al. reported the finding of an additional small heat shock protein, HspB3.[53] In a northern blot they showed that HSPB3 is also expressed mostly in the heart and in skeletal muscle (see Figure 1.3). Analysis by western blot showed that the new sHSP was found in heart and prostate tissues (see Figure 1.4). They also showed that HspB2 was found in rat heart and skeletal muscle tissues, in addition to a small amount found in rat thymus and colon tissues. Of all the other proteins analyzed, the researchers remarked that HspB2 and HspB3 show the most similar distribution patterns to each other. They also demonstrated that both HspB2 and HspB3 are induced at a similar time during a late phase of muscle differentiation. Notable is the fact that their expression was not induced by stress but by the myogenic factor MyoD. This provides evidence that sHSPs are not solely involved in stress-management but may also contribute to normal cellular differentiation and development. Continuing to develop the possible relationship between HspB2 and HspB3,

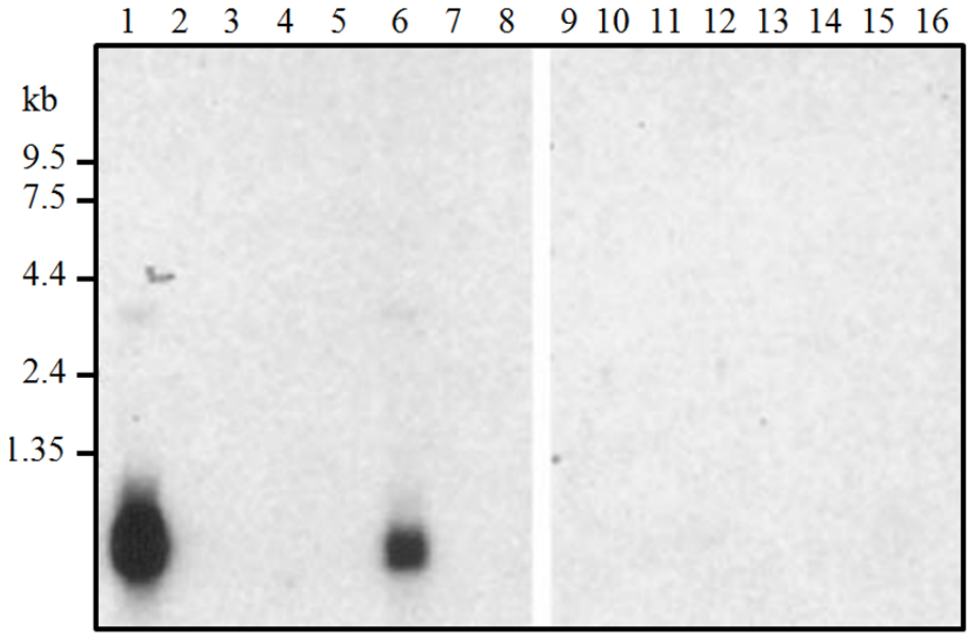

Figure 1.3. Northern blot showing the various expression levels in adult human tissue types. Tissues are as follows: 1, heart; 2, brain; 3, placenta; 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; 8, pancreas; 9, spleen; 10, thymus; 11, prostate; 12, testis; 13, ovary; 14, intestine; 15, colon; 16, blood. Adapted from Sugiyama et al., 2000.

Sugiyama et al. provide evidence of protein-protein interaction via the yeast two-hybrid. Importantly, they also report that HspB2 binds to HspB6, HspB3, but not CryAB. This was confirmation of a previous finding.[31] Sugiyama et al. further support the idea that sHSPs have differential function as they reported that several other sHSPs localize to cellular myotubes while HspB2 and HspB3 do not. In the final experiment of the paper, researchers studied whether these six sHSPs were induced by heat shock. They reported that while many sHSP's were, HspB2, HspB3, and HspB6 were not, also in part a confirmation of previous data.[31] They conclude that in order to identify the function of individual sHSPs we must understand their oligomerization states and how they translocate to the insoluble cellular fraction in the presence of certain stresses. In addition to understanding the binding partners of sHSPs it is important to understand the location of sHSPs within the cell. Nakagawa et al. continued to shed light on the important subject as they show where HspB2 localizes within the cell.[33] They chose cell lines KNS-81,

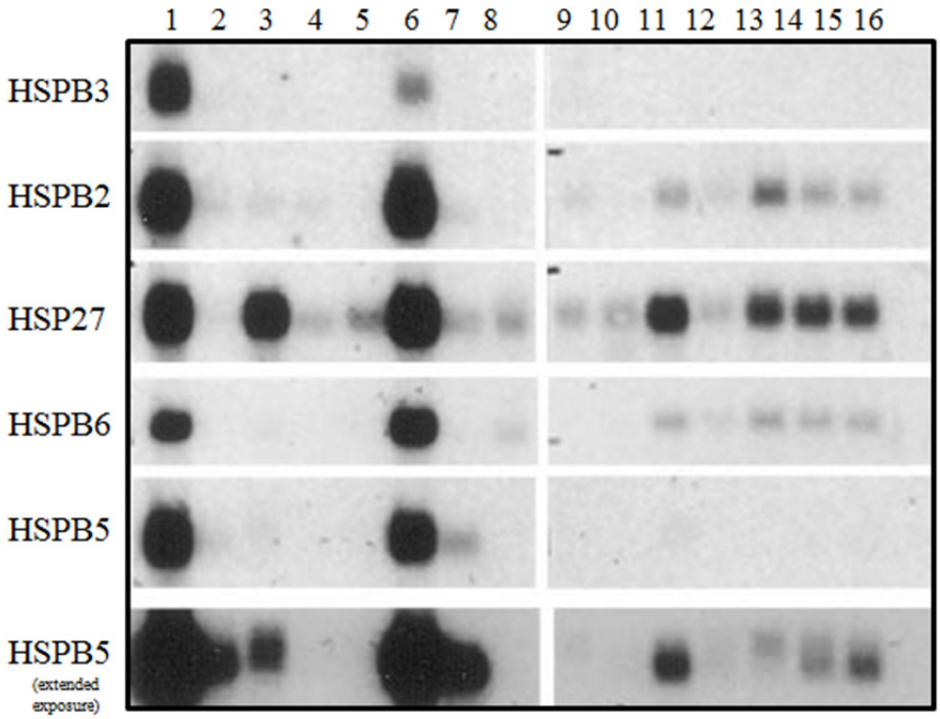

Figure 1.4. Comparison of expression levels for various sHSPs for human adult tissues. Note that HSPB5 is ubiquitously expressed, as shown by the extended exposure in the last row. Tissues are as follows: 1, heart; 2, brain; 3, placenta; 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; 8, pancreas; 9, spleen; 10, thymus; 11, prostate; 12, testis; 13, ovary; 14, intestine; 15, colon; 16, blood. Adapted from Sugiyama et al., 2000.

C2C12, and NIH3T3—cells derived from human malignant glioma, mouse myoblast cells, and mouse fibroblasts, respectively—as their model for subcellular localization. Using immunofluorescence microscopy and staining with a mitochondrial marker along with anti-Hsp60 and anti-HspB2 fluorescent antibodies, they showed that HspB2 localized to the mitochondria of KNS-81 cells (see Figure 1.5). They noticed general fluorescence around the

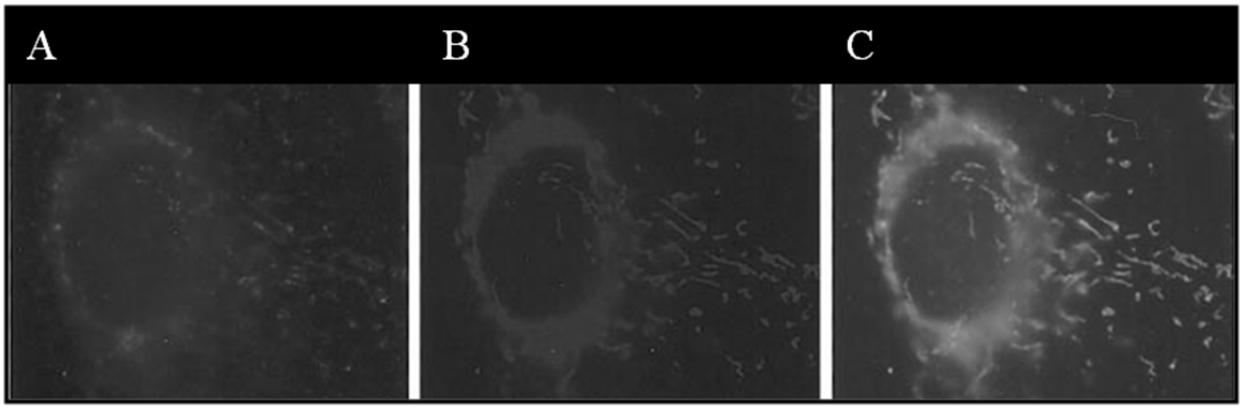

Figure 1.5. HSPB2 localizes to the outer mitochondrial membrane. Immunofluorescence imaging shows non-cardiac KNS-81 cells treated with 1μM colchicine at 37°C for two hours. A shows staining with anti-HSPB2 antibody; B shows staining with anti-human mitochondrial monoclonal antibody; C is the merged image showing colocalization of the two sHSPS. Adapted from Nakagawa et al., 2001.

mitochondria with patches of greater fluorescence within this background. To determine if these were showing HspB2 bunching around microtubules, they treated cells with colchicine, a microtubule depolymerizing reagent, and the clusters remained, suggesting mitochondrial association. To study the localization to the mitochondria more specifically Nakagawa et al. took the mitochondrial fraction and concluded that HspB2 most likely attaches loosely to the outer mitochondrial membrane rather than being transported inside the mitochondria, although they do not completely rule out the possibility. Other sHSPs locate to the mitochondrial matrix in other organisms, but there is no known human sHSP possessing a targeting signal specific to the mitochondrial matrix.[54-56] Importantly, Nakagawa et al. also note that exposure to increased heat (42°C) prompted a greater level of HspB2 localization to the mitochondria. They demonstrated that cells constitutively expressing HSPB2 lived through periods of heat shock that

were otherwise lethal to cells not expressing the sHSP. Interestingly, they also showed by northern blot that HSPB2 expression is favored more in slow-twitch muscle than in fast-twitch. They report that these levels coincide with levels of mitochondria in the different types of muscle cells, slow-twitch muscles having more mitochondria. A role in mitochondrial maintenance would not be unfounded for sHSPs since sHSPs have been shown to lend a protective role to NADH ubiquinone oxidoreductase during cellular stress.[57] In another study, Hsp27 associated with the mitochondrial membrane interacted with cytochrome c oxidase in apoptotic pathways, negatively regulating cell death.[58] In conclusion Nakagawa et al. state that to discover the function of HspB2, including any mitochondrial role, we must uncover its binding partners in the cell.[33]

Departing from localization and binding studies, Morrison et al. utilized a recently developed knockout (KO) mouse model for HSPB2 and CRYAB.[59] As discussed previously, CryAB is another small heat shock protein that is located nearby and shares a promoter region with HspB2.[32] In order to identify the function of sHSPs we must look at phenotypes following gene deletion. Unfortunately, in the first mouse models the HSPB2 gene deletion included disruption of the promoter region of HSPB2, likewise disrupting the promoter for CRYAB. Although this mouse model was imperfect at showing the individual sHSP functions of HspB2 and CryAB, it was the best model available at the time. Even though the authors could not show individual functions, they hoped to demonstrate the importance of these two sHSPs in cardiac development, structure, and function.[59] To begin to understand the importance of these sHSPs, Morrison et al. observed life span of wild type (WT) and knockout (KO) mice. They observed that KO mice had similar survival rates through 10 months, after which the KO mice survival rates took a steep decline (see Figure 1.6). The hearts of the deceased mice appeared normal, and they report that

their deaths were most likely related to malnourishment.[16] Although the hearts were actually of greater mass in the KO mice in comparison to their body weight, they discovered that this was not due to hypertrophy, but rather because the KO mice had a decreased weight. Surprisingly, the KO mice also had normal heart morphology at the microscopic level, showing the same organization of myofibrils and sarcomeres as found in WT mice. Moving into functional studies, they did not find a significant difference between WT and KO hearts using echocardiography, except for increased left ventricular contractility in KO mice. After subjecting the hearts to ischemia/reperfusion (periods of restricted blood flow followed by periods of resumed flow), they found that KO mice had a decreased recovery of contractility following the stress. Morrison et al. also observed greater levels of apoptosis and necrosis among KO mouse hearts following ischemia/reperfusion.

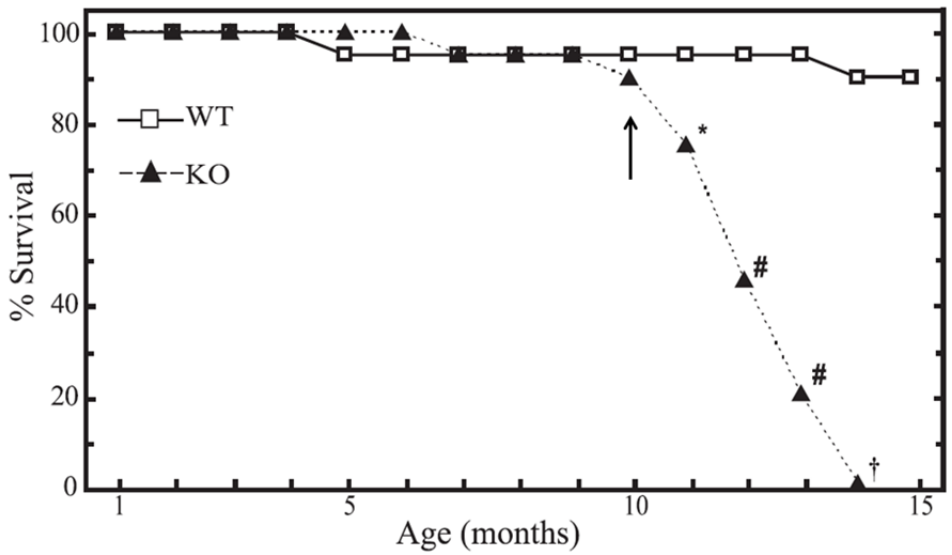

Figure 1.6. Percent survival rates for wild type mice and those without HSPB2 or CRYAB (KO). *, #, and † denote significance compared to wild type mice. Arrow denotes 10 month mark decline. Adapted from Morrison et al. 2004.

Reactive oxygen species form during periods of reperfusion and Morrison et al. hypothesized that during these times sHSPs may play an important protective role to the cells and tissues. Indeed, previous research shows that CRYAB overexpression protected the heart during periods of ischemia/reperfusion.[60-62] Additionally, Morrison et al. report that levels of glutathione

(GSH), a protective molecule that neutralizes reactive oxygen species, were lower in the KO mice, suggesting a potential role of the sHSPs regulating its production.[63] In short, this study helps identify the protective role of two sHSPs in the heart, however due to the lack of a mouse with either gene knocked out alone, we are unable to deduce if the effects are due to one gene or another or to some kind of relationship between the two.

Benjamin et al. suggest that the lack of structural or histological abnormalities in KO hearts may be due to redundancy among the sHSPs or that it may show a need for more sensitive detection of phenotypes.[15] In addition, they found beneficial phenotypes of the combined HSPB2 and CRYAB deletion mice. In light of the report by Morrison et al., these findings are controversial, however Benjamin et al. make different observations, so we can discount neither study. Firstly, Benjamin et al. observed cardiac morphology between WT and KO mice hearts and found no tissue abnormalities in the KO hearts, agreeing with previous data.[16, 59] Secondly, they report that KO hearts show a lesser infarct size in response to ischemia/reperfusion when compared to WT hearts. They also observed that in the KO mice, levels of some of the other heat shock proteins remained the same while others were lower than in WT. HspB1, for instance, was decreased in the cytosolic fraction and increased in the nuclear/pellet fraction compared to WT. This finding supports the idea that sHSPs work together to fill the needs of the cell during stress, and perhaps some can fulfill the need when others are absent. Very interesting to our current study is an observation regarding mitochondrial respiration. They report that mitochondria in KO mice respire at a much lower level than in WT and that significantly lower amounts of ATP are produced compared to WT (see Figure 1.7). Although we cannot be certain whether HspB2 or CryAB is responsible—or if they act in concert—this observation supports the idea of a

relationship between sHSPs and cardiac metabolism, and is the first paper to suggest such a relationship.

A similar study by Golenhofen et al. specifically looked at papillary muscles isolated from mice without HSPB2 and CRYAB.[64] Similar to Morrison et al., they observed increased rates of mortality and decreased body weight in mice lacking HSPB2 and CRYAB. They also observed that between 35 and 40 weeks of age the mice developed a hunchback phenotype, signifying degeneration of skeletal muscle. Because of this particular phenotype and for the fact that increasing numbers of mice died as they approached 40 weeks, Golenhofen et al. selected papillary muscles from these aged hearts. They first measured contractility of the papillary muscles and found no significant difference between the WT and KO hearts. In addition, they also note support for the lack of significant difference by the fact that there is no apparent histological difference between the two mice strains. However, they observed a marked difference between the two when placed in solution simulating ischemia reperfusion. KO hearts contracted quicker and possessed a higher resting tension, but there was less affect on twitch force. After reperfusion, hearts from the two groups recovered in similar manners. They discuss several possible mechanisms for the

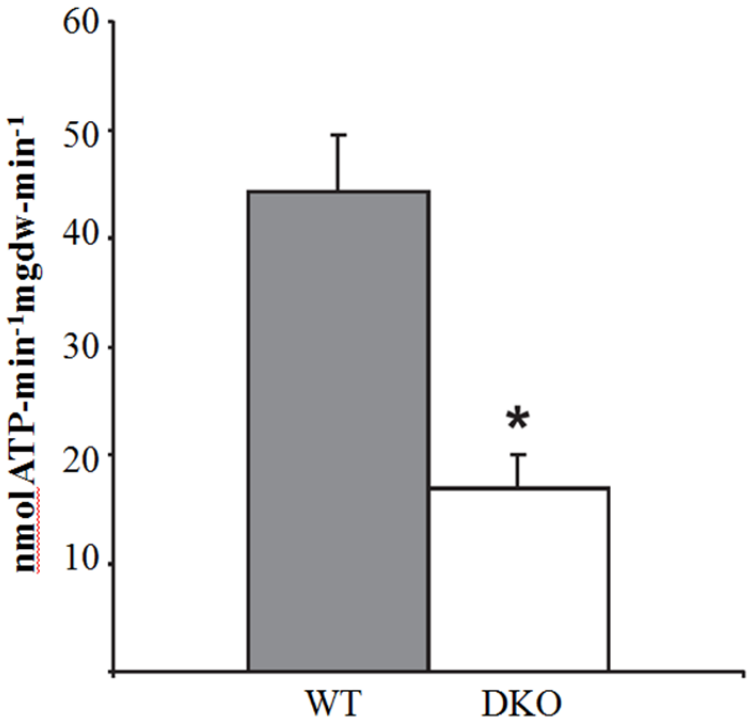

Figure 1.7. ATP production rates in cardiac fibers for wildtype (WT) and double knockout (DKO) mice. Adapted from Benjamin et al. 2006.

increased resting tension in KO mouse hearts and favor a mechanism in which HspB2 and/or CryAB acts on the elastic titin filament system.

Of particular interest to our research are studies performed by Kadono et al. because it relates HSPB2 and CRYAB to metabolism.[65] Using the mouse model lacking HSPB2 and CRYAB, they attempted to provide an explanation for increased apoptosis and necrosis in response to ischemia and reperfusion.[59] They first assessed levels of Ca^{2+} because increased calcium concentration can lead to a greater extent of injury due to ischemia/reperfusion.[66] Observing cytosolic calcium levels, Kadono et al. showed that KO myocytes uptake less calcium than WT myocytes when submitted to simulated ischemia. Later experiments further established that mitochondrial levels of calcium increased in KO myocytes as compared to wild type and showed that these organelles took in calcium from the cytosol, an important method of cytosolic calcium regulation (see Figure 1.8).[67, 68] Additionally, they determined that sHSPs may regulate nitric oxide production, a known regulator of mitochondrial Ca^{2+} uptake.[69, 70] Increased calcium uptake, along with increased ROS production, is a characteristic of mitochondria permeability transition, a contributor of the damage due to ischemia/reperfusion.[71] Measuring myocyte contracture (a method of measuring mitochondria permeability transition) while manually opening the permeability transition pore, Kadono et al. found that the KO mice developed mitochondria permeability transition sooner than WT myocytes.[72, 73] In short, the research performed by Kadono et al. suggests that the lack of HspB2 and/or CryAB preconditions myocytes for damage due to increased calcium sequestration by the mitochondria, a known step in the inducement of mitochondria permeability transition. This may provide an answer to why there is increased apoptosis and necrosis among these cells during ischemia/reperfusion, as noted previously.

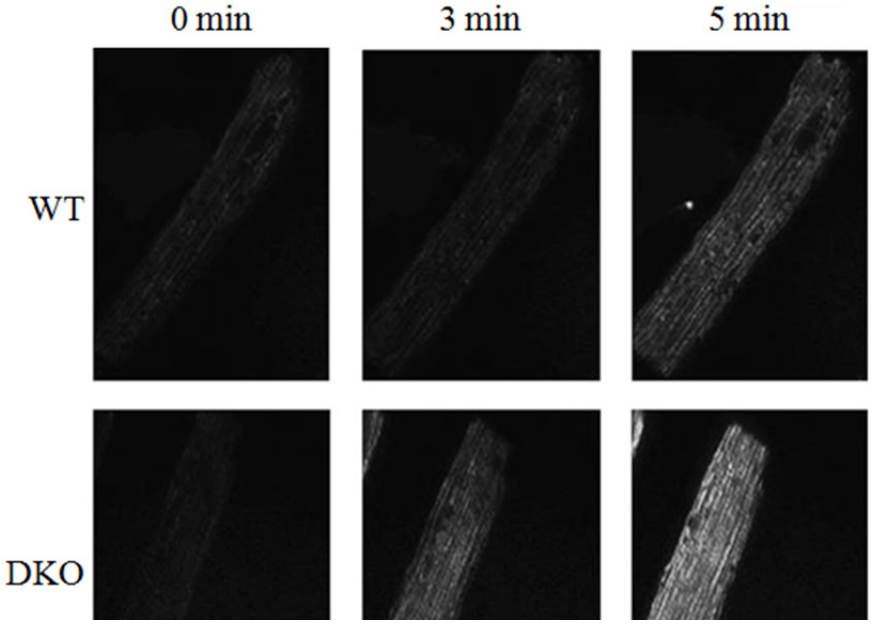

Figure 1.8. Increased fluorescence in mice lacking HSPB2 and CRYAB shows that mitochondria uptake of calcium is greater in DKO mice. Fluorescence is caused by the mitochondrial calcium indicator Rhod-2. The experiment was conducted where all Rhod-2 was intramitochondrial. Adapted from Kadono et al. 2006.

In an attempt to provide a better understanding of the individual function of HspB2, separate from that of CryAB, Pinz et al. transgenically expressed CRYAB in the mouse model lacking both HSPB2 and CRYAB.[74] We note that, while this offers valuable insights, they show by western blot that the transgenic expression of CRYAB far exceeds the level of CRYAB in the WT mice. For this reason, we must be careful about how we view this data because overexpression of CRYAB is known to have phenotypic effects.[75] They observed possible functions for HSPB2 by comparing several strains of mice—WT, double KO, WT mice with transgenic expression of CRYAB, and mice with the double KO and transgenic expression of CRYAB. After a period of ischemia, hearts that did not contain HSPB2 lost significantly more ATP and had a slower recovery than hearts with HSPB2 intact (see Figure 1.9). This may be because hearts lacking HSPB2 also have a lower contractile reserve than the controls, suggesting that HspB2 is necessary for an increased workload on the heart. In addition, they found that hearts lacking HSPB2 are unable to properly couple energy from ATP hydrolysis, leading to energy defects in the cell. While their experiment is imperfect in its "deletion" of CRYAB, it does contribute potentially valuable information that agrees with data relating HspB2 to mitochondrial function.

In addition to relationships with muscle and metabolism, Oshita et al. report that HspB2 confers an anti-apoptotic quality in a certain line of breast cancer cells.[76] This is particularly important because removal of HspB2 seems to increase levels of apoptosis and necrosis.[59] In this paper Oshita et al. suggest mechanisms for its ability to confer resistance to apoptosis. Working in a breast cancer cell line ectopically expressing HSPB2 they show that HspB2 inhibits the extrinsic apoptotic pathways initiated by TNF-related apoptosis-inducing Ligand (TRAIL) and TNF-α. By so doing, it inhibits downstream events leading to eventual apoptosis.

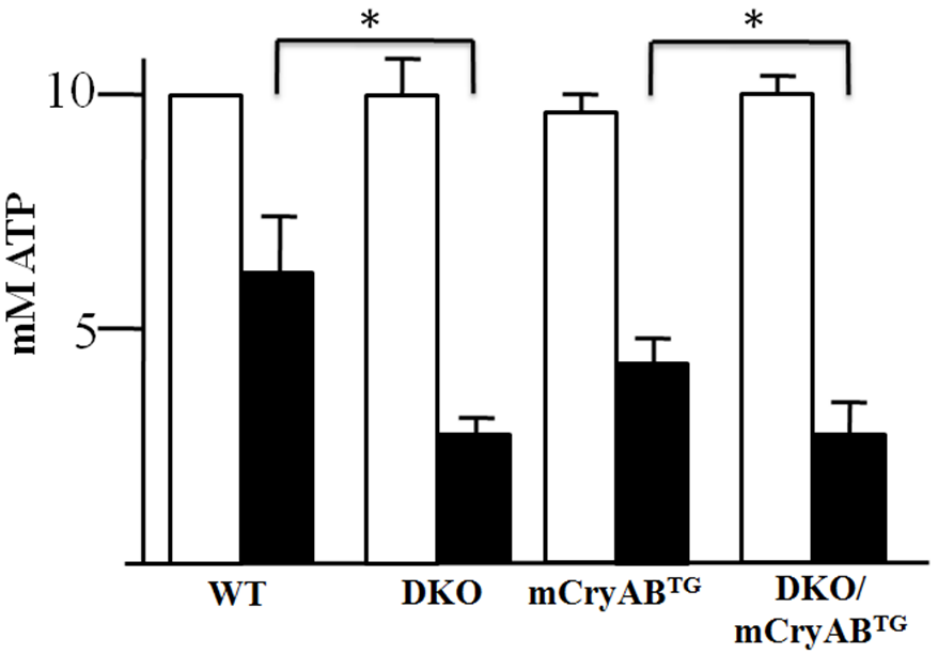

Figure 1.9. ATP levels in the heart after ischemia (white bars) and after reperfusion (black bars). Adapted from Pinz et al., 2008.

While researchers have done much to discover the function of HspB2, current models are not able to explain its precise role in the cell. They have shown, however, that it has possible relation to muscle and mitochondrial function in the heart,.. Furthermore, unpublished data from a yeast two-hybrid screen reports an interaction between R120G CryAB—a mutated form of the small heat shock protein CryAB conferring cardiomyopathy—and HspB2 (Julianne Grose, unpublished results). As stated before, in order to gain a more complete understanding of the function of HspB2 we must find out what it binds.[33] By performing an exhaustive screen for

proteins in the heart that interact with HspB2 we hope to better understand the role played by HspB2 in cardiac metabolism and muscle maintenance.

Specific Aims: A Summary

SHSPs play an important role in maintaining intracellular proteins by providing protective effects to the cell, mainly through the maintenance of protein folding during times of stress. Although HspB2 is less understood that other sHSPs it has been implicated in the maintenance of energy production and involvement in muscle function. Little is known about the mechanisms of its involvement in these capacities. A study to identify HspB2 substrates will greatly contribute to the current knowledge on HspB2 and on the general role of sHSPs within the cell.

Aim One: Discover and identify putative HspB2 binding partners using the yeast two-hybrid.

We will identify putative binding partners in yeast through the yeast two-hybrid screen using HspB2 as our "bait" and a cDNA library from human heart as our "prey". We screened over 20 million library plasmids from which approximately 9,000 interactions were identified, 600 of which were sequenced. The yeast two-hybrid screen may identify key HspB2 substrates underlying its cellular function.

Aim Two: Verify putative HspB2 binding partners in yeast through the HspB2 dependency test.

The sensitivity of the yeast two-hybrid can potentially lead to high false-positive rates. Thus, proteins identified as putative HspB2:human heart interactions in Aim One will be tested for their dependency on HspB2 . Briefly, plasmids expressing the proteins identified in Aim One will be purified and transformed into naïve yeast with our without HSPB2, and scored for their ability to activate the yeast two-hybrid system. This Aim will provide a second proof that the interaction is occurring in yeast before confirmation in mammalian cells (Aim Three).

Aim Three: Verify putative HspB2 binding partners in mammalian cells (HEK 293).

Although the yeast two-hybrid system we use in the fulfillment of the first two aims is very sensitive and false positive incidence is low, we must verify interaction in mammalian cells. We used HEK 293 cells to verify two key interactions discovered in Aims One and Two.

To our knowledge, a comprehensive yeast two-hybrid screen has never been performed for any of the sHSPs. Study into the molecular binding partners of HspB2 through yeast two-hybrid with a cDNA library will provide valuable insight into an understudied member of the sHSP family.

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CHAPTER TWO: Aim One: Discover and identify putative HspB2 binding partners using the yeast two-hybrid screen.

Introduction and Methods

Saccharomyces cerevisiae serves as an excellent model organism for identifying protein-protein interactions through the yeast two-hybrid method. Until Fields and Song developed the yeast two-hybrid method in 1989, researchers had to use such biochemical tools as crosslinking, co-immunoprecipitation, and co-fractionation by chromatography to detect protein-protein interactions.[1] Chien et al remark that this novel method allows researchers to verify interaction without the preparation or purchase of expensive antibodies, and does not require the purification of protein for biochemical analysis.[2] Although reviewers of the first grant application of what was to become the two-hybrid method did not believe the technology was feasible, Fields said that he and others began work on the method because of its beauty and elegance.[3] The yeast two-hybrid has been refined over the years although the fundamental science behind the method is the same.

The yeast two-hybrid method relies on the ability to split the GAL4 transcription factor into two separate units that, when brought together again, function as a transcriptional activator (see Figure 2.1). The GAL4 transcriptional activator binds to specific sequences of DNA upstream a series of genes necessary for galactose utilization.[1, 4] The activator itself is composed of two main domains—a DNA-binding domain located at the N-terminus of the protein and an activation domain at the C- terminus.[1] The N-terminus contains a nuclear localization sequence that allows its entry into the nucleus, as well as a domain that binds to specific sequences of DNA; the C-terminal domain of the GAL4 transcriptional activator contains acidic regions and is

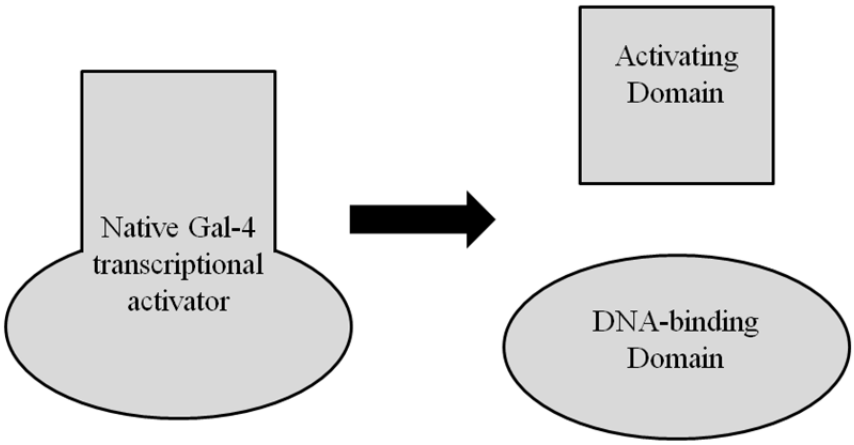

Figure 2.1. The yeast two-hybrid system works because the Gal-4 transcriptional activator can be split into the activating domain and the DNA-binding domain, yet still remain functional when brought back into close proximity to each other.

required to initiate transcription.[5-7] These two domains are essential for transcription to occur. In essence, two "hybrid" proteins are created with one possessing the GAL4 DNA-binding domain (the "bait") and another protein or library of proteins possessing the GAL4 activating domain (the "prey"). Due to their close proximity to one another, the DNA-binding and activating domains initiate transcription when the hybrid proteins interact with one another (see Figure 2.2). Transcription of various reporter genes allows for detection of these interactions.

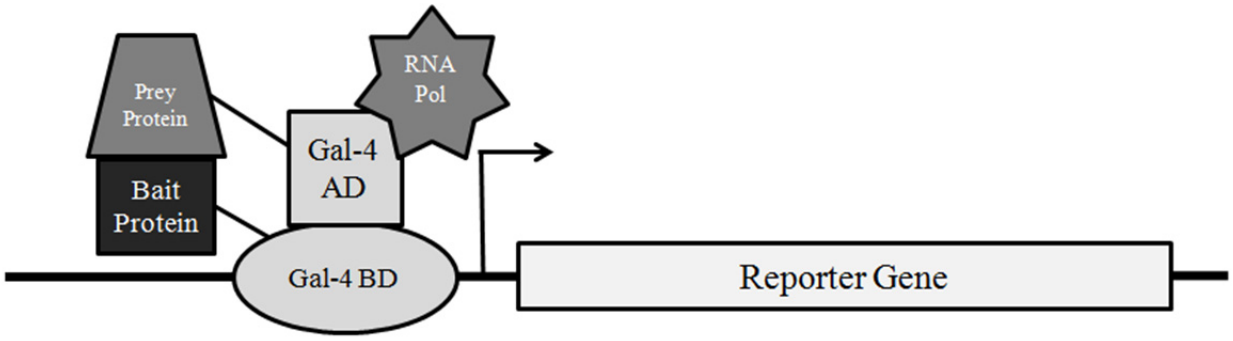

Figure 2.2 The yeast two-hybrid system for detection of protein-protein interactions. Two hybrid proteins are created: the bait protein is fused to the Gal-4 DNA binding domain and the prey protein is fused to the Gal-4 activating domain. Interaction between the bait and prey proteins brings the activating domain and binding domain of the Gal-4 transcriptional activator into proximity, allowing the recruitment of RNA polymerase and transcription of the reporter gene.

Use of the yeast two-hybrid model of detecting protein-protein interactions has historically experienced issues with false-positives. Recent advances, however, have developed the yeast two-hybrid system into a sensitive and reliable method for detecting such interactions. For our yeast two-hybrid screen we are using the Matchmaker™ Gold Yeast Two-Hybrid system developed by Clontech Laboratories, Inc..[8] The Matchmaker™ system utilizes four reporter genes (AUR1-C, HIS3, ADE2, and MEL1) situated on various yeast chromosomes. When expressed in yeast, AUR1-C confers resistance to Aureobasidin A, a drug toxic to yeast. The HIS3 gene allows the yeast to synthesize histidine, an essential amino acid, permitting it to grown on media lacking histidine (-His). The ADE2 marker allows Matchmaker™ Gold yeast to synthesize adenine, a necessary nucleotide, which permits the yeast to grow on media lacking adenine (-Ade). MEL1 encodes α-galactosidase, which, in the presence of substrate X-α-Gal, turns the yeast colonies blue. Each reporter gene is controlled by one of three unrelated GAL4 promoters, the G1, G2, and M1 promoters. Except for the 17-nucleotide sequence bound by the GAL4 DNA-binding domain, each promoter is very different.[9, 10] Three of the four reporter genes must be expressed in order for growth to occur on selective media. If a certain protein initiates transcription independently—without the GAL4 activating and DNA-binding domains—it would be highly unlikely to do so at the other two promoters as well, due to their different sequences. Thus, this system has a very low false positive rate; a less than 5-10% false positive rate is common for most screens (Clontech, personal communication).

The yeast two-hybrid system affords researchers two abilities: to study putative interactions as well as to perform a screen on a library of proteins in order to find protein-protein interactions.[2] We are utilizing the yeast two-hybrid in both capacities, as outlined in Specific Aims One and Two. We first perform a screen on a library of proteins (Aim One). To perform such a screen,

we utilize certain “bait” and “prey” proteins as briefly introduced above. In our screen, HspB2 serves as the bait protein. The bait protein is a hybrid protein fused to the GAL4 DNA-binding domain. The constitutively active, medium strength ADH1 promoter on pGBKT7 controls expression of the gene encoding the hybrid protein. Also notable is the tryptophan biosynthesis gene, TRP1, found in the plasmid. When this plasmid is transformed into yeast, the yeast expresses the TRP1 gene, allowing the yeast to grow on selective media lacking tryptophan (-Trp media). Transcription and then translation of HspB2 is coupled to transcription and translation of the GAL4 DNA-binding domain to create the hybrid fusion protein, a single polypeptide chain (HSPB2:GAL4 DNA-binding domain). The second hybrid protein—the prey protein—is created from a library of human cDNA fragments made available by Clontech as a “Mate and Plate” library. The necessary mRNA was recovered from three male hearts aged 33, 55, and 55 years of age whose cause of death was trauma. The mate and plate library comes pre-cloned into the pGADT7 vector. This plasmid, different from pGBKT7, contains LEU2, a leucine biosynthesis gene, allowing the yeast to grow on media lacking leucine (-Leu media).

Yeast exist in haploid form as one of two mating types, a and α. A and α yeast containing either bait or prey plasmids are mated, creating new diploid cells that contain both plasmids. To select for both plasmids, yeast are grown in minimal SD media (synthetic dextrose) lacking the essential amino acids leucine and tryptophan (-Leu-Trp). Cells that have not undergone proper mating lack the pair of plasmids that allow for growth in the selective media. Growth of the yeast on SD-Leu-Trp with Aureobasidin A selects for potential protein-protein interactions. At a molecular level, if bait and prey proteins interact, the fused DNA-binding and activating domains will be in adequate proximity to allow transcription of the reporter genes. If they grow on media positive for Aureobasidin A, they are expressing the AUR1-C reporter gene. The compound X-

α-Gal is also added since blue colonies on media containing X-α-Gal denote colonies expressing the MEL1 reporter (α-galactosidase), a second reporter. Hypothetically, however, these may not be true protein-protein interactions because it is possible that the prey is able to initiate transcription of both reporter genes without interacting with the bait. Thus, further testing is necessary on media lacking three amino acids and one essential nucleotide (SD-Leu-Trp-His-Ade), termed quadruple dropout media, because it lacks four essential elements necessary for growth and selects for the expression of two other Gal4-regulated promoters. Note that we do not select for all reporter genes at the same time because it stresses the yeast detrimentally. The selection of yeast in this manner allows us to retain our bait and prey plasmids by always using plates lacking leucine and tryptophan as well as selecting for the various reporter genes as detailed above. Since it is highly unlikely that a protein can activate transcription of all four reporter genes (three promoters) by binding to a GAL4 promoter and recruiting RNA polymerase, we screen very stringently for false-positives.

Our basic method was to select yeast colonies from the mated plates SD-Leu-Trp+Aureobasidin and patch them to SD-Leu-Trp-His-Ade+X-α-Gal plates divided into a 91-square grid (see Figure 2.3). Each mated -Leu-Trp+Aureobasidin plate contains approximately 1200 colonies, giving us approximately 10-15 91-square grid patch plates per mated plate. Each plate is given a unique letter and number as means of identification and all information is recorded on an Excel spreadsheet. All of our colonies turned blue when grown on X-α-gal plates, so we stopped the use of this substrate in the plates after their initial isolation. We also noticed decreased viability of yeast on plates containing X-α-gal after storing at 4'C.

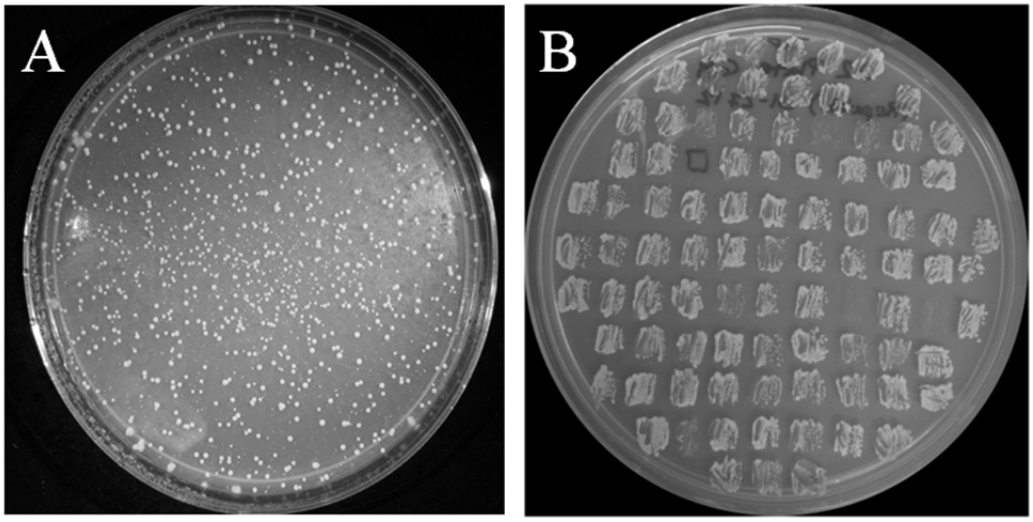

Figure 2.3. Yeast two-hybrid plates. Plate A is the initial mated plate (SD-Leu-Trp+Aureobasidin). Each individual colony signifies a putative interaction between HspB2 and a human heart protein from a cDNA library. Colonies were chosen and patched onto a 91-grid plate (B) made of SD-Leu-Trp-His-Ade+Aureobasidin or +X-α-Gal.

Each colony growing on selective media is a potential interaction between hybrid proteins. To identify the target protein encoded by the prey plasmid we must seqeunce the insert on the plasmid. Colony PCR is performed by simple lyticase treatment as detailed in the appendix. Briefly, we select a small streak of cells from each of the gridded squares and treat with lyticase, which degrades the yeast cell wall, followed by a period of boiling which releases the DNA into solution. The tubes are briefly centrifuged to separate cell debris from supernate and a small amount of supernate is used as template DNA in PCR amplification. The primers used in the PCR reaction anneal to portions flanking the multiple cloning site of the pGADT7 plasmid, thus amplifying the gene from the human heart library. Following PCR, samples are run on an agarose gel electrophoresis apparatus in order to determine PCR product size. Band sizes are recorded and correspond to each colony. We typically see a very diverse assortment of band sizes indicating many different

binding partners (see Figure 2.4).

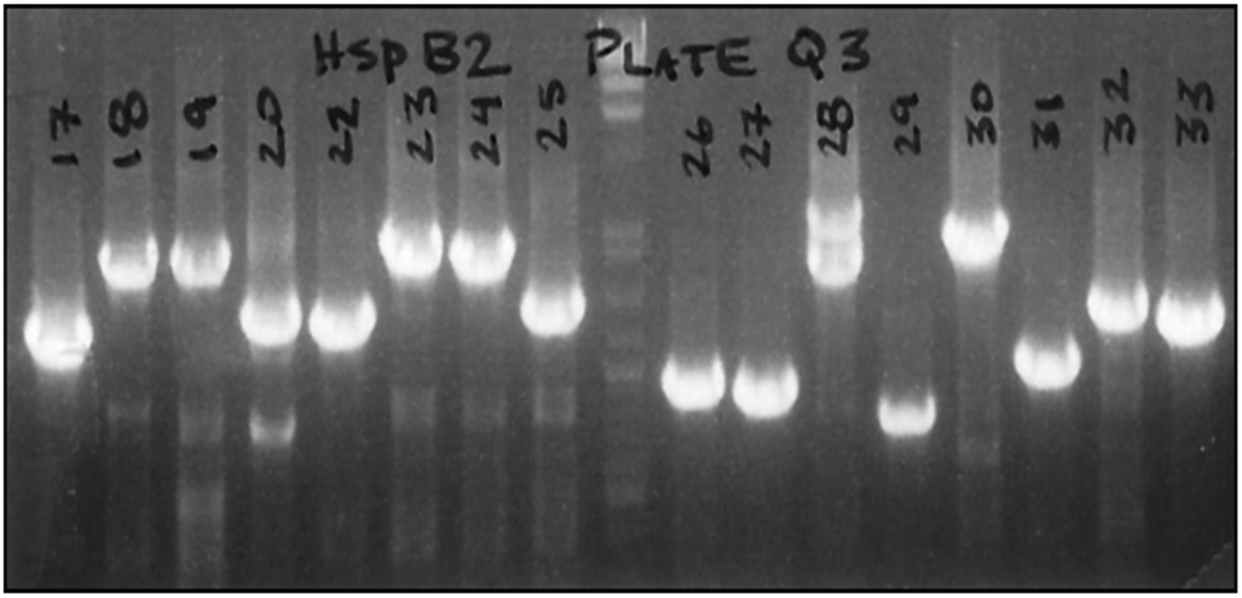

Figure 2.4. PCR band sizes for prey plasmid inserts. Typically a wide assortment of band sizes is seen. Note the occasional double banding (sample 28). This most likely occurs from accidentally patching two colonies from the mated plate to the 91-grid repatch plate.

Occasionally, double banding occurs in electrophoresis which may be due to accidentally picking two colonies when patching from the mated plates. Clear, bright single bands are sent for sequencing at the BYU Sequencing Center and identification of genes is performed using the BLAST alignment tool provided by NCBI.[11] We recorded each gene into the Excel database specific to each plate and colony. Exhaustive attention to detail in recording information is required in order to keep the results of each colony easily accessible.

Results

Introduction

From this library screen we observed approximately 24 million matings (see Table 2.1). Initial plating onto synthetic glucose media lacking, leucine, tryptophan, and including Aureobasidin A (AbA) (SD-Leu-Trp+AbA), yielded approximately 10,000 colonies. Restreaking onto synthetic glucose media lacking leucine, tryptophan, histidine, and adenine (SD-Leu-Trp-His-Ade), yielded approximately 9,130 colonies.

Summary of HSPB2 Screen					
Bait	**No. of Matings**	**SD-Leu-Trp+AbA**	**Patch SD-Leu-Trp-Ade**	**Currently Sequenced**	**No. of unique protein hits**
HspB2	24, 200,000	~10,000	~9,130	~593	206

Table 2.1 Summary of the HspB2 yeast two-hybrid screen. Units are in number of colonies.

Thus, we estimate that HspB2 binds to approximately one of every 2,000 clones in the library. We have currently sequenced approximately 600 samples and have recorded approximately two hundred unique hits in the protein library. Of these hits, approximately 54 of these proteins are our "high confidence hits" in that they have been hit two or more times, with some being hit over 100 times. Note, however, that the number of times a particular protein is hit may not reflect the strength or importance of interaction as much as it reflects the abundance in the library. These 54 unique proteins account for 383 of the ~600 sequenced putative interactions (see Table 2.2). The other proteins have only been hit once; although many are of interest, they are less reliable as a single hit. Of these 54 protein-encoding genes, we observe that a large percentage of them relate to muscular and mitochondrial function (see Table 2.2). Other genes encode proteins involved in electrolyte-related activity, phosphorylation (kinases), maintenance of the cellular redox state, assistance in stress (sHSPs), and many other proteins falling into various

categories. Understanding the significance of these hits from the screen relies on our knowledge of their various functions in the cell. Below I provide a brief synopses of the different functions known for the genes identified in our screen. I briefly present information regarding some of their pathogenic roles, but a more detailed discussion of the significance of the hits is provided in the discussion. I first cover muscle proteins, then mitochondrial, followed by electrolyte-related, kinases, redox-related, small heat shock proteins, and lastly I address the list of varied proteins found in the "other" category. It is of note that our largest number of hits are specific mitochondrial and muscle proteins, known pathways affected by HSPB2 deletion.

Muscle

Approximately 50 hits from the yeast two-hybrid screen are to genes encoding muscle-related proteins. A list of the gene abbreviations, full names, and functions follows the gene descriptions (see Table 2.2). Muscles are highly structured and rely on a vast network of interacting proteins to maintain form and function. The proteins we hit in the screen fulfill contractile, regulatory, and structural roles in the sarcomere.[12]

By way of introduction, skeletal muscle is organized into several groups, beginning with the whole muscle (see Figure 2.5). Whole muscle is comprised of fasiculi—groupings of muscle fibers. Muscle fibers are also known as muscle cells. Within each muscle cell are many myofibrils—elements that extend the length of the muscle cell. Within the myofibrils we observe a network of cytoskeletal elements called the thick and thin filaments.[13, 14] Under the microscope, the myofibrils appear striated as a result of the overlapping thin and thick filaments (see Figure 2.6). Thin filaments anchor to the Z-line and the space from Z-line to Z-line is

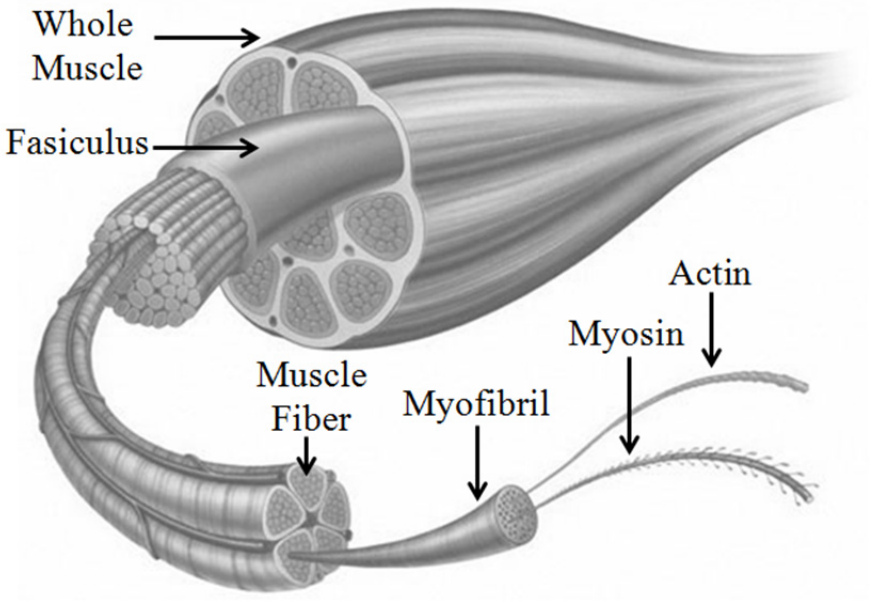

Figure 2.5.Composition of skeletal muscle. Whole muscle is made of fasiculi, which are composed of muscle fibers (muscle cells). Myofibrils, made partially of actin and myosin filaments, help make up the components of the muscle fibers. http://www.wellcome.ac.uk/Education-resources/Teaching-and-education/Big-Picture/All-issues/Exercise-energy-and-movement/Image-galleries/WTDV033068.htm

known as the sarcomere. The sarcomere is the functional unit of muscle and is responsible for the contraction of muscle.[13, 14] Many proteins compose the sarcomere, and the results discussed below describe the 14 muscle proteins we hit two or more times in the yeast two-hybrid screen.

Of the myofibrillar proteins, actin comprises approximately 20%.[15, 16] Actin plays an important contractile role in the sarcomere. In the yeast two-hybrid screen we hit two of the six actin isoforms—skeletal muscle α actin 1 (ACTA1) and cardiac α actin 1 (ACTC1)—both of which are implicated in pathogenic roles.[17-21] Both skeletal and cardiac α actin are very similar, sharing 99% amino acid identity in the mature protein.[22] Within the heart, the skeletal isoform is expressed, although most of the actin (80%) is the cardiac isoform[23]. Acta1is cleaved and folded into a 375 amino acid protein termed G actin. G actin polymerizes into F actin, which is the filamentous form we observe in muscle.[24] Functionally, actin forms the thin filament of the sarcomere and interacts with the thick filament, composed of myosin.[12] In concert with other proteins, actin and myosin comprise a major part of the sliding filament mechanism, a theory by which muscle contracts (see Figure 2.7).

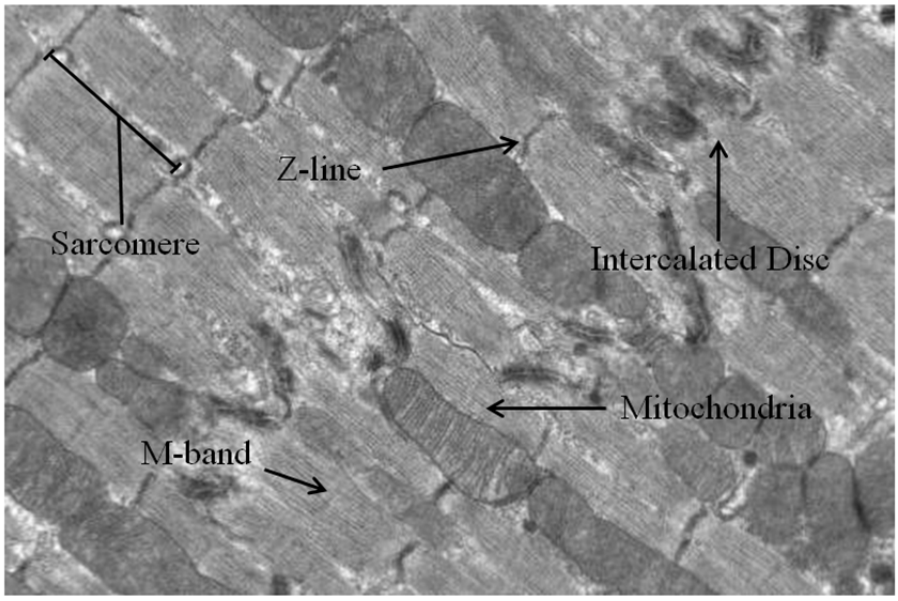

Figure 2.6 Transmission electron micrograph of cardiac muscle stained with uranyl acetate and lead citrate. Significant structures are labeled. Note the close association between mitochondria and muscle cells. Image courtesy of Drs. Toshitaka Yajima and Mark Murphy of the Veterans Medical Research Foundation's Microimaging Core Facility. **http://www.vmrf.org/researchcenters/confocal/transmission-electron-microscope-images.html**

Myosin forms the thick filament of the sarcomere and makes up approximately 50% of the total myofibrillar proteins.[15, 16] Myosin molecules are composed of six proteins: two heavy chains and two pairs of light chains—alkali or essential, and regulatory chains.[12, 25, 26] Approximately 300 myosin molecules compose one myosin filament.[14] Alongside actin, myosin fulfills an important contractile function in the sarcomere and is called the "thick" filament. The sliding filament theory hypothesizes that myosin heads form cross bridges that interact with actin filaments, shortening the sarcomere. Myosin cross-bridges (heads bridging from the thick to the thin filaments) were first observed by Huxley in 1957, one study upon which the sliding filament theory was established.[27] In our yeast two-hybrid screen, we observed multiple hits to different isoforms of myosin, including light chain 2 (MYL2), light chain 3 (MYL3), and heavy chain 7 (MYH7). MYL2—whole name myosin, light chain 2, regulatory, cardiac, slow—which was hit four times in the yeast two-hybrid screen, serves a regulatory role in addition to its role as a contractile protein. Muscle contraction is regulated by Ca^{2+} triggering the phosphorylation of the myosin regulatory light chain.[28] Additionally, we hit

MYL3 twice, another of the myosin light chains. This protein also fulfills a dual regulatory/contractile role, however its function is less understood aside from providing structural support to the myosin head.[29] Researchers suggest possible pathogenic roles for MYL3 in decreased myocyte function due to Myl3 cleavage by caspase-3.[30] The heavy chain we hit, MYH7—myosin, heavy chain 7, cardiac muscle, beta—helps compose the contractile unit of the thick myosin filament and is primarily expressed in the normal human ventricle.[31] Like the others, it has been implicated in various muscle defects; a mutation in MYH7 was the first hypertrophic cardiomyopathy-causing gene to be discovered.[32] In addition to slight regulatory roles by the light chains of the myosin, other proteins in the sarcomere are involved in regulating contraction. Our yeast two-hybrid screen also revealed interactions between HspB2 and the contractile regulators troponin I type 3 (cardiac) (TNNI3) and troponin T type 2 (cardiac) (TNNT2). As a molecule, troponin is comprised of troponin-C, troponin-I, and troponin-T subunits to make a three-subunit complex. The C subunit provides a binding site for calcium, the T subunit provides an attachment site for the protein tropomyosin, and the I subunit attaches

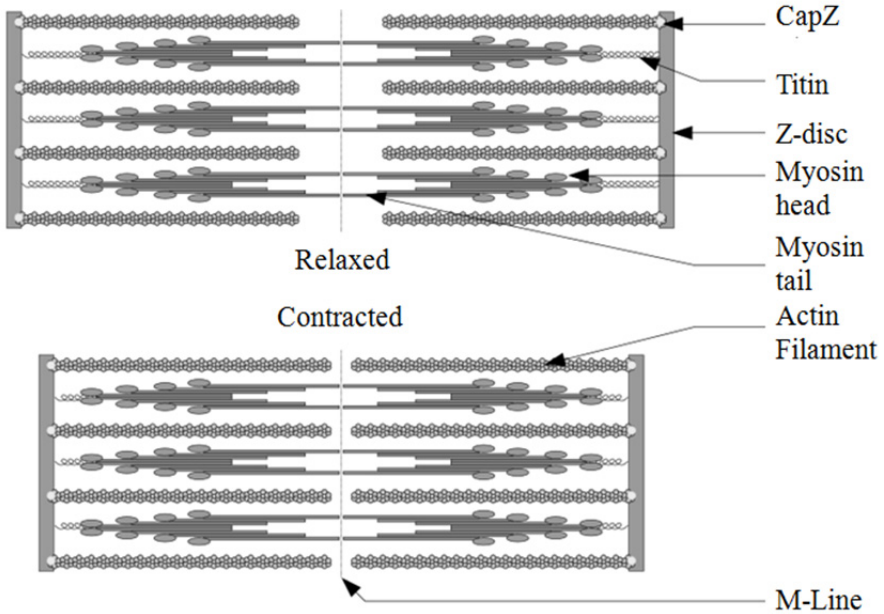

Figure 2.7. Model of muscle filament contraction. Myosin heads within the sarcomere contract and move along actin filaments to pull Z-lines closer to each other. By contraction of the sarcomere the muscle fibers shorten, thus shortening the muscle as a whole. Image credit: **http://en.wikipedia.org/-wiki/File:Sarcomere.svg**

troponin to actin. To clarify, tropomyosin is a long filamentous protein that winds its way around the actin filament. Together, the troponin protein complex holds the tropomyosin filament onto the actin, blocking the active sites of the actin molecules.[13, 14] Calcium (Ca^{2+}) binds to troponin C and facilitates removal of tropomyosin from the active site of actin, allowing myosin heavy chain cross bridges to bind at one of its active sites. When ATP binds and is cleaved at the other site on myosin, the energy is thought to be stored in the hinge region and allows the myosin to pull itself along the actin filament. This, in turn, shortens the sarcomere and the muscle as a whole.[13, 14] With their significant roles in mind, and the constant contraction and relaxation of heart muscle, it is not surprising that mutations of troponins I and T have been linked to hypertrophic cardiomyopathy.[33]

In addition to the contractile and regulatory proteins mentioned above, our yeast two-hybrid screen also suggests interactions between HspB2 and muscle proteins that serve structural roles. Before discussing these proteins it is important to understand another element of the cell—the Z-line. The Z-line is the dark line appearing at the border of each sarcomere (see Figure 2.6). Composed mostly of the protein actinin, the Z-line anchors the actin filaments from the adjoining sarcomeres.[13] Many proteins interact at the Z-line, including several we pulled from the yeast two-hybrid screen. These include titin (TTN), titin-cap (TCAP), desmin (DES), and myomesin 2 (MYOM2). Spanning from the Z-line to the M-line, the point halfway through the sarcomere, titin serves the sarcomere by conferring elasticity to the muscle as well as helping to properly situate the heavy filaments to the thin filaments.[13, 14] TCAP, also called telethonin, binds two parallel titin filaments to each other, and furthermore to the Z-line, in what is known as the strongest observed protein-protein interaction.[34, 35] Desmin, another protein found at the Z-line and hit in our yeast two-hybrid screen, plays an integral role in the organization of the muscle

fiber by joining adjacent sarcomere Z-lines to one another.[14] Thus desmin enables the cell to keep distinct organization of intermediate filaments as well as cross-bridging. Desmin also plays a variety of alternate crucial roles in the muscle cell including interactions with the mitochondria.[36] Because of its varied roles in the cell, mutations to desmin are particularly deleterious to the heart and cause what is termed desminopathy.[37, 38]

Not interacting at the Z-line, but nonetheless crucial to the function of the sarcomere, is the protein myomesin 2 (MYOM2), a protein localized at the M-line.[39] While its function is not completely understood, myomesin 2 is found only in cardiac and fast skeletal muscle fibers and is thought to help stabilize the myofibrillar thick filaments.[12, 40] On a related note, four and a half lim domains 2 (FHL2) is putatively thought to have a possible structural role in muscle due to its localization to the Z-line, its expression solely in the heart, and a study showing an increased hypertrophic response in mice lacking FHL2.[41, 42]

In addition to hits relating to the structural aspects of muscle, we also note important hits from our yeast two-hybrid screen to several other muscle proteins. The cardiac isoform of myosin binding protein (Mybpc3) has been implicated in contractile regulation by interaction with actin or myosin filaments.[43] Particularly interesting to our study is its relation to hypertrophic cardiomyopathy—one study reports that greater than 40% of all hypertrophic cardiomyopathy cases stem from MYBPC mutations.[44] Of particular interest is the protein cardiomyopathy associated 5 (CMYA5). CMYA5, also called myospryn in the literature, has been shown to interact with several proteins including desmin and dysbindin. In addition, myospryn is in close proximity to M-band titin, to intercalated discs in adult mouse heart, and is near lysosomes.[45-47] Of course, as its name suggests, CMYA5 is thought to have an association to cardiomyopathy, although Saparanta suggests that the name was given on purely hypothetical grounds.[47] In

support of its name, however, Nakagami et al have shown the relationship between a CMYA5 polymorphism and left ventricular wall thickness.[48]

Mitochondria

Most of our trillions of cells contain a copy of all of the genetic information which directs multitudinous reactions and happenings throughout our body. In addition to our DNA, eukaryotic cells contain various organelles that play an array of roles in the cell. Included among these organelles are mitochondria, traditionally known as the cellular ATP powerhouses. Human mitochondria, interestingly, possess unique DNA approximately 16,000 bp long and typically contain between two and ten copies of their DNA, but can contain hundreds of copies in some cases, such as in differentiating embryonic cells.[49, 50] While the mitochondria have the information (mitochondrian DNA, mtDNA) and machinery at hand to make proteins, most (~99%) of the mitochondrial proteins are coded on nuclear DNA and are imported into the mitochondria through several different protein import pathways.[51-55] Our yeast two-hybrid screen hit integral metabolic proteins with relation to the mitochondria. In an attempt to categorize the proteins, I have grouped them into pathways in which they are involved. Of particular interest are two proteins that we have hit nearly two hundred times in the screen, both of which align to mitochondrion DNA with high similarity to the chromosomal genes encoding NADH-ubiquinone oxidoreductase chain two-like and pterin-4-alpha-carbinolamine dehydratase-2. Below I provide a basic overview of the processes involved and specify where the various locations of protein hits from the yeast two-hybrid screen. I will discuss the significance of the mitochondrial protein hits in Chapter 5, Discussion and Conclusions.

Several of the proteins found to putatively interact with HSPB2 are key players in the glycolysis pathway, namely hexokinase 1 (HK1), aldolase A (ALDOA), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (see Figure 2.8). Glycolysis, while not the most efficient method for ATP production, provides an important source of energy for many cells in the body and forms the precursors necessary for respiration. Its prevalence as an energy source is particularly of interest in hypertrophied and failing hearts, as discussed more in Chapter 5.[56] Starting with a glucose molecule, the glycolytic pathway creates two molecules of pyruvate in two phases. In the body, pyruvate is either converted to acetyl-CoA and used in the citric acid cycle or it undergoes lactic acid fermentation. This later fermentative route is particularly appropriate for tissues experiencing hypoxia. Hexokinase 1 is the first enzyme in the preparatory phase of the glycolytic pathway. In this phase two molecules of ATP are expended to produce molecules that lead to the payoff phase, the part of glycolysis in which four molecules of ATP and two molecules of NADH are produced. Hexokinase 1 phosphorylates glucose to create glucose 6-phosphate for use in further glycolysis reactions (see Figure 2.8). From this point, glucose 6-phosphate is converted into fructose 6-phosphate and then fructose 1,6-bisphosphate by phosphohexose isomerase and phosphofructokinase-1, respectively. From this point, aldolase A cleaves fructose 1,6-bisphosphate into glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. Triose phosphate isomerase converts dihydroxyacetone phosphate into glyceradehyde 3-phosphate. Both molecules of glyceraldehydes 3-phosphate are converted into two molecules of 1,3-bisphosphoglycerate by the enzyme glyceraldehyde 3-phosphate dehydrogenase. This reaction is the first energy-yielding reaction of glycolysis because it produces two molecules of NADH. Also involved in glycolysis is enolase 3, beta, muscle (ENO3). It is muscle specific and plays a metabolic role by catalyzing the conversion of 2-

phosphoglycerate to 2-phosphoenolpyruvate, a pivotal step in glycolysis.[50, 57] We will discuss the significance of these putative interactions with HspB2 in Chapter 5.

In addition to the conversion of carbohydrates into usable forms of cellular energy, lipids are also an important starting point in the production of cellular energy. Fats undergo hydrolysis into fatty acids and are then oxidized by α-, ω-, or β-oxidation. Following oxidation, the fatty acids move from the cytosol to the mitochondria. The majority of fatty acids undergo β-oxidation, and

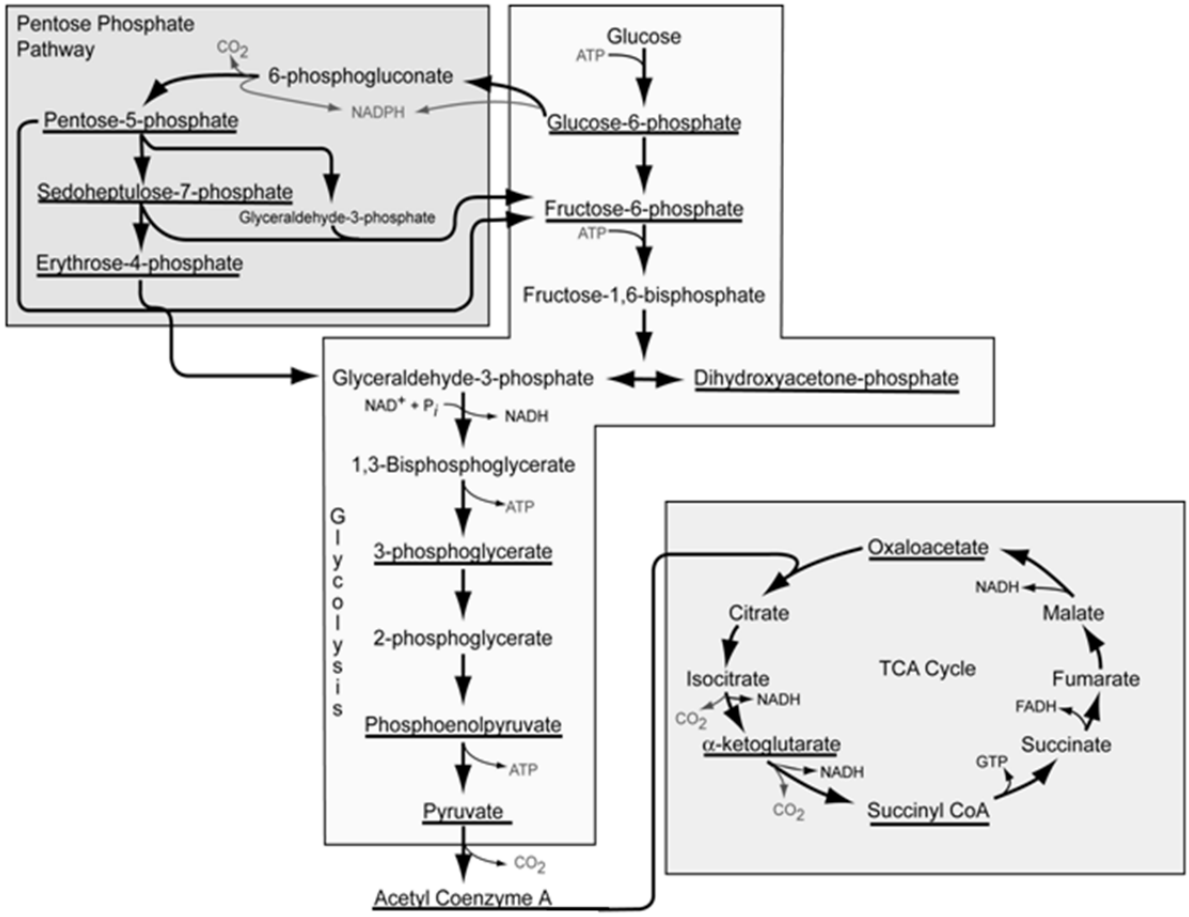

Figure 2.8. The pentose phosphate pathway, glycolysis, and citric acid (i.e. TCA) cycle. For details as to which enzymes HspB2 has been found to putatively interact, please see the text .

this pathway is of particular interest because we hit a protein in the pathway in the yeast two-hybrid experiment. This protein, electron-transfer flavoprotein, alpha polypeptide (ETFA), accepts electrons from a variety of dehydrogenases, including acyl-CoA dehydrogenase, an enzyme catalyzing the first step of β-oxidation.[58-60] The last stage of β-oxidation of fats occurs with the help of acetyl-CoA acyltrasferase (ACAA2), another protein we hit twice in the yeast two hybrid screen.[60] Acyl-CoA molecules are further converted into acetyl-CoA for use in the citric acid cycle.

The citric acid cycle (also known as the TCA or Kreb's cycle), is the pathway by which energy is produced via the conversion of acetyl CoA to carbon dioxide and water.[61] The energy output of the citric acid cycle varies greatly with the length of the carbon chain, but provides a substantially greater contribution than glycolysis to the production of ATP.[60] Note from above that acetyl CoA is not solely derived from carbohydrates, but can be derived from other sources such as lipids and proteins. Occurring in the mitochondrial matrix, the citric acid cycle is a series of conversions from one molecule to another. One step in the citric acid cycle converts one molecule of succinate to fumarate via catalysis by succinate dehydrogenase. This is of interest to us because we detected putative interaction between succinate dehydrogenase complex, subunit A, flavoprotein (Fp) (SDHA), and HspB2 in our yeast two-hybrid screen. As discussed below, SDHA also plays an important role in the electron transport chain. Oxidative phosphorylation, put simply, is the process of transferring electrons from energy molecules such as NADH and $FADH_2$ through a chain in which oxygen is the final electron acceptor.[61] Electrons pass through a series of electron carriers and complexes in the inner mitochondrial membrane, the carriers and complexes forming a "chain" of events, thus giving this process the name "electron transport chain" (see Figure 2.9).[61] Complex I is known as NADH

dehydrogenase.[62] Its function is to transfer electrons from NADH to the carrier coenzyme Q. We have hit several parts of this complex, although each was hit only one time (see Table 2.2). Complex II is the succinate-Q reductase complex. This complex donates electrons from $FADH_2$ to coenzyme Q. One of the three enzymes which composes complex II is succinate dehydrogenase (SDHA). Next in the electron transport chain, coenzyme Q transfers electrons through the inner mitochondrial membrane to complex III, known as cytochrome c reductase. Electrons are then donated to cytochrome c, which transfers electrons from complex III to complex IV, cytochrome oxidase. We hit different subunits of cytochrome c oxidase, (COX6C,and COX 7B), Complex IV transfers electrons from cytochrome c to O_2. Throughout this process, protons have been moved through the complexes I, III, and IV into the intermembrane space, creating a proton gradient of 1:10; 10 intermembrane space protons for every one matrix proton.[62] The last complex in the chain, ATP synthase, allows protons to flow back into the matrix and uses the energy from their return to create ATP.[62] Part of this complex, the ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1, cardiac muscle (ATP5A1), was hit three times in the

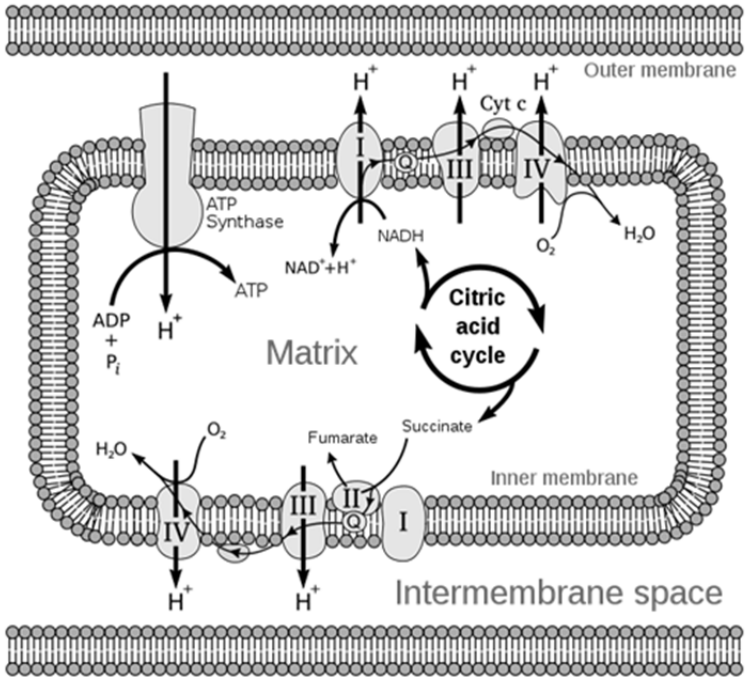

Figure 2.9. Summary of electron transport chain. Electrons are carried along a chain of protein complexes within the inner mitochondrial membrane. The yeast two-hybrid screen has hit several proteins imperative in this process. For details, see text.. Image credit: http://en.wikipedia.org/ wiki/File :Mitochondrial_electron_transport_chain%E2-%80%94Etc4.svg

yeast two-hybrid screen. Since the electron transport chain generates large amounts of energy for the cell, dysfunction of any of the involved proteins is detrimental to the energy levels of the cell, and many pathological conditions exist. Chapter 5 discusses the possible pathologic implications the involvement of HspB2 with the various proteins that help to produce energy for the cell and links our results to previously discovered defects in ATP production in HspB2-deficient mice. Adenylate kinase 4 (AK4) was also hit in the yeast two-hybrid screen. It has been shown to localize in the mitochondrial matrix.[63] AK4 has extensive ability to phosphorylate and has been shown to retain its enzymatic function once imported into the mitochondria.[64]

Several other mitochondrial proteins of interest have been hit that do not correspond to energy production in the cell. Methylmalonic aciduria (cobalamin deficiency) cblD type with homocystinuria (Mmadhc), has been hit twice in the screen. This protein is involved in the metabolism of vitamin B12, a molecule essential for human development and survival.[65] Its name stems from the fact that mutations in the gene cause either methylmalonic aciduria, homocystinuria, or both.[66] Another protein, mitochondria translation initiation factor 3 (MTIF3) is involved in initiation complex formation on mitochondrial ribosomes.[67] We hit MTIF3 twice in the yeast two-hybrid screen. Of particular interest are two proteins we have hit 186 times. They align to two categories, each with homology to mitochondrion DNA: pterin-4-alpha-carbinolamine dehydratase-2 (PCBD2) as well as NADH ubiquinone oxidoreductase chain 2-like. PCBD2 has been shown to have similar enzymatic and coactivator activities to PCBD1. PCBD1 serves as a coactivator of transcription as well as pterin-4-alpha-carbinolamine dehydratase activity, a process playing a role in amino acid hydroxylases and nitric oxide synthase.[68-70] Interestingly, a role for sHSPs in the regulation of nitric oxide production, a

known regulator of mitochondrial Ca^{2+} uptake, has already been hypothesized.[71, 72] By homology, NADH ubiquinone oxidoreductase chain 2-like probably functions as a subunit of complex I in the respiratory electron transport chain.[73] As discussed in chapter 5, these intriguing findings could suggest an intimate relationship between HSPB2 and several potentially uncharacterized proteins. Overall, more hits from our yeast two-hybrid are of mitochondrial association than any other category.

Electrolyte-Related

Maintenance of electrolyte levels in the cell is crucial for maintaining cellular function. Several of the electrolyte-related proteins were hit in our yeast two-hybrid screen. We hit CLIC6, chloride intracellular channel 6, twice in the screen. The CLIC family of proteins is thought to code for intracellular chloride channels, however a study in 2003 failed to find any chloride channel activity by voltage clamp studies.[74] Thus, the function of this protein is unknown. We also hit several ATPase genes that code for channel proteins (see Table 2.2). The most interesting channel proteins we hit in the yeast two-hybrid screen are voltage-dependent anion channels 1 and 2. The VDAC proteins are the most abundant proteins in the outer mitochondrial membrane and are of particular interest because of their functions relate to apoptosis.[75] VDAC1 typically is pro-apoptotic, while VDAC2 has been shown to prevent apoptosis.[75-80] The significance of these proteins is discussed in further detail in chapter 5.

Kinases

Two protein kinases have been hit in the yeast two-hybrid screen. Adrenergic beta receptor kinase I, ADRBK1—also called GRK2 in the literature—is thought to play an important role with relation to heart failure. Studies show that it is a regulator of the heart's inotropic response and that it is upregulated in heart failure with nonfailing myocardium.[81-84] Additionally, we hit mitogen-activated protein kinase-activated protein kinase 3 (MAPKAPK3). Interestingly, MAPKAPK3 was shown to be activated in response to cellular stress, a particular finding that perhaps has relation to HSPB2. Researchers also showed that this kinase performs in vivo phosphorylation of HSP27, another of the sHSPs.[85] The significance of potential interactions with kinases is discussed more in chapter 5.

Redox

The yeast two-hybrid screen has yielded putative interactions between HspB2 and two redox proteins, namely, glutathione peroxidase 3 (GPX3), and thioredoxin interacting protein (TXNIP,). Many diseases, including rheumatoid arthritis, reperfusion injury, cardiovascular disease, immune injury, and cancer, are associated with the production and presence of free radical species of oxygen.[86, 87] Of particular interest to us is the importance of radical oxygen species production by the heart, especially during times of ischemia and reperfusion.[87] Glutathione peroxidase 3 protects the heart by its antioxidant ability to scavenge hydrogen peroxide and other hyperoxides produced in the cell.[88, 89] TXNIP interacts with and inhibits

thioredoxin antioxidant function and is suppressed by mechanical strain. Overexpression of TXNIP results in greater levels of reactive oxygen species in the cell.[90]

Small Heat Shock Protein

As discussed in detail in chapter 1, sHSPs confer stress resistance to the cell. In the yeast two-hybrid screen we hit two sHSPs: αβ-crystallin (CRYAB, 17 hits). As discussed previously, CRYAB is highly expressed in the lens of the eye as well as in the heart where it serves as a protein chaperone.[91-93] Mutations in CRYAB have been shown to cause cardiomyopathy, however the mechanisms of action are currently unknown. Further significance of the relationship between HspB2 is discussed in Chapters 4 and 5.

Other

While there are several other proteins we hit in the yeast two-hybrid screen, they do not all fit into the categories discussed above. For a brief list and description see Table 2.2.
The primarily mitochondrial and muscle hits from the yeast two-hybrid screen for interactions with HspB2 confirm a role for HspB2 in muscle ATP production and identify key potential substrates. As mentioned previously, hits from the yeast two-hybrid screen do not necessarily denote protein-protein interactions, however they do suggest the possibility of such. Further tests are necessary to come to the conclusion that the aforementioned proteins interact with HspB2, including the HspB2 dependency test (see chapter 3) and coimmunopurification in

mammalian cells (see chapter 4).

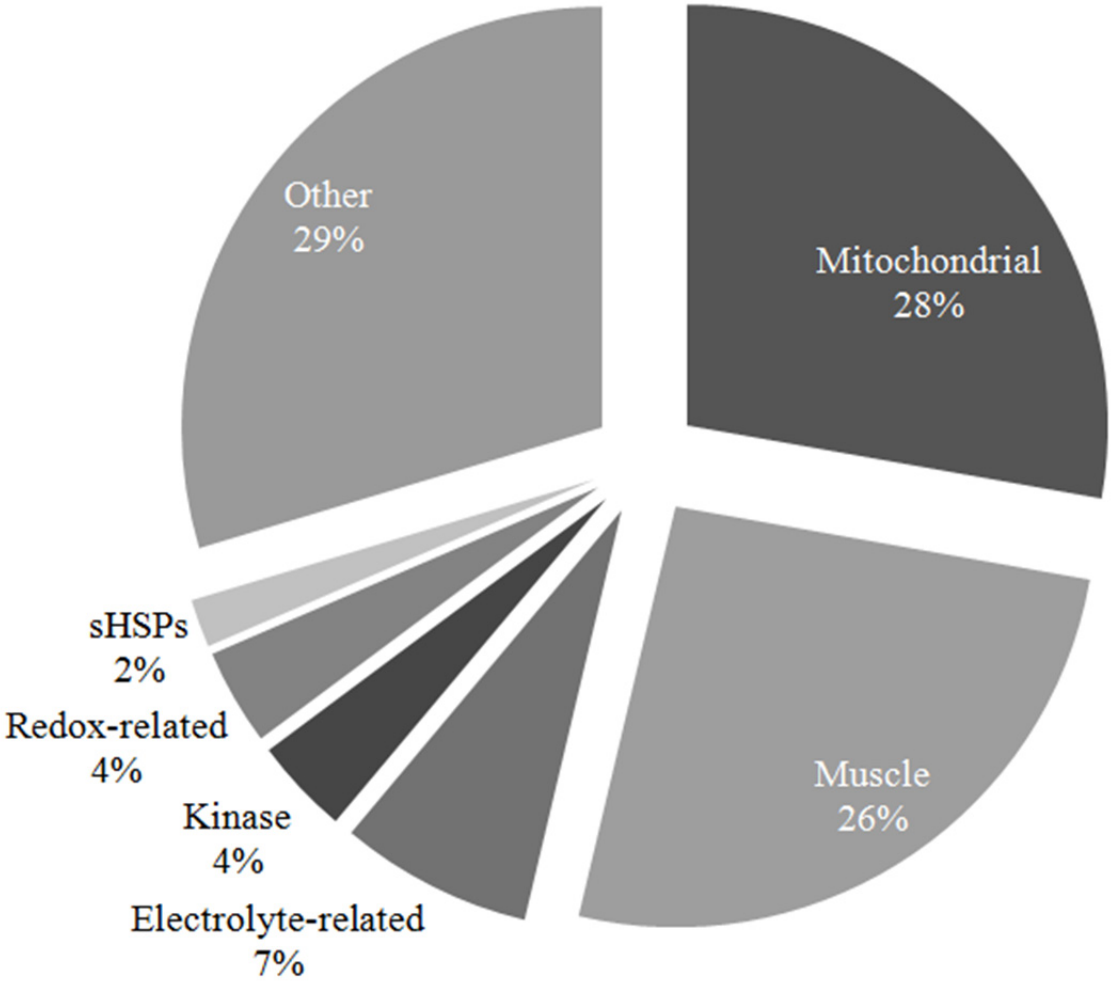

Figure 2.10. Pie chart of the yeast two-hybrid protein hits. Results were tabulated using the information from Table 2.2. Note that several of the proteins were batched together and treated as one protein: i.e. NDUF proteins and ATPases.

Table 3.2. (below) Table of proteins hit more than once from the yeast two-hybrid screen. These 55 proteins are of particular interest because of their involvement in various cellular processes. Brief summary descriptions come largely from in-text citations, however in cases wherein the protein was not discussed in-text short descriptions are summaries of entried found at genecards.org/Wikipedia.org/OMIM for the respective proteins.

Muscle Proteins			
Gene Abbrev.	**Full Gene Name**	**Hits**	**Summary**
ACTA1	Actin, alpha 1, skeletal muscle	3	Thin filament; contractile function
ACTC1	Actin alpha, cardiac muscle 1	16	Thin filament; contractile function
CMYA5	Cardiomyopathy associated 5	2	Possible muscle structural maintenance
DES	Desmin	2	Structural muscle protein, found at Z-line
FHL2	Four and a half LIM domains 2	2	Structural muscle protein, found at Z-line
MY0M2	Myomesin (M-protein) 2	2	Putative role in muscle stabilization
MYBPC3	Myosin binding protein C, cardiac	6	Contractile, regulation/myosin filaments
MYH7	Myosin heavy chain 7 cardiac muscle, beta	5	Composes contractile heavy chain
MYL2	Myosin light chain 2 regulatory cardiac slow	4	Regulatory and contractile roles
MYL3	Myosin, light chain 3, alkali; ventricular, skeletal, slow	2	Regulatory and contractile roles
TCAP	Titin-cap (telethonin)	2	Structural muscle roles
TNNI3	Troponin I type 3 (cardiac)	2	Attaches troponin to actin
TNNT2	Troponin T type 2 (cardiac)	2	Attaches troponin to tropomyosin

TTN	Titin	2	Confers elasticity to muscle; organization
Mitochondrial Proteins			
Gene Abbrev.	**Full Gene Name**	**Hits**	**Summary**
ACAA2	Acetyl-CoA acyltransferase 2	2	Involved in β-oxidation of fatty acids
ALDOA	Aldolase A, fructose-bisphosphate	6	Involved in glycolysis
ATP5A1	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1, cardiac muscle	3	Involvement in the electron transport chain
COX6C	Cytochrome c oxidase subunit Vlc	8	Involvement in the electron transport chain
COX7B	Cytochrome c oxidase subunit 7B	2	Involvement in the electron transport chain
ENO3	Enolase 3 (beta, muscle)	3	Muscle protein with involvement in glycolysis
ETFA	Electron-transfer-flavoprotein, alpha polypeptide	4	Electron acceptor in β-oxidation
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	6	Involved in glycolysis
HK1	Hexokinase 1	2	Involved in glycolysis
MMADHC	Methylmalonic aciduria (cobalamin deficiency) cblD type, with homocystinuria	2	Vitamin B12 metabolism
MTIF3	Mitochondrial translation intitiation factor 3	2	Initiation complex formation
--	Mitochondrion with NADH-ubiquinone oxidoreductase chain 2-like	102	Putative subunit of complex I in electron transport chain
NDUFA3	NADH dehydrogenase (ubiquinone) 1 alpha	1	Subunit of complex I in electron

	subcomplex, 3, 9kDa		transport chain
NDUFA6	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 6, 14kDa	1	Subunit of complex I in electron transport chain
NDUFAF1	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, assembly factor 1	1	Subunit of complex I in electron transport chain
PCBD1-like	Mitochondrion with pterin-4-alpha-carbinolamine dehydratase-2	84	Coactivator of transcription and other enzymatic function
SDHA	Succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	5	Involvement in citric acid cycle and electron transport chain
Electrolyte-related Proteins			
Gene Abbrev.	**Full Gene Name**	**Hits**	**Summary**
ATP1A1	ATPase, Na+/K+ transporting, alpha 1 polypeptide	1	Integral membrane protein Na+/K+ exchanger
ATP1B1	ATPase, Na+/K+ transporting, beta 1 polypeptide	1	Integral membrane protein Na+/K+ exchanger
ATP1B3	ATPase, Na+/K+ transporting, beta 3 polypeptide	1	Exact function unknown; non-catalytic part of the enzyme
ATP2B4	ATPase, Ca++ transporting, plasma membrane 4	1	Assists in removal of calcium ions from the cells
CLIC6	Chloride intracellular channel 6	2	May form chloride ion channels
VDAC1	Voltage-dependent anion channel 1	4	Forms channel through outer mitochondrial membrane; pro-apoptotic
VDAC2	Voltage-dependent anion channel 2	2	Forms channel through outer

			mitochondrial membrane; antiapoptotic
Redox-related Proteins			
Gene Abbrev.	**Full Gene Name**	**Hits**	**Summary**
GPX3	Glutathione peroxidase 3	2	Neutralization of hydrogen peroxide
TXNIP	Thioredoxin interacting protein	2	Inhibitor of thioredoxin antioxidant function
Kinase Proteins			
Gene Abbrev.	**Full Gene Name**	**Hits**	**Summary**
ADRBK1	Adrenergic beta receptor kinase I	1	Regulates inotropic response of heart
MAPKAPK3	Mitogen-activated protein kinase-activated protein kinase	1	Activated in response to stress
Small Heat Shock Proteins (sHSPs)			
Gene Abbrev.	**Full Gene Name**	**Hits**	**Summary**
CRYAB	Crystallin, alpha B	**17**	Highly expressed in heart and skeletal muscle; R120G mutation confers cardiomyopathy
Other Proteins			
Gene Abbrev.	**Full Gene Name**	**Hits**	**Summary**
B2M	Beta-2-microglobulin	3	Serum protein associated with MHC heavy chain
DAZAP2	DAZ associated protein 2	3	RNA-binding protein
DBNDD2	Dysbindin (dystrobrevin binding protein 1) domain containing 2	2	Putative role in apoptosis
EEF1A1	Eukaryotic translation elongation factor 1 alpha	7	Elongation factor subunit

GPR146	G protein-coupled receptor 146	2	Involvement in membranes/G-protein receptors
GUK1	Guanylate kinase 1	3	Guanine metabolism
LGALS1	Lectin, galactoside-binding, soluble, 1	4	Possible apoptosis/cell proliferation/cell differentiation protein
MALAT1	Metastasis associated lung adenocarcinoma transcript 1 (non-protein coding)	2	Possible links to lung cancer
MTRNR2L2	MT-RNR2-like 2	4	Neuroprotective and antiapoptotic function
NPPA	Atrial natriuretic peptide A	5	Helps in the regulation of natriuresis/diuresis/ vasodilation
PSAP	Prosaposin	5	Precursor for cleavage products
RPL11	Ribosomal protein L11 transcription variant 2 mRNA	3	Subunit of 60S ribosomal subunit
RPL36A	Ribosomal protein L36A	6	Subunit of 60S ribosomal subunit
RPN2	Ribophorin II	2	Membrane protein associated with rough ER
SEC62	SEC62 homolog (*S. cerevisiae*)	3	Preprotein translocation (by similarity)
SNRPG	Small nuclear ribonucleoprotein polypeptide	2	Possible histone 3'-end processing

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CHAPTER THREE: Aim Two: Verify putative HspB2 binding partners in yeast by the HspB2 dependency test.

Introduction

As mentioned previously, the yeast two-hybrid screen generated a large list of possible protein-protein interactions between HspB2 and the library of human heart proteins. While these findings are valuable, further verification in yeast, and later in mammalian cells, is required for results to be credible. The HspB2 dependency test allows us to determine whether an interaction garnered from Aim One is a true or false interaction in yeast. This additional means of verification show us if expression of the reporter genes is dependent on the presence of the protein and its interaction with HspB2. Due to the labor-intensive nature of these tests, we perform them only with proteins we have recovered two or more times from the screen. As discussed in chapter two, the yeast two-hybrid system can serve two purposes. It can be used as a method of screening for interactions with a cDNA library as well as its use to test putative protein-protein interactions.[1] The HspB2 dependency test is an example of the latter. HspB2 dependency tests required us to first purify each prey plasmid. Following purification the plasmids are transformed into yeast, the yeast are allowed to grow under selection for both plasmids, and then are streaked to media that will confirm an interaction as explained below.. We purify the plasmids from yeast by first selecting from the 91-grid plate the appropriate yeast harboring the protein that putatively interacts with HspB2. From the 91-grid plate the yeast should, in theory, contain two types of plasmids—the bait and the prey plasmids. Recall from Chapter Two that the bait plasmid contains the gene for HspB2 fused to the GAL4 transcriptional activator DNA-binding domain. On this same plasmid is TRP1, a tryptophan biosynthesis gene. The prey plasmid contains cDNA made from a library of mRNA collected

from three normal male hearts. The library gene is fused to the GAL4 transcriptional activator activation domain. Recall also that the prey plasmid contains a gene for leucine biosynthesis (LEU2). In order to select for only the prey plasmid and ensure that we have plenty of yeast for purification, we grew yeast for two to three days in synthetic glucose media lacking leucine (SD-Leu media). When grown in SD-Leu, media that lacks leucine but includes tryptophan, the yeast will jettison the bait plasmid because it is energetically expensive to maintain a plasmid that is not necessary for its survival. . Next, we centrifuged the yeast, aspirated the media, and treated the yeast with lyticase and 10% sodium dodecyl sulfate (SDS) (see Protocols in Appendix). The lyticase procedure is necessary to lyse the yeast due to the presence of the robust yeast cell wall. Next, lysed yeast go through the Sigma-Aldrich GenElute™ Plasmid Miniprep Kit. Since yeast do not typically maintain a high copy number of its plasmids per cell like in *E. coli*, after the purification procedure we have recovered a very low concentration of plasmid—hardly detectable by spectrophotometric analysis. In order to amplify the concentration of plasmid available we transform a portion from the yeast plasmid prep into the DH5α strain of *Escherichia coli* and grow on LB+amp (LB is lysogeny broth, amp is the antibiotic ampicillin) plates since the prey plasmid contains an Amp^r marker. Single *E. coli* colonies growing on the LB+amp plates are chosen and are grown in LB-amp liquid overnight. We next perform plasmid preps on the *E. coli* using the same kit used previously. We sequence library genes contained on the plasmids in order to verify their identity. Following sequencing, the plasmid is entered into the Grose Lab plasmid database by giving it a number that is unassociated with its identity (i.e. pJG 745). Renaming the plasmids allows us to perform the HspB2 dependency tests blindly, helping to eliminate potential bias.

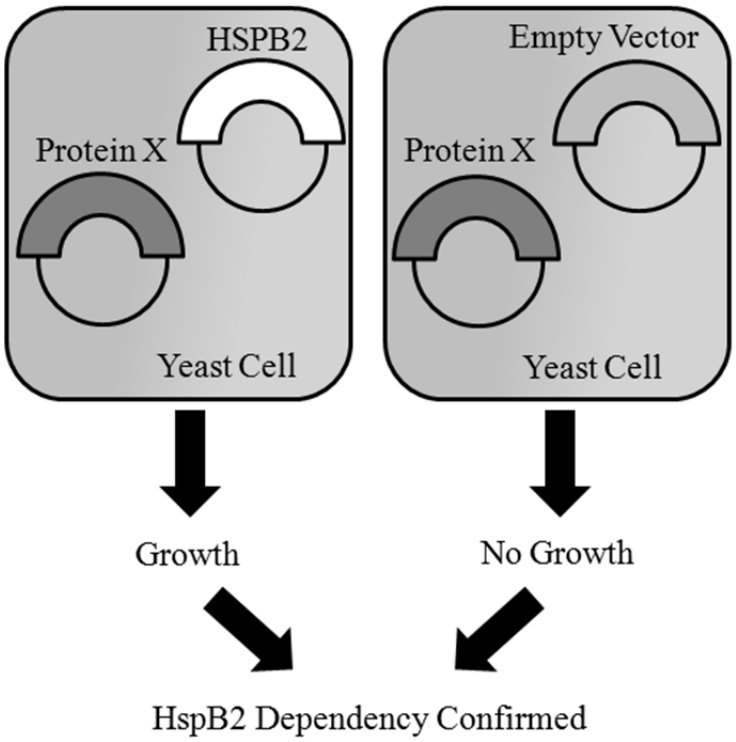


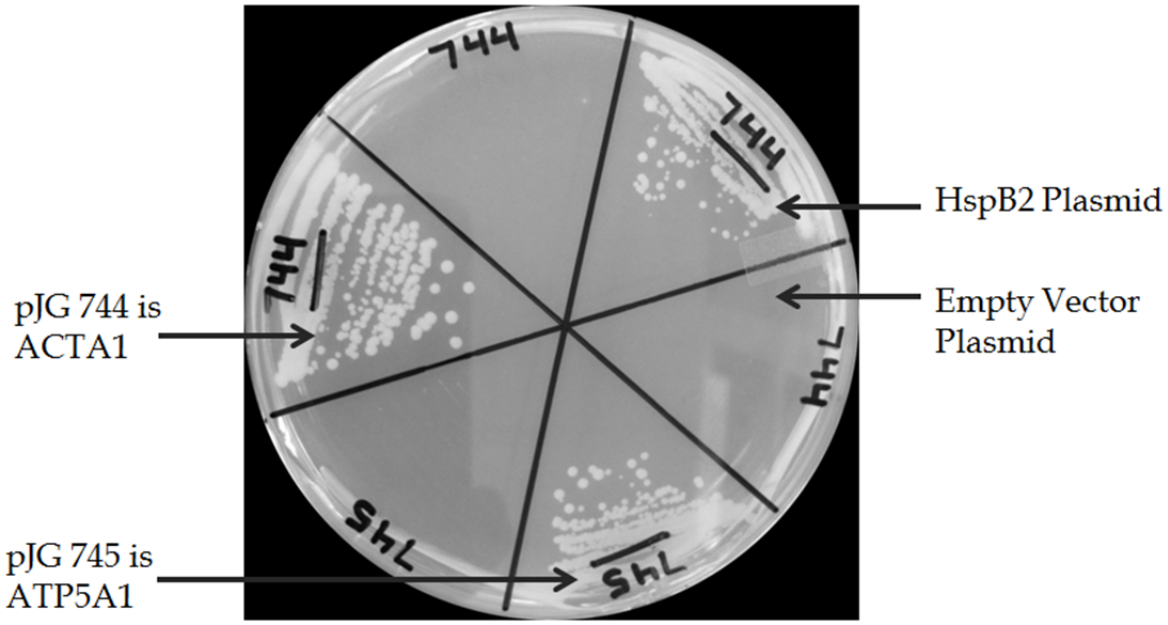
Figure 3.1. HspB2 dependency test. Plasmids are transformed into yeast as shown above. A plasmid containing a gene from the library) (Protein X) and the HSPB2 plasmid are co-transformed into the Y2HGold yeast strain (Clontech). In addition, protein X plasmid is transformed along with an empty vector into the same strain in a separate transformation. Growth on selective media will verify expression of reporter genes due to protein X/HspB2 interaction. In order for dependency to be established, yeast possessing HSPB2 plasmid must grow on selective media while the Protein X and Empty Vector plasmid-containing yeast will not grow. Thus the cell is "dependent" upon the presence of HspB2 in order for expression of reporter genes.

We transform each prey plasmid into two different yeast strains: a yeast strain containing the bait plasmid encoding HspB2 (pJG593A) and a strain containing an empty vector plasmid that does not express HspB2 but that does encode the tryptophan biosynthesis gene (pJG485) (see Figure 3.1). Both strains of yeast are created from the same Matchmaker™ Gold (Y2HGold) strain of yeast used in the yeast two-hybrid screen in Aim One. After the yeast is transformed with the bait plasmid we grow it on SD-Leu-Trp plates to select for yeast in which the transformation has occurred. Yeast that grow on this media contain each of the two plasmids, however growth at this stage does not denote a protein-protein interaction. In order to determine whether the proteins interact with each other and thereby initiate transcription of various reporter genes, we streak a colony containing the library (Protein X) and HspB2 plasmid onto synthetic glucose plates lacking leucine, tryptophan, histidine, and adenine (SD-Leu-Trp-His-Ade plates) alongside a colony containing both the library and the empty vector plasmids.

Colonies that grow regardless of whether the library plasmid is transformed along with the bait- or empty vector are deemed false-positives. This is because they activate transcription regardless of the presence of HspB2. Colonies that do not grow in either situation are deemed true negatives and most likely arise from chromosomal mutations or other artifacts. Colonies that grow in the presence of the HSPB2 plasmid but do not grow in the presence of the empty vector plasmid are deemed HspB2 dependent because growth is dependent on the expression of HspB2 (see Figure 3.2). We perform each HspB2 dependency test in duplicate from two independently isolated library plasmids as determined in Aim One. If growth on selective media is decidedly dependent on HspB2 with one copy of a gene and we find the other copy of the same gene differ in its dependency status, the transformations and dependency tests are repeated and the results compared again.

Yeast provide a simple eukaryotic environment in which we can test protein-protein interactions. We use the yeast two-hybrid system to detect protein-protein interactions as well as to verify them in yeast. Although these results do not always translate into interactions in mammalian cells, a previous study reports that approximately 80% of yeast two-hybrid results are then verifiable in mammalian cells.[2]. Verification first by yeast two-hybrid and then by HspB2 dependency tests provide credible data ready for further testing in mammalian cells, as follows in Aim Three.

Figure 3.2. (see below) HspB2 dependency test plate containing yeast streaked onto selective media (SD-Leu-Trp-His-Ade). Colonies that have grown on this selective plate are determined to be dependent upon the presence of HspB2 in the cell since only yeast containing both library plasmid and HspB2 grow. This plate confirms that expression of yeast two-hybrid reporter genes is dependent on an interaction between Acta1 and Atp5a1 with HspB2.



Results

Results for the HSPB2 dependency tests can be found in Table 3.1. For a short description of the various functions, please see the in-text protein descriptions in Chapter 2 or refer to Table 2.2.

Proteins confirmed to be HspB2 Dependent			
ACAA2	CRYAB	GUK1	RPL36A
ACAA2	DES	Mito/pterin	RPN2
ACTA1	EEF1A1	Mito/pterin	SDHA
ACTA1	ENO3	Mito/NADH	SEC62
ATP5A1	ENO3	Mito/NADH	SNRPG
ATP5SL	GAPDH	MTIF3	TNNI3
CMYA5	GAPDH	MTRNR2L2	TNNI3
CMYA5	GPR146	MYBPC3	TNNI3
COX6C	GPR146	MYL3	TXNIP
COX7B	GPX3	MYL3	
CRYAB	GPX3	PARVA	

Table 3.1. Proteins confirmed to be HspB2 dependent. Each gene is tested in duplicate from two independent plasmids isolated from the screen. Note that some have been done more than twice.

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CHAPTER FOUR: Aim Three: Verify putative HspB2 binding partners in mammalian cells (HEK 293).

Introduction

HSPB2 dependency tests were conducted after identifying putative interactions between HSPB2 and a library of human heart proteins in the yeast two-hybrid screen (Chapter Three). Following HSPB2 dependency testing we moved on to verification in mammalian cells in order to gain more credible results. Many methods exist for the detection of protein-protein interactions, however, one of the most trusted methods for verification is coimmunopurification.[1] Since co-immunoprecipitation is laborious, generally only a subset of yeast two-hybrid hits are tested. We began our verification by testing HspB2 with itself (a known interaction), and HspB2 with CryAB (a hit from our screen).

Co-immunopurification has been used widely in the literature to identify protein-protein interactions. In essence, the experiment is comprised of purifying a target protein from cell lysate and testing to see if another protein copurified through association with the target protein. After purification the samples are run on Western blot, which consists of running a protein gel, blotting to a nitrocellulose membrane, and imaging with antibodies to detect proteins of interest. Our co-immunoprecipittion experiment to verify the yeast two-hybrid interactions was set up as follows. An empty pCMV-myc as well as pCMV-HA vector set was purchased from Clontech, which would allow expression of our fusion proteins of interest from the CMV promoter. Plasmids (pCMV-myc) containing HspB2-myc were provided to us by our collaborators, the Ivor Benjamin lab. Plasmids containing CryAB-HA were created by subcloning CRYAB obtained from one of the "prey" plasmid isolated in our screen through the restriction enzymes SfiI/XhoI, which allows direct, in-frame cloning into pCMV-myc. The resulting plasmids were

then transfected in HEK 293 cells. Plate A contained the following transfections: pJG550 (HspB2-myc) with pJG552 (HspB2-HA), with pJG904 (CryAB-HA), and with the empty HA vector. Transfections were performed in duplicate and two wells from the 6-well plate were combined in order to ensure that adequate protein was available for CoIP. Plate B was transfected in the same manner, but with pJG554, an empty pCMV-myc clone, instead of pJG550. This was used as a negative control.

We chose to conduct our experiment in HEK 293 cells because of their ease of culturing and transfecting. Our cells were graciously given to us by the Laura Bridgewater lab at Brigham Young University, to whom we owe thanks. We cultured cells in Gibco DMEM high glucose (Life Technologies Cat. No. 11960-051) supplemented with 4mM sodium pyruvate, 4mM L-glutamine, and 10% fetal bovine serum (FBS). Cells were cultured in 75 cm^2 Greiner filter-capped flasks. When split, cells were released from the flask surface using .05% trypsin-EDTA and light bumping of the flask. Transfection was performed with Lipofectamine LTX per the instructions specific for transfecting HEK 293 cells.[2]. The media was changed approximately five hours after transfection was performed to reduce toxic effects from Lipofectamine. Cells were allowed to grow for approximately 48 hours after transfection and were harvested with 0.5 mL lysis solution (0.5% NP-40, 20mM Tris-HCl [pH 7.8], 180mM NaCl, 1mM EDTA, and complete protease inhibitor cocktail). We placed the cells on ice for approximately 20 minutes with gentile shaking and collected them in 1.5mL tubes after which they were put in a rotator for 10 minutes. Tubes were then centrifuged at 15,000rpm for 15 minutes to remove debris. The supernate was removed and placed into a newly-labeled tube that had chilled on ice. Note that it is critical to keep everything as cold as possible throughout the process so as to inhibit potential proteases. The contents of the tubes at this point contained what is called the crude lysate. 50μL

of crude lysate is prepared for gel electrophoresis by the addition of 12μL of 5x SDS loading buffer followed by boiling for 5 minutes. These samples are then placed in the freezer. 20μL of washed and buffer equilibrated anti-myc antibody beads were added to the remaining .45mL that is then incubated at 4^{o}C for approximately four hours. The washing and equilibration of antibody beads is a crucial step and must be performed well in order to achieve good binding of protein to the beads (see the appendix for a precise protocol). After beads have been incubated with the cell lysate, the beads are pelleted then washed three times with 1mL cold NP-40 lysis buffer. After the third wash, the lysis buffer is removed and 30μL of 4x SDS loading buffer is added. The tubes are then boiled for 5 minutes, spun down for 30 seconds, and kept on ice or in the refrigerator/freezer for the protein gel and Western blot. Results for our CoIP experiment are detailed below.

Results

The co-immunoprecipitation experiment successfully verified the known interaction between HspB2 and itself as well as a novel interaction between HspB2 and CryAB (Figure 4.1). The plasmids pJG550 (HspB2-Myc) and pJG552 (HspB2-HA) were co-transfected as a positive control because sHSPs have been shown to interact with themselves to form dimers.[3] The second set of transfections was pJG550 (HspB2-Myc) with pJG904 (CryAB-HA), this was our interaction in question. Since CryAB was hit multiple times in the yeast two-hybrid screen and it was shown to interact in the HSPB2 dependency test (Chapter Two), this co-immunoprecipitation was the final step in verification.

It is necessary in co-immunoprecipitation experiments to have adequate controls to make sure you have detected a true interaction between proteins. The third transfection was with pJG550 (HspB2-Myc) and the empty HA vector. This was our negative control and allowed us to make sure that it wasn't the HA that bound the myc beads or the HspB2-Myc protein. A duplicate plate was transfected with pJG554 (empty pCMV-myc) replacing pJG550 (HspB2-Myc) as the other necessary negative control, although it is unlikely that CryAB would bind to the myc-sepharose beads. This plate included pJG551 (empty pCMV-myc) transformed with pJG552 (HspB2-HA), pJG904 (CryAB-HA) and empty pCMV-HA.

Results of the co-immunoprecipitation are shown in Figure 4. The first image is a 20 minute exposure using anti-HA beads to detect protein. Since we can see bands corresponding to the sizes of HspB2-HA (lane 1), and CryAB-HA (lane2) from the transfections done with HspB2-Myc, but not empty pCMV-myc this tells us that 1) HspB2 dimerizes, 2) HspB2 and

CryAB bind one another, and 3) none of the proteins bound to Myc or HA alone. The significance of this novel HspB2-CryAB interaction will be discussed in Chapter 5.

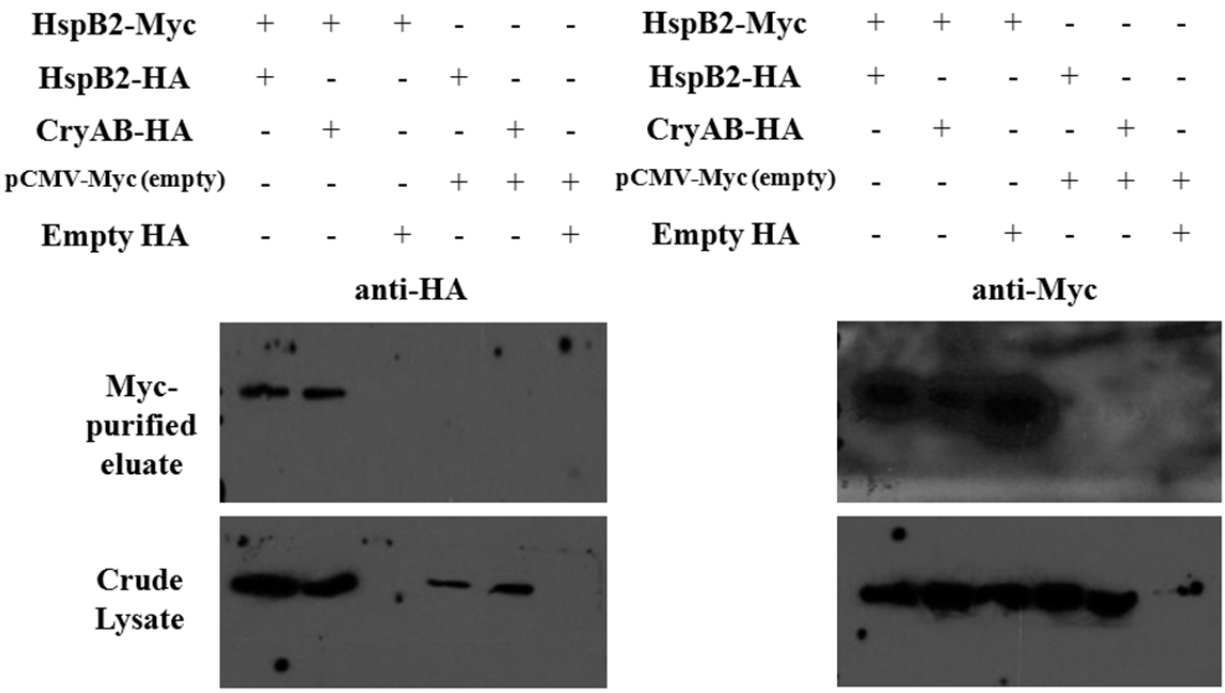

Figure 4.1. Western blot of coimmunopurification with anti-Myc beads. The left panel is an anti-HA blot of the anti-Myc purification. The right panel denotes an anti-Myc blot of the anti-Myc purification. This figure identifies a novel interaction between HspB2 and CryAB. See text for additional information.

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CHAPTER FIVE: Discussion and Conclusions

Discussion and Conclusions

Characterizing protein-protein interactions within the cell is a powerful method for understanding protein function. Studies to date had not sought the identification of interacting pairs on a library-wide scale for any of the small heat shock family, but rather with proteins already suspected to bind to HspB2. In fact, only three proteins had previously been shown to interact with HspB2, HspB3 and Hsp20.[1, 2] The yeast two-hybrid screen afforded us a look into the normal binding partners of HspB2, allowing a chance to characterize the protein at a broad level.

Although the yeast two-hybrid screen is quite sensitive and false positive rate is relatively low, HspB2 dependency tests allowed us to verify suspected interactions uncovered by yeast two-hybrid. We had a verification rate of approximately 80% in yeast. In the case of one of the important proteins in the heart (CryAB), additional verification was performed by co-immunoprecipitation. These additional forms of verification produced a set of results with varying surety: those only confirmed through yeast two-hybrid are slightly less sure of a true interaction than those undergoing the HspB2 dependency tests. These, even, are less sure that the protein interaction confirmed by co-immunopurification.

In this section of discussion and conclusions I will recall important findings from the project, ending with the interaction of which we are most certain. As I discuss the results it will be apparent that the data gathered is significant in that it characterizes the role of HspB2 as a protein involved in both muscle and mitochondrial function. HspB2 plays an important role in helping the cardiac myocytes maintain structural, contractile, and regulatory muscle function while also

maintain the energy resources and process of catabolism necessary for running such an energy-intensive system as the heart. Finally, possible sources of error and suggested roads for future experiments are addressed.

Fortunately, the yeast two-hybrid system purchased and used in these experiments is one of the most advanced and sensitive systems available for research. Especially of interest is the Mate and Plate library that was available for purchase which afforded us a method of detecting interactions with a library of human heart proteins. Proteins which were pulled out of the screen represent a putative group of HspB2:protein interactions. Further steps of verification allow a more complete view of the role of HspB2 in the heart. Although results conducted thus far with yeast two-hybrid alone offer important insights into HspB2 function within the heart, further verification in mammalian cells is vital.

Important in characterizing HspB2 in the heart is, as mentioned above, its prevalence in maintenance of muscle and mitochondrial function. We will discuss proteins which were found to interact with HspB2 through yeast two-hybrid alone, followed by interactions verified in the HspB2 dependency tests, and finally the important finding verified by coimmunopurification. An effort will be made to relate protein hits to potential pathological relationships, especially cardiomyopathy. In this manner we will move through the results, finishing with the protein-protein interaction of which we are most sure occurs within the cell.

A large quantity of proteins have been found to interact with HspB2, although many of them have only been pulled out only a single time from the screen, suggesting possible false positives. The yeast two-hybrid uncovered proteins involved in structural, contractile, and regulatory roles within the muscle cell. This finding helps to characterize HspB2 as being heavily involved in the

maintenance of muscle. While the system of detection is not particularly "stressed" in terms of cellular stress inducing expression of sHSPs, it is likely that during times of stress the interactions observed in yeast two-hybrid would only become more pronounced rather than being different from the observed interactions.

In light of the interactions observed in Chapter Two under the "Muscle" heading, it is not surprising that many of the proteins found to putatively interact with HspB2 are also implicated in disease, specifically in cardiomyopathy. This fact gives significance to the study because cardiomyopathy, while not a widespread problem, is one of concern and great importance in public heath circles.[3] Behind coronary artery disease and hypertension, for instance, dilated cardiomyopathy is the United States' leading cause of heart failure and hypertrophic cardiomyopathy—the leading cause of sudden death in athletes.[4, 5] Thus, an increased understanding of potential players in the disease may lead to better treatments.

Of the muscle proteins hit in our yeast two-hybrid screen, many have relation to cardiomyopathy. Up to three-quarters of all cases of hypertrophic cardiomyopathy originate from mutations in two genes, MYH7 and MYBPC3, two proteins which were hit multiple times in the screen. Recall that these myosin proteins comprise the thick filaments of muscle and are necessary for muscle contraction. Also involved in cardiomyopathy are mutations in actin, another of the proteins we hit in the yeast two-hybrid screen.[6, 7] Actin, myosin, desmin and many others proteins discussed in chapter 3 are required for maintenance of proper muscle structure and contraction. Many of these have been implicated in cardiomyopathy when mutated, however, other sources of cardiomyopathy have been observed.

Crucial to the function of muscle is the ability for mitochondria to produce ATP and fuel the contraction of myosin heads. ATP is produced by the catabolism of various sources of energy: carbohydrates, fats, and even proteins. Converting foods to energy is a network of enzymes catalyzing a variety of reactions, many of which occur in the mitochondria and which have been hit in the yeast two-hybrid screen. HspB2 has been shown to localize to the outer membrane of mitochondria.[8] We hit VDAC1 and VDAC2, genes that encode proteins that are situated within the outer mitochondrial membrane, suggesting that perhaps this is one location to which HspB2 localizes. Because we hit many proteins found to localize to the mitochondrial matrix and inner mitochondrial membrane this study suggests that HspB2 also functions inside the mitochondria or helps fold proteins entering the mitochondria. Indeed, nearly 200 hits had homology to uncharacterized mitochondrion DNA, possibly suggesting that HspB2 plays an intimate role with an inner mitochondrial protein. This association may help explain the ATP defects in muscle cells found in HspB2 knockout mice. In addition, the HspB2 association with proteins involved in apoptosis such as cytochrome C and GAPDH may help explain the anti-apoptoic properties of cells overexpressing HspB2.

N o mitochondrial chaperone has been reported however it seems likely since large quantities of reactive oxygen species are produced inside the mitochondria. This study shows that HspB2 associates with Gpx3 and Txnip, proteins known to neutralize hydrogen peroxide and other harmful species in the heart. Ischemia followed by reperfusion is a time of particular ROS generation, and it makes sense that mitochondria would be damaged to the point that energy production decreases due to a result of misfolded proteins, as may occur during this time of stress. This could, in part, explain previous findings as discussed in chapter one regarding ATP

production in KO mice.[9, 10] In addition, it could help explain the previous finding that glutathione levels are greatly affected in mice harboring mutant CryAB.[11]

As stated previously, HspB2 dependency tests offer an additional level of confidence in our yeast two-hybrid results. The HspB2 tests conducted by this study report confident interactions between HspB2 and 28 proteins hit by our yeast two-hybrid screen.

Perhaps one of the most intriguing findings of this study is that HspB2 binds CryAB both in yeast and mammalian cells. One of the reasons HSPB2 was chosen for a yeast two-hybrid study was because of its relation to CRYAB as well as the fact that it was detected in a previous yeast two-hybrid screen using R120G CRYAB as bait (Julianne Grose, unpublished results)., a mutant CryAB that confers cardiomyopathy in mice and man. As presented in Chapter One, HSPB2 and CRYAB share regulatory elements because of their proximity to each other on chromosome 11 and share high amino acid conservation. Thus our verification of a CryAB/HspB2 interaction confirms a close relationship between these proteins.

This study shows that HspB2 is involved in multiple cellular processes. As a small heat shock protein, HspB2 serves to confer stress resistance to the cell and may work closely with other well-studied sHSPs such as CryAB. Particularly prevalent are the putative interactions between HspB2 and muscle and mitochondrial proteins which may help elucidate the mechanisms by which HspB2 affects muscle ATP levels and apoptosis. Further research will be necessary to perform co-immunoprecipitation analysis with the rest of our "high confidence" hits. As part of a larger study protein-protein interaction study composed of several other sHSPs known to be involved in cardiomyopathy, these results may also lead to a deeper understanding of the role of individual heat shock proteins as well as the amount of redundancy within the family. By

constructing a protein interaction network we hope to better understand the mechanism of disease of cardiomyopathy in addition to basic muscle regulation and maintenance.

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APPENDIX A: Protocols—In order of application

Y2H Mating (120X screen) (see also Matchmaker™ Gold Yeast Two-Hybrid User Manual)

Before starting, make sure:

a) Plate of bait-Y2H Gold is freshly streaked (<1month in fridge)
b) Enough plates have been poured (SD–leu–trp–his–ade) (~50 big plates 150 x 15mm)

Steps:

1. Grow up 50mL O/N of bait in SD-Trp
2. Pellet and re-suspend in 50 mL 2x YPAD with 166.7 µL kanamycin
3. Add to flask along with 1mL of yeast library (thaw library at room temp before adding)
4. Slow shake (RPM 40) for 24 hours
5. Pellet 1,000 x g for 10 minutes
6. Rinse Flask with 50 Ml 0.5xYPAD and use to re-suspend pellet
7. Pellet 1,000 x g for 10 minutes
8. Remove supernatant and re-suspend in 10 mL 0.5x YPAD
9. Make dilution series to test mating efficiency starting with 100 uL of mating mix (1/10, 1/100, 1/1,000, 1/10,000)
10. Plate out each dilution on to SD –leu, SD –trp, and SD –leu-trp plates
11. Plate the rest of yeast cells onto SD-leu-trp +Auerobasidin
12. Incubate in 30^{o} incubator (dilution plates 2-3 days, screen plates ~ 7 days)
13. Count and **Record dilution on plates to determine # of matings**
14. Colonies that grow up on screen plates, continue on to patching protocol

Y2H Patching

Before starting make sure:

a) Have plates to patch on (SD –leu – trp – his –ade)
b) Have autoclaved toothpicks

Steps:

1. Make sure to use sterile technique and have a flame on.
2. With a sterile toothpick, pick colony from transformation/mating plate (SD –leu – trp – his –ade) and patch to appropriate plate.
3. Incubate at 30^{o} C for ~2 days or until colonies have grown up
4. Number each colony that grow up and **record these as # of putative interactors.**
5. **Record Strength of interactions (i.e. S=strong, M=medium, W=weak, VW=very weak).**

Lyticase (To lyticase yeast colony to prep for use as template DNA for PCR)

1. Combine in a pre-labeled PCR tube the following:
    a. 10µL 0.5M Tris-HCl (pH. 7.5)

b. 5µL lyticase

2. Use a pipet tip to gently scrape some of a colony from the 91-grid plate and swish into the solution in the corresponding PCR tube. The solution should now be turbid.
3. Vortex the PCR tube.
4. Incubate tubes at 37^{O}C with shaking for 30 min.
5. Boil at 95-100^{O}C for 5 min.
6. Freeze samples at -20^{O}C or use immediately in PCR reaction. If using as template DNA for PCR reaction, spinning the tubes in a

PCR

Note that we used various primers ing the prey plasmid Gal4 activation domain (forward primter) as well as the terminator region (reverse primer) to optimize the PCR reaction

1. Make master mix for the number of lyticase samples plus a negative control.
   Mix (For one 25µL sample):
   a. 18.55µL ddH_2O
   b. 2.63µL NEB Thermopol buffer (New England Biolabs, Inc., Cat. No. B9004S)
   c. 0.79µL 10mM dNTPs
   d. 0.4µL forward primer
   e. 0.4µL reverse primer
   f. After mixing the reagents above, add .25µL Taq DNA polymerase (New England Biolabs, Inc., Cat. No. M0267X)
2. Place 2µL template DNA (to lyse yeast to get template DNA from 91-grid plate see *Lyticase Protocol*) into pre-labeled tube. Note that if you are using template from lyticase protocol of yeast it will be very dirty and must be kept on ice to avoid degradation.
3. Aliquot 23µL into each PCR tube containing template DNA.
4. Cap PCR tubes and place in thermocycler asap.
   a. Thermocycler program times depend on primer.

Plasmid Isolation from Yeast (For transforming plasmid into *E. coli*)

*Denotes points at which you can stop if you do not have time for the entire protocol.

1. Pick a colony from 91-grid plate and inoculate into 5mL SD-Leu (for prey plasmid isolation) or appropriate media for proper plasmid isolation
2. Vortex for 1 min. Leave to grow for ~3 days at 30^{O}C.
3. Spin yeast culture at 13,000rpm for 2 min. in 1.5-2.0mL. Discard supernatant. Repeat until all 5mL are spun down.
4. *Pour off supernate and resuspend the pellet in residual liquid (i.e. do not spin tube and remove all liquid).
5. Add 20µL of 0.5M Tris-HCl (pH 7.5). Mix thoroughly by vortexing/pipetting.
6. Add 10µL lyticase. Mix thoroughly by vortexing/popetting.
7. Incubate samples form 30-60 min. at 37^{O}C in shaker.
8. Add 20µL of 10% SDS and vortex for 1 min.
9. *Freeze samples at -20^{O}C. This usually takes ~15 mins.
10. Thaw at room temperature, but don't let them sit out too long or DNA will degrade.
11. Vortex.
12. Start Sigma-Aldrich GenElute™ Plasmid Miniprep Kit (Sigma, Cat. No. PLN350-1KT)

13. *Freeze yeast plasmid prep at this point or continue to transform into *E. coli*.

Making *E. coli* Plasmid Prep from Yeast Plasmid Transformation:

Steps:

1. Inoculate a colony from transformation plate into 5 mL selective media (LB+AMP)
2. Place in 37^{o} C shaker for 24 hours
3. Spin down at 13,000 rpm for 1 minute to pellet. Remove supernatant. Repeat until all 5 mL is spun down.
4. *You can freeze at this point or continue on to the next step
5. Start SIGMA miniprep kit at step 1 (re-suspend in 200 µL re-suspension buffer). Continue through rest of steps.
6. Check concentration/quality of your prep. You should have at least 100 ng/µL and a sharp clean peak at 260.
7. Place *E. coli* plasmid prep in appropriate box in -20^{o}C freezer for dependency tests to be performed.

Transformation for DH5α *E. coli*

Steps:

1. Retrieve DH5α cells from -80^{O}C freezer and place immediately on ice.
2. Allow cells to thaw on ice for a few minutes.
3. Add 2-5µL of plasmid prep to 25µL DH5α. Keep on ice for 2-30 min.
4. Heat shock for 1 min. at 42^{O}C.
5. Place tube immediately back into the ice bucket to cool for 2-5 min.
6. Add 500µL LB and incubate at 37^{o} C for 30 minutes (if selection is AMP)
7. Plate on selective media (i.e. LB+AMP) and incubate for 24 hours at 37^{o} C.
8. *Place plate with colonies in fridge until ready to grow up an overnight for an *E.coli* plasmid prep.

Transfection and Coimmunoprecipitation

Guidelines for Transfection

- Maintain same seeding conditions between experiments
- Use low-passage cells; make sure they are healthy and more than 90% viable before transfection
- Can be performed with or without serum media
- **Opti-MEM I Reduced Serum Medium (Cat. No. 31985-070)** to dilute DNA Lipofectamine LTX reagent before complexing.

Following Reagents Necessary

- HEK 293 cells in **DMEM (Cat. No. 11960-044)** supplemented with **4mM L-Glutamine (Cat. No. 25030-081)**, **10% fetal bovine serum (Cat. No. 16000-044).**
- Plasmid DNA of interest
- Lipofectamine LTX Reagent
- **Opti-MEM I Reduced Serum Media (Cat. No. 31985-070)**
- Tissue Culture Plates and Supplies
    - Phosphate-buffered saline (PBS)

DAY 1

Transfection Protocol

1. The day before transfection, trypsinize and count the cells. Plate 6.25 x 10^5 cells per well in 2.0 ml of complete growth medium. Cell density should be 50-80% confluent on day of transfection.
2. (Optional) The day of transfection, remove growth medium from cells and replace with 2.0 ml of complete growth medium.
3. For a six-well transfection: dilute 2.5µg DNA into 500µl Opti-MEM Reduced Serum Media without serum.
4. Add 3.75-8.75µl Lipofectamine LTX Reagent to the diluted Opti-MEM:DNA solution (6 uL is the standard), mix gently and incubate 30 min at room temperature to form DNA-Lipofectamine LTX Reagent complexes.
5. After 30 minute incubation, add 500 µl of the DNA-Lipofectamine LTX Reagent complexes directly to each well containing cells and mix gently by rocking the plate back and forth.
6. Complexes do not have to be removed following transfection. Incubate the cells at 37°C in a CO_2 incubator for 48 hours post-transfection performing copurification or expression experiments.

DAY 3

Coaffinity Purification Experiments in HEK 293T Cells

a) Aspirate off media and wash 1 time with cold 1x PBS (2 ml per well of 6 well plate)
b) Add 0.5 ml (0.5% NP-40, 20mM Tris-HCl [pH 8.0],180mM NaCl, 1mM EDTA, and complete protease inhibitor cocktail [Roche]) for 15min on ice. Place on ice for 20-30 minutes with occasional shaking. Collect cells into a 1.5 ml tube using p1000 pipetman. After complete lysis, no cells should be left on the plate.
c) Continue to rotate in fridge for 10 minutes.
d) Centrifuge for 15min at 13,000rpm at 4°C.
e) Take out supernate and transfer to a new tube that has been chilled on ice.
f) Take out 50 µL and add 12 µL of 5X SDS-buffer. Boil 5 minutes. Store in freezer for later (this is the lysate control to check for gene expression).
g) Add anti-myc antibody to the remaining 0.45 mL. Incubate overnight at 4°C while rotating.

DAY 4

h) Add 20 µL of protein G beads that have been washed and equilibrated. Be sure to cut-off the yellow tip of the pipet to be sure it pipets easily. Rotate at 4°C for 1.5 hr, then centrifuge at 4°C at 2000 rpm for 3 minutes to pellet beads.
    a. **Washing and equilibrating protein G beads.**
        i. **Resuspend beads well by inverting. Pipet out the desired volume and mark the side of the eppendorf with a marker to indicate the volume.** Centrifuge at 2000 rpm / 3 minutes at 4°C, then *carefully* remove supernatant with needle and syringe. Replace with 1 mL water and repeat

centrifugation and aspiration. Resuspend in 1 mL NP-40 lysis buffer. Repeat centrifugation, aspiration, and resuspension in buffer but this time add buffer to the mark on the eppendorf so the volume is the original volume.

ii. ******Do not centrifuge protein G beads above 2000rpm******

i) Wash 2x with 1 mL cold lysis NP-40 buffer without proteases. Each time centrifuge at 2000 rpm for 3 minutes in the cold room or fridge, then *carefully* remove supernatant with needle and syringe.
j) Remove lysis buffer after third wash.
k) Add 30 µL of 4x sample loading buffer. Boil 5 min. Spin down 30 seconds. Keep on ice/fridge/freezer until western run with anti-myc and anti-HA antibodies as well as the lysate controls!

Buffers:

a. <u>NP-40 Lysis buffer:</u>

88.5 ml H_2O	(to final volume 100 ml)
5 ml 1 M Tris-HCl (pH 7.5)	to final concentration 50 mM
5 ml 3 M NaCl	to final concentration 150 mM
0.5 ml NP-40	to final concentration 0.5%
0.21 g NaF	to final concentration 50 mM

b. Before use add to each 1 ml of lysis buffer (always add PMSF last):

10 µL 0.1 M Na_3VO_4	to final concentration 1 mM
10 µL 0.1 M DTT	to final concentration 1 mM
10 µL 100x protease inhibitor	to final concentration 1x
10 µL 100 mM PMSF	to final concentration 1 mM

Western Blotting

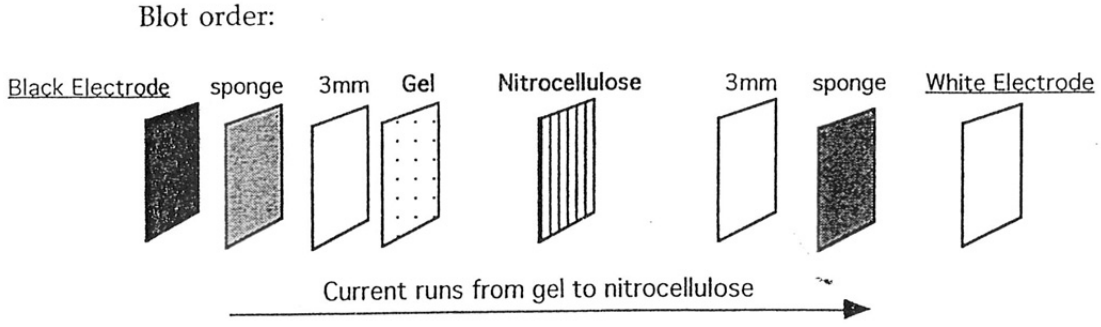
I. Materials

- **Transfer buffer:** 4L = 57.64g glycine; 12.12g Tris base; 800ml Methanol; ddH_2O to 4 liters. Store at 4°C. Recycle 5 times.
- **TBS** (1x): (20 mM Tris, 500 mM NaCl)
  2L = 4.84g Tris; 58.48g NaCl; ddH_2O to 2 liters adjust pH to 7.5 with conc. HCl.
- **TTBS:** 1L = 0.5ml of Tween-20 to 1 liter of TBS
- **Blocking buffer:** 50ml of TBS with 5% dry milk
- **Antibody buffer:** make up 10ml – antibodies are expensive so use as little as possible. Antibody solutions can be saved in the fridge for ~1 month and reused.
  - Primary: use as per manufacturer's instructions. This is usually a 1:500 dilution in 1x TBST with 1% BSA.
  - Secondary: Use either anti-rabbit or anti-mouse depending on primary antibody. Secondary antibodies are usually used at a 1:10,000 dilution in TBST with 1% dry milk.

- **HRP color development solution:** Mix together equal amounts of the reagents right before use. DO NOT MIX EITHER REAGENT INTO THE STOCK VIALS!
- **Stripping Buffer:** 67.5mM Tris. Cl pH 6.7; 2% SDS; 100mM β-ME

II. Method

1. Prepare samples for gel. See preparation of yeast cells for western blotting if necessary. Prepare protein MW markers for gel by boiling the desired amount to be loaded in gel.
2. Pour a SDS-PAGE gel.
3. Western Blot: With gloves, carefully measure and cut nitrocellulose rectangles the size of the western blot sponges. Pour transfer buffer into a large Tupperware tray. Slip one rectangle of nitrocellulose in at a 45° angle. Leave for a few minutes.
4. Open up glass plates of SDS-PAGE gel. Remove stacking gel by scraping off. Carefully bind a rectangle of 3mm paper on top. Roll out air bubbles gently with a plastic pipette. Wet sponges in transfer buffer and put one under and one over the sandwich. Roll out the air bubbles gently. Place sandwich in plastic "cage" with black side on gel side.



5. Set up Western blot apparatus with stir bar inside and ~1/2 full of transfer buffer. Put ice block (or water cooler for large transfer) and sandwich cage(s) inside electrode box. When it's all set up, the transfer buffer should be at the top of the chamber. Place on stir plate and stir rapidly. Set amperage at < 500 mA. As the temperature increases, amperage will increase and the power supply will buzz if it goes over 500mA. Transfer for 1.5 hours. (If using the 200/2.0 power supply, run mini-blot at 300V or full size blot at 800mA.) Be careful – high amperage kills!
6. Open up sandwich and check that all visible markers have transferred to the nitrocellulose.
7. Wearing gloves put nitrocellulose in 50 ml blocking buffer and shake for 30 minutes. Pour out blocking buffer. Rinse blot once with TTBS.
8. Put nitrocellulose into primary (1°) Ab buffer. Incubate with shaking for 2 hours -> overnight.
9. Wash blot 3 times with TTBS for 10 minutes each.
10. Soak blot in 10mls of antibody buffer + secondary (2°) Ab. Incubate 1 hour.
11. Wash blot 3 times in TTBS for 10 minutes each. Then wash once in TBS. Pour off TBS and add color development solution made immediately prior to using. Quickly blot off nitrocellulose paper (NP) on paper towel. Then incubate NP in reaction mix for 1-2 min (up to 10 if you suspect a low signal). Then blot off and wrap in syran wrap, tape into cassette.

Expose on film. There should be bands forming within 1 minute. Once bands are clearly visible, rinse blot with water, dry and photograph, if desired.

Note: If you need to strip and reprobe use ~20-30ml Stripping buffer. Incubate at 50° for 30 minutes. Rinse, blot well, and reprobe.