THE USE OF *C. ELEGANS* TO IDENTIFY MUTATIONS THAT CONFER DRUG RESISTANCE, USING HEMIASTERLIN AS AN EXAMPLE.

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DEDICATION

My journey here at UTSouthwestern has allowed me to engage with a number of incredible people. I would like to thank my mentor Michael Roth for his advice, guidance, his incredible patience and for allowing me the independence to grow as a critical thinking scientist. I would like to thank my thesis committee members, Melanie Cobb, Michael White, and especially Leon Avery for volunteering his time and expert knowledge in genetics and *C. elegans*.

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I dedicate everything positive I have done in my life including the successful completion of my Ph.D. thesis to my first son Isaak Eduardo Cardenas, my youngest son Elias Benjamin Cardenas and to my wife Erica Lee Garza. They inspire me to be the best person I can be.

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Hemiasterlin is a natural product isolated from the several species of marine sponges. Hemiasterlin inhibits microtubule polymerization resulting in cell cycle arrest and apoptosis. Cells over-expressing efflux pumps associated with multidrug resistance are not resistant to the toxic effects of hemiasterlin. Because of these characteristics, hemiasterlin or hemiasterlin derivatives have been involved in several clinical trials.

The Roth lab set out to discover novel modes of hemiasterlin resistance. To that effect, a mutagenic screen to identify hemiasterlin resistant mutant *C. elegans* was performed. As a result of this screen, seven independent true-breeding hemiasterlin-resistant worms were identified.

Studies in this lab have identified hemiasterlin resistance in these worms is due to a mitochondrial dysfunction that results in elevated levels of reactive oxygen species. Interestingly this mitochondrial dysfunction also results in a multi-drug resistant phenotype.

This dissertation describes the identification of the previously unmapped hemiasterlin resistant worm *har-2*. There is no known homolog or function of *HAR-2* outside of other nematode species. HAR-2 has been predicted to be an E3 ligase. Through genetic, biochemical and pharmacological analysis, it is determined that *har-2* is not within the same class of previous hemiasterlin-resistant mutants. *har-2* does not exhibit a mitochondrial dysfunction leading to elevated reactive oxygen species and a multi-drug resistant phenotype. Interestingly it is hypersensitive to the microtubule polymerizer Paclitaxel.

In order to identify possible interacting partners in the hopes of gaining insight into HAR-2 function, a yeast-two-hybrid screen of a *C. elegans* cDNA library was performed using HAR-2 cDNA as the bait. The yeast-two-hybrid experiment identified Rps-1 most frequently as an interactor with HAR-2.

Rps-1 is the homolog to the human S3A small ribosomal protein. Published reports show that increased expression of S3A is correlated with drug resistance and that S3A interacts with the anti-apoptotic factor Bcl-2(Hu *et al.* 2000). The yeast-two-hybrid result identifying S3A as an interactor of HAR-2 suggest a possible mechanism of hemiasterlin resistance in which HAR-2 is involved in the modulation of the interaction of S3A with Bcl-2.

TABLE OF CONTENTS

I. Introduction – Natural Products 1

A. Natural Products and Marine Sources 2
- i. Natural Products as Medicines
- ii. Diverse Compounds
- iii. The Future in Natural Product Discovery

B. The Natural Marine Product hemiasterlin 7
- i. Hemiasterlin initial discovery and research
- ii. Hemiasterlin resistance in *C. elegans*-Mitochondrial mutants

C. The Multidrug Resistance Dilemma 12
- i. Implications of Multidrug Resistance
  - a. Mechanisms of Drug Resistance
    1. Efflux pumps conferring drug resistance
    2. Adaptive mutations lead to drug resistance
  - b. Strategies to combat drug resistance
    1. High-Throughput Compound Screening
    2. The adaptive immune response (vaccines)
    3. Increased knowledge through model organisms

D. Questions addressed in this thesis 26
- i. *C. elegans* genetics
- ii. Major discoveries through *C. elegans* research

II. Genetic Mapping of a hemiasterlin resistant *C. elegans* mutant 27

A. Locating the mutation conferring hemiasterlin resistance 29

B. Results 30
- i. Strategy I: 2pt & 3pt mapping
- ii. Strategy II: Deficiency mapping
- iii. Confirmation of point mutation in gene T24D1.5

C. Discussion 37

III. The determination of har-2 function 41

A. Introduction 41

B. Results 42
i. har-2 does not exhibit MDR phenotype
ii. har-2 respiration rates are wildtype
iii. N-acetylcysteine does not abrogate har-2 resistance
iv. har-2 resistance is independent of pkc-1
v. har-2 resistance is independent of brc-1
vi. the beta-tubulin mutant ben-1 is hemiasterlin desensitized
vii. har-2 microtubule patterns are similar to wt in the presence of hemiasterlin

C. Discussion 53
i. har-2 differs from previously identified hemiasterlin mutants

IV. Yeast Two Hybrid 54

A. Introduction 54

B. Results 56

C. Discussion 59

V. Conclusions and Future Directions 63

A. Conclusions 63

B. Future Directions 65

VI. Materials and Methods 69

A. Genetic Mapping and Complementation 69
i. SNP mapping (linkage mapping)
ii. 3pt mapping

iii. Deficiency mapping
iv. T24D1.5 sequencing
v. RNAi
vi. *C. elegans* microinjection

B. Toxicity assays- 74
i. Toxicity curves
ii. Mitochondrial purification and respiration analysis
iii. pkc-1;har-2 and brc-1;har-2 double mutant constructs
iv. N-acetylcysteine treatment
v. Embryo Microtubule staining

C. Material and Methods – Yeast-two-hybrid screen 79

D. Acknowledgements 84

LIST OF FIGURES

Chapter II: Genetic Mapping of a hemiasterlin resistant *C. elegans* mutant

Figure 1: Dose response curve of hemiasterlin 29

Figure 2: *ad2306* linkage to chromosome I by RFLP 31

Figure 3: Deficiency Mapping 35

Figure 4: Wildtype *HAR-2* DNA resensitizes hemiasterlin mutant..... 37

Figure 5: The HAR-2 Ring domain is similar to BRCA-1 38

Chapter III: The determination of har-2 function

Figure 6: *har-2* does not exhibit a multidrug resistant phenotype 42

Figure 7: *har-2* is hypersensitive to Taxol 43

Figure 8: Basal level respiration comparison of *har-2* and wildtype 44

Figure 9: *har-2* hemiasterlin resistance is not lost after NAC treatment 46

Figure 10: *har-2* hemiasterlin resistance is independent of PKC-1..... 48

Figure 11: The BRCA-1 E3 ligase does not mediate HAR-2 hemiasterlin Resistance 50

Figure 12: Microtubule staining of wildtype and *har-2* embryos 52

List of Tables

Chapter IV: Yeast Two Hybrid

Table 1: Table 1. Potential *har-2* interacting proteins…….... 58

I. Introduction – Natural Products

The majority of compounds (>70%) used to treat diseases are derived from natural sources.(NEWMAN and CRAGG 2007) An explanation for why such a large percentage of medicines should be derived from natural sources is the diversity of species found throughout nature, and the large variety of chemicals produced by such a vast population. To survive extinction, for example, from predation or habitat loss, a plethora of defense mechanisms have emerged in different organisms(DERBY 2007; THERY and CASAS 2009). An example of one defense mechanism is the production of a selectively toxic compound, such as the accumulation of the plant-derived heart poison cardenolide by the monarch butterfly to prevent predation by vertebrates.(BROWER and GLAZIER 1975)

Throughout history, toxic plants have been used as medicines. Early records that show the use of indigenous plants being used to treat parasites found in the intestines date back to the early 6th century BC(OLSNES 2004). Natural product resources have long been sought after for their healing characteristics, specifically in Asia where many Eastern medicines use plants and herbs to combat ailments ranging from inflammation to pain and anxiety.(GOSSLAU *et al.* 2011; LAKHAN and VIEIRA 2010; OLSNES 2004; RAINSFORD 2007)

The success of using natural products for the treatment of various diseases has encouraged many to expand the search for new natural products in previously

unexplored environments. Intriguingly, most of the planet's ocean environment has remained unexplored. The discovery of new natural products from marine sources has great potential; its depths have prohibited its exploration in the past but with the increasing threat of pathogens resistant to current drugs it has become essential to explore the vast ocean resource.

A. Natural Products and Marine Sources

Until recent technical advances in underwater ocean exploration, there had been little progress in marine natural product discovery, thereby leaving a huge resource virtually untapped. It is estimated that 70% of the earth's surface is covered by our oceans, and that oceans contain 90% of the biosphere. Remarkably, as recent as 15 years ago less than 0.1% of the ocean volume had been sampled for scientific purposes.(O'DOR *et al*. 2010a; O'DOR *et al*. 2010b) With the advent of deep-sea robotics and cameras, the improvement in manned and unmanned submersibles has allowed exploration where depths once prevented the scientific study of marine life. The increase in marine exploration has resulted in identifying new species and gives the potential of finding new and interesting compounds to combat disease(AMADOR *et al*. 2003; MOLINSKI *et al*. 2009; NEWMAN and CRAGG 2004a; NEWMAN and CRAGG 2004b)

Some of the organisms surviving for millennia in isolated marine niches would be expected to have developed unique toxic chemical compounds to either

avoid predation or insure successful capture of prey. Such chemicals, as the result of a large scale, long-term natural experiment, should provide highly potent leads for developing novel therapeutics. Indeed, in 2004 Prialt became the first marine derived drug approved by the FDA.(DENOON 2004)

i. Natural Products as Medicines

One interesting study on the benefits of using natural products as medicinal leads, as opposed to drug discovery through combinatorial chemical libraries, is the observation that chemical structures of clinically approved drugs more closely resemble those of natural products than those of the purely synthetic compounds used in most high-throughput screening campaigns.(MOLINSKI *et al.* 2009) Thus, the natural product is usually a starting point for drug discovery that is closer to the desired endpoint. Some examples of natural products or natural product derivatives commonly used today include Aspirin, penicillin, artemisinin, or the anticancer drugs Taxol, Halaven and Tarceva. The development of new therapeutics based on natural products is continuing. Epigallocatechin-3-gallate (EGCG) a polyphenol found in green tea has been shown to significantly inhibit tumor growth in animal studies and clinical trials(CHEN *et al.* 2011). Reports indicate that EGCG reduced levels of the oncogenes c-myc and c-H-ras(CHEN *et al.* 2011). C-myc and c-H-ras are known to be over-expressed in many cancers and are associated with evasion of apoptosis and expression of detoxifying

proteins(PORRO *et al*. 2011; SOMMER *et al*. 2007). As other examples, polyphenols extracted from black tea are used for treating inflammation. The *Streptomyces roseosporus* fermentation product daptomycin is used as an antibiotic against Gram-positive bacteria. Daptomcyin depolarizes the bacterial cell membrane causing loss of membrane potential and eventual cell death(TALLY and DEBRUIN 2000). The potent activator of protein kinase C (PKC), bryostatin-1 isolated from the marine invertebrate *Bugula neritine,* has been shown to inhibit tumor growth by inhibiting the production of components of the matrix metalloproteinases family, down-regulating multidrug-resistance 1 (*MDR1*) gene expression, modulating expression of the antiaptotic protein *bcl-2* and tumor suppressor *p53* and inducing apoptosis(AMADOR *et al*. 2003).

In the treatment of cancer a major challenge is the heterogeneity of cell phenotypes seen within tumors(GERLINGER and SWANTON 2010). This heterogeneity suggests that a diverse set of compounds may be needed to target the multiple pathways and functions that have been altered. A study by Yao et. al, illustrates the extent of heterogeneity in a lung cancer tumor within a single patient. Yao et. al, discovered that treatment of epidermal growth factor receptor (EGFR) inhibitor erlotinib is initially effective in patients harboring an oncogenic EGFR mutation. However, over time many patients will develop resistance to erlotinib. In half of these patients, the acquired resistance is due to a secondary EGFR mutation. Yao et. al, identified a subpopulation of cells harboring EGFR

mutations, derived from tumors that had yet to be treated with erlotinib. Furthermore, these cells showed an increase in expression of TGF-β. Knocking down TGF-β resulted in cells becoming sensitive to erlotinib(YAO *et al.* 2010).

In addition, heterogeneity within single tumors is not the only source of drug resistance in cancer cells. As stated earlier fifty percent of erlotinib resistant tumors have an EGFR mutation, for the remainder of resistant tumors, the mechanism of resistance is unknown. Cellular changes induced by chemotherapeutics can result in altered gene expression, changes in cellular signaling, mutated drug targets and evasion of apoptosis(HIGGINS 2007; PIDDOCK 2006a; PIDDOCK 2006b; WONG and GOODIN 2009; YAO *et al.* 2010). Because of this, it is likely that combinations of compounds that can target different pathways will be needed for successful treatment of cancers. This further emphasizes the need for the continued exploration for natural compounds and medicines as well as innovative methods for the identification of novel species that can potentially synthesize novel compounds.

ii. The Future in natural product discovery

The increase in marine exploration and the discovery of novel organisms and compounds will require new and existing methods to determine their potential as a therapeutic for cancer and other disease therapy. In the past, newly discovered bacteria in the marine environment proved to be difficult to culture

since the nutrient and habitat requirement of the bacteria could not be replicated in cultures as is found in nature. In this regard, innovative techniques continue to be developed in order to isolate novel microorganisms. One example of new innovations in culture techniques is the attempt to mimic the natural environment of an organism of interest. Living cells are used as the source of food, and as a possible source of key signaling mechanism needed for survival of hard to grow cultures(LIU *et al*. 2010). The use of seawater based media to cultivate marine cultures and developing novel high-throughput methods for culture isolation have been recently developed. In addition, the isolation of newly cultured bacteria will increase the likelihood of novel compound discovery(LIU *et al.* 2010). The development of high-quality microbial natural product libraries has been developed by the use of the combinatorial analysis of repetitive sequence based-PCR sequencing (REP) and single-strand conformation polymorphism (SSCP) mapping or DNA fingerprinting. Using this method it has been shown that strains similar in sequence analysis but unique according to REP analysis could produce different surfactant mixtures under the same growth conditions(LIU *et al.* 2010), these results serve as an example that bacterial cultures morphologically identical can produce different compounds and be identified by their REP and DNA fingerprint classification. As new technologies become available, the number of natural products is bound to increase.

Some organizations such as The National Cancer Institute (NCI), pharmaceutical companies and academic institutions have already begun to develop innovative assays and compound screens to evaluate the potential of novel compounds. Academic institutions and pharmaceutical companies have and will continue to invest time and effort in drug discovery in hopes of achieving a better outlook on cancer patient treatment and survival, with the ultimate goal of finding a cure.

B. The Natural Marine Product hemiasterlin

i. Hemiasterlin discovery and research

The tripeptide hemiasterlin (MW:527), is a natural product consisting of 3 heavily modified amino acids found in the marine sponges *Hemiasterella minor* (Kirkpatrick), *Siphonochalina sp., Cybastela sp*. and *Auletta c.f. constricta* discovered in Sodwana Bay in South Africa (TALPIR *et al.* 1994), and Papua New Guinea(COLEMAN *et al.* 1995; GAMBLE *et al.* 1999). After treating MCF-7 human mammary carcinoma cells with hemiasterlin, Coleman et al, observed microtubule depolymerization similar to nocodazole and vinblastine(COLEMAN *et al.* 1995). It was later shown that cell cycle arrest occurs at the G2-M phase with hemiasterlin treatment(KUZNETSOV *et al.* 2009; LOGANZO *et al.* 2003; VASHIST *et al.* 2006). Hemiasterlin has since been identified as highly cytotoxic to a wide range of cancer cell lines, including P388 leukemia, MCF-7 breast cancer (LD_{50}

0.5 nM), U373 glioblastoma/astrocytoma, HEY ovarian, and numerous prostate and bladder caner cell lines(ANDERSON *et al.* 1997; COLEMAN *et al.* 1995; HADASCHIK *et al.* 2008a; HADASCHIK *et al.* 2008b; TALPIR *et al.* 1994).

In 2003 and 2004, Loganzo et. al. probed the activity of the hemiasterlin analog HTI-286 to determine the potential of this compound as a chemotherapeutic(LOGANZO *et al.* 2003; YAMASHITA *et al.* 2004; ZASK *et al.* 2004). Results from those studies included Xenograft models of skin, breast, prostate, brain and colon cancer, and all resulted in tumor growth inhibition.

Cell lines resistant to paclitaxel by overexpressing the p-glycoprotein pump remained responsive to HTI-286. A stepwise increase in HTI-286 resulted in the resistant KB-3-1 epidermoid carcinoma cell line KB-HTI. KB-HTI cells were cross-resistant to DNA-damaging agents while remaining sensitive to paclitaxel. Further investigation revealed highly resistant KB-HTI cells had an alanine^{12} to serine mutation in α-tubulin. These cells became resensitized to HTI-286 after drug removal from the media. Although sensitivity was nearly identical to the parent cell line, the alanine^{12} to serine mutation was not lost. The Loganzo studies revealed that both mutations in α-tubulin and a not yet identified ABC transporter could be involved in KB-HTI acquired resistance to HTI-286. It should be noted that opposing theories of HTI-tubulin binding exist; a later study based on a combination of computational analysis of photolabeling studies, structure activity relation (SAR) studies, biological data and biophysical data,

revealed a pocket within β-tubulin in the αβ-tubulin interface(RAVI *et al*. 2005). In contrast, the hemiasterlin analog E7974 primarily bound α-tubulin (KUZNETSOV *et al*. 2009). It is likely that a crystal structure of hemasterlin bound to an αβ tubulin dimer will be required to identify the binding site of hemiasterlin on tubulin. It has become evident that there are multiple ways of evading cytotoxicity with most compounds and HTI-286 is no exception. Innovative ways of uncovering modes or resistance are needed to fully understand the potential for any compound of chemotherapeutic interest.

ii. Hemiasterlin resistance in C. elegans-Mitochondrial mutants

Hemiasterlin has been shown to target microtubules and evade multidrug efflux pumps. Because hemiasterlin has strong potential as a cancer chemotherapeutic and we now have the capability to chemically synthesis this compound, a forward genetic screen was carried out in *C. elegans* as an unbiased approach to uncover all possible mechanisms of resistance to hemiasterlin that still render an organism viable*(ZUBOVYCH et al. 2006)*. One possible drawback in using *C. elegans* as a model organism is that mechanisms normally not found in mammals could predominate. For example nematodes have an expanded family of genes encoding efflux pumps, presumably to export toxic compounds found in soil or in their bacteria food. However, preliminary data determining that mammalian efflux pumps were poor transporters of hemiasterlin and the finding

that hemiasterlin is quite toxic to worms gave encouragement that a forward genetics approach would be successful(HADASCHIK *et al*. 2008a; HADASCHIK *et al*. 2008b; KUZNETSOV *et al*. 2009; LOGANZO *et al*. 2003; SHEPS *et al*. 2004; ZUBOVYCH *et al*. 2006; ZUBOVYCH *et al*. 2010).

Dr. Patrick Harran synthesized a hemiasterlin analog nearly identical to HTI-286 (compound-3) that was used for the forward genetic screen. Wild-type worms treated with 1 μM of the HTI-286 analog exhibited a dumpy paralyzed phenotype. Seven independent hemiasterlin-resistant mutants were isolated that were capable of reproducing in 1 μM HTI-286 analog. Of these seven, a prohibitin-2 mutant *phb-2(ad2154)* was the first hemiasterlin resistant mutant to be identified. *phb-2(ad2154)* mutant worms were not only resistant to HTI-286 but cross-resistant to compounds that target non-tubulin proteins, such as, the DNA-topoisomerase inhibitor camptothecin (ZUBOVYCH *et al*. 2006). Prohibitin-2 localizes to the inner-mitochondrial membrane, but has multiple functions, some of them in the nucleus; it is a repressor of transcription in an estrogen dependent manner and has been shown to inhibit apoptosis(KASASHIMA *et al*. 2006). *spg-7(ad2249)* and the dominant *har-1(ad2155dm)* mutants were subsequently identified and shown to exhibit a similar multidrug resistant phenotype. SPG-7 gene encodes for the paraplegin protein. Mutations in SPG-7 are associated with the neurodegenerative disease hereditary spastic paraplegia(HEDERA *et al*. 2002; ZUBOVYCH *et al*. 2010). SPG -7 and PHB-2 were previously identified genes

shown to function in mitochondria, in contrast to har-1, an uncharacterized mitochondrial gene with no known function(HEDERA *et al*. 2002; KASASHIMA *et al*. 2006; ZUBOVYCH *et al*. 2006; ZUBOVYCH *et al*. 2010).

Assuming that the mutations in mitochondrial genes that caused *C. elegans* to be resistant to multiple toxins also caused a loss of function of the mitochondrial protein, Zubovych et. al, hypothesized that mitochondrial dysfunction in worms might lead to protection from multiple toxins, including the natural product hemiasterlin. To test this hypothesis, *C. elegans* mitochondrial mutants *isp-1, clk-1* and *eat-3* were tested for hemiastasterlin resistance. *isp-1, clk-1* and *eat-3* have defects in oxidative phosphorylation and all survived lethal concentrations of hemiasterlin. Increased reactive oxygen species (ROS) are a byproduct of defective oxidative phosphorlyation. Paraquat is commonly used to increase superoxide ($\bullet O_2^-$)(COCHEME and MURPHY 2008). Paraquat treatment was able to rescue wildtype worms from hemiasterlin toxicity. Zubovych et. al, tested *C. elegans* mitochondrial superoxide dismutase (mnSOD) mutants and determined that these mutants were also resistant to hemiasterlin. The mutations identified in these worms have uncovered a mechanism in which increased ROS rescues worms from hemiasterlin and other toxic compounds. The findings from these experiments give new insights into the mechanisms of MDR, a phenotype seen not only in cancer but other human disorders.

C. The Multidrug Resistance Dilemma

i. Implications of Multidrug Resistance

Multidrug resistance (MDR) arises when a biological system has acquired resistance to the toxic effects of a wide range of drugs or chemicals. MDR is commonly seen in the treatment of cancer or bacterial infection as both cancer cells and bacteria possess a high mutation rate, and as a consequence any mutation conferring resistance to treatment will be selected. MDR is largely associated with the expression of efflux pumps in bacterial pathogens and cancer. Alternatively, mutations within apoptotic signaling pathways can lead to a multidrug resistant phenotype. In addition, in cancer cells, a significant portion of cancers become drug resistance through unknown mechanisms(YAO *et al*. 2010). Therefore, understanding the various mechanisms that lead to MDR is important for devising ways to combat it.

a. Mechanisms of Drug Resistance

1. Efflux pumps conferring drug resistance

Classical MDR results from an increase of cell surface efflux pumps that export therapeutic compounds(OZBEN 2006). A major group of efflux pumps are the ATP-binding cassette transporters (ABC-transporters) that use the energy from ATP to actively transport molecules out of the cell. Fourteen ABC-transporter genes have been identified to be involved in MDR in humans(OZBEN

2006). The normal physiological role of transporter pumps are to excrete toxins and other small molecules from the liver, kidneys and gastrointestinal tract. However, in tumors, cancer cells have been shown to upregulate ABC-transporter pumps and confer resistance to multiple anticancer drugs(OZBEN 2006; WONG and GOODIN 2009).

2. Adaptive mutations lead to drug resistance

a. Mutation of drug target

Efflux pumps are recognized as a mechanism for MDR, but pathogens or cancers have developed multiple strategies to evade drug treatment. Although many drugs can be effective initially, evidence shows with time, dynamic systems will adapt(FERNANDEZ *et al.* 2007; OETHINGER *et al.* 1998; OGERT *et al.* 2010; POMMIER *et al.* 2004; YAO *et al.* 2010). For example, in lung cancer it has been shown that erlotinib responsive tumors inevitably become refractory to treatment. Erlotinib is a tyrosine kinase inhibitor that is effective for controlling non-small cell lung cancers that have an oncogenic mutation in the epidermal growth factor receptor (EGFR). Erlotinib inhibits tumor growth by reversibly binding to EGFR within the ATP binding pocket. Erlotinib-resistant tumors develop a secondary mutation termed EGFR T790M that decreases the affinity of the receptor for the drug(YAO *et al.* 2010) and this alteration of the drug target results in the failure of

erlotinib and chemotherapy. This mutation renders tumors resistant to gefitinib as well, another commonly used EGFR inhibitor.

An example of a viral adaptation to an initially effective drug is the human immunodeficiency virus (HIV). HIV binds target cells by attachment through the viral glycoprotein 120 (gp120) of the chemokine co-receptors-CC motif chemokine receptor 5 (CCR5) to enter its target cells. Binding of gp120 to the co-receptor is needed for a conformational change in viral proteins required for fusion of the virus and cell membranes and subsequent viral entry and infection. In the treatment of HIV, vicriviroc (VCV) is a small molecule antagonist of the HIV-1 (CCR5) co-receptor. VCV binds CCR5 and locks the receptor into a conformation that prevents gp120 from attaching and entering host cells. Six amino acid changes in the viral gp120 confer HIV-1 by altering the viral proteins contact points with the CCR5 co-receptor, resulting in resistance to VCV and continued viral entry and replication(OGERT *et al*. 2010). Although this does not represent an MDR phenotype, it is a mutation rendering drug treatment ineffective.

In addition to changes in drug targets rendering treatment ineffective, there also exist more complex mechanisms of drug resistance, such as activating cell survival pathways or inhibiting apoptotic pathways.

b. Compensation through a different pathway, or compensation with an alternate protein targeting the same pathway. (KAISER 2011)

Multiple examples are known of alternate pathways compensating for the inhibition of a cellular function or an alternate mechanism releasing a pathway from drug inhibition. As an example, the majority of Non Small Cell Lung Cancers (NSCLCs) expressing an oncogenic EGFR, identified by having a deletion in exon 19 or a missense substitution, are erlotinib sensitive(YAO *et al.* 2010). EGFR signaling can be the result of EGFR forming homo or heterodimers with other family members, including v-erb-b2 erythroblastic leukemia viral oncogene homolog (erbB)2, 3 and 4. ERBB3 activation, similar to EGFR activation, leads to the downstream activation of phosphatidylinositol 3-kinases (PI3K)/AKT signaling independent of EGFR. Activation of the PI3K/AKT pathway is associated with cell survival and proliferation. One of the downstream targets of the PI3K/AKT pathway is the oncogene c-MET. c-MET confers resistance to erlotinib in a number of erlotinib sensitive cancers due to the activation of the PI3K/AKT pathway through ERBB3, bypassing the need for EFGR activation(NAKACHI *et al.* 2010; YAO *et al.* 2010),(ENGELMAN and JANNE 2008).

There is a correlation between altered functions in mitochondria and an MDR phenotype(PARK *et al.* 2004; ZUBOVYCH *et al.* 2006; ZUBOVYCH *et al.* 2010). *C. elegans* mutants identified through a mutagenic screen for survival in

the presence of the microtubule poison hemiasterlin, show resistance to unrelated poisons. Hemiasterlin mutants have altered mitochondrial function resulting in elevated levels of ROS, and ROS induction in wildtype worms results in hemiasterlin resistance(ZUBOVYCH *et al*. 2006; ZUBOVYCH *et al*. 2010). Other *C. elegans* mitochondrial mutants *isp-1, eat-3* and *clk-1* also exhibited hemiasterlin resistance. A study in which Park et. al studied the multidrug-resistant, mitochondrial-depleted hepatomacellular carcinoma cells (SK-Hep1) showed SK-HEP1 cells to be resistant to various forms of cell death. Studies have also shown that altering the mitochondrial permeability transition pore (mPTP) resulted in increased apoptosis in SK-Hep1 cells(LING *et al*. 2010).

The PIM kinase family has also been identified as a pathway to MDR. PIM kinases have been linked to the activation of survival pathways and expression of drug efflux transporters MDR-1 and BCRP(ISAAC *et al*. 2011). The signal transducer and activator of transcription-3 (STAT-3) has been shown to be positively regulated by PIM in prostate cancer and pancreatic cancer(ISAAC *et al*. 2011). STAT-3 is associated with gene transcription and cell cycle progression and activated STAT3 has been correlated with drug resistance in a variety of tumors(STEPHANOU and LATCHMAN 2005). STAT3 activity and evasion of drug toxicity is due to activation of anti-apoptotic proteins B-cell lymphoma-2 (Bcl-2) and Bcl-x. A connection between efflux pump MDR-1 transcription and STAT3

activation has also been reported(ISAAC *et al*. 2011; STEPHANOU *et al*. 2000; STEPHANOU and LATCHMAN 2005).

WNT signaling: Wingless/integration (WNT) signaling is crucial during embryogenesis for cell fate determination and development, but in adults this signaling acts to promote cell renewal, cell proliferation and differenatiation(TAKEBE *et al*. 2010). In cancer, aberrant WNT signaling is another pathway leading to multidrug resistance. Activation of Wnt signaling prevents β-catenin degradation and allows it to translocate to the nucleus where it binds and activates transcriptional repressor T-cell factors (TCF's) resulting in target genes including human MDR-1(TAKEBE *et al*. 2010).

Gene amplification of detoxifying genes has also been reported in the MDR phenotype(SANDEGREN and ANDERSSON 2009). Gene amplification or duplication in bacteria by plasmid alteration was first seen in bacteria resistant to chloramphenicol and streptomycin. It was later determined that bacteria acquired antibiotic resistant duplications through homologous recombination(SANDEGREN and ANDERSSON 2009).

One mechanism proposed to confer resistance to microtubule poisons is the differential expression of tubulin isotypes. Studies have shown differences in binding affinities between microtubule poisons and specific tubulin isotypes. For example, Burkhart et. al determined that a class II β-tubulin isotype was increased in Taxol resistance cells when compared to the Taxol sensitive parental cell

line(BURKHART *et al*. 2001). The mechanisms involved in drug resistance and MDR are diverse and complicated, and it is likely new mechanisms will be discovered. Identifying new compounds to inhibit or antagonize these pathogenic changes will continue to be important to extend the use of existing therapeutics(POMMIER *et al*. 2004).

b. Strategies to combat drug resistance

The increased discovery of natural products and the increase in compound libraries has allowed for innovative methods to identify effective strategies for the treatment of drug resistant disease. Some innovative methods that hold promise include: high-throughput compound screening (HTS), immunotherapy and the continued use and innovative development of model organisms.

1. High-Throughput Screening (or HTS)

In 2007 Nouiery et. al designed a screen of 80 thousand compounds to identify any that could inhibit west nile virus (WNV) infection. Ten promising compounds were identified that could inhibit WNV infection in neurons. Compound AP30451 inhibited WNV, yellow fever and dengue fever replication in cell based assays(NOUEIRY *et al.* 2007). The authors concluded that further modification of AP30451 would allow a more promising therapeutic profile, such as increased lipophilicity and biodistribution(NOUEIRY *et al.* 2007).

A high-throughput RNAi based compound screen was developed by Whitehurst et. al to identify gene targets that would sensitize the human non small cell lung cancer cell line NCI-H1155 to the microtubule poison paclitaxel(WHITEHURST *et al.* 2007). The screen resulted in multiple hits showing the diverse pathways involved in a Paclitaxel response and revealed multiple

mechanisms in which a cancer cell can survive or be sensitized to paclitaxel and possibly other chemotherapeutics.

2. The adaptive immune response (vaccines)

Although immunotherapy or vaccine treatment is not thought of as a treatment for multidrug resistant disease, in theory this approach would bypass known mechanisms of multi-drug resistance. The identification of tumor-associated antigens has provided the basis for antigen-specific vaccination(ABERN *et al*. 2011). Two promising approaches for cancer vaccination are: adaptive T-cell therapy, an approach where cancer-antigen specific T-cells are expanded ex vivo and reinfused to patients, or T-cells expansion in vivo through vaccination(PALUCKA *et al*. 2011). A great success story in cancer vaccination is the development of the human-papillomavirus (hpv-16) vaccine, a strain shown to cause the development of cervical cancer(PALUCKA *et al*. 2011). In 2006 GARDASIL® was approved by the U.S. Food and Drug Admistration(FDA) for the treatment of human papilloma virus-6,11,16 and 18(MCLEMORE 2006). In 2010 Provenge® was approved by the Food and Drug Administration for the treatment of advanced prostate cancer. Many promising vaccinations for prostate cancer, metastatic melonoma and follicular lymphoma are currently under clinical trials with initial promising results. In another example of prostate cancer immuno-therapy, the PROSTVAC-VF® vaccine system was shown to increase

median survival by 8.5 months compared to control(ABERN *et al.* 2011). Breakthroughs such as GARDASIL and an increasing interest in immunity as it related to cancer will hopefully lead to more cancer vaccine development and immuno-based therapy(COUZIN-FRANKEL 2010).

3. Increased knowledge through model organisms

Drug discovery and development has long benefited from the use of model organisms. Model organisms in science allow for researchers to ask questions through controlled experiments using genetics. The mouse model is perhaps the most well known animal model with sophisticated genetic tools and genetic resources. The mouse is a small mammal with a life span of 1.5-2 yrs in which it is possible to generate transgenic and knockout animals to create disease models. Mouse models of disease range from prostate and lung cancer, to diabetes and alzheimers(BARIBAULT 2009; JOHNSON *et al.* 2011; KIM *et al.* 2011; SOMPOL *et al.* 2008) Through the use of animal models, efficacy of new and promising drugs can be tested for therapeutic significance and evaluated for toxic side effects. Although not as popular for drug studies as the mouse, the fruitfly *Drosophila melanogaster*, and the soil nematode *Caenorhbaditis elegans* are also useful for studying the therapeutic potential of natural compounds. As an example, drosophila has been used to develop new combined modality therapy in cancer, which involves a dual treatment with radiation and chemotherapy(EDWARDS *et al.*

2011). By developing a natural compound screen Edwards et. al studied the fly and identified the microtubule poison maytansinol, which enhanced the killing effect of radiation in *Drosophila(EDWARDS et al. 2011)*.

Caenorhbaditis elegans disease models exist for aging, development and for the elucidation of drug mode of action(AVERY and HORVITZ 1987; LUCANIC *et al*. 2011; ZUBOVYCH *et al*. 2006; ZUBOVYCH *et al*. 2010). Zubovych et. al uncovered a novel mode of drug resistance by identifying and characterizing mutants resistant to the natural compound hemiasterlin(ZUBOVYCH *et al*. 2006; ZUBOVYCH *et al*. 2010). With the increasing use of phenotype-based HTS identifying compounds of interest that have unknown mechanisms of action, the use of genetics in model organisms will continue to be important for identifying drug targets and mechanisms of resistance.

D. Caenorhabditis elegans as a model organism

Important discoveries in fundamental biological processes of aging, development, RNAi-mediated gene silencing, cell cycle control, sensory physiology and synaptic transmission, have been made with the use of the nematode *C. elegans(STRANGE 2006)*. *C. elegans* has some of the characteristics that one would consider ideal when choosing a model organism for the purpose of asking fundamental biological questions, including small size, short life cycle and ease of maintenance. The study of *C. elegans* can and has had major implications

not only to understanding biology and disease of humans but also of our livestock and crops. According to Strange et. al, parasitic nematode infection of plants and animals cause an estimated 80 billion dollars worth of crop damage annually(STRANGE 2006). Basic worm research can thus have substantial implication on the human condition beyond cancer or developmental research. To this extent, Sydney Brenner was awarded the 2002 Nobel Prize in Physiology or Medicine, alongside John Sulston and H. Robert Horvitz, for their work using *C. elegans* as a model organism for the study of organ development and cell death. In 2006 Andrew Fire and Criag Mello were awarded the Nobel Prize in Physiology or Medicine for the discovery of RNAi in C. elegans. And in 2008, Martin Chalfie shared the Nobel Price in Chemistry for developing the use of green fluorescent protein to probe protein expression in *C. elegans*.

i. C. elegans genetics

The uniqueness of *C. elegans* and the tools available make this nematode an ideal genetic model. An adult hermaphrodite measures approximately 1 mm in length and feeds on *E. coli.* This allows one to grow, maintain and observe a large quantity of worms, which makes it relatively easy to identify mutants when attempting a forward genetic screen. In theory, all one needs to perform a screen is an assay to detect a given mutation. In the case of *C. elegans,* when performing a forward mutagenic screen one can look at worm movement, body morphology,

life span, feeding, or as was the case for this project, a mutation conferring survival in the presence of an otherwise toxic compound.

Forward genetics in *C. elegans* is straightforward. There are many tools that facilitate the mapping of a genetic mutant. From the first F2 genetic screen done by Brenner in 1967, strains with visible markers and known genetic locations have been available(BRENNER 1974). Deficient strains, strains that can be maintained with a significant deletion within one chromosome, are available that cover most of all six chromosomes. With the completion of the genome sequence for both the *N2 Bristol* and *Hawaiian* wild-type strains, thousands of single nucleotide polymorphisms (SNP) have been identified throughout the genome with an average of 1 SNP per one thousand base pairs. Research groups such as those of Jorgenson, Waterson and Hobert have developed and used SNP mapping to efficiently identify mutated genes(DAVIS *et al.* 2005; DOITSIDOU *et al.* 2010; WILLIAMS *et al.* 1992).

C. elegans reverse genetics using RNAi has been employed with great success. First discovered by Andrew Fire and Craig Mello(SEYDOUX *et al.* 1996) and further developed with the help of Julie Ahringer and her RNAi feeding library(FRASER *et al.* 2000); RNAi has been used for *C. elegans* in high-throughput screens(FRASER *et al.* 2000)and for the identification of proteins involved in organelle morphology and embryogenesis(ICHISHITA *et al.* 2008; SONNICHSEN *et al.* 2005).

The ability to create mutants of interest, to maintain them with little cost and room, the ability to not only create transgenic worms through microinjection, but to be able to store any strain of interest indefinitely in liquid nitrogen make this model organism an attractive model for scientific research.

ii. Major discoveries through C. elegans genetics

The works of Mello and Fire and the discovery of RNAi have been well documented. RNAi has changed the landscape of research and has allowed for knockdown of mammalian cultures and has led to the development of whole genome RNAi screens. Other landmark discoveries in worm genetics include work done by Horvitz in programmed cell death(AVERY and HORVITZ 1987; ELLIS and HORVITZ 1986). The discovery of the EGF/RAS homologs let-23 and let-60 has given insights and a model system to address questions of cancer signaling(AROIAN *et al.* 1990; HAN and STERNBERG 1990). Research in the antidepressant fluoxetine by Dempsey et al, led to the discovery of an alternate fluoxetine target, which has improved our understanding of the mode of action of this antidepressant.

The use of *C. elegans* does not come without caveats. *C. elegans* is a nematode and not human, or mammalian for that matter. With an estimated 30-40% human homology, discovery of a new gene or pathway might not be relevant to higher organisms. One has to take into consideration that, ultimately, a better

understanding of any system is the root of all science. The importance can lie in the eye of the beholder, or discoverer.

D. Questions Addressed in this Thesis

Three drug-resistant *C. elegans* mutants, *phb-2(ad2154), spg-7(ad2249)* and *har-1(ad2155dm),* identified through a forward genetic screen, have been classified as functioning in mitochondria (ZUBOVYCH *et al.* 2006; ZUBOVYCH *et al.* 2010). These mutations are hypothesized to have increased levels of ROS resulting in resistance to multiple toxic compounds. An unmapped hemiasterlin-resistant mutant worm from this forward genetic screen was also identified. One aim of this project was to identify the gene that was mutated in this Worm. A second aim was to determine if this mutant exhibited the same mitochondrial phenotype as previous mutants. A third aim of this work was to characterize the function of this mutant, in the event that its function was unknown.

II. Genetic Mapping of a hemiasterlin resistant *C. elegans* mutant

Hemiasterlin is a natural product that causes cell death to a diverse group of cancers by inhibiting polymerization of microtubules and preventing mitotic spindle formation, leading to apoptosis. Hemiasterlin has been shown to be a poor substrate for the efflux pumps that are upregulated in many multidrug resistant cancers. There is interest in hemiasterlin as a chemotherapeutic and it has been tested in several clinical trials. However, the upregulation of the MDR efflux pumps is not the only form of drug resistance. In an attempt to investigate modes of hemiasterlin drug resistance, a mutagenic screen on the wildtype *N2(Bristol)* strain of *C. elegans* was performed. Based on previous knowledge that hemiasterlin is not a good substrate for efflux pumps and mutations within tubulin can transform sensitive cells into becoming resistant, a hypothesis was formed that a mutagenic screen could identify alternate modes of hemiasterlin resistance. The strength of a forward genetic approach is its lack of bias. Any mutation conferring hemiasterlin resistance that allows worm viability can be identified.

To this effect an F2 screen on mutagenized worms was performed to identify those that could survive a lethal concentration of hemiasterlin. To eliminate false positive survivors, surviving candidate worms from the initial screen were retested for hemiasterlin resistance. As a result of this screen, seven hemiasterlin-resistant-worms were identified. The mutants with highest resistance were chosen for mapping and several were identified(ZUBOVYCH *et al.* 2006;

ZUBOVYCH *et al*. 2010). The last unmapped strong survivor was the true-breeding recessive *C. elegans* hemiasterlin resistant mutant *ad2360*. In order to locate the site of genetic mutation conferring resistance, we wanted to identify the concentration range in which hemiasterlin was toxic to wildtype *C. elegans* compared to the *ad2306* mutant. Therefore, a hemiasterlin toxicity curves were established for the *N2* wildtype strain and the *ad2360* mutant to determine the hemiasterlin concentration range in which subsequent mapping strategies and experiments would be designed. (Figure 1) As expected, the hemiasterlin mutant *ad2306* resulted in a significantly higher tolerance to hemiasterlin compared to the wildtype.

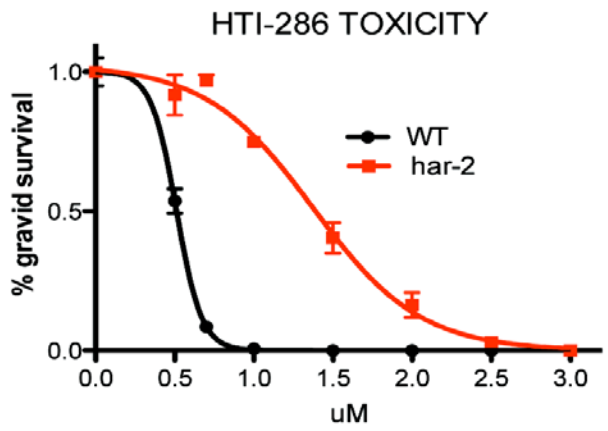

Figure 1. Dose response curve of hemiasterlin.

Hemiasterlin LD_{100} for *har-2* is over 2 fold higher than wt. Both wt and *har-2 (ad2306)* synchronized L1's were seeded in triplicate for the concentrations tested to establish strain tolerance of hemiasterlin. Dose curves in figure are a representation of multiple assays.

A. Locating the mutation conferring hemiasterlin resistance

Since the *ad2306* mutant strain resulted in a two-fold difference in toxicity compared to the wildtype strain, the graph shown in Figure 1 establishes a window to select for hemiasterlin resistant progeny resulting from mating experiments. Using these drug concentrations, a standard forward genetic approach that included SNP two-point and 3-point mapping was employed to identify the genetic mutation conferring hemiasterlin resistance in *ad2360(Davis et al. 2005; Fay and Bender 2006)*. This resulted in the identification of a point

mutation in the T24D1.5 gene located on chromosome I. To date, there is no known function for T24D1.5 and sequence analysis failed to identify a human homolog for this gene. A previously mapped hemiasterlin-resistance worm *ad2155dm* conferring hemiasterlin resistance was named *har-1* for ***h****emi****a****sterlin* ***r****esistant-****1***(ZUBOVYCH et al. 2010). For ease of recognition, the mutation conferring hemiasterlin resistance in *ad2360* was named *HAR–2*.

B. Results

i. Strategy I: 2pt & 3pt mapping

Once establishing the hemiasterlin toxicity curve in *ad2306* (fig.1), linkage mapping through the use of restriction fragment length polymorphisms (RFLP's) or single nucleotide polymorphisms that modify restriction sites (snip-SNP's) was used to identify the linkage group in which the hemiasterlin resistant mutation is located(DAVIS *et al.* 2005; FAY and BENDER 2006). To prevent selection of self fertilizing F2 hemiasterlin survivors from unmated *ad2306* hermaphrodites, *ad2306* males were mated with the Hawaiian strain *CB(4856)* virgin hermaphrodites. This insured that all surviving progeny were from a successful mating between the two strains. The F2 progeny from this mating were screened for hemiasterlin resistance and the surviving worms were isolated for

propagation, resulting in clonogenic populations derived from the initial worm isolates. Genomic DNA of cloned worms was analyzed by RFLP's.

SNP identification resources are listed in materials and methods. RFLP's were identified within each chromosome followed by PCR amplification of genomic DNA spanning the region containing the SNP. Digest DNA for each clonogenic population was loaded onto individual wells and analyzed by gel electrophoresis.

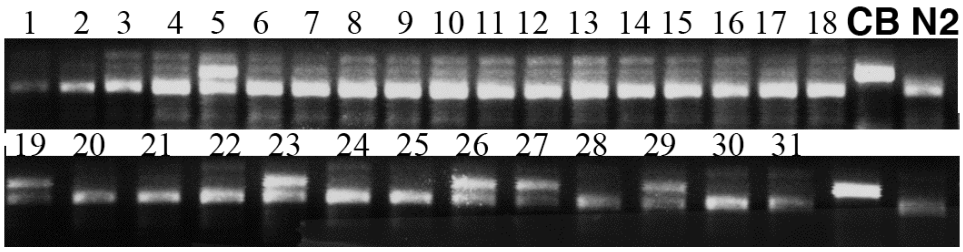

Figure 2. *ad2306* linkage to chromosome I by RFLP. Mapping reveals a 90% *N2* genetic background of mated *ad2306* x *CB (4556)* hemiasterlin surviving progeny for chromosome I. Lanes 1-31 of each gel is representative digested DNA of clonogenic worm populations, the last two rows of each gel are the controls *CB* or *N2*.Mated gravid *ad2306* x *CB (4856)* progeny were placed in 1 μM hemiasterlin. Surviving gravid F2 progeny were isolated and cloned. DNA lysates of surviving clones were PCR amplified for the region containing a RFLP at allele *pkP1057*. Endonuclease digested DNA was separated on a 1% agarose gel and

A SNP within chromosome I was inherited more than 90 percent of the time from the drug-resistant *N2* parent, indicating linkage of the mutation conferring hemiasterlin resistance to chromosome I. (Figure 2)

To determine the mutated genetic locus within chromosome I conferring hemiasterlin resistance, RFLP's located at both ends of chromosome I were individually analyzed to identify worms in which recombination had occurred on chromosome I Results revealed a higher percentage of *N2* DNA on the right hand of chromosome I. With this same strategy of 2-pt mapping with RFLP's, the mutation conferring resistance was narrowed to a 4-centiMorgan (cM) region spanning +1.0cM and +5cM of chromosome I. This 4cM region contained roughly 1000 genes. SNP mapping using RFLP's becomes less efficient as the region being mapped decreases due to the lower probability of a recombination event occurring between the mutant *ad2306* DNA and *CB(4856)* wildtype strain DNA within an increasingly narrow genetic locus. When SNP mapping is no longer an efficient mapping strategy to further decrease the region containing a loss-of-function recessive mutation, microinjecting cosmid DNA into adult worms to rescue the phenotype can be attempted. A cosmid is a type of vector isolated from lambda phage capable of holding up to 50 kilobases of DNA therefore cosmid microinjections can effectively map a region containing 2-30 genes. A less technical approach to increase SNP mapping efficiency is three-point mapping using visible markers.

Three-point mapping can help visually identify worms that have recombined within a region of interest, allowing much larger numbers of progeny to be scored and infrequent recombinants to be identified. To this effect, the

visible marker strains *DR210: dpy-5(e61) daf-16(m26) unc-75(e950)I* and *DA568: dpy-5(e61) eat-5(ad464) unc-29(e1072)I* were mated with *har-2(ad2306)*. Progeny of *har-2*/*DA568* did not appear healthy and many dead embryos were visible within the mated hermaphrodite worms.

Successful mating was achieved with *DR210* and *har-2*. An insufficient number of recombinants resulted from this cross, which did not allow for further narrowing of the locus containing the drug-resistance mutation. However, both *Dpy-5, Har-2* and *Har-2, Unc-75* worms were isolated and cloned. These double mutants were used for 2-pt mapping by crossing with the *CB(4856)* strain and picking F2's that had lost the visible marker but were still resistant to hemiasterlin. RFLP mapping by this method narrowed the locus of *har-2* to within 3cM between CHI $^{+}$2cM and $^{+}$5cM, a region containing roughly 800 genes.

The hemiasterlin-resistant mutants previously identified by my laboratory have a mitochondrial function(ZUBOVYCH *et al*. 2006; ZUBOVYCH *et al*. 2010). To identify candidate mutant genes within the 3cM region, known mitochondrial genes and genes predicted by the online software GeneOrienteer to interact with Prohibitin-2 were sequenced(ZHONG and STERNBERG 2006). Prohibitin-2 is one of the hemiasterlin mutants identified from the mutagenic screen(ZUBOVYCH *et al*. 2006). Genes in this region that were sequenced for mutations included: the Complex I subunit NDUF-7, the dehydrogenase DHS-4 and Complex IV CCO-1. However, none of the genes sequenced had a mutation within exons.

i. Strategy II: Deficiency mapping

Considering the relatively large region in which the *ad2306* mutation was located, sequencing of the remaining genes was abandoned in favor of a deficiency mapping strategy. *C. elegans* deficient strains harboring deleted regions of a chromosome often are only viable when heterozygous for that region. A number of deficient strains are available through the Caenorhabditis Genetics Center (CGC). Mating a loss of function recessive mutant with a strain containing a deficiency within the same chromosomal location as the mutation results in non-complementation. *har-2* males were mated to the deficient strains *JK1534* and *JK1547* that had deletions in the interval of chromosome I containing the drug-resistant mutation. A lack of complementation would result in a loss of function phenotype (hemiasterlin resistance) in the F1 generation. The F1 progeny of both *JK1534*/*har-2* and *JK1547*/*har-2* failed to compliment, resulting in F1 progeny surviving in hemiasterlin. (Figure 3) Both deficient strains span a region of 42 (*JK1534)* and 48 *(JK1547)* genes respectively. Upon further analysis a region on chromosome I between $^{+}$4.054cM and $^{+}$4.60cM, containing 40 genes was identified as the locus for the *ad2306* mutation conferring hemiasterlin resistance. The results from the deficiency mating confirmed that *har-2* is a recessive loss of function mutation causing hemiasterlin resistance.

0uM	1uM
N2	*N2*
har-2	*har-2*
JK1534	*JK1534*
JK1547	*JK1547*
NA	*har-2 x JK1534*
NA	*har-2 x JK1547*

Figure 3. Deficiency Mapping. *JK1534, JK1547* deficient strains fail to compliment *har-2*, signifying the mutation conferring hemiasterlin resistance is located within the genetic region. *JK1534*or *JK1547* deficient hermaphrodites were mated with *har-2* males. Gravid hermaphrodites were placed in 1μM hemiasterlin and scored for survival. Strains that did not survive hemiasterlin are indicated in red. All worms were placed 1worm/well in liquid culture. All concentrations tested were done in triplicate except mated deficient strains. 10-wells were seeded for mated deficient strains, with no less than 10 gravid progeny/well with expected number of male survivors.

iii. Confirmation of point mutation in gene T24D1.5 by complementation

The coding regions of the 40 genes within this region were sequenced and only one nucleotide change was found. The T24D1.5 gene locus is on one margin of the region mapped by both deficient strains. Sequence analysis revealed a guanine

to adenine point mutation within the first exon of T24D1.5 at nucleotide position 139, resulting in a glutamic acid to lysine amino acid change (D47K).

To verify the point mutation in T24D1.5 resulted in worms surviving lethal concentrations of hemiasterlin, the T24D1.5 gene was amplified from wildtype *N2* genomic DNA and inserted in the TA cloning vector pCR2.1® from Invitrogen and used for microinjecting *har-2* mutants. Loss of hemiasterlin resistance after T24D1.5^{wt} DNA would indicate that the mutation in T24D1.5 conferred the resistant phenotype.

Recombination of microinjected DNA in *C. elegans* can form large extrachromosomal arrays that occasionally become stable and can be transmitted to progeny. The progeny of microinjected pCR2.1:T24D1.5 and the co-injection dominant marker pRF4 plasmids were analyzed to identify worms with a rolling phenotype that is associated with the expression of the pRF4 plasmid. Stable roller transgenic strains were subsequently tested for hemiasterlin resistance. Stable expression of microinjected T24D1.5^{wt} DNA by *har-2* resulted in loss of hemiasterlin resistance, a clear indication that the mutation in T24D1.5 was responsible for drug-resistance. (Figure 4)

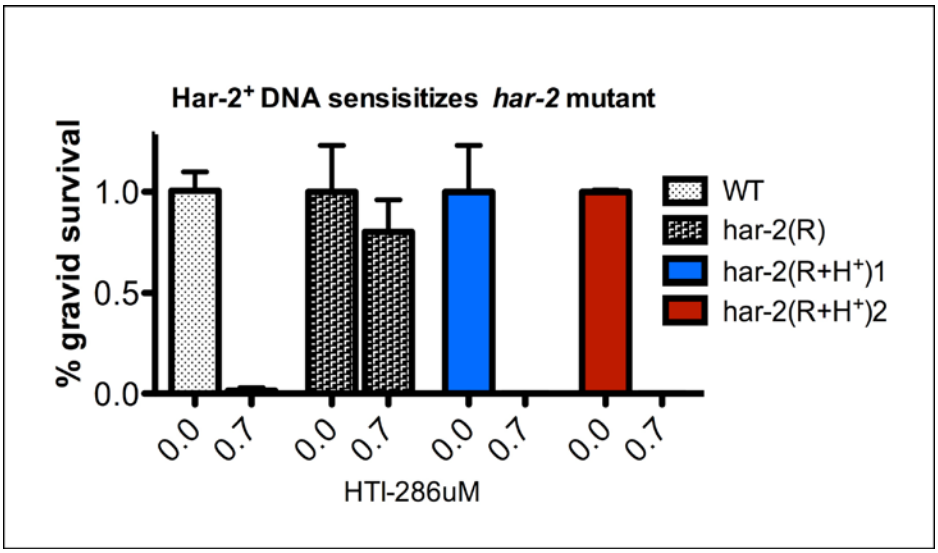

Figure 4. WT har-2 DNA resensitizes hemiasterlin mutant.

Transgenic *har-2* worms expressing a wildtype copy of T24D1.5 did not survive hemiasterlin treatment, strong evidence that the T24D1.5 point mutation confers drug resistance. Gravid worms were placed singly in liquid culture with or without 0.7 μM of hemiasterlin. The surviving progeny from the untreated samples were normalized to one hundred percent survival. R=worms expressing the pRF4 *rol-6* plasmid, R+H=worms expressing both pRF4 + wt T24D1.5. Numbers 1 & 2 after the strain name is used to label two independent strains.

C. Discussion

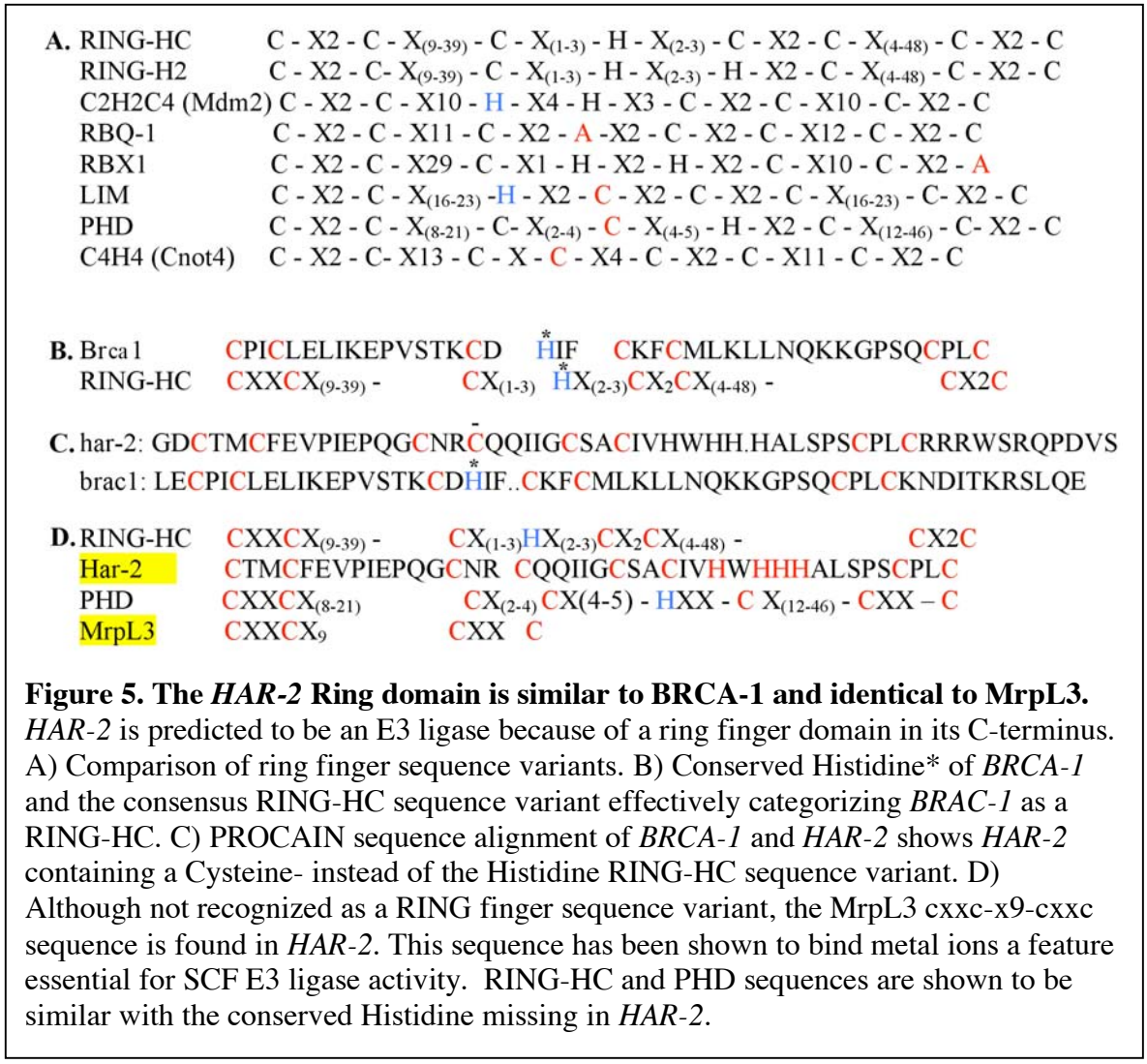

Figure 5. The *HAR-2* Ring domain is similar to BRCA-1 and identical to MrpL3. *HAR-2* is predicted to be an E3 ligase because of a ring finger domain in its C-terminus. A) Comparison of ring finger sequence variants. B) Conserved Histidine* of *BRCA-1* and the consensus RING-HC sequence variant effectively categorizing *BRAC-1* as a RING-HC. C) PROCAIN sequence alignment of *BRCA-1* and *HAR-2* shows *HAR-2* containing a Cysteine- instead of the Histidine RING-HC sequence variant. D) Although not recognized as a RING finger sequence variant, the MrpL3 cxxc-x9-cxxc sequence is found in *HAR-2*. This sequence has been shown to bind metal ions a feature essential for SCF E3 ligase activity. RING-HC and PHD sequences are shown to be similar with the conserved Histidine missing in *HAR-2*.

Two independent and stable *har-2* transgenic strain lines exhibiting a roller phenotype that had been co-injected with wild-type T24D1.5 DNA lost hemiasterlin resistance (Figure 4), indicating that the mutation in T24D1.5 in *har-2* confers hemiasterlin resistance. The T24d1.5 gene has therefore been renamed *HAR-2*. *HAR-2* is 1248bp in length encoding 415 amino acids. *HAR-2* homologs exist in *C. remain* and *C. briggsae* but are not obvious in other organisms and there is no data on the function of HAR-2. Although HAR-2 has no known function, it has been predicted to be an E3-ligase largely due to containing a ring finger domain(KIPREOS 2005)(Figure 5). One observation supporting *har-2* as a loss of function, ribonucleic acid interference (RNAi) knockdown of *HAR-2* in wildtype *N2* worms resulted in a slight increase in hemiasterlin resistance. (see appendix)

The sequence identified as a Ring finger within *HAR-2* was analyzed using the structure/function prediction software PROCAIN(WANG *et al*. 2009). *BRCA-1* and *BARD-1* where the two top hits resulting from this analysis. *BRCA-1* is known to have E3 ligase activity, and ligase activity is increased when dimerizing with *BARD-1*(BRZOVIC *et al*. 2001). A conserved histidine containing sequence (c-x1-3-h) within *BRCA-1* effectively classifies it as a RING-HC sequence variant. Upon further analysis the conserved histidine is not found within *HAR-2*, which decreases confidence in labeling *HAR-2* as an E3 ligase as previously predicted(KIPREOS 2005). It must be noted that a subset of E3 ligases

require the ability to coordinate metal ions for functional E3 ligase activity, and *HAR-2* contains the conserved sequence cxxc-x9-cxxc established to be critical for metal binding in MrpL32(BONN *et al*. 2011; DESHAIES and JOAZEIRO 2009). Therefore it is still possible that *HAR-2* does function as an E3 ligase as predicted. There is no antibody available for *HAR-2,* preventing pull down assays to identify binding partners and possible proteins to assay for *HAR-2*-mediated ubiquitylation. Over 600 genes are predicted to be E3 ligases controlling many cellular processes. Assaying if HAR-2 functions as an E3 ligase will require more knowledge of the possible pathways or mechanisms in which HAR-2 is involved. Further analysis is needed to make a prediction of *HAR-2* function.

III. The determination of har-2 function

A. Introduction

Little experimental data is available to determine the function of HAR-2 or to confidently classify it as an E3 ligase. To determine if *har-2* could be categorized with previous hemiasterlin-mutants it was necessary to establish if *har-2* exhibited similar characteristics. Previous hemiasterlin-mutants exhibited a multidrug resistance phenotype and mitochondrial dysfunction that resulted in elevated levels of ROS(ZUBOVYCH *et al.* 2010). In this regard *har-2* was analyzed in a similar manner as previous hemiasterlin-mutants to determine if it too was resistant to multiple drugs and exhibited mitochondrial dysfunction.

B. Results

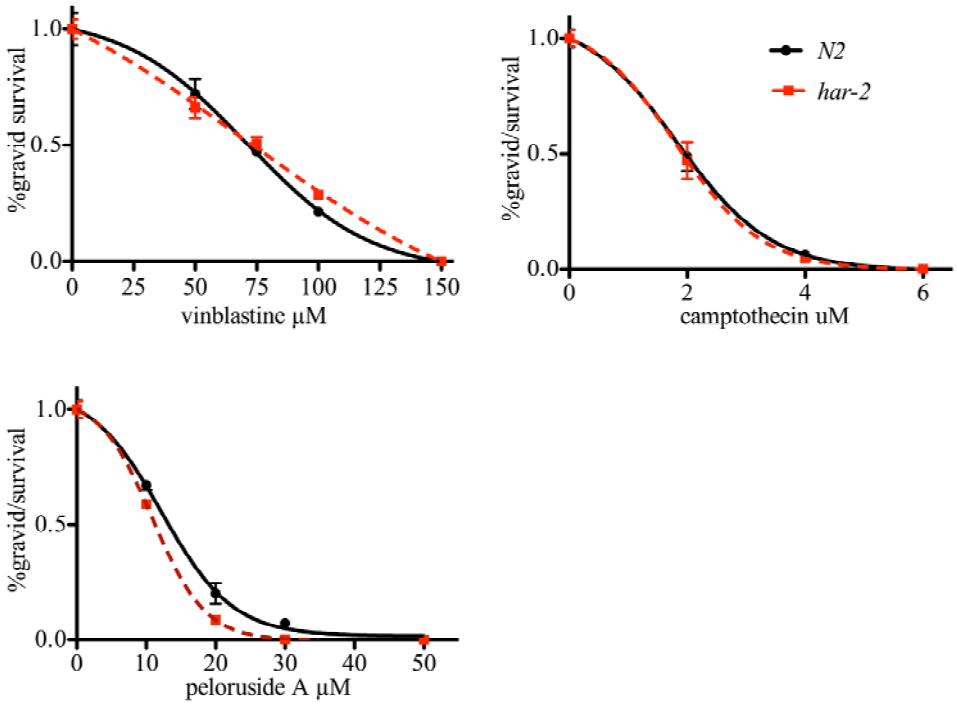

Figure 6. *har-2* does not exhibit a multidrug resistant phenotype. *har-2* dose response is similar to the *N2* wildtype, unlike previous hemiasterlin-resistant-mutants. Worms were synchronized at the L1 stage before being seeded into 48-well plates containing worm media with the indicated compound concentrations. These assays were repeated a minimum of two times, and were done in triplicate. The number of worms counted per strain in the untreated triplicate sample were averaged and considered to be one hundred percent survival.

i. har-2 does not exhibit the MDR phenotype of previous hemiasterlin mutants.

The response of synchronized L1 *har-2 ad2306* and wildtype *N2* worms to a series of concentrations of the tubulin targeting compounds vinblastine, peloruside A and paclitaxel, or the DNA topisomerase I inhibitor camptothecin, was measured (Figure 7 and 8). Compared to wild type worms, *har-2(ad2306)* did not exhibit resistance to any additional compounds. In fact, *har-2* exhibited a hypersensitivity to Paclitaxel as compared to wildtype (Figure 8).

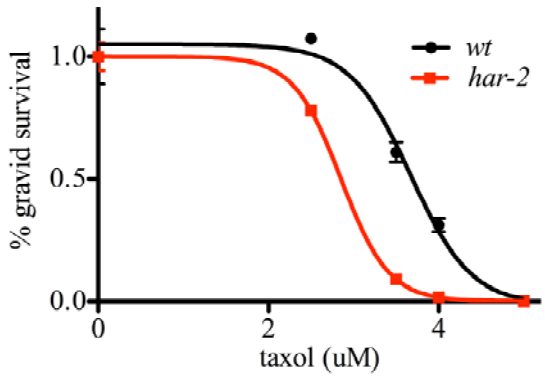

Figure 7. *har-2* is hypersensitive to Taxol. Unlike previous hemiasterlin-mutants, *har-2* is hypersensitive to the microtubule polymerizer Paclitaxel (Taxol). Graph is representative of multiple experiments done in triplicate. Error bars indicate the mean $\pm$ SE of triplicate measurements, SEM bars are smaller than symbols for most data points.

ii. har-2 respiration rates are similar to wildtype

Previous hemiasterlin-mutant worms have been established to have a mitochondrial dysfunction phenotype indicated by elevated levels of ROS conferring hemiasterlin resistance(ZUBOVYCH *et al*. 2010). Although respiration measurements have not been measured in previous hemiasterlin mutants it is a fundamental function of mitochondria, and therefore important to determine if *har-2* respiration was altered compared to wildtype. If *har-2*'s hemiasterlin resistance was due to mitochondrial dysfunction, it was hypothesized that differences in basal levels of respiration could be detected. To investigate this, purified mitochondria of mixed-staged wildtype and *har-2* worms were analyzed for differences in basal levels of respiration.

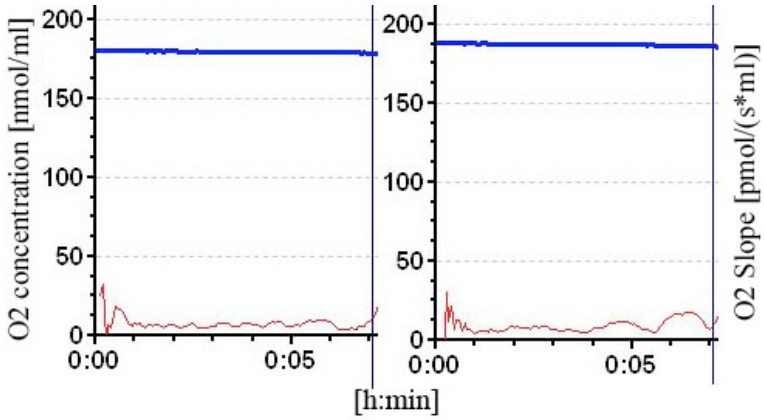

Figure 8. Basal level respiration comparison of *har-2* and the wildtype strains. Purified mitochondria from mixed populations of *N2* or *har-2* strains were analyzed in a sealed chamber for basal respirations levels. Blue bar represents total oxygen available in chamber, red bar represents oxygen concentration rate of change.

(Figure 8) Basal level respiration measurement results indicated that there is no difference in the level of respiration between wildtype and *har-2*. Because there was no difference in mitochondrial basal respiration levels, and previous hemiasterlin-mutants have not been established to be defective in respiration further analysis was required.

iii. N-acetylcysteine does not abrogate har-2 resistance to hemiasterlin

C. elegans mitochondrial mutants *clk-1* and *isp-1* have respiration defects, are long lived, have elevated levels of ROS and are resistant to hemiasterlin(LEE *et al*. 2010; ZUBOVYCH *et al*. 2010). Although no respiration defect had been established in any of the hemiasterlin mutants and lifespan has been observed to be like wildtype; previous hemiasterlin mutants do have elevated ROS levels and this has been correlated with hemiasterlin resistance(ZUBOVYCH *et al*. 2010). Hemiasterlin-resistant-mutants when treated with hemiasterlin in the presence of the reactive oxygen species scavenger N-acetylcysteine (NAC) lose hemiasterlin resistance(ZUBOVYCH *et al*. 2010). In sharp contrast, *har*-2 hemiasterlin resistance was not changed when ROS was depleted with NAC (Figure 9), indicating that ROS signaling is not required for *har-2* hemiasterlin resistance.

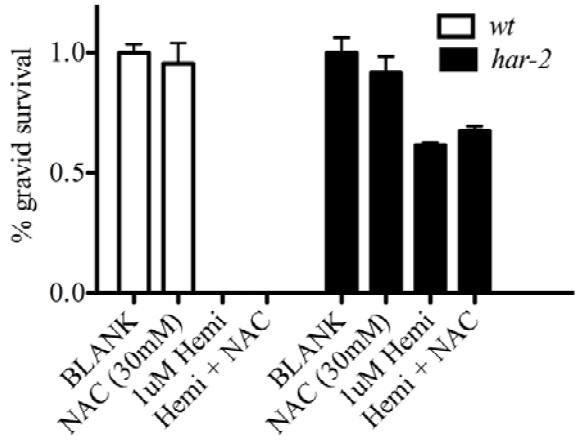

Figure 9. *har-2* hemiasterlin resistance is not lost after NAC treatment.

Synchronized L1 worms were placed in liquid culture containing hemiasterlin with or without 30mM of NAC. NAC alone had no significant effect on either *N2* or *har-2* viability. In contrast to previous hemiasterlin-mutants, NAC treatment had no effect on *har-2* hemiasterlin resistance. Error bars indicate the mean $\pm$ SE of triplicate measurements,

iv. har-2 resistance is independent of pkc-1

ROS serves as an activating signal for protein kinase C (PKC) ε resulting in a reduced mitochondria permeability transition pore and the activation of mitochondrial potassium ATP channels(BAINES *et al.* 2003; HANLEY and DAUT 2005; LIU *et al.* 2001; ZUBOVYCH *et al.* 2010). Since depletion of ROS by NAC sensitized previously identified hemiasterlin-resistant mutants to hemiasterlin, and, in contrast, treating wildtype worms with the mitochondrial uncoupler Carbonyl cyanide p-trifluoro-methoxyphenylhydrazone (FCCP) increased ROS levels and conferred hemiasterlin resistance to wildtype worms, it seemed reasonable that functional PKCε was required for ROS signaling resulting in hemiasterlin resistance in hemiasterlin-resistant worms(ZUBOVYCH *et al.* 2010). To test this hypothesis, worms with a loss-of-function mutation, *pkc*-1, in the ortholog to human PKCε, were crossed with worms bearing each of the har-*1, spg-7* or *phb-2* mutations. All double mutants lost hemiasterlin resistance, suggesting that ROS signaling requires a functional PKC-1(PKCε) for hemiasterlin resistance(ZUBOVYCH *et al.* 2010).

Although NAC treatment did not abrogate *har-2* hemiasterlin resistance, PKC-1 could still be required for hemiasterlin resistance independent of ROS signaling. To determine if *har-2* resistance required a functional PKC-1, a *har-2;pkc-1* worm was constructed. *har-2;pkc-1* resistance to hemiasterlin was not

lost, indicating that *har-2* is not functioning by the same mechanism as previous hemiasterlin-mutants (Figure 10) .

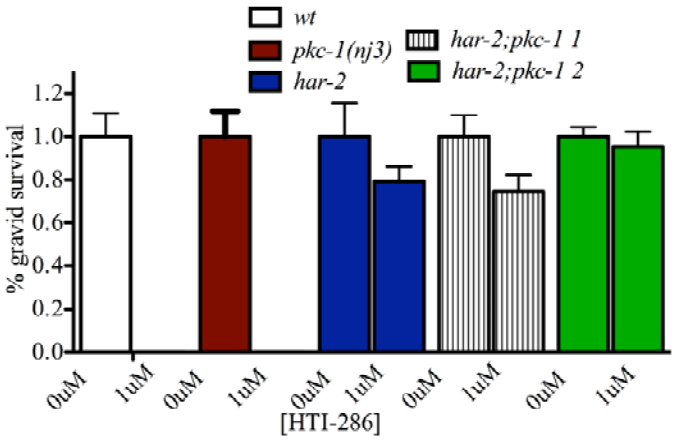

Figure 10. *har-2* hemiasterlin resistance is independent of *pkc-1*. Two independent *har-2;pkc-1* mutants were constructed, denoted on the legend by a 1 or 2 at the end of strain name. Unlike previous hemiasterlin mutants, *har-2* does not require functional PCK-1 for hemiasterlin resistance. Error bars indicate the mean ± SE of triplicate measurements.

v. har-2 resistance is independent of brc-1

As discussed above, HAR-2 is predicted to be an E3 ligase, due to a RING finger domain(KIPREOS 2005). Using the structure/function prediction software

PROCAIN, the amino acid residues spanning the proposed ring finger domain of HAR-2 were analyzed(WANG *et al*. 2009) (Figure 5). BARD-1 and BRCA-1 were two of the top scoring orthologs resulting from this analysis. Both BARD-1 and BRCA-1 have ring finger domains and BRCA-1 is a known E3 ligase. *brc-1* and *brd-1* are the BRCA-1 and BARD-1 loss of function mutants in *C. elegans*. To determine if *brc-1* or *brd-1* worms were functioning in a similar or redundant pathway of hemiasaterlin resistance as *har-2,* the dose-response of each of these strains to hemiasterlin was measured (Figure 11). Both *brc-1* and *brd-1* were sensitive to hemiasterlin, similar to wildtype worms, indicating that loss of function of either protein did not phenocopy hemiasterlin resistance. To determine if epistasis exists between HAR-2 and BRC-1, a double mutant (*har-2;brc-1)* was constructed and analyzed for a change in hemiasterlin resistance. Figure 11 shows the hemiasterlin dose response of *har-2;brc-1* double mutant to be equally resistant to hemiasterlin as *har-2,* establishing that *har-2* does not require the BRCA-1 E3 ligase for hemiasterlin resistance.

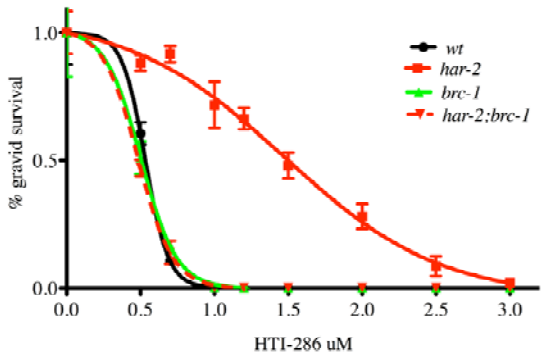

Figure 11. The BRCA-1 E3 ligase does not mediate HAR-2 hemiasterlin resistance. The RING finger containing proteins BARD-1 and BRCA-1 are not resistant to hemiasterlin. The *brc-1;har-2* response to hemiasterlin shows that BRCA-1 E3 ligase activity is not needed for *har-2* hemiasterlin resistance. Error bars indicate the mean ± SE of triplicate measurements,

vi. har-2 microtubule morphology is similar to wt in the presence of hemiasterlin

har-2's hypersensitivity to the microtubule stabilizer Paclitaxel and resistance to the microtubule depolymerizer hemiasterlin led to the hypothesis that there might be differences in microtubule dynamics between wildtype and *har-2*

worms that could be detected through microtubule staining. Visualizing defects in worm microtubules is simplified by staining isolated embryos due to their high rate of cell division; any defect in microtubule dynamics or mitotic spindle formation would be augmented at this stage in worm development. Figure 12 shows alpha-tubulin stained wildtype and *har-2* embryos to have no discernable differences after 1μM hemiasterlin treatment.

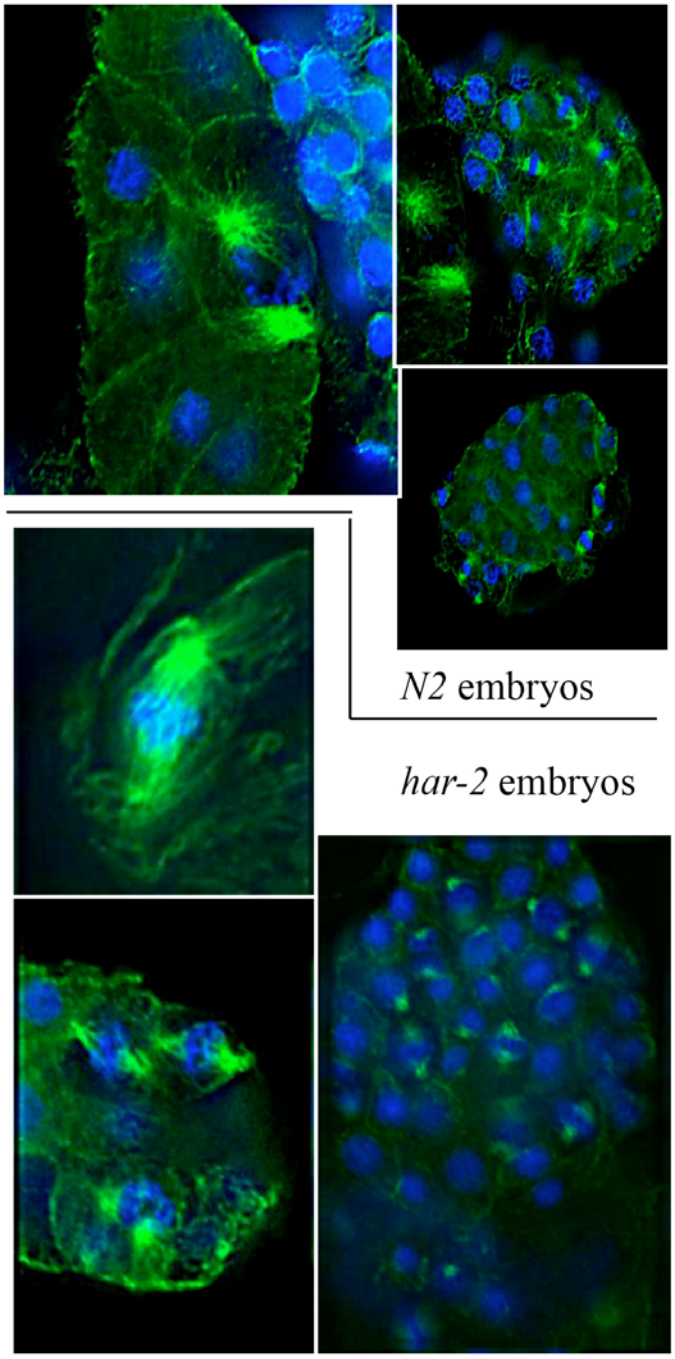

Figure 12. Microtubule staining of wildtype and *har-2* embryos. Alpha-tubulin staining of purified egg embryos of both wildtype and *har-2* embryos after 1μM hemiasterlin treatment resulted in a diffuse microtubule staining pattern. No difference was detected between the strains before or after hemiasterlin treatment.

C. Discussion:

Experimental data reveals *har-2* as a unique hemiasterlin-resistant-mutant distinct form the previously identified hemiasterlin-resistant-mutants *har-1, phb-2* and *spg-7(ZUBOVYCH et al. 2006; ZUBOVYCH et al. 2010). har-2* does not appear to have an MDR phenotype nor does ROS signaling appear to be necessary for hemiasterlin resistance in this mutant. (Figures 9 & 10) This was evidenced by the fact that the NAC treated *har-2* mutant retained hemiasterlin resistance, and by the double mutant *har-2;pkc-1* retaining resistance to hemiasterlin. (Figures 9 & 10)

Within the cellular environment proteins perform various functions through extensive networks, often forming protein-protein complexes. Thus, identifying binding partners of *har-2* is one approach for elucidating possible HAR-2 function. Using HAR-2 as bait in a pull-down assay or yeast-two-hybrid are methods to identify binding partners. In *C. elegans,* constructing a transgenic worm expressing a protein of interest as a green fluorescent protein (gfp) fusion is another method to further elucidate the function of a protein by determining its location and temporal expression.

IV. Yeast Two Hybrid

A. Introduction

Yeast-two-hybrid cDNA library screens have been used numerous times to identify protein-protein interactions that help give mechanistic insights into key biological functions(DEL BENE *et al*. 2004; JING *et al*. ; POLANSKI *et al*. 2011). In one example, by searching for proteins that bound to the protein Six/Sine oculis (Six3), a transcription factor required for proliferation of retinal cells during early development, Del Bene et. al, identified the DNA replication/cell cycle inhibitor Geminin. Through their work, Del Bene et. al, were able to establish the mechanism by which Six3 competes with the DNA replication licensing factor Ctd1 for Geminin binding. The Geminin/Six3 interaction leads to cell proliferation and, in contrast, the Geminin/Ctd1 interaction inhibits cell cycle progression. Thus, this work elucidated an intricate balance in early eye development between cell proliferation and cell differentiation(DEL BENE *et al*. 2004).

A recent yeast-two-hybrid experiment was performed to identify potential Prion Protein (PrP) interactors. PrP^{C} isoform expression is strongest in the neurons of the central nervous systerm (CNS), where abnormally folded PrP (PrP^{Sc}) molecules tend to aggregate. This aggregation is associated with many diseases including Creutzfeldt-Jakob disease, fatal familial insomnia and bovine spongioform encephalopathy(JING *et al*. 2011). At the time of the study no

cellular function had been assigned to PrP^C, therefore, a yeast-two-hybrid screen of an adult human cDNA library was performed to identify potential PrP^C interactors. As a result, the strong PrP^C binding protein, HS-1 associated protein X-1 (HAX-1) was identified. Further studies by Jing et. al, determined that PrP^C and HAX-1 co-localized in the cytoplasm and that co-expression of the two proteins led to enhanced cellular resistance to oxidative stress induced by hydrogen peroxide. Previously PrP^C expression had been established as a requirement for prion disease onset. There was speculation that PrP^C existed within a multi-protein complex modulating various cellular functions(JING *et al.* 2011). Through their work Jing et al, determined that the interaction of HAX-1 and PrP^C resulted in enhanced resistance to H_2O_2 induced apoptosis. Co-immunoprecipitation experiments revealed that HAX-1 also bound to PrPs that are asociated with prion disease (PrP-PG9 & PrP-PG14), and unlike the binding of HAX-1 to PrP^C, did not protect against toxicity induced by oxidative stress. Apoptosis is one mechanism in which abnormal cells with damaged DNA or aggregated proteins (prion disease) are removed. Jing et. al therefore identified novel interactions in the pathway of apoptosis induced by oxidative stress that could have implications in human disease such as cancer and Creutzfeldt-Jakob disease.

Since very little is known about the function of HAR-2, a yeast-two-hybrid *C. elegans* cDNA library screen using HAR-2 as the bait protein was

performed to identify candidate-binding partners of HAR-2. Many proteins function in complexes to perform their biological roles within the cell. Identifying binding partners is informative because it can identify the protein network needed for a particular protein to execute its function(s). A yeast-two-hybrid cDNA library screen can therefore shed light on newly discovered proteins when no prior functional data is available.

B. Results

Co-transformation of MaV203 cells with the *HAR-2* bait plasmid pGBKT7:HAR-2 and the pACT2 based cDNA *c. elegans* library resulted in hundreds of colonies after growth on minimal selection media agar plates. Yeast colonies with rigorous growth in plates containing multiple selective media were isolated and assayed for beta-galactose activity, an indicator of the *LACZ* gene that is also part of the MaV203 yeast-two-hybrid system. After selection, positive colonies were grown and plasmids were isolated from them and subsequently sequenced to identify the *C. elegans* cDNA whose protein product interacted with HAR-2 in the yeast-two-hybrid system. Genes identified in this way are shown in **table 1**.

The protein identified most frequently in the yeast-two-hybrid experiment was the small ribosomal protein subunit S3A (rps-1). Ribosomal proteins are often identified as interactors in a yeast-two-hybrid screen due their high

abundance within the cell or their inherent non-discriminant binding to bait proteins(MAPLE and MOLLER 2007). Four of the seven proteins identified in the yeast-two-hybrid screen as interacting with HAR-2 either form a complex with the ribosome or bind RNA. The other multiple hit proteins included the ribosomal/ubiqutin fusion protein, ubiquitin-like protein UBL-1, DNA excision repair protein XPC-1 and SRGP-1 a protein involved in chromosome segregation during meiosis.

Table 1. Potential *har-2* interacting proteins.

Interacting protein	NCBI ID	# of clones	Description
rps-1, ribosomal protein, small subunit	F56F3.5.1	5	encodes a small ribosomal subunit S3A protein
rps-26, ribosomal subunit S26	F39B2.6	2	encodes a small ribosomal subunit S26 protein
ubl-1, ubiquitin/ribosomal protein S27a	H06I04.4	2	similar to Drosophila ubiquitin/ribosomal protein S27a
xpc-1, xeroderma pigmentosa	Y76B12C.2	2	DNA excision repair homolog
gpd-3, glyceraldehydes 3-phosphate dehydrogenase	K10B3.7	2	A predicted glyceraldehydes 3-phosphate dehydrogenase
Srgp-1, Slit-Robo GAP homolog	F12F6.5	2	Cdc42-interacting protein CIP4
vig-1, VIG (Drosophila Vasa Intronic Gene) ortholog	F56D12.5	2	RNA-binding protein, VIG-1 is a component of the RNA- induced silencing complex (RISK)
F23C8.5	F23C8.5	1	Ortholog of the human gene Electron Transfer Flavoproteins Beta Subunit (ETFB)
pab-2, PolyA Binding protein	F18H3.3	1	Encodes a polyadenylate-binding protein 1 homolog similar to human PABP 1
mig-32, abnormal cell MIGration	F11A10.3	1	RING domain-containing protein
rnp-7, (RNP (RRM RNA binding domain) containing)	K04G7.10	1	U1 small nuclear ribonucleoprotein (RRM superfamily)
Y49F6B.2	Y49F6B.2	1	Uncharacterized protein
vit-2, (VITellogenin structural genes (yolk protein genes))	C42D8.2	1	A vitellogenin homolog YP170, a predicted lipoprotein
rpl-25.2, Ribosomal Protein, Large subunit	F52B5.6	1	60s ribosomal protein L23
ttr-15, TransThyretin-Related family domain	T07C4.5	1	Uncharacterized proteins with conserved cysteine
rps-7, Ribosomal Protein, Small subunit	ZC434.2	1	40S ribosomal protein S7

C. Discussion

A yeast-two-hybrid screen of a *C. elegans* cDNA library using HAR-2 cDNA as the bait identified several hits (Table 1), S3A was identified as a positive interactor in five separately derived yeast clones and therefore a brief analysis follows. A brief analysis of a second interactor, the UBL-1 protein, is also discussed.

It was previously shown that treating acute myeloid leukemia (AML) cells with all-trans retinoic acid (ATRA) resulted in increased drug sensitivity(HU *et al.* 2000; YANG *et al.* 1994). It had also been reported that protein S3A supported growth in malignant tumors(HU *et al.* 2000). Hu et al were able to show that U937 cell lines that had increased phopshorylated S3A had reduced tolerance to cytosine arabinoside and doxorubicin(HU *et al.* 2000). Hypothesizing that the increased apoptotic response seen after ATRA treatment might involve bcl-2, they immunoprecipitated bcl-2 and identified S3A as a binding partner. Hu et al showed that the drug sensitivity induced by ATRA required S3A and proposed a possible mechanism in which the anti-apoptic effects of bcl-2 and the growth promoting activity of S3A are due to their interaction. This might be an intriguing finding, since the har-2 mutant has a drug resistant phenotype and my yeast two-hybrid results suggest that HAR-2 binds S3A. Since *har-2* is a recessive mutation, it is likely to cause a loss of protein function. For a loss of HAR-2 function to increase drug resistance by a mechanism involving S3A, the loss of HAR-2

function would cause a decrease in the interaction of S3A and Bcl-2. Har-2 is predicted to be an E3 ligase. If ubiquitylation of S3A by a ubiquitin ligase containing Har-2 normally regulates S3A, it must not be for the purpose of degrading S3A, but rather serve a regulatory function.

UBL-1 was identified twice as interacting with HAR-2 in the yeast-two-hybrid experiment. UBL-1 is the ortholog to the human ribosomal/ubiquitin fusion protein S27a. S27a is post-translationally processed to form free ubiquitin and the ribosomal protein S27a. A complex of ribosomal protein S27a with MDM2 and p53 seems to function in a ribosomal-stress-specific response(SUN *et al.*). Sun et. al, identified S27a as interacting with MDM2 to increase p53 activity. It was shown that S27a was required for p53 activity specifically after ribosomal stress was induced by actinomycin D (Act-D). Sun et. al were able to determine that MDM2 ubiquitylated S27a, marking it for degradation after 12hrs of Act-D treatment. Thus, the initial response to ribosomal stress leads to p53 activity, resulting in cell cycle arrest and increased MDM2 expression. Increased expression of MDM2 leads to the eventual degradation of S27a and decreased p53 activity. These results are consistent with a method of cellular recovery after ribosomal stress induces a period of cell cycle arrest.

In the context of the identification of UBL-1 as a possible HAR-2 binding partner, it can be speculated that HAR-2 could work in a stress specific death/survival pathway. *har-2(ad2306)* is likely to be a loss of function mutation

resulting in hemiasterlin-resistance, although the mutated amino acid residue is at the C-terminus of HAR-2 and not at the N-terminal Ring Finger domain that is the signature of E3 function. It is possible that the Har-2 mutation resulted in a loss of an S27a binding domain. It is possible that S27a binding to the HAR-2, similar to MDM2, results in Har-2's degradation of p53, resulting in decreased p53 activity and apoptosis. Loss of HAR-2 binding to S27a would then lead to decreased p53 activity and drug-resistance. Of note there are no known homologs of MDM2 identified in *C. elegans*. It has also known the MDM2 regulation can be modulated depending on the ribosomal protein(s) bound. rps-26, another protein identified in the yeast-two-hybrid experiment, might be one such possible candidate that could regulate HAR-2.

Other genes identified multiple times in the yeast-two-hybrid include: Slit-Robo GAP-1 (Srgp-1) and Glyceraldehyde dehydrogenase-3 (Gpd-3), xeroderma pigmentosa group C-1 (Xpc-1) and the Vasa Intronic Gene (Vig-1). Functions reported for these genes include neuronal migration and axonal branching(ENDRIS *et al*. 2002), glycolysis(SIROVER 1997), DNA repair(THOMA and VASQUEZ 2003) and RNA-Induced Silencing, Vig-1(WU *et al*. 2006). Connections between these proteins and hemiasterlin resistance involving HAR-2 are not evident at this time.

Follow-up experiments to determine if HAR-2 is modulating S3A and Bcl-2 interactions or if Har-2 modulates p53 activity similar to MDM2 may shed light into the hemiasterlin resistance pathway in the *har-2* mutant. Future

experiments to address these and other questions are further discussed in the following chapter.

V. Conclusions and Future Directions

A. Conclusions

To uncover novel modes of resistance to the microtubule depolymerizer and natural product hemiasterlin colleagues in my laboratory performed a screen of *C. elegans* mutagenized with EMS. As a result of this screen seven independent true-breeding hemiasterlin mutants were isolated for further study.

Forward genetic mapping of the two recessive mutations *(ad2154)*, *(ad2249)* and the dominant mutation *(ad2155dm)* resulted in the identification of *phb-2, spg-1* and *har-1* respectively, as the mutated genes conferring hemiasterlin resistance. Pharmacological experiments were carried out to determine if these mutants were resistant specifically to hemiasterlin or if resistance was to a broader class of compounds. Surprisingly results from the pharmacological experiments revealed hemiasterlin-resistant mutants were resistant to a diverse group of compounds, revealing a potential novel mode of an MDR phenotype.

Previous studies of PHB-2, SPG7, and the HAR-1 ortholog, CHCHD2, revealed either a mitochondrial function or localization, suggesting a possible mitochondrial dysfunction resulted in hemiasterlin resistance and MDR.

In studies of *C. elegans*, paraquat hypersensitivity is generally considered an indicator of a mitochondrial/respiration deficient phenotype(COCHEME and MURPHY 2008; GRAD *et al.* 2007; ZUBOVYCH *et al.* 2006; ZUBOVYCH *et al.* 2010). *phb-2, spg-1* and *har-1* were shown to be paraquat hypersensitive when compared

to wildtype worms(ZUBOVYCH *et al*. 2010). This suggested hemiasterlin-mutants had elevated levels of ROS; a hypothesis that was strengthened when it was discovered that treating worms with the ROS scavenger NAC resulted in loss of hemiasaterlin resistance(ZUBOVYCH *et al*. 2010). Further genetic studies revealed that the mitochondrial-hemiasterlin-resistant mutants required a functioning protein kinase C (PKC-1), the ortholog to the human protein kinase C epsilon. PKCε has been implicated in cardioprotection during ischemia(CROSS *et al*. 2002).

For my dissertation research, a recessive mutation *ad2306* conferring hemiasterlin resistance was to be mapped and classified as to whether it was in the same category as previous hemiasterlin-resistant mutants. Genetic mapping of *ad2306* using SNPs, and deficient strains led to the identification of a point mutation within the first exon of the predicted gene T24D1.5 that resulted in a glutamic acid to lysine amino acid substitution.

Microinjections of the wildtype *T24D1.5* gene into *har-2* worms resulted in loss of hemiasterlin resistance (Figure 4.) *T24D1.5* has been renamed *HAR-2* for **h**emi**a**sterlin **r**esistant-**2.** Furthermore, results from the microinjections and deficient mapping suggest the *har-2(ad2306)* mutation results in a loss of function. There is no homolog or known ortholog for HAR-2 outside of other nematodes, although it has been predicted to be an E3 ligase(KIPREOS 2005).

Pharmacological data determined that *har-2* was not an MDR mutant, as resistance was specific to hemiasterlin (Figure 2). Both ROS depletion of *har-2* worms and loss of PKC-1 function did not result in loss of hemiasterlin resistance. Finally biochemical analysis of purified mitochondria did not reveal any differences in respiration levels between *har-2* and wildtype worms. *har-2* was found to be hypersensitive to paclitaxel. This suggested that *har-2* might have changes in microtubule dynamics that might be seen in the mitotic spindle. To address this, *har-2* and wildtype embryos were purified and stained with an alpha-tubulin antibody before and after treatment. Although microtubule staining after hemiasterlin treatment revealed a more diffuse microtubule staining pattern there was no difference observed between wildtype and *har-2* (Figure 12).

To determine if the *har-2* mutant fell into the same category as previous hemiasterlin-resistant mutants, several approaches were taken, these included pharmacological, genetic and biochemical. The results from this data suggest *har-2* to be in a separate pathway from a mitochondrial-MDR phenotype.

B. Future directions:

Considering how little is known of HAR-2 function beyond what has been shown here, a yeast-two-hybrid experiment was performed using *HAR-2* cDNA as the bait against a *C. elegans* cDNA library. This identified ribosomal protein

subunit-1 (Rps-1) as interacting with HAR-2 five times, the most of any other protein.

The *C. elegans* Rps-1 protein is the ortholog to human S3A, which is one of over 30 proteins that bind the 40S small ribosomal. Experimental data identified S3Ato interact strongly with the anti-apoptotic protein Bcl-2 after all-*trans* retinoic acid (ATRA) treatment(HU *et al.* 2000). This interaction correlated with increased sensitivity to Ara-C and doxorubicin and resulted in increased apoptosis. Interestingly, both S3A and Bcl-2 are known to be involved in cell growth and cell survival (anti-apoptotic), respectively(HU *et al.* 2000; PATTINGRE *et al.* 2005). Yet ATRA treatment favors an S3A and Bcl-2 interaction, which results in increased sensitivity to the toxic compounds Ara-C and DNR(HU *et al.* 2000). In light of these findings, a mechanism can be proposed for *har-2* hemiasterlin resistance. Data suggest that the *har-2(ad2306)* mutation results in a loss of function, and this results in hemiasterlin resistance. As stated earlier if a normal response to hemiasterlin treatment is S3A/Rps-1 modification by Har-2 resulting in an interaction between S3A/Rps-1 and Bcl-2 leading to apoptosis; then a loss of Har-2 would result in the loss of the S3A/Rps-1 interaction with Bcl-2, leading to increased survival after treatment specifically with hemiasterlin. A transgenic *rps-1::gfp* expressing *C. elegans* is available. The GFP allows co-immunoprecipitation assays with anti-GFP antibody to determine if interaction between Har-2 and 3Sa/Rps-1 exists *in-vivo* and determine if this interaction is

increased with hemiasterlin treatment as was the case with ATRA treated AML cells(HU *et al*. 2000). Further, it could be determined if the interaction of Bcl-2 with Rps-1 is increased after hemiasterlin treatment (a ced-9 antibody is available). The double mutant *har-2(ad2306);rps-1::Gfp* could be constructed to repeat the Co-IP experiments and determine if the *har-2* mutation is affecting any of these interactions, or the expression levels of Rps-1 or Bcl-2/Ced-9.

Ubiquitin like protein (Ubl) was also identified as interacting with Har-2 in the yeast-two-hybrid screen. Ubl is the ortholog to the human ribosomal/ubiquitin fusion protein S27a. As stated previously, Har-2 could have a similar function to the human anti-apoptotic factor MDM2. Har-2 could ubiquitinylate the *C. elegans* ortholog of p53, Cep-1, and mark it for degradation, and this interaction could be mediated by S27a. If the Har-2 mutation prevents it from binding S27a, then after hemiasterlin treatment mutant Har-2 would be free to bind Cep-1/p53 leading to degradation and increased survival.

Ongoing experiments include the *Uba*-1 mutant, a temperature sensitive E1 ligase mutant in *C. elegans*. If the double mutant *har-2;uba-1* results in synthetic lethality or a change in hemiasterlin resistance, an argument could be made that Har-2 is functioning within a similar ligase pathway. I have constructed the *har-2;uba-1* double mutant and can answer these questions in the near future.

One common experiment done in *C. elegans* research when trying to determine protein function is the constructions of a transgenic GFP fusion protein.

A GFP fusion protein allows for the identification of protein localization, levels of expression and temporal expression. In this regard, I created a C-terminal Har-2::Gfp worm expression vector and have microinjected many worms. Lack of a *har-2::gfp* worm suggest a possible lethality is associated with this construct, and this is further supported by the isolation of a transgenic F1 roller worm that produced dead F2 embryos. A possible solution could be to create an N-terminal Gfp::Har-2, or construct a vector with an alternate epitope tag such as Myc.

In conclusion, a new hemiasterlin-resistant mutant, *har-2* has been identified. A function for Har-2 cannot yet be assigned. Yeast-two-hybrid results have allowed us to form testable hypothesis for future experiments with the hopes of assigning Har-2 function and the mechanism for *har-2* hemiasterlin resistance.

V. Materials and Methods

A. Genetic Mapping and Complementation

i. Single Nucleotide Polymorphism (SNP) Mapping

The hemiasterlin resistant mutant *har-2(ad2306*) parent strain is N2 Bristol. Worms are maintained at a constant 20°C environment unless otherwise specified. To identify the mutation in *ad2306* that leads to hemiasterlin resistance, *ad2306* males were crossed with *Hawaiian(CB4856*) virgin hermaphrodites. Mated Hawaiian hermaphrodites were placed as individuals on NGM 6 cm agar plates and allowed to lay F1 eggs. Hermaphrodite F1's were isolated from males and allowed to grow into gravid adults. Gravid adults where placed in liquid culture (M9 solution, cholesterol 5 ng/ml, *E. coli HB101* 1:50) containing 1um of the hemiasterlin analog HTI-286 in 48 well tissue culture plates. At 1 um adult worms survive and lay eggs. Viable (gravid) F2 progeny surviving in HTI-286 were isolated and allowed to propagate. Worms were washed and collected with M9 solution. Collected worms were spun down at 3,000 rpm for 1 minute. M9 solution was aspirated and pellet was washed two more time with M9 solution. Worm DNA lysates were prepared for restriction fragment length polymorphism mapping (snip-SNP mapping) with DNA lysis buffer: *(50mM KCL, 10mM Tris-CL (ph 8.3), 2.5mM $MgCl_2$, 0.45% Nonident P-40, 0.45% Tween 20 autoclaved) + protein kinase 2 ug/µl* as described in Fay et al.(FAY and BENDER 2006) with

the following modification: worms were placed in a 58°C water-bath for 90 minutes, vortexing for 30 seconds at the one hour time point. Worm lysates were then centrifuged at 13,000 rpm for 5 minutes. Lysates were placed in PCR tubes and heated at 95°C for 15 minutes; 3.5 μl of DNA lysate was used for subsequent PCR reactions. PCR primers were ordered to amplify a region within the center of each chromosome identified to contain a snip-SNP. The PCR products were digested with appropriate restriction endonucleases and run on 1.5% TAE agarose gels and stained with ethidium bromide for strain identification.

ii. 3pt Mapping

C. elegans strain LG I: *dpy-5(e61) daf-16(m26) unc-75(e950)* was used to flank the region identified by SNP mapping that contains the mutation conferring HTI-286 resistance. *har-2(ad2306)* males were crossed with virgin *dpy-5(e61) daf-16(m26) unc-75(e950)* hermaphrodites. Mated hermaphrodites were separated and allowed to produce F1 progeny. F1's were allowed to produce F2's; F2's with a dpy non-unc or unc non-dpy phenotype were singly plated and cloned. Cloned F2's were harvested for eggs by washing plates with M9 solution and collecting worms in 15ml conical tubes. Standard egg preparation; worms are resuspended and vortexed for 1 minute in autoclaved dH_2O, 40% bleach (v/v) and 100 mM NaOH. Following worm egg prep lysed worms were washed 3 times with M9 solution. Eggs were allowed to synchronize at the L1 stage overnight (9-14 hrs)

by incubating in a 15 ml conical tube with 3 ml M9 solution at room temperature. The following day synchronized worms were placed in liquid culture with or without 0.7 uM HTI-286. Viable adults were isolated; cloned and classified as either *dpy-5(e61) har-2(ad2306)* or *har-2(ad2306) unc-75(e950). dpy-5(e61) har-2(ad2306)* and *har-2(ad2306) unc-75(e950)* worms were cloned to identify and verify if strains bred true. Either *dpy-5(e61) har-2(ad2306)* or *har-2(ad2306) unc-75(e950)* males were crossed with *Hawaiian(CB4856)* virgin hermaphrodites. Mated hermaphrodites were isolated and allowed to produce F1 progeny. F1 gravid worms were singly seeded into liquid culture containing HTI-286. Surviving F2's that no longer displayed the visible marker (dpy or unc) were isolated and cloned. Cloned worms were lysed for DNA isolation and SNP mapping.(TIMMONS 2000)

iii. Deficiency mapping

Strains JK1534 - *[ces-1(n703) qDf5/unc-29(e193) mec-8(e398) dpy-24(s71)I]*, JK1547 - *[ces-1(n703) qDf10/unc-13(e1091) lin-11(n566)I]*, SP1540 – *[mnDf111/unc-13(e1091) lin-11(n566)I,* and JK1553 – *ces-1(n703) qDf9/unc-29(e1072) lin-11(n566)I* were used for deficiency mapping. *har-2(ad2306)* males were crossed with deficient virgin hermaphrodites. Deficient mated hermaphrodites were allowed to grow gravid and placed in liquid culture with or without HTI-286. Cultures were observed for surviving F1 progeny.

iv. T24D1.5 sequencing

Primers used to sequence location of point mutation are: forward primer 5'-caacaagttggatggatcc-3'; reverse primer 5'-tcatcactgaacacaactcgt-3'. *har-2(ad2306)* gDNA was used to PCR amplify region within T24D1.5. PCR product was run on 1% TAE agarose gel and gel purified using *Qiagen Qiaquick® Gel Extraction kit*, sample was confirmed by sequencing. Sequence trace was analyzed with CodonCode Aligner software from the CodonCode Corporation, download available online.

v. T24D1.5 RNAi

Primers used to amplify a 522 bp fragment of T24D1.5 insert are: forward primer with bglII cut site: 5'-ctagagatcttcaacaagttggatggatcc-3', reverse primer with knpI cut site: 5'-ctagggtaccgccttcatgtgctcgatgat-3'. *har-2(ad2306)* gDNA was amplified using the PFU high fidelity DNA polymerase. PCR product was PCR purified using QIAGEN QIAquick® PCR Purification Kit. The RNA worm expressing plasmid L4440 and purified T24D1.5 PCR product were double digested with BglII and KpnI for 3 hours. Digested DNA was run on agarose gel and gel purified as described above. *Ligation:* 28ng of T24D1.5 insert and 26ng of L4440 plasmid was used in a standard ligation reaction following manufactures instructions and incubated at 16°C for 4 hrs. Five microliters of ligation reaction

was diluted with 10 μl ddH_2O. Ten microliters of ligation reaction was used to transform C-max 5α competent cells following standard protocol. Transformed cells were plated and incubated overnight at 37°C. Colonies were picked after 24 hr growth on ampicillin (100 mg/ml) LB plates, and grown in 5 ml minicultures overnight. Plasmid purification was performed using the Promega WizardPlus SV Miniprep DNA purification system following manufactures instruction. After plasmid purification, double digestion with BglII and KpnI was performed for 2 hrs at 37°C and run on a 1% TAE agarose gel for correct insert identification. Plasmid L4440 with T24D1.5 insert (L4440:T24) was transformed into competent HT115(DE3) bacterial cells resulting in bacteria expressing T24D1.5 dsRNA (HT115:T24) when induced with 0.4 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). The Timmons(TIMMONS *et al*. 2001) RNAi protocol was followed to knockdown T24D1.5. N2 worms were synchronized at the L1 stage as previously described. N2 worms synchronized at the L1 stage were fed IPTG to induce the production of har-2 dsRNA, control experiment included feeding worms non-induced bacteria. Gravid worms feeding on induced or non-induced HT115:T24D1.5 where placed in liquid culture containing induced or non-induced HT115:T24D1.5 with or without 1 uM HTI-286.

vi. C. elegans T24D1.5 complementation

Primers used to amplify 3.7 kb region of T24D1.5 were: forward 5'-tggcgatgatgcgagaacata-3' and reverse 5'-agctctgtttcgacctcttcg-3'. The proofreading DNA polymerase Phusion® was used in a PCR reaction using genomic DNA according to manufactures guidelines. To add dTTPs after PCR reaction, 1 ul of GoTag® DNA polymerase was added to the PCR mix and incubated for 10 minutes at 72°C before gel purifying. After gel purification the 3.7 kb T24D1.5 fragment was ligated into the pCR®2.1 TOPO vector from Invitrogen following manufactures instructions. The ligation reaction was transformed into OneShot® compotent cells from Invitrogen and plated onto X-β-gal LB ampicillin plates. White colonies were isolated and grown for plasmid purification and enzyme digestion to determine insert size. The pCR®2.1 TOPO vector with T24D1.5 insert (pCR:T24) was sequence verified before use in microinjections. *Microinjection:* For microinjections worms were immobilized by placing on injection pads: 24X60 microscope cover glass covered with dried 2% agarose. A DNA mixture of *pCR:T24 50 ng/µl* and *pRF4(rol-6)150 ng/µl* was microinjected into the gonads of *har-2(ad2306)* young adult worms.

B. Toxicity assays-

i. Toxicity curves

To generate toxicity curves worms are grown at 20°C on NGM agar plates*[3 g NaCl, 17 g agar, and 2.5 g peptone are added to 975 ml DH_2O and autoclaved.*

Flask was allowed to cool to 55℃ before adding 1 ml of 1 M CaCl, 1 ml cholesterol (5 mg/ml) in ethanol, 1 ml of 1 M $MgSO_4$, 25 ml of 1 M KPO_4 and Streptomycin at 0.1 g/L] seeded with *HB101 E. coli*. Worms were then harvested 2-3 days for eggs and synchronized at the L1 stage. L1 synchronized worms were seeded in 48-well tissue culture plates with liquid culture with or without compound of interest. Assays were all done in triplicate. Worms were scored when untreated controls reached gravid stage. Survival percentage was normalized to identical strain control (no treatment) and taken as 100% survival. Prism software was used to analyze and graph results.

ii. Mitochondrial purification and respiration analysis

Method was slightly modified from Grad et. al.(GRAD *et al*. 2007) To prepare purified mitochondria from mixed stage *C. elegans,*, N2 Bristol and *har-2* strains were seeded into 1ml flasks containing 150ml S media. To make 100 ml of S media, add *97 ml S basal: [5.85 g NaCl, 1 g K_2HPO_4, 6 g KH_2PO_4, 1 ml cholesterol(5 mg/ml in ethanol), H_2O to 1 liter, autoclave], 1 ml of 1M potassium citrate pH. 6, 1 ml of trace metals [trace metals: 1.86 g disodium EDTA, 0.69 g $FeSO_4$-$7H_2O$, 0.025 g $CuSO_4$-$5H_20$, add H_2O to 1 liter store in dark at 4℃], 300 µl of 1 M $CaCl_2$, and 300 µl of 1 M $MgSO_4$.* A on liter culture pellet of HB101 *E. coli* was added and placed on an culture incubator at 21°C and 110 rpm. On the third day, one HB101 pellet was added to liquid culture flask,

worms were ready to harvest the following day, can wait two more days if desired. Worms were centrifuged at 3000 rpm for 5 minutes in 50 ml tubes and washed 4X with M9 buffer. Worms were resuspended in M9 buffer and placed on rocker for 30 min. Worms were spun down at ~1000 rpm for 4 min and washed with ice-cold 0.1 M NaCl and centrifuged once again before resuspending in 100 ml of 0.1 M NaCl and chilled on ice for 2 min. An equal amount of 60% sucrose was added to sample and spun for 5 min at 1000 rpm. Worms were removed from top layer and diluted four fold. Sample was then washed twice with 0.1 M NaCl. After final wash worms were spun down and supernatant was removed. The worm pellet was resuspended in 2 vol. of worm lysis buffer (*0.8 M sucrose, 1 mM EDTA and 1 M Tris-HCL pH 7.4 + 1 tablet of Roche complete protease cocktail per 100 mls),* worms were lysed in a bead beater containing 0.7 mm Zirconia Beads from BioSpec Products Inc. thrice in 1 min intervals. Lysed worms were homogenized in a glass-homogenizer. Homogenate was centrifuged at 2500 g for 10 min at 4°C. Supernatant was aspirated and pellet was resuspended in a small volume of worm lysis buffer and briefly homogenized in a glass-teflon homogenizer. To isolate pure mitochondria, 18.5 ml of 2 M sucrose was added on top of 18.5 ml 1 M sucrose in a 38.5 ml Ultra-cleartube™ from Beckman. Worm homogenate was carefully added on top of sucrose gradient and centrifuged at 80,000 g for 1 hr and 45 min at 4°C using a Beckman SW-28 swing bucket. Mitochondria will form a band at interface of 1 M and 2 M sucrose

gradient. Mitochondria were removed and diluted with 3 volumes of ice-cold worm lysis buffer and centrifuged at 30,000 g for 30 min at 4°C to pellet mitochondria. The supernatant was removed and the pellet was resuspended in MiR05 buffer. MiR05 buffer consisting of *0.5 mM ethylene glycol tetra-acetic acid (EGTA), 3 mM magnesium chloride hexa-hydrate ($MgCl_2$-$6H_2O$), 60 mM K-lactobionate, 20 mM Taurine, 10 mM mono-potassium phosphate (KH_2PO_4), 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 110 mM sucrose, 1 g/L bovine serum albumin (BSA) pH 7.1 with 5 N KOH at 30*°C store at -20°C. High-resolution mitochondrial respiration measurements were performed with the Oroboros Oxygraph-2K and graphed using Oroboros software by following manufactures instructions.

iii. pkc-1(ik130)V; har-2(ad2306)I and brc-1(tm1145)III; har-2(ad2306) double mutant construction

To construct both *pkc-1(ik130)V; har-2(ad2306)I* and *brc-1(tm1145)III; har-2(ad2306)* double mutants, male *har-2(ad2306)* worms were crossed with either *pkc-1(ik130)* or *brc-1(tm1145)* virgin hermaphrodites. F1 virgin hermaphrodites were isolated and allowed to become gravid. Gravid F1 worms were placed in 48-well plates with liquid culture (+) or (–) HTI-286. F2 gravid survivors were isolated and placed on NGM plates at 1worm/plate. Cloned worms were genotyped by preparing DNA lysate from whole plates and PCR amplification of

brc-1 or pkc-1 sequence. Primers for *pkc-1(ik130)* sequencing are: fwd-5'-ttcgagcgactgatattca-3', rev-5'-tgtaatctatgattcatctacgc-3', primers for *brc-1(tm1145)* are: fwd-5'-ccagcgggaatactcgactt-3' and rev-5'-cgatgcgctccagattttgct-3'. PCR products were PCR purified as previously described and sequenced and confirmed by sequencing. *brc-1(tm1145)* and *pkc-1(ik130)V* double mutants were cloned and verified to be *har-2(ad2306)I* homozygous.

iv. N-acetylcysteine treatment

N2 bristol and *har-2(ad2306)* worms were synchronized in the L1 stage and placed in 48-well plates with liquid culture containing 0mM or 20 mM of N-acetylcysteine, with or without 0.9 uM HTI-286. Worms were scored and graphed three days later. The average of the untreated strain was taken as 100% survival.

v. Embryo microtubule staining

Worm staining protocol was slightly modified from Pelletier et. al,.(PELLETIER *et al.* 2004) Worm eggs were placed on poly-prep microscope slides (Sigma P0425) and covered with microscope cover slips before being submerged in liquid nitrogen for 2 minutes. Cover-slip was pried off with a razor and immediately submerged in -20°C methanol for 20 minutes in -20°C freezer. Egg slides were washed 3 times for 5 minutes/wash in PBST solution to rehydrate. PBST: *[10 ml*

phosphate buffered saline (PBS), 500 µl Tween-20, 20 mg sodium azide, to 100 ml].

Slides were blocked for 10-30 minutes in blocking buffer. Blocking buffer: *[10 ml fetal bovine serum (FBS), 1 g BSA and 90 ml PBST].* Slides were incubated for 1-2 hrs at room-temperature with YL1/2 tubulin (abcam: ab6160) primary antibody diluted in blocking buffer at 1:200. After primary antibody incubation, slides were washed 4 times with PBST for 5 minutes. Alexa Fluor® 488 goat anti-rat IgG(H+L) (Invitrogen A11006) secondary antibody was diluted with blocking buffer to a final concentration of 1:1000 and incubated at room-temperature for 1-2 hrs. ProLong® Gold antifade reagent with DAPI (Invitrogen P36931) was placed on slide and covered with coverslip. Slides were placed in dark at room-temperature and allowed to dry overnight. The Applied Precision DeltaVision RT deconvolution microscope and *softWoRx* software were used to visualize and image capture stained embryos(LUBY-PHELPS). Image J software was used to edit images(HEALTH 2010).

C. Materials and Methods -Yeast two hybrid

The plasmid base used for the *C. elegans* yeast two-hybrid library is pACT2, provided by Dr. Zheng Zhou. *har-2 bait plasmid construct:* One 10 cm NGM plate of *N2* worms was washed twice with M9 solution. Worms were pelleted and 250 μl of packed worms was resuspended in 1 ml of TRrizol® reagent

(Invitrogen) in a 15 ml conical tube. 0.7 mm Zirconia beads (BioSpec) were added to worm suspension and vortexed for 2 minutes. Worms were freeze-thawed twice in liquid N_2 (can stop and store at -80°C). After the second freeze-thaw cycle 500 microliters of TRizol® was added to worms and vortexed for 1 minute. 500 microliters of chloroform ($CHCl_3$) was added to worm solution and tubes were shaken by hand for 15 seconds. Tubes were incubated at room temperature for 3 minutes prior to centrifugation at 12,000 g for 15 minutes at 4°C. The top aqueous layer was carefully removed and an equal volume of isopropanol was added, mixed and incubated at room temperature for 15 minutes.

Worms were centrifuged at 12,000 g for 15 minutes at 4°C. The pellet was washed with 75% ethanol and centrifuged at 7,500 g for 5 minutes at 4°C. Supernatant was decanted and pellet was air dried for 10 minutes. Air-dried pellet was resuspended in 150 μl of DEPC-H_2O (.5 ml/1ml packed worms). Concentration was measured using the NanoDrop spectrophotometer (Thermo Scientific), and RNA concentration was determined to be 3.7 μg/μl. RNA sample was loaded into a spin-cartridge (spin cartridge was from Invitrogen PureLink™ micro-to-midi total RNA purification system) with 350 μl of wash buffer I and centrifuged for 15 minutes at 12,000 g at room temperature. In separate tube 10 μl of DNase I was added to 70 μl of DNase buffer and mixed gently before a quick spin down. DNase was transferred to spin cartridge and centrifuged at 12,000 g for 15 minutes. Flow-through was discarded and 700 μl of washer buffer

I was added to column before centrifuging at 12,000 g for 15 minutes. Cartridge was placed in new collection tube and 500 μl of washer buffer II with ethanol was added to spin cartridge and centrifuged at 12,000 g for 1 minute. Cartridge was placed in new recovery tube, 50 μl of RNase-free H_2O was added to cartridge, after a 1 minute incubation cartridge was centrifuged at 12,000 g for 2 minutes.

cDNA synthesis. The Invitrogen ThermoScript™ RT-PCR system to make cDNA was used following manufactures instructions with slight modifications. The RT-PCR reaction was as follows: 1 μl of oligo $(dT)_{20}$, 1 µl of RNA, 2 μl of 10 mM dNTP mix, DEPC-H_2O to 12 μl. 4 μl of 5X cDNA synthesis buffer, 1 μl of 0.1 M DTT, 1 μl of RNase OUT, 1 ul of DEPC-treated H_2O, and 1 μl of ThermoScript RT was added to the RT-PCR reaction mix. The reaction mix was incubated at 50°C for 50 minutes followed by 5 minutes at 85°C. 1 μl of RNase H was added to the reaction mix and incubated at 37°C for 20 minutes. cDNA was immediately used for har-2 PCR. The Phusion® high fidelity DNA polymerase was used to amplify har-2 cDNA using the following primers: forward primer with NdeI cut site-5'-gatccaatgcgtggatctcgtcaa-3', reverse with XmaI cut site-5'-aaaacgacgatgagcgatag-3'. GoTaq® was added to the PCR product and prepared for cloning by PCR purification. The resultant purified product was ligated into the pCR2.1® TA cloning vector following manufactures instructions. The ligation reaction was transformed into One Shot® compotent cells from Invitrogen. Transformed cells were plated onto LB plates with ampicillin (100 ng/ml) and X-

β-gal (20 ng/ml) added. White colonies were isolated and grown for plasmid purification. Plasmids were digested to determine correct DNA insert size, followed by DNA sequencing.

To clone har-2 cDNA into the bait vector pGBKT7, both pGBKT7 and pCR2.1:har-2 cDNA vectors were double digested with XmaI and NdeI. Digested plasmids were gel purified; pGBKT7 was treated with Shrimp Alkaline Phosphatase (SAP) before being gel purified. Digested bait vector and har-2 cDNA were ligated as previously described and transformed into competent *E. coli (HT115 DE3)*. The transformed bacteria were plated on kanamycin LB plates. Positive colonies were picked and digested to determine correct band size. Clones with correct band size after digest were sequence verified. Resulting har-2 cDNA bait plasmid was used in yeast-two hybrid library screen.

Yeast-two hybrid screen: MaV203 Competent Yeast Cells from Invitrogen were used for the library screen. pGBKT7:har-2 bait was transformed into the yeast strain PJ69-4A (MYA-563) as described by Gietz et, al. (GIETZ and WOODS 2001). All plates contain 100 ng/ml ampicillin. A yeast colony was resuspended in 5 ml of YPDA medium consisting of 20 g/L peptone, 10 g/L yeast extract, 20 g/L glucose and 0.003% adenine and grown overnight at 30°C and 230 rpm. The following day an aliquot of culture was added to 15 ml of YPDA with starting optical density (OD) at 600 nm reading at 0.005-0.01. Cells took between 4-6 hrs to reach OD 0.6. Cells are then centrifuged at 1000 g for 3 minutes. Supernatant

was removed and pellet was washed with 1 ml of distilled water. One milliliter of 0.1 M LiAc was used for a second wash. Cells were pelleted at 1000 g for 3 minutes and resuspended in 240 μl of 50% polyethylene glycol (PEG) with an average molecular weight of 3000-4000, 36 μl of 1 M LiAc, 25 ul of boiled single stranded salmon testes DNA (1 mg/ml) and 1 ug of DNA in 50 μl of distilled water. For the positive control co-transformation of pGBKT7-53 bait plasmid and pGADT7-T prey plasmid were contransformed in a separate transformation reaction. Cells in transformation mix were incubated at 30°C for 30 minutes. Cell were heat-shocked at 42°C for 30 minutes and centrifuged at 1000 g for 3 minutes. Cells were resuspended with 1 ml YPDA medium and incubated for 4 hrs at 30°C. After incubation cells were diluted at 1:20 in distilled water before plating onto LB plates made of synthetic drop out media lacking tryptophan, leucine, adenine and histadine (-T-L-H-A), to determine transformation efficiency, cells were also incubated in –T-L plates. Plates were incubated for 3 days at 30°C. After 3 days plates were scored to determine if pGBKT7:har-2 bait auto-activated by scoring –T-L-H-A plates. To determine transformation efficiency –T-L-H-A plates seeded with pGBKT7-53+pGADT7-T co-transformation reaction.

For yeast-two-hybrid screen, MaV203 competent yeast cells from Invitrogen (11281-011) were used. Transformation procedure was as described by the manufactures protocol. Plating was done on 10 mM 3-aminotriazole (3-AT).

After 4 days of incubation, master-plates were created and incubated at 30°C for 1 day. Master plates with colony growth were replicated onto increasing amounts of 3-AT (25 mM, 50 mM and 100 mM) in the –T-L-H-A plates. Positive colonies at 50 mM were streaked onto –T-L-U minimal media plates, and x-β-gal LB plates. Plasmid purification of positive colonies was done with the Zymoprep yeast plasmid purification kit by following manufactures protocol. To determine the sequence of the plasmid insert within the positive clones, primers flanking the insert were used in a PCR reaction. Primers are: forward primer-5'-ctattcgatgatgaagataccccacc-3', reverse primer-5'-gtgaacttgggggttttttcagtatc-3'. After PCR, PCR purification as described above was performed before sequence determination.

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