UNIVERSITY OF CALIFORNIA SAN DIEGO

The Role of the microRNA Pathway During Recovery After Heat Shock in *Caenorhabditis elegans*

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Biology

by

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2021

The dissertation of Delaney Catherine Pagliuso is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2021

DEDICATION

I would like to dedicate this thesis to my incredibly supportive family and to Garrett, who provided unwavering love, encouragement, and patience throughout this time.

EPIGRAPH

“I believe that accurate knowledge is very, very important, but find that out in free time. Don’t let it take over every hour of the day. Perhaps most important, talk about it.”

-Jane Goodall

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ACKNOWLEDGMENTS

I would like to thank my phenomenal thesis committee and my advisor, Amy Pasquinelli, for her mentorship and guidance. I feel incredibly fortunate to say that I truly enjoyed graduate school and this is largely due to joining a wonderful lab. Amy advocated for me throughout my time as a graduate student while I explored my interests in teaching, mentoring, and outreach and I feel lucky to have an advisor who recognized the importance of these experiences in addition to my research. My lab mates also deserve special recognition for their friendship, generosity, and willingness to collaborate. Will, thank you for mentoring me when I first joined the lab. Laura, thank you for your positive attitude and contagious laughter, even during our two-year CLIP troubleshooting woes. Angela, thank you for your kindness, attention to detail, and always making time to lend an ear. Erin, you have made tremendous contributions to all of our work through your excellent questions and constant willingness to help troubleshoot experiments. I always enjoy talking to you about science, hiking, triathlon, and new restaurants. Corrina, you are one of the most considerate people I know and I'm lucky to have you as a lab mate and friend. Ian, I am grateful for your organization as our lab manager and technical support with all things worm related that I never fully mastered, i.e., injections. Finally, Dev, thank you for being such an outstanding undergrad mentee. Your dedication to research is tremendous and I am so fortunate to have worked with you these last

few years. Your questions and ideas always challenged me to be a better scientist and mentor and I am very thankful for that.

I also owe a great debt of gratitude to Garrett who always helped remind me of life beyond the lab. Thank you for always making time for weekend adventures on the trails, patiently listening and supporting me through ups and downs, and keeping the chocolate stocked at home - a necessary ingredient for all late-night bioinformatics analysis and paper writing. Thank you to my supportive and loving parents, Beth and John, and my siblings, Mitchell and Grace. I am privileged to be able to pursue higher education and would not have been afforded this opportunity without the support and selflessness of my parents and the encouragement from family and friends along the way.

Chapter 2 contains material from the paper “Remodeling of the *Caenorhabditis elegans* non-coding RNA transcriptome by heat shock “, Schreiner, W. P., Pagliuso, D. C., Garrigues, J. M., Aalto, A. P., Chen, J. S., Pasquinelli, A. E. *Nucleic Acids Research*, 2019. Pagliuso, D. C. was a co-author on this paper.

Chapter 3, in full, is a reprint of material as it occurs in *PLoS Genetics*, “Recovery After Heat Shock Requires the microRNA Pathway in *Caenorhabditis elegans.*” Pagliuso, D.P., Bodas, D.M., and Pasquinelli A.E., PLOS, 2021. I was the primary author.

Chapter 4 contains unpublished material coauthored with Bodas, D.M. and Pasquinelli, A.E. I was the primary researcher and author of this material.

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D. C. Pagliuso, D. M. Bodas, and A. E. Pasquinelli. “Recovery After Heat Shock Requires the microRNA Pathway in *Caenorhabditis elegans.”* *PLoS Genetics.* 2021.

ABSTRACT OF THE DISSERTATION

The Role of the microRNA Pathway During Recovery After Heat Shock in Caenorhabditis elegans

By

Delaney Catherine Pagliuso

Doctor of Philosophy in Biology

University of California San Diego, 2021

Professor Amy Pasquinelli, Chair

The heat shock response has been studied for nearly sixty years. How organisms respond to stress relies on this highly conserved cellular response. However, once a stress is ameliorated, little is understood about how an organism re-sets its molecular landscape. In my thesis, I uncover a role for the microRNA pathway in regulating the molecular re-setting after stress and provide evidence to support its function in promoting survival post stress.

The heat shock response (HSR) is a cellular response that has been identified in all species in which it has been studied. The HSR is elicited in response to stressful events including infection, oxidative stress, and heat stress.

In my second Chapter, I will discuss how non-coding RNAs, including microRNAs, change expression in response to heat stress in *C. elegans*. MicroRNAs are small non-coding RNAs that post-transcriptionally repress target mRNA expression. I will detail my work focused on understanding how a specific HS-induced miRNA, miR-239a/b is regulated. As well, I will discuss functional analysis of miR-239a/b performed in an effort to characterize its regulatory roles.

In Chapter 3, I will discuss the role of the microRNA pathway during heat shock (HS) recovery. I identified that ALG-1, the predominant Argonaute protein in *C. elegans,* contributes to survival after heat shock. Furthermore, I unveil a new role for miR-85 in HS recovery in which it acts to downregulate *hsp-70,* a highly conserved chaperone that is transcriptionally upregulated in response to stress but detrimental when misregulated after stress. Chapter 4 will present evidence of miR-85 mediated regulation of fertility in *C. elegans* and analysis aimed at addressing mechanistic understanding of this phenotype. Overall, this work deepens our understanding of the role of miRNAs in response to stress.

Chapter 1

Introduction

1.1 The Heat Shock Response

In their native environments, organisms can face a barrage of stressful events. Stress can be pathophysiological, including pathogen infection and inflammation, or environmental assaults, such as UV rays and heat stress. Despite the wide range of stress an organism might face, one common outcome of these stresses involves widespread protein misfolding and aberrant protein aggregation which, if left unresolved, can be fatal to an organism (Gomez-Pastor, Burchfiel, and Thiele 2018). To maintain proteostasis upon stress, the heat shock response (HSR) is deployed to perform damage-control and promote cell survival (Figure 1.1). Central to the HSR is the production of heat shock proteins (HSPs). HSPs are rapidly transcribed and act as molecular chaperones, preventing further protein misfolding and aiding in dismantling aggregated proteins (Richter, Haslbeck, and Buchner 2010). Misregulation of the HSR has been associated with a variety of pathologies including protein misfolding diseases, aging, and cancer (Richter, Haslbeck, and Buchner 2010). The HSR

has been identified in organisms spanning from bacteria to yeast to humans and has been found in every cell and tissue type (Lindquist).

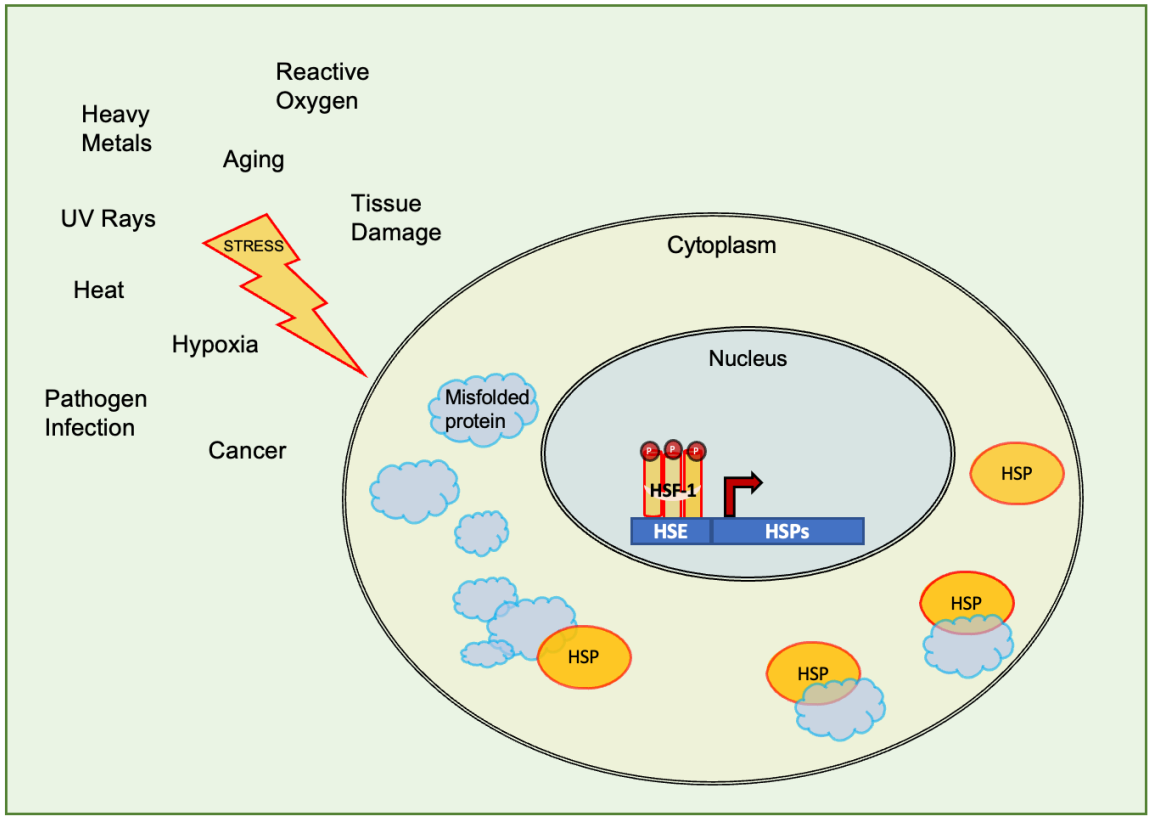


Figure 1.1: The Heat Shock Response. Various types of stress can lead to protein misfolding and elicit the heat shock response. HSF-1 associates with HSEs in the nucleus to promote the production of HSPs which act as cellular chaperones to mitigate protein misfolding.

Upon stress, a robust yet transient cellular reprogramming occurs. A hallmark of this reprogramming is the global reduction in transcription and translation, which allows for the shunting of resources towards producing large amounts of HSPs (Morimoto 1993). The induction of HSP expression is largely driven by the transcription factor, Heat Shock Factor HSF-1. HSF-1 is a highly conserved, essential protein, that is constitutively expressed throughout the

cytoplasm and nucleus (Morton and Lamitina 2013). Upon stress, HSF-1 undergoes trimerization and hyperphosphorylation, prompting its activation and accumulation in the nucleus where it can interact with Heat Shock Elements (HSEs) within the promoter region of heat-shock induced genes (Richter, Haslbeck, and Buchner 2010). HSF-1 then drives the transcription of HSPs which acts as molecular chaperones and aid in recovering proteostasis. In mammalian cells, HSF-1 induces *Hsp* transcription by increasing promoter-proximal pause release of RNA Polymerase II (Mahat et al. 2016). HSF-1 can also recruit co-factors that aid in the dramatic upregulation of *Hsps* (Åkerfelt, Morimoto, and Sistonen 2010).

Importantly, although HSF-1 and HSPs are named based on their function related to heat shock, HSF-1 also induces HSP expression upon ER stress, oxidative stress, and heavy metal stress (Gomez-Pastor, Burchfiel, and Thiele 2018). Outside of its role in the stress response, HSF-1 broadly contributes to organismal development and prevention of protein folding maladies, including those associated with aging (J. Li et al. 2016; Hsu, Murphy, and Kenyon 2003). Furthermore, HSF-1 misregulation has been identified across multiple tumor types, where it can aid in the overexpression of HSPs to promote the proliferation of otherwise toxic cancer cells (Mendillo et al. 2012). Given these critical functions, it is logical that HSF-1 activity is subject to careful regulation. One proposed model of HSF-1 regulation has been termed the chaperone titration model (Figure 1.2). In this model, the concentration of chaperones such as HSP70 can modulate the activation of HSF-1. In permissive temperatures,

HSF-1 is thought to be bound by chaperones which repress HSF-1 activation. During heat stress, chaperones are titrated away from HSF-1 to assist with protein folding, allowing for HSF-1 to trimerize, enter the nucleus, and drive transcription. This aligns with our understanding that HSF-1 concentrations remain constant in stressful and non-stressful conditions yet can assume enhanced activity levels through changes in cellular localization and post-translational modification (J. Li, Labbadia, and Morimoto 2017). When a cell returns to ordinary functions, the excess of now unemployed chaperones can reengage with HSF-1, downregulating its transcriptional activation (Mendillo et al. 2012). In addition to the chaperone titrations model, *C. elegans* have additional modes of HSF-1 regulation including repression by Heat Shock Factor Binding protein (HSB-1) and Daf-16-Dependent Longevity 1 and 2 Proteins (DDL-1/2). Both mechanisms of repression reduce the HSF-1 dependent transcription of heat shock proteins (HSPs) (Chiang et al. 2012; Abravaya et al. 1992).

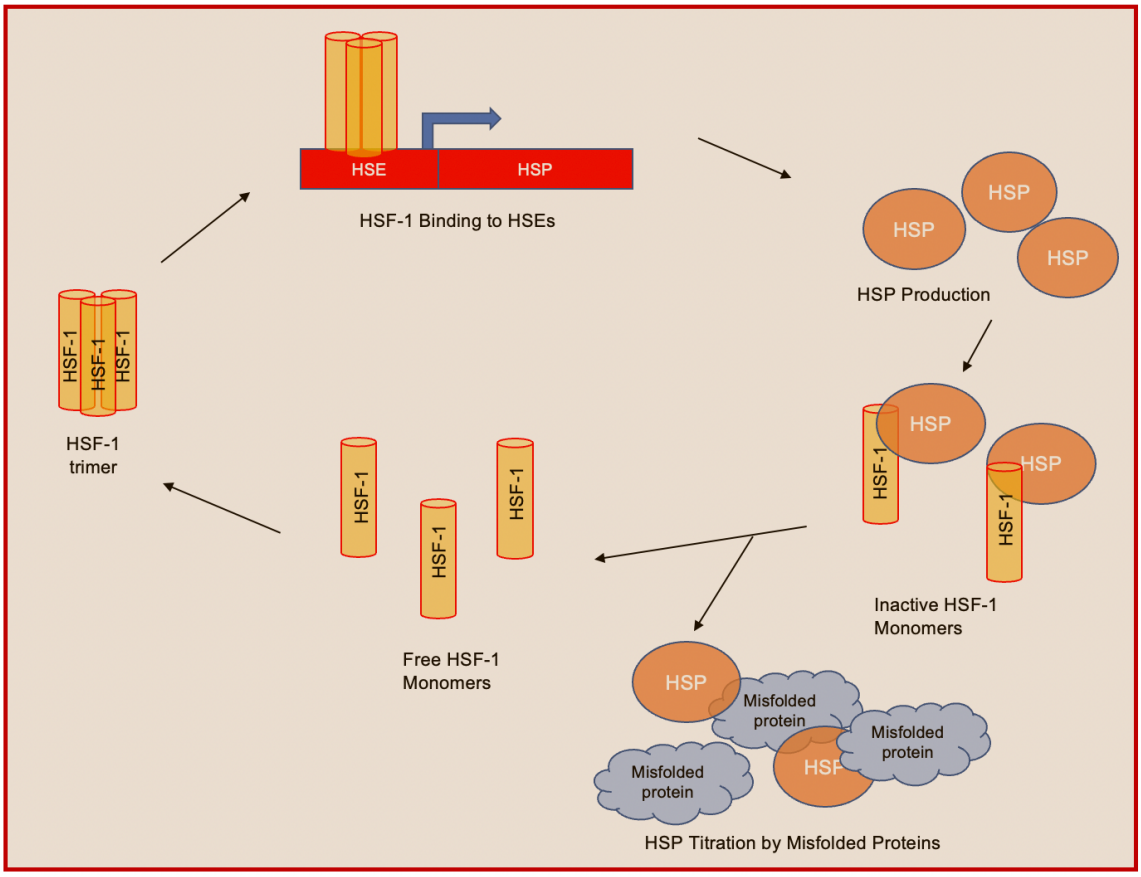


Figure 1.2: The Chaperone Titration Model. HSPs (chaperones) negatively regulate HSF-1 activity. Perturbations to proteostasis titrate away chaperone, allowing for the activation of HSF-1. HSF-1 induces a negative feedback loop by stimulating production of HSPs.

HSPs were discovered in the fruit fly *Drosophila* when researchers identified expanded chromosomal puffs in salivary glands, indicative of localized enhanced transcription (Ritossa 1964). While the effects of heat were first studied in the 1960s, it wasn't until the late 1970s that the specific genes transcribed from these sites were elucidated and named HSPs. At this time, scientists also began to appreciate that the response to heat was an ancient and universal mechanism as it was observed in other eukaryotes and prokaryotes (Richter, Haslbeck, and Buchner 2010). HSPs act as chaperones and help return

proteins to their native folding which is often disrupted in high temperature conditions. HSPs can also target misfolded proteins and protein aggregates for recycling through the proteosome (S Lindquist and Craig 1988). In general, the rapid production of HSPs is imperative for responding to widespread protein misfolding that occurs upon stress and is integral to an organism's viability.

HSPs can be divided into six major families: HSP100, HSP90, HSP70, HSP60, HSP40, and small HSPs (sHSPs), according to their molecular weight, structure and function. Hsp100 is induced upon HS and has been best studied in bacteria, yeast, and plants. Organisms lacking Hsp100 suffer a high risk of death when challenged with high temperatures (Singh et al. 2020). Hsp90 is highly conserved and has broad cellular roles both in non-stressful and stressful environments. Hsp90 is one of the most highly abundant cytosolic proteins in eukaryotes under physiological conditions and can interact with a broad range of client proteins including steroid hormone receptors and transcription factors (Schopf, Biebl, and Buchner 2017). Additionally, it has been identified as an essential protein under all growth conditions in yeast (Borkovich et al. 1989). Hsp90 has been of great interest in targeted cancer therapeutics as it contributes to the stability and activity of oncoproteins including Her-2 and EGFR (Trepel et al. 2010). Hsp70 has many similarities to Hsp90. It can be classified as either constitutively expressed (Hsc70) or heat induced (Hsp70), like Hsp90, and it is also upregulated by Hsf-1 upon HS, although typically to a greater extent because of the higher basal levels of Hsp90 (Whitley, Goldberg, and Jordan 1999). Constitutively expressed Hsp70 contributes to *de novo* protein folding

during mRNA translation and protein trafficking, interacting nonspecifically with hydrophobic regions of proteins and relying on a balanced cellular stoichiometry. Heat can induce Hsp70 upregulation over 100-fold to help with aberrant protein folding but overall, it assumes similar roles to its constitutively expressed counterpart (Nollen and Morimoto 2002). Hsp70 binds to substrate proteins through an ATPase cycle directed by co-chaperones where substrate binding occurs in high-affinity ADP-bound states and release occurs in low-affinity ATP bound states (Mayer and Bukau 2005). I will elaborate on Hsp70 activity and regulation further in a later section. Hsp60 is a smaller but similar chaperone to Hsp70 and is also induced upon HS but generally to a lesser extent than Hsp70 (Caruso Bavisotto et al. 2020). Hsp40, also known as a J-Domain Protein or DnaJ, operates as a co-chaperone of Hsp70, facilitating the ATP exchange activity along with nucleotide exchange factors (Ahmad et al. 2011). The abundance of Hsp40, therefore, is integral to Hsp70 protein folding activity (Faust et al. 2020). Small HSPs encompass a multitude of HSPs of low molecular weight, and can also operate as co-chaperones, associating with other classes of HSPs that are swiftly upregulated upon HS to aid in chaperoning activities (S Lindquist and Craig 1988). Hsp70 typically incurs the greatest fold change upon HS but is usually transcribed in a synchronous fashion along with other HS-inducible HSPs. However, the repression of HSPs after HS is less synchronous, suggesting the resetting of HSP expression after HS is regulated through a separate mechanism (DiDomenico et al.)

Hsp70 is a robustly stress induced HSP and is highly conserved, making it of great interest to researchers interested in studying stress. The role of Hsp70 under both physiological and stressful conditions implies the importance of careful regulation. One might predict that an abundance of Hsp70 would confer some benefits, perhaps priming an organism for a future stressful assault. However, mice overexpressing Hsp70 in physiologic conditions had delayed development and shortened lifespans (Vanhooren et al. 2008). Flies showed similar results where Hsp70 overexpression proved immediately helpful for surviving stress but long term, was detrimental to growth, development, and lifespan (Krebs and Feder 1997). Hsp70 is characteristically overexpressed across many tumor types and has been a facet of cancer research. High levels of Hsp70 can inhibit apoptosis of proliferating cancer cells and generally correlates with poor prognoses (Murphy 2013). Developing tools to fine-tune the overexpression of Hsp70 has been of therapeutic interest for a variety of protein aggregation disease including Alzheimer's. Undoubtedly, the balance of Hsp70 expression has far-reaching implications for the health of an organism. In Chapter 3, I will discuss new evidence for Hsp70 regulation by a microRNA, miR-85, in *C. elegans.*

1.2 Heat Shock Recovery and Regulation of Hsp70

The dynamics of heat shock induced Hsp expression as well as the subsequent restoration of Hsps back to basal levels has been assessed in a few

different organisms. Remarkably, overall patterns of induction and restoration are relatively similar across organisms, suggesting a conserved regulatory mechanism (Richter, Haslbeck, and Buchner 2010). We can consider the events in *Drosophila* as an example, as they were the first organism utilized to carefully study the temporal regulation of Hsps during and after heat treatment. Within just fifteen minutes of exposure to high temperatures, *Drosophila* cells shift cellular resources entirely to heat shock synthesis (DiDomenico et al.). This is a highly specialized program as most other mRNAs are no longer spliced or translated upon HS (Yost et al.). To allow for the specific and rapid translation of newly made Hsps, pre-existing messages are cleared from the polysome, freeing up the translation machinery to maximize production of heat shock proteins (DiDomenico et al.). But Hsp70 has also been reported to circumvent bulk 5' cap dependent translation through an internal ribosome entry site (IRES), which recruits the ribosome to an internal region of the mRNA (Silver and Noble 2012). Once translated, unlike most other proteins, HSPs are thermodynamically stable at high temperatures, allowing for their chaperoning activity almost immediately after synthesis (Silver and Noble 2012). The abundance of HSPs produced upon stress directly correlates with the extent of heat treatment but appears to plateau after 3 hours of exposure. Once the temperature returns to tolerable levels, the selective translation of *Hsps* ceases and normal patterns of transcription and translation are restored within a few hours of recovery (Petersen and Lindquist 1988). Importantly, this restoration appears to be regulated through a different mechanism than what drives selective transcription and translation upon first

encountering stress. However, the precise mechanism of regulation and sequence specify is unknown (DiDomenico et al., Bönisch et al.).

A return to conventional modes of protein synthesis after stress hinges on repression of Hsp70. After stress, Hsp70 is the first HSP to decline while other HSPs are sustained and slowly return to low levels, sometimes hours after Hsp70, and often asynchronously (Yost et al.). This observation begged the question as to how Hsp70 was regulated during recovery after stress. One possible mode of regulation after heat shock could be that *hsp70* messages remain translationally inactive and stored in the cell. A second possibility is that *hsp70* messages are targeted for degradation. When researchers investigated these possibilities in *Drosophila* cells, they found *hsp70* mRNA levels rapidly declined during recovery, which suggested targeted degradation (DiDomenico, Bugaisky, and Lindquist 1982). Furthermore, this observation opens the possibility that Hsp70 expression levels could provide regulatory feedback to the larger network of HSPs, perhaps directing their subsequent repression. While it remains unclear how Hsp70 might coordinate HSP expression after heat stress, I will provide possible strategies to address this hypothesis in my conclusion.

The *hsp70* mRNA of *Drosophila* is very unstable under normal growth temperatures. It's half-life is estimated to be around 10 minutes in flies and 50 minutes in human cells (N G Theodorakis and Morimoto 1987). Upon HS, transcripts are stabilized, which contributes to the rapid induction of Hsp70 protein synthesis. It's hypothesized that the same targeted degradation that results in high turnover of *hsp70* in physiologic conditions is responsible for

degrading transcripts during recovery after heat shock. Researchers set out to identify a specific sequence in *hsp70* that directed its rapid degradation. Though mutations to various regions of the coding sequence did not alter stability, mutations made in the 3' untranslated region (3'UTR) of *hsp70* did confer enhanced stability, suggesting sequence specific features that regulated turnover existed within this region (Petersen and Lindquist 1989; Simcox et al. 1985). Reporters carrying the 3'UTR of *hsp70* were rapidly deadenylated and degraded after HS treatment, likely targeted by the CCR4-NOT complex (Bönisch et al. 2007). The rapid deadenylation appeared to be inhibited by HS which contributed to the stabilization of *hsp70* messages. However, further analysis of the 3'UTR of *hsp70* in *Drosophila* failed to identify the specific region that conferred regulation. Deletion of the three AU-rich elements (ARE) within the 3'UTR, which commonly correspond to destabilization of transcripts, did not alter stability (Bönisch et al. 2007). The link between *hsp70* stability and a specific sequence remained unsolved. Notably, the *hsp-70* 3'UTR sequence in *C. elegans* is about 75% identical to *Drosophila hsp70.* It is relatively short, only about 100nts long, and is very AU-rich. This presents a possible conserved mechanism of regulation shared between *Drosophila* and *C. elegans.* Chapter 3 will discuss *hsp-70* regulation in *C. elegans* and explore the role of the microRNA pathway in this process.

1.3 The microRNA Pathway

The microRNA (miRNA) pathway is a mechanism of post-transcriptional regulation in which small, ~22nt non-coding RNAs target and repress mRNA expression. Through imperfect base-pairing, miRNAs guide Argonaute (AGO) proteins to their targets. AGO, and in complex with GW182 proteins, can recruit deadenylases and destabilize target transcripts (Bartel 2018). Typically, miRNAs associate with the 3'UTR region of target mRNAs (Figure 1.3). Given our understanding of Hsp70 regulation in flies, miRNAs remain an intriguing possible mechanism of hsp70 regulation during HS recovery.

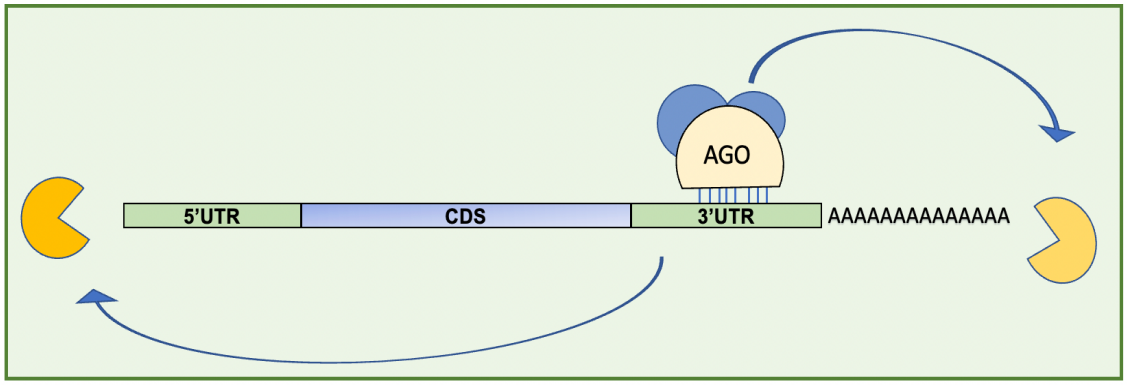


Figure 1.3: MicroRNA Targeting. Argonaute (AGO) proteins are guided to their targets by miRNAs. miRNAs direct targeting through imperfect base pairing with the 3'UTR of target transcripts. AGO and associated proteins primarily promote RNA degradation.

The biogenesis of a miRNA begins with the transcription of a capped and poly-adenylated primary miRNA (pri-miRNA) sequence by RNA Polymerase II (Ha and Kim 2014). Drosha and DGCR8 (Pasha), termed Microprocessor, then cleave the 5' and 3' pri-miRNA regions leaving a ~65nt pre-mRNA that is folded into a stem-loop structure or hairpin (Ha and Kim 2014). The pre-miRNA hairpin is exported to the cytoplasm where it is further processed by Dicer to form a

mature miRNA duplex. The RNA complex is unwound, and one strand is loaded into Argonaute (AGO) while the other strand, termed passenger, is usually degraded. The loaded Argonaute is called the miRNA silencing complex (miRISC) and is able bind to and repress and its targets. In *C. elegans,* ALG-1 and ALG-2 are considered the primary AGO proteins to associate with miRNAs but ALG-5 has recently been reported to function exclusively in the miRNA pathway in the germline (Brown et al. 2017; Grishok et al. 2001). ALG-1 is thought to serve as the predominant Argonaute in *C. elegans* as deletion of *alg-1* can slow development and result in sick animals, whereas *alg-2* mutants do not have overt phenotypes under controlled laboratory conditions (Grishok et al. 2001; Vasquez-Rifo et al. 2012). I will explore the role the ALG-1 during and after HS in Chapter 3, primarily through phenotypic analyses of an *alg-1* loss of function mutant and through targeted ALG-1 degradation.

The discovery of miRNAs stems back to the early 1990s. In *C. elegans*, genetic screens revealed a novel regulator of development, lin-4, which unlike other genes, did not give rise to a protein product. Instead, lin-4 appeared to be a small RNA that inhibited the production of LIN-14 (Lee, Feinbaum, and Ambros 1993; Wightman, Ha, and Ruvkun 1993). Subsequent studies revealed lin-4 used imperfect base-pairing within the 3'UTR sequence of *lin-14* to mediate its repression (Olsen and Ambros 1999). It wasn't until the discovery of let-7, a miRNA also integral to *C. elegans* development, that the miRNA field flourished (Reinhart 2000). Not only was let-7 found in nematodes but it was highly conserved in metazoans including humans (Pasquinelli et al. 2000).

Today, over 400 miRNAs have been identified in *C. elegans* (Kozomara, Birgaoanu, and Griffiths-Jones 2019). However, most miRNAs do not have known targets or functions as the vast majority of miRNA mutants lack observable phenotypes (Miska et al. 2007). One possible explanation for the lack of phenotypes in single miRNA mutants could be attributed to functional redundancy as each mRNA can be targeted and regulated by multiple miRNAs, making this system of regulation quite robust to deletion of a single miRNA. mRNAs can be targeted by multiple miRNAs and every miRNA targets multiple mRNAs, making for a complex web of regulation. The specificity of miRNA targeting largely depends on the seed sequence. The seed sequence is generally found between nucleotides 2-8 at the 5' end of a miRNA. When miRNAs share similar seed sequences, they are considered sisters and part of the same family (Bartel 2018). It's therefore predicted that most miRNA families regulate a similar pool of mRNAs. One example of this redundancy is miR-35, where deletion of individual family members does not result in overt phenotypes but deletion of the entire cluster is lethal (McJunkin and Ambros 2014). In Chapter 2, I will discuss the miR-239 family and its role in the heat shock response.

1.4 Stress Responsive miRNAs and Non-coding RNAs

Another important consideration underlying studies of miRNA mutants is that most phenotypic analyses of *C. elegans* miRNAs have been performed in

carefully controlled laboratory conditions. However, seemingly dispensable miRNAs have recently been identified to respond to stress (Victor Ambros and Ruvkun 2018). MiR-34, for example, is induced upon irradiation and was subsequently identified to regulate DNA damage responses (Kato et al. 2009). In response to heat stress, miR-71 and miR-239 have been reported to increase in expression (Schreiner et al. 2019). But, deletion of miR-71 resulted in decreased heat shock survival whereas deletion of miR-239 resulted in an increased survival (Nehammer et al. 2015; De Lencastre et al. 2010). How misregulation of specific miRNA targets relates to these phenotypes remains poorly understood and future work aimed at elucidating specific miRNA targets during stress are warranted. Recently, sequencing-based experiments during heat shock have uncovered cryptic miRNAs that are virtually undetected in controlled laboratory temperatures such as miR-4936 (Schreiner et al. 2019). Functional analysis of these conditionally expressed miRNAs may reveal previously unappreciated functions. However, inconsistencies of experimental stress conditions and subtle variances in developmental timing can result in dramatic differences in miRNA expression profiles. While sequencing represents a powerful tool for whole genome expression analysis, in depth genetic analysis of specific miRNAs remains the gold standard for identifying functions and mRNA targets.

In addition to addressing specific roles of miRNAs in response to stress, it remains unclear as to how the miRNA pathway as a whole functions during stress. Researchers have tracked the expression of *alg-1* during HS using qRT-PCR and observed a 40% decrease, which could explain broad changes in

miRNA abundance during stress (Nehammer et al. 2015). However, our lab has not identified dramatic differences in ALG-1 protein in HS. Regardless, reduced *alg-1* levels during heat stress are unlikely to explain why some miRNAs are upregulated and it is likely that other, miRNA-specific regulatory mechanisms exist. In Chapter 2, I will explore the regulation of miR-239 during HS and provide evidence to support that it is upregulated by HSF-1. In Chapter 3, I will discuss the importance of maintained ALG-1 expression during HS and after HS, while the organism recovers.

While stress can induce or suppress specific miRNA expression, most miRNAs remain unchanged. However, miRNAs that do not change in expression should not be overlooked for playing important regulatory functions. It's possible, for example, that expression of a specific miRNA is unaffected by stress, but the targeting of that miRNA could be altered, perhaps through changes in tissue specific expression or availability of binding sites. By leveraging the predictive power of TargetScan, software that allows for the identification of potential miRNA targets, we can identify trends among predicted targets of a miRNA of interest. Using this approach, we were eager to identify possible miRNAs responsible for regulating heat shock response transcripts and identified miR-85 target sites within the 3'UTRs of multiple heat shock genes (Jan et al. 2011). Upon further evaluation, we determined that miR-85 expression is unchanged before, during, and after HS yet when deleted, reduces HS viability. We further define miR-85 function in HS viability as necessary for reducing Hsp-70 expression after heat shock. In Chapter 3, I will elaborate on this miRNA's

integral role in regulating *hsp-70* in *C. elegans* as well as other stress response genes.

Importantly, miRNAs are not the only form of non-coding RNAs that appear to respond to heat stress. In fact, nearly every level of gene expression contributes to the regulation of the HSR (Richter, Haslbeck, and Buchner 2010). However, there is still much to learn in the realm of non-coding RNA regulation of the HSR. This gap in our understanding could be explained by the fact that much of the non-coding genome was considered junk until the last couple decades. Now, with the advent of total RNA sequencing, it's clear that the majority of the human genome is transcribed and this has sparked far-reaching efforts to assign to functions to the non-coding genome (Hangauer, Vaughn, and McManus 2013).

Non-coding RNAs (ncRNAs) have been appointed to various classes depending on characteristics and roles, as associated phenotypes and molecular functions have been uncovered. The first class of ncRNA discovered was the transfer RNAs (tRNAs), which were identified in the late 1950's. This was followed by ribosomal RNAs (rRNAs), small nucleolar RNAs (snoRNAs) and small nuclear RNAs (snRNAs) which were discovered in the following decade and all before the advent of next-generation sequencing (Fu 2014). In the 1990s, miRNAs were discovered in *C. elegans* and subsequently identified to be conserved in humans (Pasquinelli et al. 2000). Through more recent RNA sequencing experiments, additional classes of ncRNAs have been identified. For example, long non-coding RNAs (lncRNAs) and long intergenic non-coding

RNAs (lincRNAs), which are usually 200nt or longer, were classified in the 2000s. LincRNAs are similar to lncRNAs as they do not produce a protein product, but lincRNAs, by definition, do not overlap PCG sequences. Like humans, *C. elegans* transcribe both lincRNAs and lncRNAs (Nam and Bartel 2012). Transcripts derived from pseudogenes, or non-coding genes that have homology to PCGs but are not translated, also make up a large portion of the ncRNA transcriptome and are classified under the broad category of lncRNAs. Although, some transcribed pseudogenes are recognized by the nonsense mediated decay pathway in *C. elegans* and are quickly degraded, making it challenging to capture these sequences by RNA sequencing methods (Milligan and Lipovich 2015). Additionally, repetitive-sequence-derived RNAs such as those transcribed from transposons are also included as part of the ncRNA transcriptome. One example is the ncRNA produced from the *C. elegans* rolling circle transposable element, *Helitron1_CE.* While the list of ncRNA classifications continues to expand as new functions are defined, I will primarily focus on examples from each class described here and our efforts to characterize specific thermoregulated ncRNAs.

After observing global changes in the *C. elegans* non-coding transcriptome upon HS, we began to wonder if any of these ncRNAs were subject to regulation by HSF-1. HSF-1 is known to direct the upregulation of HSPs in response to stress, leading to rapid accumulation of genes such as *hsp-16.2* and *hsp-70* within just 15 minutes of exposure to high temperatures. Notably, our study expands the role of HSF-1, as we identified ncRNAs that also

accumulate rapidly upon HS in an HSF-1 dependent manner. In Chapter 2, I will discuss changes in the non-coding RNA transcriptome during HS and role of HSF-1 in driving some of these changes.

1.5 microRNA Function in Fertility

In addition to the role of miR-85 in regulating *hsp-70* during recovery after HS, we have identified this miRNA as an important regulator of *C. elegans'* fertility. Chapter 4 will detail what we have learned thus far. The miRNA pathway has been central to understanding *C. elegans* germline development, gametogenesis, and fertility. Loss of function strains for factors involved in miRNA biogenesis and targeting have displayed mild to severe developmental and fertility defects. This includes reduced brood sizes in *alg-1* mutants and sterility in Microprocessor mutants (Vasquez-Rifo et al. 2012). Specific miRNAs have also been identified to impact brood size such as miR-35, miR-51, miR-72, and miR-61 (Minogue et al. 2018). However, direct targets of these miRNAs and downstream regulation of cellular processes remain incompletely understood. In addition, developmental timing is influenced by miRNAs such as lin-4 and let-7. These miRNAs influence the expression of heterochronic genes which act as developmental switches, relying on the integration of intrinsic and extrinsic signals to control post-embryonic development from larval stage 1 (L1) to larval stage 4 (L4) and continuing into adulthood (Rougvie 2005). Lin-4 negatively regulates both *lin-14*, to promote the L1 to L2 transition, and *lin-28,* to promote

the L2-L3 transition. Let-7 represses *lin-41,* regulating the L3-L4 transition (Lee, Feinbaum, and Ambros 1993; Wightman, Ha, and Ruvkun 1993).

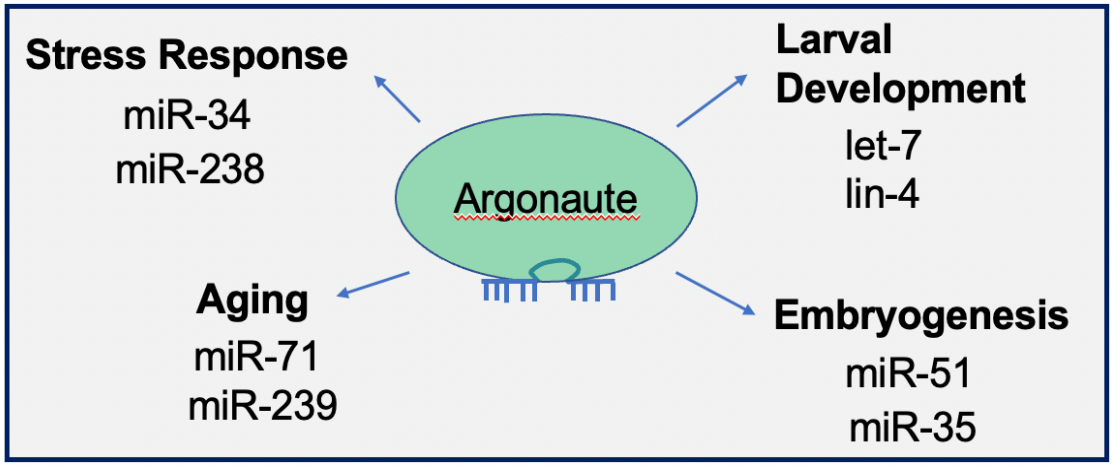


Figure 1.4: Diverse roles of miRNAs. Examples of miRNAs with known functions in diverse contexts such as stress, aging, development, and embryogenesis.

C. elegans exist as hermaphrodites (XX) and males (XO), with XO animals arising infrequently (0.1%) by spontaneous non-disjunction in the hermaphrodite germline. XX animals are capable of self-fertilization which allows for homozygous worms to generate genetically identical progeny. XO cannot self-fertilize and must mate with a hermaphrodite to reproduce. Male larvae have a similar body plan to XX animals until L2 where the posterior half diverges as the sexual organs develop (Byerly, Cassada, and Russell 1976). Hermaphrodites perform spermatogenesis during L4 before transitioning to exclusively producing oocytes in young adulthood. Ovulation occurs when proximal oocytes enter the spermathecae where they are fertilized and complete meiosis (Ward and Carrel 1979). This process is tightly regulated in a predictable

temporal manner. In Chapter 4, I will discuss our preliminary work in understanding how mir-85 regulates fertility and explore potential interactions with *C. elegans* germline morphology.

MiRNAs have also been recognized to regulate features of the RNA interference (RNAi) pathway in *C. elegans.* RNAi has broad implications for regulation of development, fertility, and maintenance of genome stability (Hoogstrate et al. 2014). In general, the RNAi pathway utilizes double stranded RNA (dsRNA) to target and silence gene expression through partial or perfect base-pairing. There are three main classes of small interfering RNAs that can serve as sources of dsRNA: piwi-interacting RNAs (piRNAs), endogenous small interfering RNAs (siRNAs), and microRNAs. All three types act through Argonaute effector proteins to recognize target transcripts, however each class varies greatly in the factors required for biogenesis (Hoogstrate et al. 2014). Despite this, there have been reports of cross talk between these pathways, for example between the miR-35-41 family and siRNAs where deletion of miR-35-41 results in reduced silencing by endogenous siRNAs but an enhanced sensitivity to exogenously derived (exo-siRNAs) (Massirer et al. 2012). In addition, deletion or mutation of RNAi factors such as DCR-1 (Dicer) or the RNA Dependent RNA Polymerase (RdRP) EGO-1 results in phenotypes that complement those observed in *miR*-85*(n4117),* including compromised fertility (Grishok 2013; Ketting et al. 2001). Furthermore, some factors utilized in the RNAi pathway are shared with the miRNA pathway, linking these processes and suggesting that these pathways may compete for resources (Ketting et al. 2001). While

attempting to understand how miR-85 regulates specific mRNA targets to affect fertility, we discovered that fertility defects could be partially or completely rescued through the introduction of non-specific RNAi. In Chapter 4, I will discuss efforts to address possible cross talk between miR-85 and the RNAi pathway in *C. elegans* and how this relates to miR-85 regulation of germline mortality and fertility.

Chapter 2

Identifying Heat Shock Responsive Non-Coding RNAs and their Functions

2.1 Introduction

When an organism encounters elevated temperatures, the heat shock response (HSR) is activated to protect cells from the cytotoxic consequences of protein misfolding and aggregation. The response is well-conserved and is largely determined by the transcriptional program driven by HSF-1. While the induction of protein coding genes (PCGs) has been well characterized, little is understood about the differential expression in the non-coding RNA transcriptome upon heat stress. PCGs only make up ~1% of the genome, yet characterization of non-coding regions has only been of recent interest. By studying how non-coding RNAs respond to heat stress, we can begin to assign functional roles to these seemingly overlooked components of the genome and narrow our focus on ncRNAs that change in expression upon stress. Global small-RNA and total-RNA sequencing methods enable the rapid identification of various classes of non-coding RNAs, including miRNAs, long intergenic-noncoding RNAs (lincRNAs), long-noncoding RNAs (lncRNAs) as well as pseudogene- and repeat-derived RNAs that change in response to stress.

Building on our observations from sequencing experiments, I will highlight my contributions aimed at the characterization of specific non-coding RNAs that are upregulated in response to heat stress. Furthermore, I will discuss how some of these changes rely on HSF-1. Overall, this work highlights the importance of ncRNAs in the HSR and expands our understanding of HSF-1 directed transcription to include some ncRNAs.

One non-coding RNA of particular interest from these studies was miR-239. The miR-239 family includes miR-238, miR-239a, and miR-239b, all of which share a similar seed sequence. It would therefore be predicted that each miRNA in the family regulates a similar pool of mRNA targets. Upon HS, the miR-239 family members were among the most highly upregulated miRNAs. Furthermore, it appeared that miR-239a/b were under direct regulation of HSF-1. To carefully study the role of miR-239 and the unique contributions of each family member, I will discuss my strategies to generate single miRNA mutants. This is significant as previously established phenotypes of miR-239 showing resistance to stress and an extended lifespan relied on a mutant (*miR-239(nDf62))* that not only disrupts both miR-239a and miR-239b but also deletes a ncRNA and snoRNA, making it impossible to distinguish how each specific non-coding RNA contributes to the HSR (De Lencastre et al. 2010). I will discuss the strains I generated which delete individual miRNAs (miR-239a and miR-239b) and the advantages of these single mutant strains. Furthermore, I will report on the phenotypic analysis I performed with each new miRNA mutant and

discuss what we have learned about the miR-239 family based on these findings.

2.2 Heat Shock Induced Non-coding RNAs

To uncover non-coding RNAs with potential functions in the heat stress response, we sought to identify candidates that appeared to be thermoregulated. If a non-coding RNA changes expression in response to stress, it is plausible that it serves a role in promoting survival. In the case of miRNAs, this is presumably accomplished by toggling the expression of target transcripts. Global sequencing approaches during stress have been used before and helped curate an initial list of candidate miRNAs that could be studied for essential heat shock related functions (Nehammer et al. 2015). Employing a similar tactic, we performed small-RNA sequencing on synchronized L4 animals after they were subjected to a 6-hour heat shock at 35°C and compared changes in miRNA expression greater than 2-fold relative to non-heat shocked, paired samples. We identified 8 up-regulated and 15 down-regulated miRNAs out of the 205 detected. This included the miR-239 family which was among the most up-regulated miRNAs (Figure 2.1A, B). We also identified changes in other classes of non-coding RNAs, including 9 up-regulated and 1-down regulated lincRNAs, 21 up-regulated and 23-down regulated repetitive RNAs (repetitive element-derived RNAs), and 94-upregualted and 23-downregulated pseudogenes (Figure 2.1A).

A

Number of RNA species with ≥ 2-fold change in expression after heat shock

	UP	DOWN	ALL
miRNA	8	15	437
piRNA	15	63	15363
lincRNA	9	1	176
unclassified ncRNA	71	1	7859
pseudogene	94	23	1922
repeat	21	23	181
mRNA	1059	801	20203

B

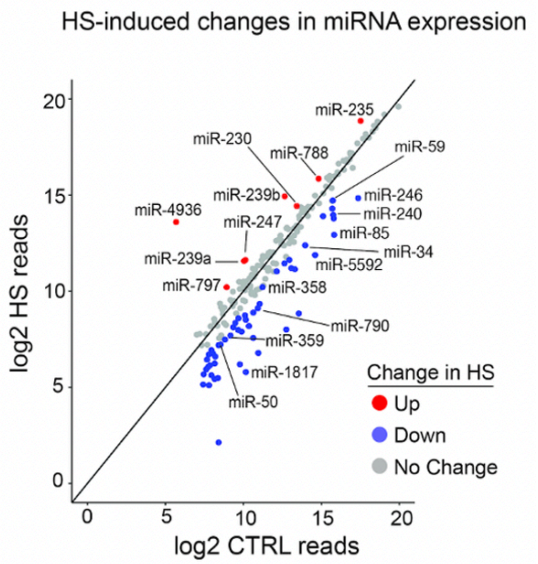


Figure 2.1: Heat shock induced non-coding and coding RNAs. (A) Table of significantly up- or down-regulated RNAs after heat shock and number of all genes detected by class. (B) Plot of significantly up- and down-regulated miRNAs after 6hr of heat shock.

To validate the changes observed, I collected RNA throughout a time course of HS treatments. Based on previous studies in *Drosophila*, we predicted that heat shock response genes, such as Hsp70, would be induced within 15 minutes of heat shock (Lindquist). Using *hsp-70* and *hsp-16.2* as controls, I assessed changes in mRNA expression at 15, 30, and 180 minutes of stress in WT *C. elegans* (Figure 2.2A, B). By RT-qPCR I found that just 15 minutes of exposure to 35°C induced *hsp-70* and *hsp-16.2* upregulation and the expression continued to rise throughout the 3 hr HS treatment. Using a similar method, I assessed the expression of select non-coding RNAs during this HS time course, beginning with the most upregulated ncRNAs from our 6 hr HS sequencing data. One hypothesis we began to consider was whether any of the upregulated non-

coding RNAs could be part of the HSR driven by HSF-1. If they were, we predicted they would follow a similar pattern of induction observed for *hsp-70* and *hsp-16.2,* which are known to be driven by HSF-1 upon HS (Figure 2.2A and B) (J. Li et al. 2016). First, I assessed the expression of *linc-7*, one of the most upregulated lincRNAs detected by RNA sequencing. Within just 15 minutes of HS, *linc-7* showed a nearly 2-fold upregulation, followed by an increase over time of HS treatment (Figure 2.2C). This expression profile mimicked that of *hsp-70.* Similarly, we assessed changes in upregulated repeat-derived ncRNAs. By re-mapping the reads from our sequencing to a list of consensus *C. elegans* repeat elements, we were able to identify previously unstudied repetitive RNA sequences induced upon HS. As validation, we re-mapped other HS data sets and confirmed the expression profiles of these repetitive RNAs. We found widespread misregulation of repetitive element derived RNAs. This finding corroborated previous work which also identified upregulation of repeat RNAs in response to HS in human cells (Jolly et al. 2003). While surveillance mechanisms usually help maintain genome integrity by repressing transposons, it appears that these silencing pathways are compromised in stressful conditions. One repetitive ncRNA of particular interest was *Helitron1_CE,* an RNA derived from a rolling circle DNA transposon. *Helitron1_CE* was highly upregulated in HS but nearly undetectable under controlled conditions (Figure 2.2D). Through semi-quantitative and quantitative RT-PCR, I confirmed that *Helitron1_CE* was rapidly upregulated within just 15 minutes of HS, mimicking the expression profile of *hsp-16.2* (Figure 2.2E).

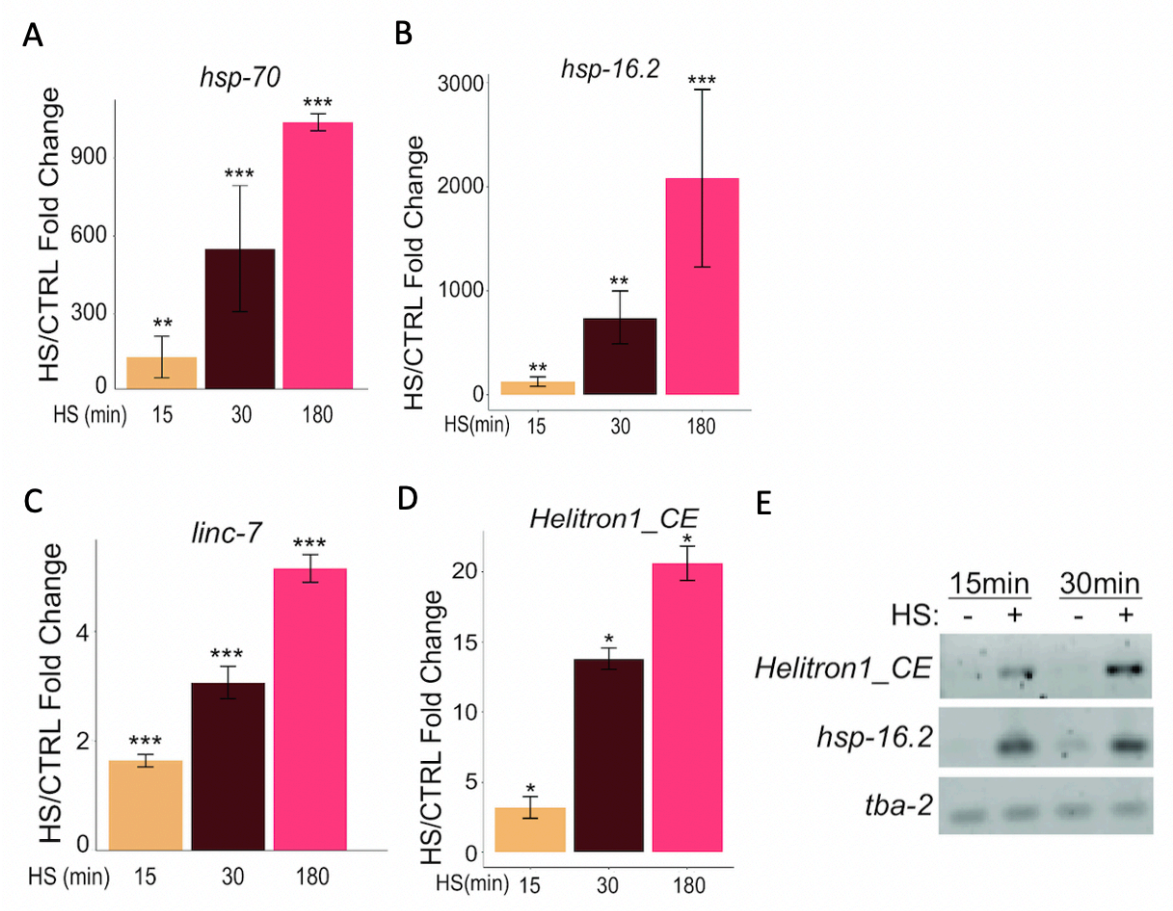


Figure 2.2: Time Course Analysis of RNA Expression During Heat Shock. (A-D) RT-qPCR analysis of *hsp-70*, *hsp-16.2, linc-7,* and *Helitron1_CE* RNA levels after 15, 30 and 180 min of HS versus CTRL conditions. Mean fold changes and SEM from three independent replicates are shown. **P* <0.05, ***P* <0.01, ****P* <0.001 (*t*-test, two-sided). (E) Semi-quantitative RT-PCR detection of the indicated RNAs in CTRL and after 15 or 30 min of HS.

Given the rapid induction of some non-coding RNAs, we sought to assess if HSF-1 could be driving their expression upon HS. While it is known that hundreds of PCGs are upregulated in response to HS, experiments in mammalian cells and yeast concluded that only a portion of these were direct targets of HSF-1 (Mahat et al. 2016; Solís et al. 2016). Looking at changes in gene expression during heat shock was not sufficient evidence to suggest these

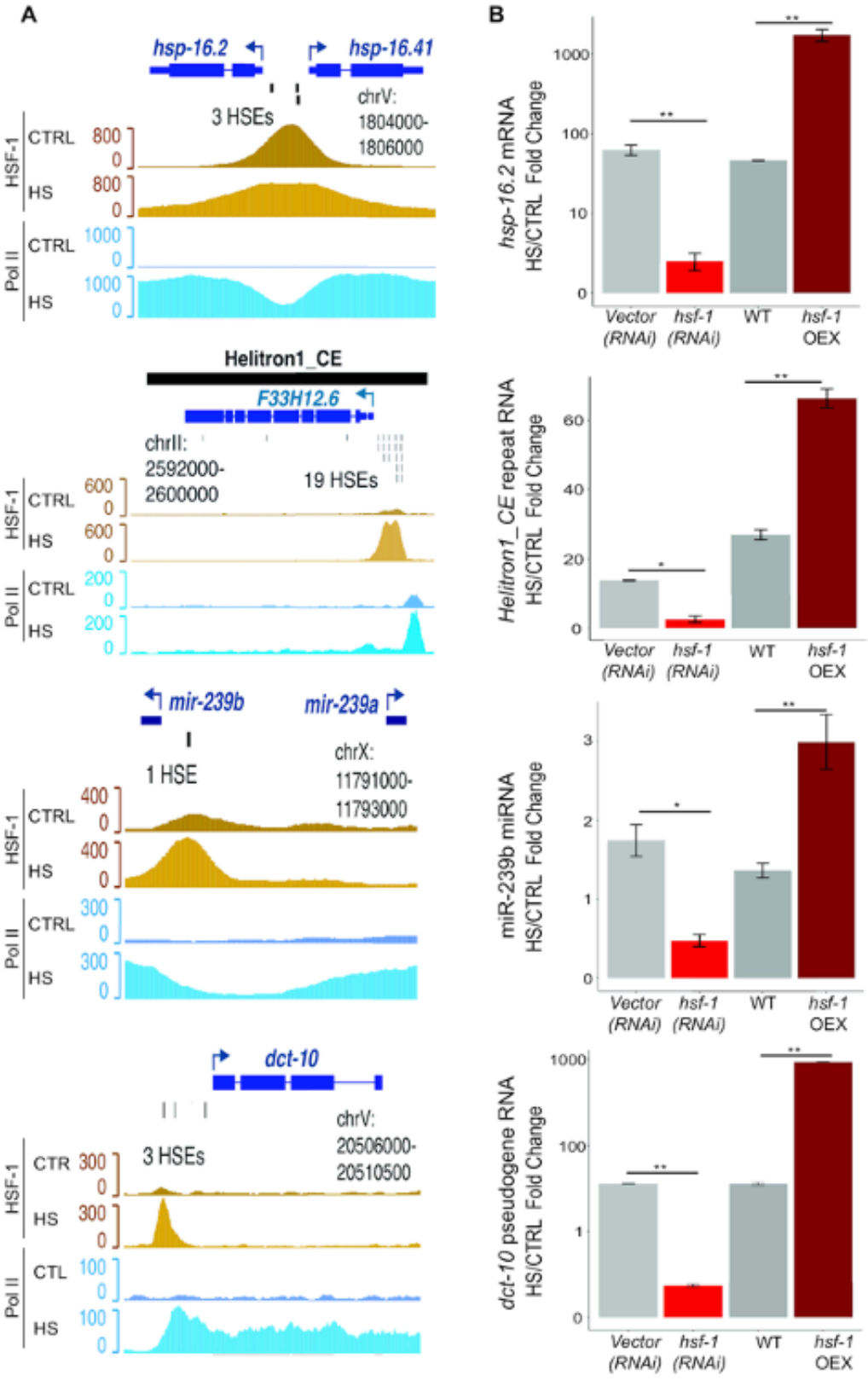
ncRNAs were regulated by HSF-1. To more carefully investigate our hypothesis, we remapped *C. elegans* HSF-1 and Pol II Chromatin Immunoprecipitation Sequencing (ChIP) data from Li and colleagues to include non-coding and repetitive RNA loci. (J. Li et al. 2016) Similar to the promoter regions of canonical HS-induced genes like hsp-16.2*,* we found HSF-1 ChIP peaks in the promoter region of Helitron1_CE, dct-10, an up-regulated pseudogene, and miR-239, each representing a class of non-coding RNA which accumulated in response to HS. Additionally, each ncRNA contained one or more HSEs within the promoter regions, supporting the potential HSF-1 interaction at those loci (Figure 2.3A).

I next asked if the induction of these candidate non-coding RNAs upon HS was dependent on HSF-1. To assess this, I used complementary approaches to knock down *hsf-1* with RNAi and overexpress *hsf-1* using a transgenic strain (*EQ87)*. I then asked how each target of interest was affected upon HS in these backgrounds through RT-qPCR. Compared to *vector*(*RNAi)*, *hsf-1(RNAi)* reduced the induction of *hsp-16.2, Helitron1_CE, miR-239b,* and *dct-10* in WT animals*.* Conversely, overexpression of *hsf-1* promoted further accumulation of each target upon HS (Figure 2.3B). Overall, these findings suggest an expanded role for HSF-1 in driving the transcription of specific ncRNA genes as part of the *C. elegans* HSR.

In summary, our comprehensive analysis of the changes in ncRNA expression upon HS found at least a two-fold change in the expression of ~9% PCGs, 5% miRNAs, 6% lincRNAs, 6% pseudogenes, and 24% of repeat families. Furthermore, our study demonstrates the importance of studying lowly

expressed genes in varied contexts as some of the most dramatically upregulated genes in HS are barely detectable in controlled temperatures. One striking example is miR-4936 which is nearly undetectable in permissive temperatures yet over 100-fold upregulated during HS. Furthermore, we found that some ncRNAs paralleled expression patterns of canonical heat shock induced genes and accumulated rapidly in the early phases of heat stress. This finding, in conjunction with their dependence on HSF-1 for upregulation in HS, suggests a functional role for some ncRNAs in the HSR. However, it remains largely unknown as to how these ncRNAs contribute to an organism's ability to survive heat shock.

Figure 2.3: NcRNAs are regulated by HSF-1 during HS. (A) Genome browser screenshots of HSF-1 (yellow) and Pol II (blue) ChIP-seq data from control (CTRL) and HS conditions (data from (Li et al., 2016)) for representative genes (*hsp-16.2* and *hsp-16.41*, mRNA; *Helitron1_CE*, repeat RNA, miR-239a and *miR-239b*, miRNA; *dct-10*, pseudogene). Individual HSEs identified using FIMO (P < 1e-04) are indicated (Grant et al., 2011). (B) Fold change in RNA levels of *hsp16.2*, *Helitron1_CE*, *miR-239b* and *dct-10* after 30 min of HS in animals subjected to empty vector or *hsf-1* RNAi, and WT versus a strain overexpressing HSF-1 (*hsf-1* OEX) determined by qRT-PCR analyses. The mean fold changes and SEM from three independent replicates are graphed. *P < 0.05, **P < 0.01 (t-test, two-sided).


A
hsp-16.2
hsp-16.41
chrV:
1804000-
1806000
3 HSEs
HSF-1
CTRL
HS
Pol II
CTRL
HS
Helitron1_CE
F33H12.6
chrII:
2592000-
2600000
19 HSEs
mir-239b
mir-239a
chrX:
11791000-
11793000
1 HSE
dct-10
chrV:
20506000-
20510500
3 HSEs
CTR
CTL
B
hsp-16.2 mRNA HS/CTRL Fold Change
Helitron1_CE repeat RNA HS/CTRL Fold Change
miR-239b miRNA HS/CTRL Fold Change
dct-10 pseudogene RNA HS/CTRL Fold Change
Vector (RNAi)
hsf-1 (RNAi)
WT
hsf-1 OEX

2.3 Assessing the Role of miR-239 in Heat Shock

As follow up to our findings that miR-239 was induced upon HS in an HSF-1 dependent manner, I speculated that it may contribute to the regulation of the HSR. Furthermore, previous studies also reported an upregulation of miR-239 in HS and found that deletion of this miRNA family improved survival after stress (De Lencastre et al. 2010). In addition, loss of miR-239 improved survival in response to oxidative stress extended lifespan compared to WT. However, the mutant (mir-239a/b(*nDf62))* used for these analyses spans both miR-239a and miR-239b as well as an unclassified ncRNA and a snoRNA. It's therefore impossible to assess the relative contributions of each feature in HS or other phenotypes of interest. Both miR-239a and miR-239b are upregulated in response to heat but miR-239b is consistently more abundant than miR-239a. Of note, I was unfortunately unable to detect either by Northern blot and relied on TaqMan probes for expression analysis by RT-qPCR. The HSE and HSF-1 ChIP peak resides between miR-239a and miR-239b which suggests that HSF-1 likely contributes to the upregulation of both miRNAs as they are transcribed in opposite directions from a shared promoter. The ncRNA and snoRNA are also upregulated in HS but it's not clear if the upregulation is a byproduct of miR-239 upregulation or independently targeted for upregulation in HS.

To begin to dissect the role of each miRNA, I generated four new strains: a *miR-293a(ap439)* (*PQ636)*, two alleles of *miR-239b(ap432 and ap433)* (PQ592 and *PQ593),* and a double mutant (*PQ600*) that disrupts *miR-239a(ap439)* and

miR-239b(ap432) but does not alter the sequence of the ncRNA or snoRNA. I used the CRISPR-Cas9 purified protein, a single guide RNA to target each miRNA, avoiding the seed regions which share a high degree of sequence similarity, and a *dpy-10* co-Crispr marker for screening. To make the double mutant, I injected the *miR-239b(ap432) (PQ592)* mutant with the single guide for miR-239a. In parallel, we attempted to cross the single mutants together to make a double mutant but because the mutations are only about 100bp apart, generating a homozygous double mutant required an immense amount of screening and we failed to obtain a double mutant through this method. After backcrossing each strain to WT (N2) three times, I performed HS viability assays using our standard protocol of 4 hrs at 35°C on L4 animals. However, my results varied, and I did not detect reproducible changes in HS viability in any of the strains compared to WT. Moreover, when I tried to reproduce previously reported phenotypes using the *miR-239a/b*(*nDf62)* strain, I did not see changes in viability compared to WT. Unlike previously published viability of 80-90%, I observed 40-50% viability of the *miR-239a/b*(*nDf62)* strain.

While the HS protocol utilized in our lab has been optimized to regularly result in WT viability between 45-60%, I wondered if different HS protocols or developmental stages of the animals could explain my failure to reproduce previously reported HS phenotypes. I first adjusted the HS treatment to mimic the 12-hour 35°C treatment used in de Lencastre et al. However, using our incubator, this killed every single animal, including WT. Next, I tried 2 hr, 6 hr and 8 hr HS treatments at 35°C. I counted animals immediately after the HS, as done in de

Lencastre and after 24 hrs of recovery at 20°C but these all had variable results with 6 and 8 hours resulting in death of every strain and 2 hours showing no reproducible difference between WT and the *miR-239* strains (De Lencastre et al. 2010). I also tested the HS protocol used by Nehammer and colleagues which counts viability over 25 hrs at 32°C. Again, this protocol produced variable results and ultimately killed all the animals before the 25 hr treatment was completed, inconsistent with the reported ~20% viability observed in WT after 25 hrs (Nehammer et al. 2015). Next, I wondered if the stage of development in which I performed the HS was contributing to the lack of HS phenotypes. Previous reports used Day 2 adults whereas our standard HS protocol uses L4 animals. However, neither L4 and Day 2 adult *miR-239a/b(ap435)* mutants showed significant changes from WT stage-matched animals after 4 hrs of HS. It should also be noted that the single mutants and double mutant did not have observable developmental delays or other overt phenotypes under normal growth conditions.

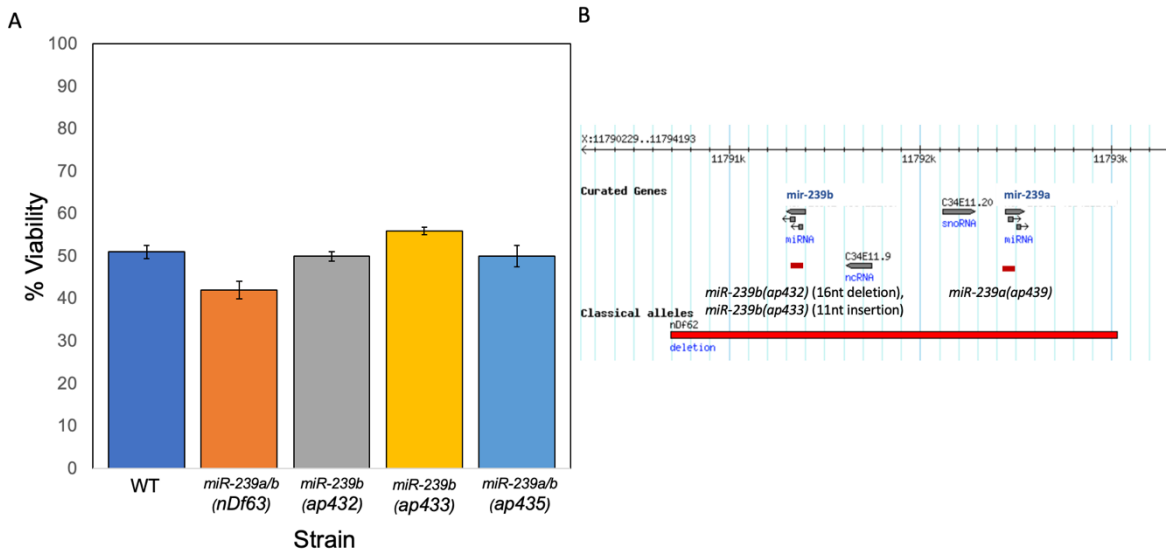


Figure 2.4: Heat Shock Viability of miR-239 Mutants. (A) Mutants did not show significant differences in viability after 4 hr HS compared to WT animals. (B) Depictions of strains.

2.4 Role of miR-239 in Lifespan and Fertility

Convinced that neither miR-239a or miR-239b contributed to HS phenotypes, I assessed potential contributions to alternative phenotypes, such as lifespan. Deletion of miR-239a/b has been reported to extend lifespan and transgenic overexpression of miR-239 can shorten lifespan at 20°C compared to WT (De Lencastre et al. 2010). In addition, miR-239 expression patterns have been evaluated as a predicter of lifespan in *C. elegans* (Pincus, Smith-Vikos, and Slack 2011*).* Through genetic studies, it's thought that mir-239 acts within the Insulin/IGF-1 Signaling (IIS) Pathway which has been long studied for its relationship with aging and stress phenotypes (Altintas, Park, and Lee 2016). More specifically, researchers found that miR-239 acts downstream of *daf-2* and

upstream of *daf-16* within the IIS to promote anti-longevity cellular programs (De Lencastre et al. 2010). Fortunately, we were able to reproduce the extended lifespan of *miR-239a/b(nDf62)* although we found the lifespan extension to be more modest. As we generated each new allele, we began to assess the lifespan of the new single miRNA mutants. Interestingly, at 20°C, we observed a slightly shortened lifespan in both *miR-239b* alleles (*PQ592, PQ593*) compared to WT and *miR-239a/b(nDf62)* (Figure 2.5A). At 25°C, we did not observe a decrease in lifespan, and *miR-239b,* WT, and *miR-239a/b(nDf62)* all behaved in a similar fashion, corroborating findings from Nehammer et al (Figure 2.5B). We have not yet tested the lifespan of *miR-239a(ap439)* (*PQ636)* or the double mutant PQ600. Further dissection of miR-239 family members' contributions to lifespan at 20°C is warranted, despite showing perhaps counterintuitive preliminary findings.

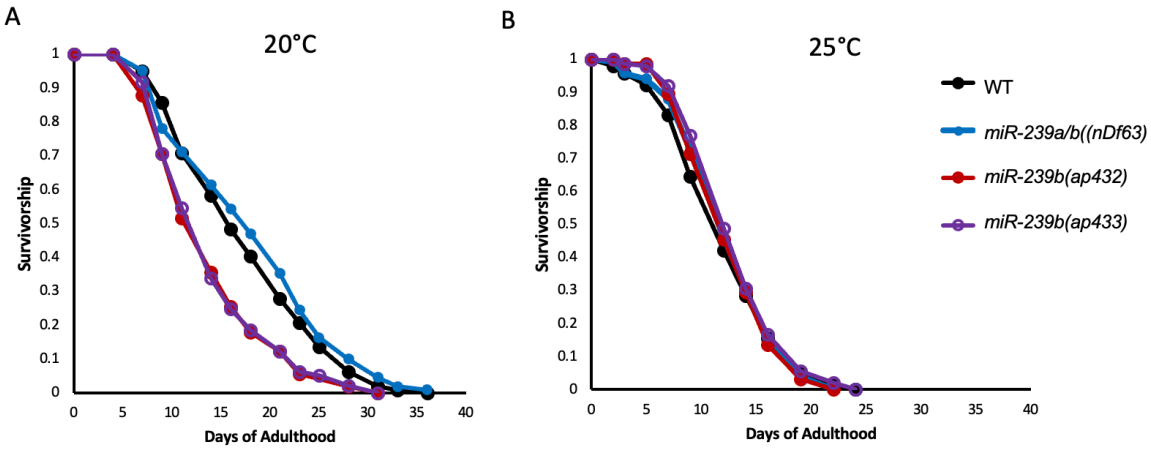


Figure 2.5: Lifespan Assay of miR-239 mutants at 20°C and 25°C. (A) Lifespan of WT, *miR-239a/b(nDf62)*, *miR-239b(ap432)*, *miR-239b(ap433)* at 20°C and (B) 25°C.

Finally, I wondered if miR-239 family members contributed to *C. elegans* fertility. At 20°C, *miR-239a/b(nDf62)* has no reported change in brood size compared to WT but at 25°C, there is a decrease from WT (Nehammer et al. 2015). Our results were consistent with this finding using our single miRNA mutants for *miR-239b(ap432)* (*PQ593*) and *miR-239a(ap439) (PQ636)* as neither presented a reduced brood size. However, unlike previously reported brood size phenotypes for miR-239 at 25°C, we did not observe a reduced brood size in *miR-239b(ap433) (PQ593)* animals. Our findings suggest miR-239 may not have a role in gamete production in mildly stressful temperatures. However, further analysis of miR-239a and the *miR-239a/b* double mutant at 25°C are warranted and may reveal novel insights into the potentially overlapping or unique contributions of miRNA family members. Finally, the ncRNA and snoRNA residing within the *miR-239a/b(nDf62)* deletion could be contributing to regulation of HS, lifespan, and brood size and would be important to study in these contexts. It remains possible that the incongruence of my findings with previously reported phenotypes could be explained by these ncRNAs.

2.5 Discussion

Multiple classes of non-coding RNAs respond to heat shock in *C. elegans*. While the heat shock response has been well characterized in bacteria through humans, the majority of studies focus on changes in PCGs. We found that specific RNAs are thermoregulated in *C. elegans* which opens up new

possibilities for functional roles of ncRNAs in regulating physiological responses. Non-coding RNAs are often considered dispensable as they rarely result in overt phenotypes in controlled laboratory conditions when deleted. However, there is mounting evidence that miRNAs play integral roles in a variety of stress response pathways (Victor Ambros and Ruvkun 2018; Miska et al. 2007; Alvarez-Saavedra and Horvitz 2010). Disruption of miR-71, miR-246, miR-80, and the miR-229, -64, -66 cluster have all been identified to increase sensitivity to heat stress (Nehammer et al. 2015; De Lencastre et al. 2010). Interestingly, disruption of miR-239 had been reported to improve survival during heat stress. Despite this finding being reported in two separate articles, we were not able to reproduce this phenotype in our lab. However, the rapid upregulation of this miRNA family upon HS was reproducible. While the upregulation of miR-239 in response to stress and the improved viability in the loss of function *miR-239a/b(nDf62)* mutant may seem counterintuitive, it could also suggest a more nuanced regulatory role for miR-239 in the HSR.

The lack of heat shock phenotypes observed in the single miR-239a and miR-239b mutants did not support our hypothesis that loss of this miRNA family would promote HS survival. While we did not directly test the role of the ncRNA or snoRNA's contributions to the improved HS viability phenotype reported in *miR-239a/b(nDf62)*, the fact that we did not see a change in viability in the *miR-239a/b(ap435)* double mutant (PQ600) might suggest a role for the ncRNA and snoRNA in HS. Additionally, both the ncRNA and the snoRNA are upregulated upon HS and it remains an open question as to whether their expression is

independent of miR-239 or might also be driven by HSF-1. A second outstanding question regarding miR-239 centers around the identification of direct target mRNAs and how those targets change in response to stress. Cross-linking and Immunoprecipitation (CLIP) experiments assessing ALG-1-associated miRNA:mRNA pairs in HS compared to controlled temperatures could help answer this question and shed light on functional roles for this miRNA (Broughton and Pasquinelli 2013).

miR-239 also has reported phenotypes related to lifespan and brood size. We generated new tools to study the contributions of each family member and performed preliminary work to identify individual contributions these miRNAs. However, our work suggests that miR-239 may not contribute to lifespan as previously described where *miR-239b* mutants actually showed a reduced lifespan compared to WT. However, the fact that *miR-239b* shows a reduced lifespan is intriguing, despite being contradictory to previous reports, it may be worth further investigation. In addition, assessment of brood size in *miR-239a* (PQ636) and *miR-239a/b* (PQ600) at 25°C may be of interest as it's possible these mutants could show sensitivity at slightly stressful temperatures of 25°C. Overall, these strains represent valuable tools for dissecting functional roles of miRNA family members and could be utilized for the evaluation of other phenotypes such as response to other forms stress. In addition, sequencing of single mutants and studying differences in gene expression may elicit divergent regulatory functions of these family members and broaden our understanding of miRNA function.

2.6 Materials and Methods

2.6.1 RNA Sequencing and Analysis

N2 wild-type (WT) worms were grown to L4 stage in a 20°C incubator under standard growth conditions (Wood, 1988). The experimental group was subjected to heat stress by raising the temperature to 35°C for 4 hr. Animals were then collected, snap-frozen and totalRNA was extracted using a standard Trizol RNA extraction protocol. cDNA sequencing libraries from three independent biological replicates were prepared from total RNA from N2 (WT) control or heat shocked worms using the standard protocol from the Illumina Stranded TruSeq RNA library prep kit. Prior to library preparations, ribosomal RNA was removed using RiboZero Gold (Illumina). cDNA libraries were sequenced on an Illumina Genome Analyzer II (100 bp paired-end reads). FASTQ reads were first trimmed using fastq-mcf (https://expressionanalysis.github.io/ea-utils/), which removed flanking Illumina adapter sequences as well as nucleotides with low quality sequencing scores. Reads were then aligned to the *C. elegans* genome WS235 using STAR (Dobin et al. 2013). Aligned reads were sorted using Samtools (H. Li et al. 2020). Reads were counted using FeatureCounts and Ensembl 88 gene annotations (Liao et al. 2014). Differential expression of gene expression was determined using DESeq2 (Love, Huber, and Anders 2014). Pseudogenes, lincRNAs and unclassified ncRNAs are included in the Ensembl 88 gene annotations. After differential expression, these classes of genes were filtered out of the results and analyzed separately. See github.com/wschrein for code and additional example graphs. Coding and non-

coding RNA gene classes are based on their annotations in Wormbase version WS266 (WS266.geneIDs.txt file). To identify false positive upregulated mRNAs that likely resulted from failure of Pol II transcriptional termination of an upstream HS-responsive gene, we used a strategy similar to that in Duarte et al., (Duarte et al. 2016). First, an intron retention score (IR score) for each gene was calculated by dividing the total normalized intron reads by the total normalized exon reads per gene. Reads were normalized by DESeq2 which normalizes to sequencing depth but not length. Next, upregulated genes were analyzed for accumulation of Intergenic Junction (IJ) reads between their annotated start site and the closest upstream gene. To do this, a 21 bp region that overlaps 11 nts into the 5' annotated start site and 10 nts upstream of the start site was obtained for each gene. The location for these regions was obtained by parsing a list of intergenic regions downloaded from the WS235 versionof the Wormbase ftp site. Reads for this region in both control and HS samples were obtained using the program FeatureCounts. The IJ Ratio for each gene was calculated by dividing the normalized (for depth) HS Intergenic reads by the CTRL Intergenic reads. Genes with an IR score >0.4 and an IJ ratio >2 were removed from thelist of upregulated PCGs. In addition, PCGs with an IR score >1 were also filtered out asthese reads derived from independently transcribed ncRNAs, such as tRNAs and snoRNAs, present in the intron of the PCG. Finally, PCGs that overlapped a repetitive element by > 50% were filtered out. A list of C. elegans repetitive elements was obtained from the UCSC genome Browser. Overlap was determined using Bedtools intersect (Quinlan and Hall 2010). To analyze the expression of different classes of Repetitive Element RNAs, RNA-

sequencing data were aligned to a set of *C. elegans* consensus repeats obtained from repbase (Bao, Kojima, and Kohany 2015). Primary aligned reads were obtained using the following com-mand: samtools view -F 260 ${s} | cut -f 3 | sort | uniq -c | awk '{printf('%s\t%s\', $2, $1)}' > ${s}counts.txt Differential expression was determined using DESeq2. More detailed information including sample code/commands can be found on github.com/wschrein.

2.6.2 Small RNA Sequencing and Analysis

Caenorhabditis elegans were grown to the L4 stage at 20°C then shifted to 35°C for 6 hrs. Animals were then collected, snap-frozen and total RNA was extracted using a standard Trizol RNA extraction protocol. Small-RNA libraries were prepared using Illumina's TruSeq Small RNA library prep kit. miRNAs were analyzed by mapping small RNA sequencing data to *C. elegans* miRNAs. DESeq2 was used to determine differential expression fromtwo independent biological replicates. piRNAs were analyzed by mapping small RNA sequencing data to a database of *C. elegans* piRNAs obtained from Wormbase using theSTAR aligner. Primary reads were obtained using samtools, and differential expression was determined using DESeq2. For miRNA seed analysis, the longest 3' UTR isoform for each gene was considered. UTR annotations were obtained from the WS263 GTF annotation from Wormbase. Cytoscape was used to generate the network analysis graphs (Shannon et al. 2003).

2.6.3 ChIP-seq Data Mapping, Peak Calling and Normalization

HSF-1 and Pol II ChIP-seq data were obtained from Li et al., (GEO Accession Number GSE81523) (Li et al., 2016). Sequencing reads were aligned to a non-repeat- masked version of the *C. elegans* N2 reference genome (ce11) using Bowtie2 with the command bowtie2 —no-unal —very-sensitive (Langmead and Salzberg 2012). HSF-1 peaks present at 34°C and their summits were called from Bowtie2-aligned reads with theMACS2 command macs2 callpeak -g ce —keep-dup auto —call-summits -q 1e-6 using combined biological replicates and the single input replicate available (Y. Zhang et al. 2008). To normalize ChIP-seq data for display purposes, Bowtie2-mapped reads from combinedbiological replicates were filtered for duplicates using macs2 filterdup -g ce —keep-dup auto, and the condition with more mapped reads after filtering was randomly sampled down using macs2 randsample so the total number of reads considered were identical between conditions. Finally, pileup of filtered reads was performed using macs2 pileup with the—extsize parameter set to the fragment lengths predicted by MACS2 during peak-calling steps.

2.6.4 HSE Identification and Scanning

Motifs enriched in 101-bp non-repeat-overlapping HSF-1 summit regions were identified using MEME with the command meme -mod zoops -dna -revcomp (Bailey et al. 2013). The most significant motif identified in HSF-1 peak summits closely resembles thepreviously-identified HSE motif using the same dataset (J. Li et al. 2016). To scan the *C. elegans* (ce11) genome for HSE locations, MEME-derived output for HSEs was used in conjunction with FIMO and its default

parameters, which reports identified HSEs with P <1e-04. HSF-1-bound HSEs were defined as those with at least 14-bp overlap with 201-bpregions centered on HSF-1 summits.

2.6.5. HSF-1 RNAi and Overexpression

RNAi was performed by feeding animals either empty vector or hsf-1(RNAi). RNAiexperiments were performed as described in (Ahringer 2006). RNAi knockdown efficiency was validated by western blotting for HSF-1 (data not shown) and analysis of *hsp-16.2* expression, a direct HSF-1 transcriptional target. The HSF-1 overexpression strain EQ87,*hsf-1p::hsf-1::gfp* + *rol-6*, was used for HSF-1 overexpression studies (Kumsta et al. 2017; Chiang et al. 2012).

2.6.6 RT-qPCR

Quantitative polymerase chain reaction (qPCR) was performed as described in (Aalto et al. 2018) except that (*ama-1*) was used as a reference gene and the Quant Studio machine was used for all experiments. Primer sequences as follows:

ama-1

Forward: CACTGGAGTTGTCCCAATTCTTG

Reverse: TGGAACTCTGGAGTCACACC

dct-10

Forward: GTCACACAGCCAACGAATG

Reverse: GTCGGAACTGTACGGATCAT

Helitron1_CE

Forward: AATCGTCGTGCCAATACCTC

Reverse: GTGCTCACCGAGATGTCTGA

hsp-16.2

Forward: GCTCTGATGGAACGCCAATTTGC

Reverse: CTGTGAGACGTTGAGATTGATGGCAAAC

hsp-70

Forward: CCGCTCGTAATCCGGAGAATACAG

Reverse: CAACCTCAACAACGGGCTTTCC

linc-7

Forward: ACCAAGCAGACCCACCCT

Reverse: GTTGATGACGAGACGAGTGT

2.6.7 Heat Shock Experiments

Animals were synchronized using hypochlorite and bleach and rocked for 24 hrs at 20°C in 5mL of M9 buffer. Animals were plated on UV-treated plates and grown at 20°C for 44 hrs before subjecting to 4 hrs at 35°C. Animals were scored for viability after 24 hrs of recovery at 20°C.

2.6.8 Brood Size Assay

Animals grown at either 20°C or 25°C were singled to small, seeded plates at L4 and moved every 24 hrs to new plates for the subsequent 5 days. The number of eggs laid on each plate was counted and totaled. At least 5 individual animals were

assayed per strain, per replicate. 3 biological replicates were performed. Student's t-test were used to determine significant differences between strains.

2.6.9 Life Span Assay

Animals were synchronized using hyperchlorite and bleach and plated as L1s onto small NGM plates seeded with OP50 as previously described (Aalto et al. 2018). At least 100 animals were assayed per stain.

2.7 Acknowledgments

The RNA sequencing experiments and analysis were performed by W. Schreiner, J. M. Garrigues, A. P. Aalto, J. S. Chen, and A. E. Pasquinelli. I performed all RT-PCR and RT-qPCR analyses, including the RNAi and HSF-1 overexpression assays.

Chapter 2 contains material from the paper "Remodeling of the *Caenorhabditis elegans* non-coding RNA transcriptome by heat shock ", Schreiner, W. P., Pagliuso, D. C., Garrigues, J. M., Aalto, A. P., Chen, J. S., Pasquinelli, A. E. *Nucleic Acids Research*, 2019. Pagliuso, D. C. was a co-author on this paper.

Chapter 3

Recovery After Heat Shock Requires the microRNA Pathway in *Caenorhabditis elegans*

3.1 Abstract

The heat shock response (HSR) is a highly conserved cellular process that promotes survival during stress. A hallmark of the HSR is the rapid induction of heat shock proteins (HSPs), such as HSP-70, by transcriptional activation. Once the stress is alleviated, HSPs return to near basal levels through incompletely understood mechanisms. Here, we show that the microRNA pathway acts during heat shock recovery in *Caenorhabditis elegans.* Depletion of the miRNA Argonaute, Argonaute Like Gene 1 (ALG-1), after an episode of heat shock resulted in decreased survival and perdurance of high *hsp-70* levels. We present evidence that regulation of *hsp-70* is dependent on miR-85 and sequences in the *hsp-70* 3'UTR that contain target sites for this miRNA. Regulation of *hsp-70* by the miRNA pathway was found to be particularly important during recovery from HS, as animals that lacked miR-85 or its target sites in the *hsp-70* 3'UTR overexpressed HSP-70 and exhibited reduced viability. In summary, our findings show that down-regulation of *hsp-70* by miR-85 after HS promotes survival, highlighting a previously unappreciated role for the miRNA pathway during recovery from stress.

3.2 Author Summary

In the natural world, organisms constantly face stressful conditions such as oxidative stress, pathogen infection, starvation and heat stress. While many studies have helped illustrate how organisms respond to stress, little is known about how organisms recover after the stress has been ameliorated. Here, we show that turning off stress-induced cellular responses is a critical feature in promoting proper recovery after stress in the nematode, *Caenorhabditis elegans* and that animals lacking the ability to restore basal levels of a highly conserved, stress-induced protein HSP-70 cannot efficiently recover from heat stress. Additionally, we identify a small non-coding RNA, miR-85, that is largely responsible for repressing *hsp-70* levels during heat stress recovery, facilitating survival. Due to the broad conservation of the cellular stress response, our work prompts further investigation of the recovery phase after stress and elicits new questions about the detriment of long-term overexpression of stress-induced genes.

3.3 Introduction

The heat shock response is a broadly conserved cellular mechanism that is activated to help organisms survive stress (Richter, Haslbeck, and Buchner 2010). Various types of stress including heat shock, oxidative stress, infection, and tumorigenesis can trigger the heat shock response (HSR) (S Lindquist and Craig 1988; Ikwegbue et al. 2017). Upon encountering stress, proteins can misfold and

form harmful aggregates, which have the potential to cause cell death if not ameliorated (Jolly and Morimoto 2000; Morimoto 1998; Zeng et al. 2004). To maintain protein homeostasis (proteostasis), the HSR stimulates a transient yet robust reprogramming of cellular activities, coupling a general decline in transcription and translation with the specific induction of molecular chaperones known as heat shock proteins (HSPs) (Duarte et al. 2016; Shalgi et al. 2013; Vihervaara, Duarte, and Lis 2018). Upon stress, activation of the transcription factor HSF1 (Heat Shock Factor 1) drives the accumulation of HSPs, which aid in restoring proteostasis and are, thus, integral to organismal survival (Åkerfelt, Morimoto, and Sistonen 2010).

Many HSPs have a diversity of cellular functions under both stressful and non-stressful conditions that revolve around mediating protein folding, stability and complex formation (Nollen and Morimoto 2002; Rosenzweig et al. 2019). This group includes HSP70, which is constitutively expressed in most cell types but is robustly induced by heat shock and other types of stress. While up-regulation of HSP70 upon stress is primarily a result of HSF1 dependent transcription, less is understood about how HSP70 levels return to basal levels after stress (Mayer and Bukau 2005). Studies in *Drosophila* and human cells have documented the rapid induction and subsequent decline of *Hsp70* transcripts during the HSR. Destabilization of *Hsp70* mRNA after return to ambient temperatures is regulated post-transcriptionally and involves yet to be defined 3'UTR sequences and trans-

acting factors (Hess and Duncan 1996; DiDomenico, et al. 1982; Yost, et al. 1990; Bönisch et al. 2007).

In addition to the transcriptional reprogramming of protein coding genes elicited upon HS, microRNAs (miRNAs) have also been reported to respond to HS. MiRNAs are short, ~22 nucleotide non-coding RNAs that guide Argonaute (AGO) proteins to target mRNAs, triggering destabilization through imperfect base pairing (Bartel 2018). In *Caenorhabditis elegans*, ALG-1 (AGO-Like Gene 1) is broadly expressed and binds most miRNAs (Vasquez-Rifo et al. 2012). Specific *C. elegans* miRNAs have been observed to be up- or down-regulated during HS (Brunquell et al. 2017; Nehammer et al. 2015; Schreiner et al. 2019), suggesting roles in the HSR. Moreover, deletion of *miR-71* or *miR-246* results in reduced HS survival (De Lencastre et al. 2010). Despite the identification of individual miRNAs that influence HS survival, much is yet to be learned about specific roles and targets of miRNAs during stress.

In this study, we demonstrate a role for the miRNA pathway in the recovery phase following heat shock in *C. elegans*. Removal of ALG-1 after HS resulted in reduced survival and perdurance of higher *hsp-70* levels. We found that efficient down-regulation of *hsp-70* after an episode of HS also requires miR-85 and the *hsp-70* 3'UTR, which contains two miR-85 binding sites. Moreover, animals lacking miR-85 or its binding sites in the *hsp-70* 3'UTR exhibited greater sensitivity to HS. The reduced survival of these strains was dependent on *hsp-70* expression,

as knock down of *hsp-70* by RNA interference (RNAi) after HS restored viability to WT levels. Altogether, we show that in *C. elegans* down-regulation of *hsp-70* after HS is facilitated by miR-85 and is important for organismal survival.

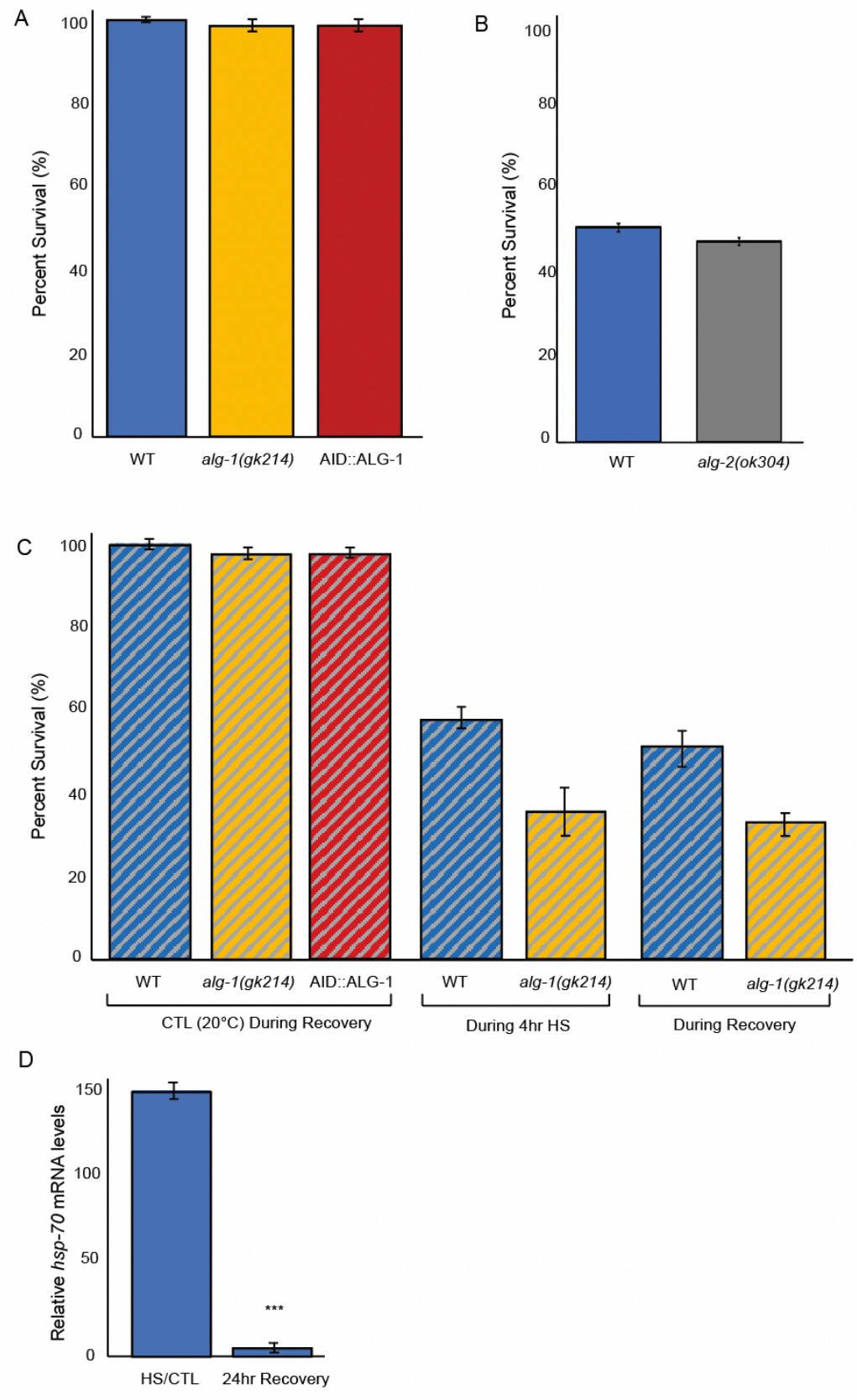
3.4 Results

3.4.1 The microRNA pathway is important for recovery after heat shock

To investigate the role of the miRNA pathway in the *C. elegans* heat shock response (HSR), we first tested if Argonaute Like Gene 1 (ALG-1) was important for survival after exposure to elevated temperatures. Populations of wild type (WT) and *alg-1(gk214)* loss-of-function mutants were subjected to 4 hrs of HS at 35°C, and the percent survival was calculated after allowing the animals to recover for 24 hrs at 20°C. Under these conditions, about 60% of the WT and only 30% of the *alg-1(gk214)* animals survived (Figure 3.2A). Loss of *alg-2*, the closest homolog to ALG-1, did not result in a HS phenotype (Figure 3.1B). Although *alg-1(gk214)* animals are entirely viable during this time course when maintained at 20°C (Figure 3.1A), this strain does exhibit moderate developmental defects (Vasquez-Rifo et al. 2012; Tops, Plasterk, and Ketting 2006; Zinovyeva et al. 2014), reduced fertility (Brown et al. 2017; Vasquez-Rifo et al. 2012; Rios et al. 2017; Bukhari et al. 2012) and a shortened lifespan (Aalto et al. 2018). To avoid pre-existing defects that might make *alg-1(gk214)* more sensitive to HS, we utilized the auxin-inducible degron (AID) system to remove ALG-1 upon HS treatment (L. Zhang et al. 2015). Four hours of auxin treatment in the CTL (20°C) or HS (35°C) conditions

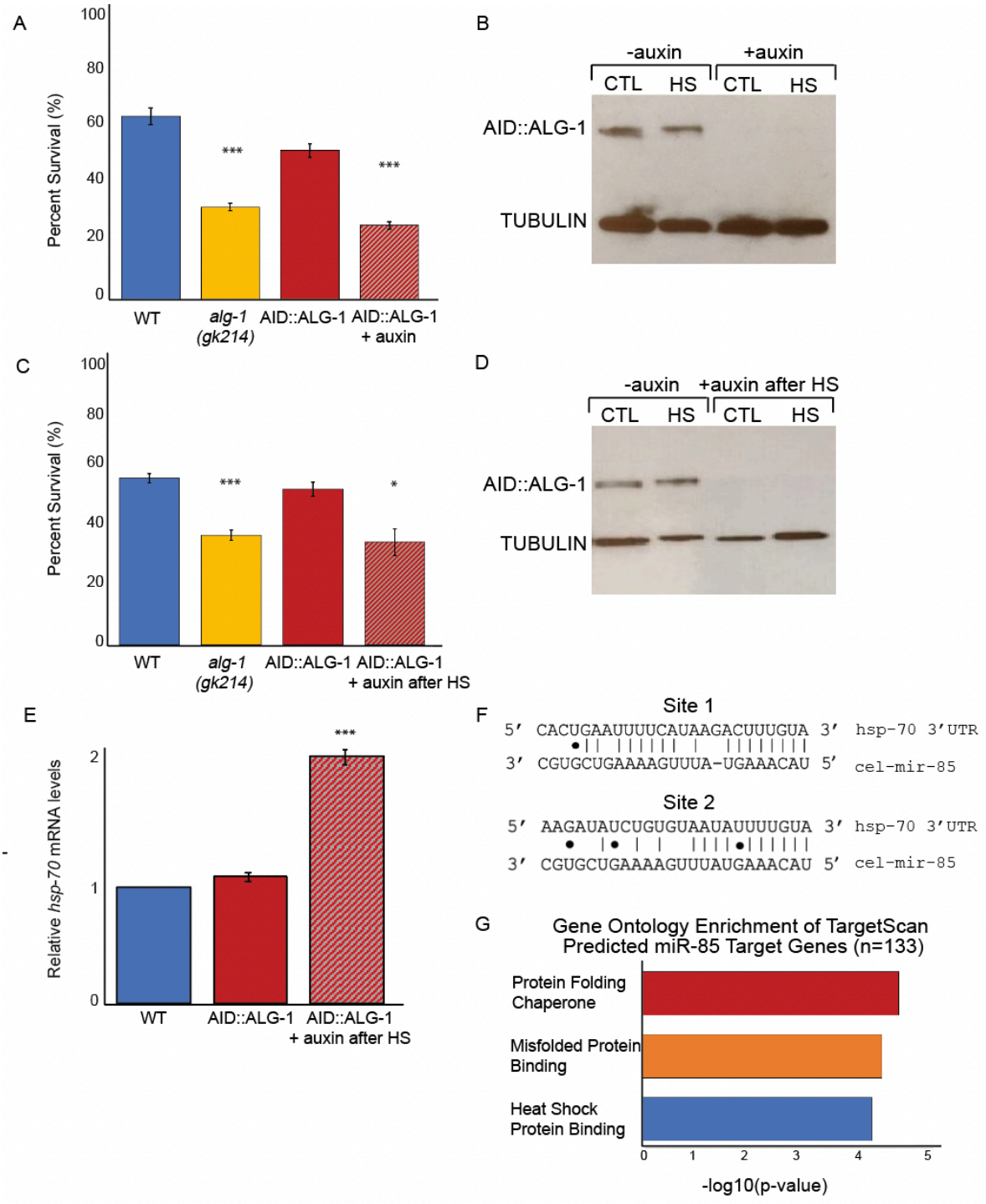
was sufficient to deplete ALG-1 protein, as judged by Western Blotting (Figure 3.2B). Removal of ALG-1 during HS resulted in reduced levels of survival, comparable to the *alg-1(gk214)* strain (Figure 3.2A). Given the dramatic auxin-induced depletion of AID::ALG-1, we considered that the reduced viability phenotype could be due to a requirement for ALG-1 during HS or in the recovery period. To test if ALG-1 is needed for recovery from HS, we performed a similar HS viability experiment but instead moved AID::ALG-1 animals to auxin-containing media for the 24 hr recovery period following HS. Loss of ALG-1 after HS resulted in decreased survival compared to WT or the AID::ALG-1 strain in the absence of auxin (Figure 3.2C). The auxin-induced depletion of AID::ALG-1 was confirmed by Western blot analysis of control and HS-treated animals (Figure 3.2D). Importantly, auxin treatment (during HS or during recovery) of WT and *alg-1(gk214)* animals did not alter average HS survival of these strains compared to average survival scores for no auxin controls (Figure 3.1C). Additionally, to control for potential stress induced by moving animals to new plates for auxin treatment, all strains, regardless of condition, were moved to fresh plates. While our results do not rule out a role for ALG-1 activity during HS, they do demonstrate that ALG-1 and, by inference, the microRNA pathway contribute to the recovery phase following HS.

Figure 3.1: Viability assay controls and *hsp-70* RT-qPCR during heat shock and recovery. (A) A. Percent survival of WT, *alg-1(gk214),* and AID::ALG-1 animals grown at 20°C for 72 hrs. Three biological replicates were performed with at least 50 worms per strain. Error bars represent SEM.(B) Percent survival of synchronized WT and *alg-2(ok304)* animals grown at 20°C for 44 hrs before heat shock for 4 hrs at 35°C. Viability was scored after 24 hrs of recovery at 20°C. Three biological replicates were performed with at least 50 worms per strain. Error bars represent SEM. (C) Percent survival of synchronized WT, *alg-1(gk214)*, and AID::ALG-1 animals grown to stage L4 before being moved to auxin-containing media either for 24 hrs at 20°C, during 4 hrs HS, or during 24 hrs recovery after HS. Three biological replicates were performed with at least 50 worms per strain. Error bars represent SEM. (D) RT-qPCR of *hsp-70* mRNA levels in WT animals subjected to 3 hr HS and allowed to recover for 24hrs. All replicates were normalized to *ama-1*. Three biological replicates were assayed. Student's t-tests were performed to determine significance relative to *hsp-70* mRNA expression compared to HS *(***P* < 0.001). Error bars represent SEM.


A
Percent Survival (%)
100
80
60
40
20
0
WT
alg-1(gk214)
AID::ALG-1
B
Percent Survival (%)
100
80
60
40
20
0
WT
alg-2(ok304)
C
Percent Survival (%)
100
80
60
40
20
0
WT
alg-1(gk214)
AID::ALG-1
WT
alg-1(gk214)
WT
alg-1(gk214)
CTL (20°C) During Recovery
During 4hr HS
During Recovery
D
Relative hsp-70 mRNA levels
150
100
50
0
***
HS/CTL
24hr Recovery

In contrast to the well-studied transcriptional induction of genes that respond to HS, much less is known about how gene expression changes during recovery from this stress (Morimoto 1993; Yost, Petersen, and Linquist 1990b). There is evidence that in *Drosophila* S2 cells the HS-induced gene Hsp70 is subject to post-transcriptional down-regulation during recovery through a yet to be defined mechanism (Yost, Petersen, and Linquist 1990b; Simcox et al. 1985; DiDomenico, Bugaisky, and Lindquist 1982b). Under our heat shock conditions, *hsp-70 (C12C8.1)* mRNA is up-regulated over 100-fold and recovers to baseline levels within 24 hrs of return to the control temperature (Figure 3.1D). However, depletion of ALG-1 after HS resulted in significantly higher levels of *hsp-70* mRNA relative to control strains that maintained expression of ALG-1 (Figure 3.2E). A requirement for ALG-1 in the down-regulation of *hsp-70* following HS raised the possibility that it could be targeted by a miRNA. Using the miRNA target prediction tool TargetScanWorm, we identified two predicted miR-85 binding sites in the 3'UTR of *hsp-70* (Figure 3.2F) (Jan et al. 2011). Furthermore, gene ontology (GO) enrichment analysis using DAVID (Huang, Sherman, and Lempicki 2009) revealed that the most enriched molecular functions of the 133 predicted miR-85 targets include protein folding chaperone, misfolded protein binding, and heat shock protein binding (Figure 3.2G). The predicted binding sites within the 3'UTR of *hsp-70* and the potential for targeting other stress response genes focused our attention on miR-85 as a candidate for functioning in the HSR.

Figure 3.2: The microRNA pathway is important for recovery after heat shock. (A) Loss of Loss of *alg-1* activity during HS results in reduced viability. Heat shock treatment was performed on synchronized L4 worms for 4 hrs at 35°C and percent survival was determined after 24 hrs recovery at 20°C. The AID::ALG-1 strain was exposed to auxin during the 4 hr HS period. Three biological replicates were performed with at least 50 worms per strain, per condition. Student's t-tests were performed to determine significance compared to WT (****P* < 0.001). (B) Western blot of ALG-1 protein levels in synchronized L4 AID::ALG-1 worms collected at CTL (20°C) or immediately after 4 hr HS (35°C) in the absence or presence of auxin during the same 4 hr period. (C) Loss of *alg-1* activity during recovery after HS results in reduced viability. Heat shock treatment was performed on synchronized L4 worms for 4 hrs at 35°C and percent survival was determined after 24 hrs recovery at 20°C. The AID::ALG-1 strain was placed on auxin immediately after HS during the 24 hr recovery period. Three biological replicates were performed with at least 50 worms per strain, per condition. Student's t-tests were performed to determine significance compared to WT (**P* < 0.05, ***P* < 0.01). (D) Western blot of ALG-1 protein levels in synchronized AID::ALG-1 worms collected after 24 hrs of recovery post HS treatment or kept at CTL (20°C) in the absence or presence of auxin during the recovery period. (E) RT-qPCR of *hsp-70* mRNA after 24 hr recovery from HS in WT and AID::ALG-1 recovered in the absence or presence of auxin. All replicates were normalized to *ama-1* mRNA levels, which are not affected by HS. Three biological replicates were assayed. Student's t-tests were performed to determine significance relative to WT *(****P* < 0.001). (F) Depiction of predicted mir-85 target sites in the *hsp-70* 3'UTR based on Target Scan Worm. Site 1 is conserved and Site 2 is poorly conserved (Jan et al., 2011). (G) Gene ontology (GO) enrichment analysis of mir-85 predicted targets from Target Scan Worm (n=133) using DAVID (Huang et al., 2009).


A
Percent Survival (%)
100
80
60
40
20
0
***
***
WT
alg-1 (gk214)
AID::ALG-1
AID::ALG-1 + auxin
B
-auxin
+auxin
CTL
HS
CTL
HS
AID::ALG-1
TUBULIN
C
Percent Survival (%)
***
*
WT
alg-1 (gk214)
AID::ALG-1
AID::ALG-1 + auxin after HS
D
-auxin
+auxin after HS
CTL
HS
CTL
HS
AID::ALG-1
TUBULIN
E
Relative hsp-70 mRNA levels
2
1
0
***
WT
AID::ALG-1
AID::ALG-1 + auxin after HS
F
Site 1
5′ CACUGAAUUUUCAUAAGACUUUGUA 3′ hsp-70 3′UTR
3′ CGUGCUGAAAAGUUUA-UGAAACAU 5′ cel-mir-85
Site 2
5′ AAGAUAUCUGUGUAAUAUUUUGUA 3′ hsp-70 3′UTR
3′ CGUGCUGAAAAGUUUAUGAAACAU 5′ cel-mir-85
G
Gene Ontology Enrichment of TargetScan Predicted miR-85 Target Genes (n=133)
Protein Folding Chaperone
Misfolded Protein Binding
Heat Shock Protein Binding
0
1
2
3
4
5
-log10(p-value)

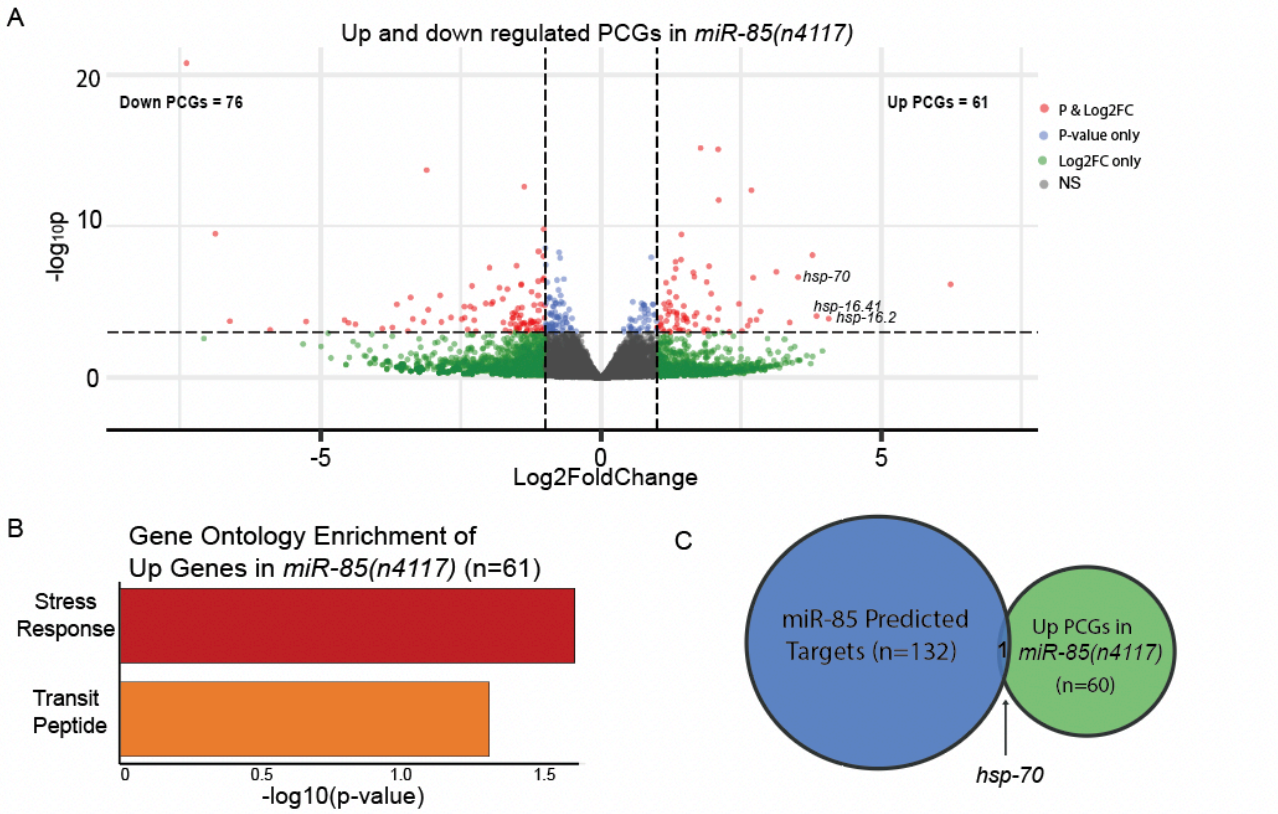
3.4.2 miR-85 regulates genes in stress response pathways

Since miRNA regulation often results in target mRNA degradation (Bagga et al. 2005; Valencia-Sanchez et al. 2006; Bartel 2018), we predicted that *hsp-70* and potentially other stress response target genes might be up-regulated in the absence of miR-85. To test this idea, we performed total RNA sequencing of RNA from last larval stage (L4) WT and *miR-85(n4117)* animals cultured at 20°C and identified differentially expressed genes using DESeq2 (Love, Huber, and Anders 2014). Consistent with the lack of obvious developmental defects in the *miR-85* deletion mutants (Miska et al. 2007), few genes were up- (61 genes) or down-regulated (76 genes) by at least 2-fold in *miR-85(n4117)* compared to WT. Strikingly, within the set of up-regulated genes, *hsp-70* and several other stress responsive genes were highly over-expressed in the *miR-85(n4117)* mutants (Figure 3.3A). Additionally, stress response and transit peptide were the only two terms identified among the upregulated genes by GO enrichment analysis (Huang, Sherman, and Lempicki 2009) (Figure 3.3B). Although the enrichment of these terms was modest, this finding is consistent with a role for miR-85 in regulating stress response genes.

The predicted miR-85 binding sites and up-regulation of *hsp-70* mRNA in *miR-85* mutant animals make *hsp-70* a candidate for direct regulation by miR-85 (Figure 3.3C). To explore this possibility, we used CRISPR-Cas9 to generate a deletion in the *hsp-70* 3'UTR that removes both miR-85 target sites (*hsp-70*Δ*TS)*

(Figure 3.3D). Like *miR-85* mutants, the *hsp-70∆TS* strain did not exhibit any obvious developmental abnormalities (Figure 3.4A). We then examined relative *hsp-70* mRNA levels by RT-qPCR in WT, *miR-85(n4177),* and *hsp-70∆TS* L4 stage animals cultured at 20°C. Consistent with the RNA sequencing results, *hsp-70* mRNA was up-regulated ~2-fold in *miR-85(n4117)* compared to WT animals (Figure 3.3E). The *hsp-70∆TS* animals exhibited a similar level of *hsp-70* mRNA overexpression relative to WT (Figure 3.3E). These results show that miR-85 and the 3'UTR of *hsp-70* are important for regulating the levels of *hsp-70* during optimal temperature (20°C) conditions in *C. elegans*.

Figure 3.3: miR-85 regulates genes in stress response pathways**.** (A) Volcano plot of differentially expressed PCGs in WT and *mir-85(n4117)* animals synchronized to L4 at 20°C. Three biological replicates were used for RNA sequencing analysis. Genes were considered significant if they had a base mean > 10, Log2FC +/- 1, and p-adj. < 0.05. (B) Gene ontology enrichment analysis of PCGs up-regulated in loss of *mir-85* animals vs WT (n=61) using DAVID (Huang et al., 2009). (C) Venn-diagram of up-regulated PCGs in loss of *miR-85* animals and predicted miR-85 targets. (D) Depiction of the *hsp-70* 3'UTR with mir-85 target sites in bold and deletion region in the *hsp-70ΔTS* animals marked in red. (E) RT-qPCR of *hsp-70* mRNA in WT, *mir-85(n4117),* and *hsp-70ΔTS* animals. All replicates were normalized to *ama-1* mRNA. Relative expression was calculated by comparing to *hsp-70* expression in WT. Three biological replicates were assayed. Student's t-tests were performed to determine significance compared to WT (***P < 0.01, ****P* < 0.001). Error bars represent SEM.

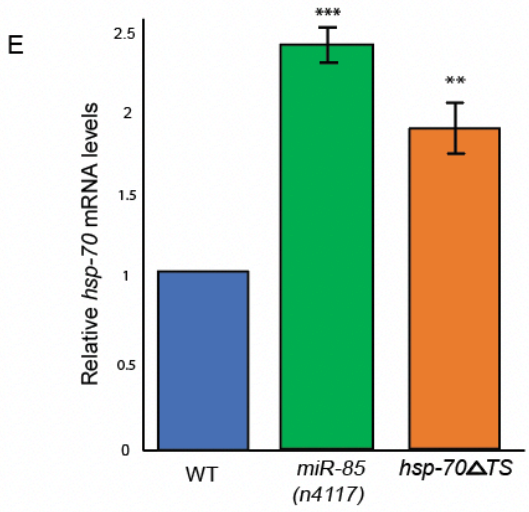


D *hsp-70 3'UTR:*

Site 1 Site 2

5′ TTATCTA**CACTGAATTTTCATAAGACTTTGTA**TTTATTTTA**AAGATATCTGTGTAATATTTTGTA**AATAAACATTGGAAAAAATAC 3′

hsp-70ΔTS



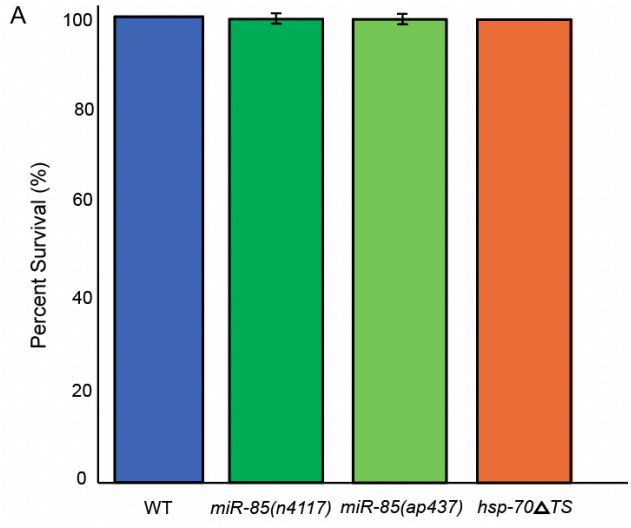


Figure 3.4: Viability assay at control temperatures (20°C). (A) Percent survival of WT, *miR-85(n4117), miR-85(ap437),* and *hsp-70ΔTS* animals grown at 20°C for 72 hrs. Three blinded biological replicates were performed with at least 50 worms per strain. Error bars represent SEM.

3.4.3 miR-85 regulates *hsp-70* during recovery from Heat Shock

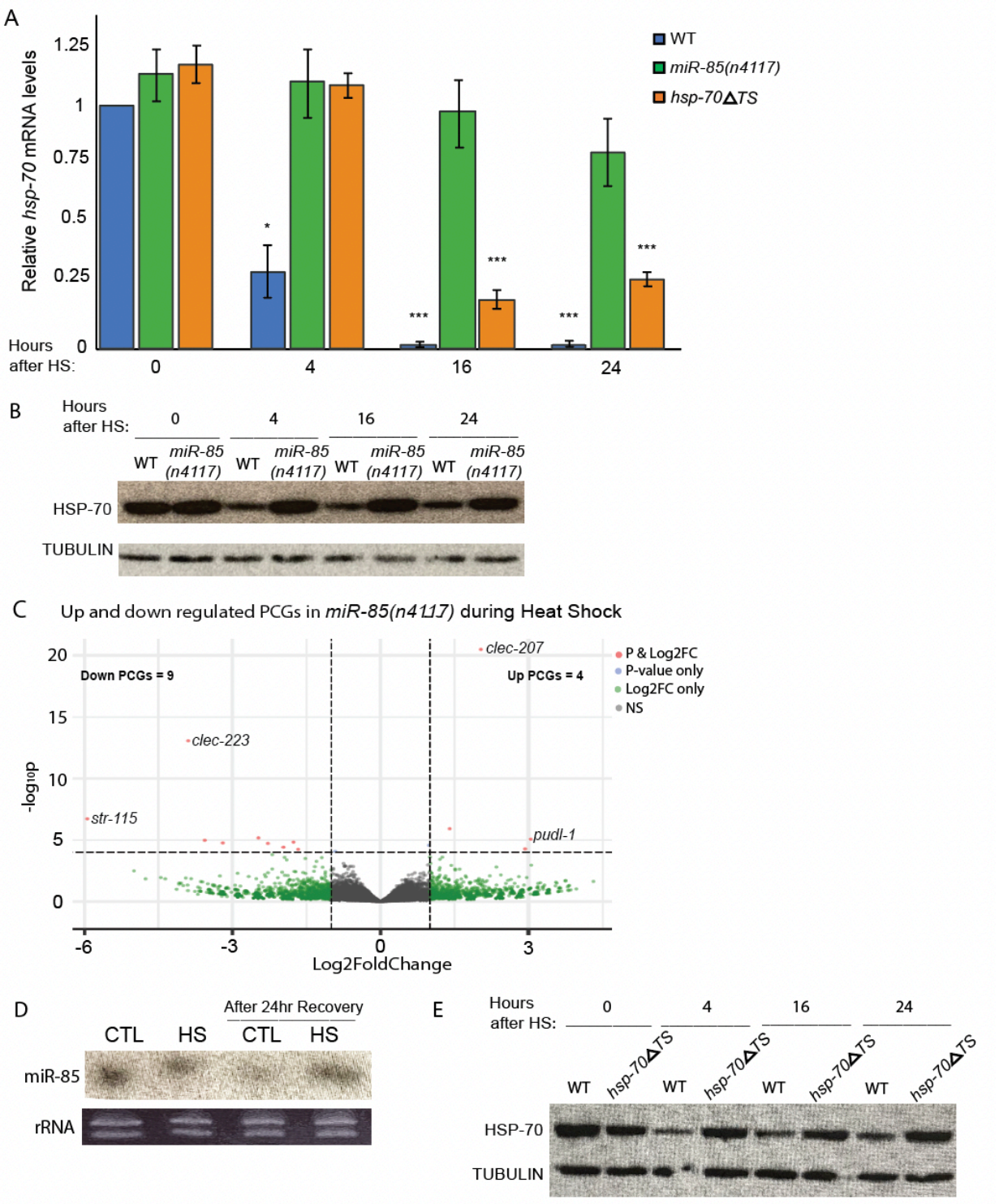
We next asked if miR-85 also plays a role in regulating *hsp-70* as part of the heat shock response. Despite higher basal levels of *hsp-70* mRNA in *miR-85(n4117)* animals (Figure 3.3E), after 3 hrs of HS at 35°C we detected no significant difference in *hsp-70* mRNA in *miR-85(n4117)* compared to WT animals (Figure 3.5, 0 hr time point). HSP-70 protein also accumulated to similar levels immediately following HS, with no detectable difference in *miR-85(n4117)* compared to WT (Figure 3.5B, 0 hr time point). In addition, RNA sequencing after 3 hrs of HS at 35°C revealed very few differences in gene expression with only 4 up- and 9 down-regulated genes (Figure 3.5C). Given the similar gene expression profiles in WT and *miR-85* strains during HS, we wondered if this congruence might be explained by reduced miR-85 levels in response to HS. This does not

seem to be the case as Northern blot analysis showed that miR-85 levels were maintained in HS conditions and through recovery (Figure 3.5D). Similar to *miR-85(n4117),* the *hsp-70*Δ*TS* animals expressed *hsp-70* mRNA and protein at levels comparable to WT (Figure 3.5A and E, 0 hr time point). These results indicate that the robust induction of *hsp-70* upon HS is not influenced by miR-85 or the *hsp-70* 3'UTR.

During HS recovery, however, WT and *miR-85(n4117)* animals showed striking differences in *hsp-70* expression. After return to 20°C, WT animals displayed a rapid decline of *hsp-70* mRNA (Figure 3.5A) and protein (Figure 3.5B), following temporal changes previously reported for *hsp-70* during recovery from HS (Prahlad, Cornelius, and Morimoto 2008). By 24 hrs after HS, *hsp-70* mRNA had almost returned to non-stress levels in WT but persisted at levels nearly as high as during HS in *miR-85(n4117)* animals (Figure 3.5A). Furthermore, we found that efficient down-regulation of *hsp-70* during HS recovery was dependent on its 3'UTR. Compared to WT, *hsp-70*Δ*TS* animals exhibited higher *hsp-70* mRNA and protein levels up to 24 hrs after return to 20°C (Figure 3.5A, E). It should be noted that the antibody used to detect HSP-70 likely reacts with homologs of this protein not expected to be regulated by miR-85 as their 3'UTRs lack binding sites for this miRNA. Thus, the Western blot results are likely an underestimate of the accumulation of HSP-70 in *miR-85(n4117)* compared to WT animals during

recovery from HS. These results demonstrate a requirement for mir-85 and the 3'UTR of *hsp-70* in down-regulating *hsp-70* levels after HS.

Figure 3.5: miR-85 regulates *hsp-70* during recovery from heat shock. (A) RT-qPCR of *hsp-70* mRNA in WT, *mir-85(n4117)*, and *hsp-70ΔTS* animals subjected to 3 hr HS at 35°C and allowed to recover at 20°C for 4, 16, and 24 hrs at 20°C. All replicates were normalized to *ama-1* mRNA. Three biological replicates were assayed. Student's t-tests were performed to determine significance relative to levels immediately after HS (0 hr) in WT animals (*$P < 0.05$, ***$P < 0.001$). Compared to the WT 0 hr time point, *mir-85(n4117)* showed no significant difference in *hsp-70* mRNA levels through the recovery time course. Error bars represent SEM. (B) Western blot of HSP-70 protein levels in WT and *mir-85(n4117)* animals subjected to 3 hr HS at 35°C and allowed to recover at 20°C for 4, 16, and 24 hrs at 20°C. Tubulin was used as a loading control. (C) Volcano plot of differentially expressed PCGs in WT and *mir-85(n4117)* animals synchronized to L4 and heat shocked for 3 hrs at 35°C. Three biological replicates were used for RNA sequencing analysis. Genes were considered significant is they had a base mean > 10, Log2FC +/- 1, and p-adj. < 0.05. (D) Northern blot of L4 synchronized WT animals at 20°C or HS treated for 3 hr at 35°C to detect mir-85-3p; ethidium bromide staining of rRNAs shows similar levels of loaded RNA for each sample. (E) Western blot of HSP-70 protein levels in WT and *hsp-70ΔTS* animals subjected to 3 hr HS at 35°C and allowed to recover at 20°C for 4, 16, and 24 hrs at 20°C. Tubulin was used as a loading control.

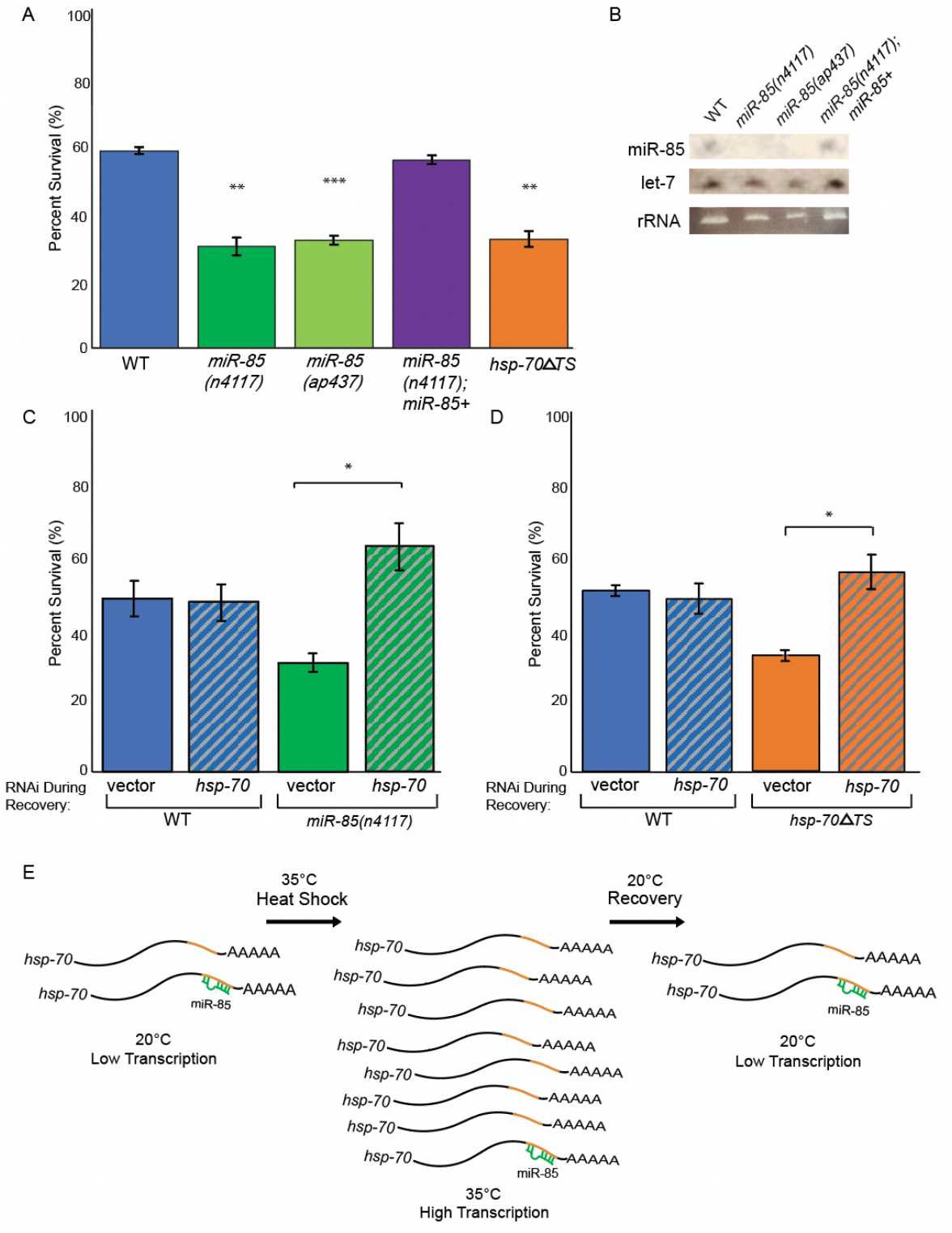

A
Relative hsp-70 mRNA levels
1.25
1
0.75
0.5
0.25
0
WT
miR-85(n4117)
hsp-70ΔTS
Hours after HS:
0
4
16
24
B
Hours after HS:
0
4
16
24
WT
miR-85 (n4117)
HSP-70
TUBULIN
C
Up and down regulated PCGs in miR-85(n4117) during Heat Shock
Down PCGs = 9
Up PCGs = 4
clec-207
clec-223
str-115
pudl-1
P & Log2FC
P-value only
Log2FC only
NS
-log10p
Log2FoldChange
D
After 24hr Recovery
CTL
HS
CTL
HS
miR-85
rRNA
E
Hours after HS:
0
4
16
24
WT
hsp-70ΔTS
HSP-70
TUBULIN

3.4.4 Down-regulation of *hsp-70* by miR-85 is important for recovery from heat shock

While the *miR-85* and *hsp-70*Δ*TS* strains exhibited no obvious developmental or viability defects (Figure 3.7A), we wondered if these animals might differ from WT in their ability to survive HS. To examine this possibility, L4 populations of WT, *miR-85(n4117),* and *hsp-70*Δ*TS* animals were subjected to 4 hrs of HS at 35°C, and the percent survival was calculated after allowing the animals to recover for 24 hrs at 20°C. Compared to the nearly 60% viability observed in WT, only about 30% of *miR-85(n4117)* and *hsp-70*Δ*TS* animals survived (Figure 3.6A). To rule out possible phenotypes associated with *F49E12.8,* which is partially disrupted in *miR-85(n4117),* we generated a new *miR-85* mutant, *mir-85(ap437).* This allele contains a short deletion within the pre-miR-85 sequence that does not overlap the *F49E12.8* coding region. The *miR-85(ap437)* animals lacked mature miR-85 (Figure 3.6B) and phenocopied *miR-85(n4117),* displaying an average survival around 32% after HS (Figure 3.6A). Additionally, Mos-1 Mediated Single Copy Insertion (MosSCI) (Philip et al. 2019) of *miR-85* in the loss-of-function *miR-85(n4117)* mutant (*miR-85(n4117);miR-85+*) restored mature miR-85 expression (Figure 3.6B) and rescued the HS viability phenotype to WT levels (Figure 3.6A). Taken together, these findings show that miR-85 and the 3'UTR of *hsp-70* facilitate HS survival.

Given the dramatic mis-regulation of *hsp-70* during recovery from HS in *miR-85(n4117)* and *hsp-70∆TS* strains (Figure 3.5A, B, and E), we predicted that the failure to down-regulate *hsp-70* levels after HS might be responsible for the decreased survival of these animals. To test this idea, we used RNA interference (RNAi) to repress *hsp-70* expression during recovery from HS. Compared to vector RNAi control, *hsp-70(RNAi)* resulted in ~10-fold reduction in *hsp-70* mRNA levels in WT, *miR-85(n4117),* and *hsp-70∆TS* strains (Figure 3.7A,B). While survival rates for WT animals recovered on vector or *hsp-70(RNAi)* were indistinguishable at about 50%, RNAi depletion of *hsp-70* rescued the reduced viability of *miR-85(n4117)* and *hsp-70∆TS* strains from ~35% to near WT levels (Figure 3.6C, D). These results indicate that overexpression of *hsp-70* during recovery from HS contributes to the diminished survival of animals lacking miR-85 or the miR-85 target sites in the *hsp-70* 3'UTR. Furthermore, they highlight the importance of down-regulating *hsp-70* levels after HS and reveal a new role for the miRNA pathway in this process (Figure 3.6E).

Figure 3.6: Down-regulation of *hsp-70* by miR-85 is important for recovery from heat shock. (A) Heat shock viability of WT, *mir-85(n4117)*, *mir-85(ap437)*, *mir-85(n4117); miR-85*+, and *hsp-70ΔTS* animals subjected to 4 hr of HS at 35°C. Percent survival was determined after 24 hrs recovery at 20°C. *mir-85(ap437)* was generated using CRISPR-Cas9 to disrupt the production of mature mir-85. The *mir-85(n4117); miR-85*+ rescue strain was generated using MosSci. The complete rescue strain genotype is *knuSi832[pNU2156 (mir-85 in cxTi10882, unc-119(+))] IV; unc-119(ed3) III; mir-85(n4117)*. Three blinded biological replicates were performed with at least 50 worms per strain, per condition. Student's t-tests were performed to determine significance compared to WT *(**P* < 0.01, ****P* < 0.001). Error bars represent SEM. (B) Northern blot of L4 synchronized WT, *mir-85(n4117)*, *mir-85(ap437)* and *mir-85(n4117); miR-85*+ strains probed for mir-85-3p and let-7 as a control to ensure strains were developmentally synchronized to the L4 stage; ethidium bromide staining of rRNA shows similar levels of loaded RNA for each sample. (C) Following 4 hr of HS at 35°C, WT and *mir-85(n4117)* animals were subjected to *vector RNAi* or *hsp-70 RNAi* and percent survival was determined after 24 hrs recovery at 20°C. Three blinded biological replicates were performed with at least 50 worms per strain, per condition. Student's t-tests were performed to determine significance for each strain relative to survival on *vector RNAi* (**P* < 0.05). (D) Following 4 hr of HS at 35°C, WT and *hsp-70ΔTS* animals were subjected to *vector RNAi* or *hsp-70 RNAi* and percent survival was determined after 24 hrs recovery at 20°C. Three blinded biological replicates were performed with at least 50 worms per strain, per condition. Student's t-tests were performed to determine significance for each strain relative to survival on *vector RNAi* (**P* < 0.05). (E) Model for the role of miR-85 role in regulating *hsp-70*. At 20°C, miR-85 represses *hsp-70* by binding to complementary sites in the 3'UTR of its mRNA. Heat shock induces transcriptional up-regulation of *hsp-70* and the very high levels of *hsp-70* mRNA override the regulatory potential of miR-85. Upon return to 20°C, the transcriptional induction ceases and miR-85 aids in the rapid down-regulation of the abundant *hsp-70* mRNAs.


A
Percent Survival (%)
100
80
60
40
20
0
**
***
**
WT
miR-85 (n4117)
miR-85 (ap437)
miR-85 (n4117); miR-85+
hsp-70ΔTS
B
WT
miR-85(n4117)
miR-85(ap437)
miR-85(n4117); miR-85+
miR-85
let-7
rRNA
C
Percent Survival (%)
*
RNAi During Recovery:
vector
hsp-70
vector
hsp-70
WT
miR-85(n4117)
D
Percent Survival (%)
*
RNAi During Recovery:
vector
hsp-70
vector
hsp-70
WT
hsp-70ΔTS
E
35°C
Heat Shock
20°C
Recovery
hsp-70
AAAAA
miR-85
20°C
Low Transcription
35°C
High Transcription
20°C
Low Transcription

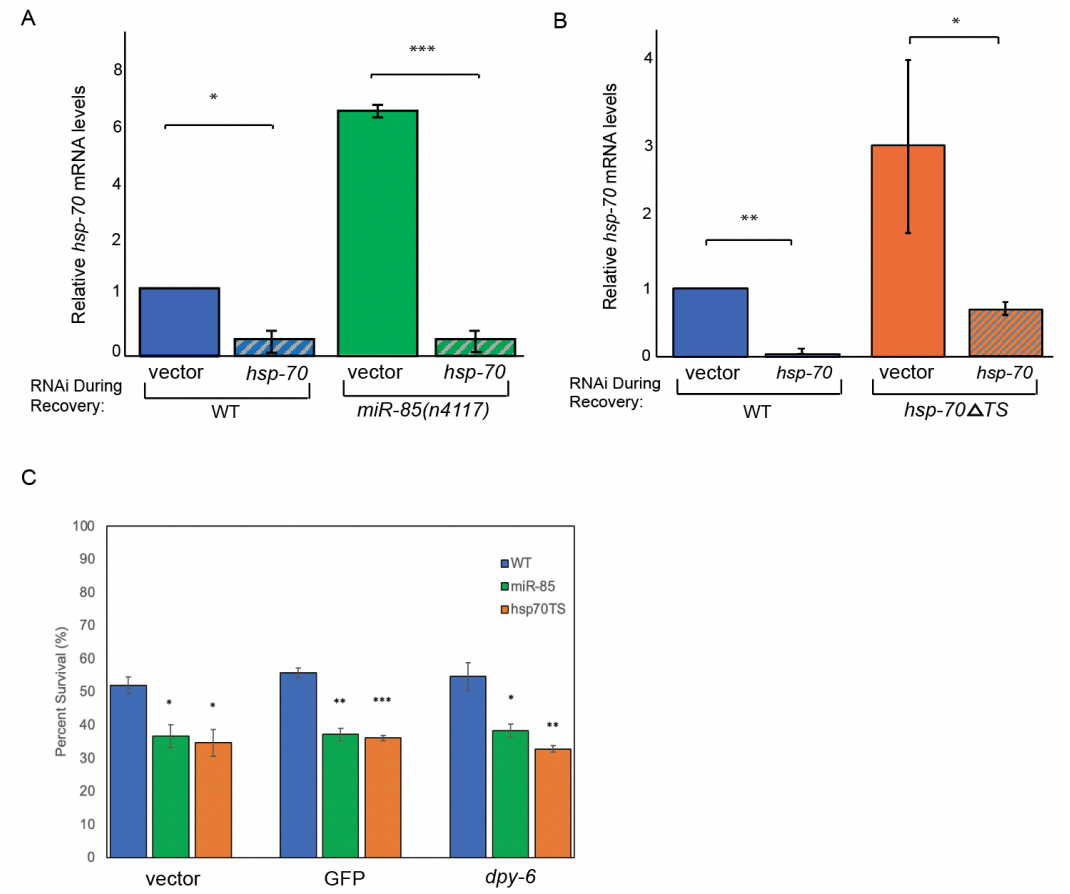


Figure 3.7: RT-qPCR of RNAi knock down and non-specific RNAi heat shock viability assay controls. (A) RT-qPCR of *hsp-70* mRNA levels in WT and *miR-85(n4117)* animals subjected to HS and allowed to recover on *vector RNAi* or *hsp-70 RNAi* for 24 hrs. All replicates were normalized to *ama-1* mRNA. Three biological replicates were assayed. Student's t-tests were performed to determine significance relative to *hsp-70* mRNA expression immediately after HS for each strain *(***P* < 0.001). Error bars represent SEM. (B) RT-qPCR of *hsp-70* mRNA levels in WT and *hsp-70ΔTS* animals subjected to HS and allowed to recover on *vector RNAi* or *hsp-70 RNAi* for 24 hrs. All replicates were normalized to *ama-1* mRNA. Three biological replicates were assayed. Student's t-tests were performed to determine significance relative to *hsp-70* mRNA expression immediately after HS for each strain *(*P* < 0.05, ***P* < 0.01). Error bars represent SEM. (C) Following 4 hr of HS at 35°C, WT, *miR-85(n4117)*, and *hsp-70ΔTS* animals were subjected to *GFP* and *dpy-6 RNAi* and percent survival was determined after 24 hrs recovery at 20°C. Three blinded biological replicates were performed with at least 50 worms per strain, per condition. Student's t-tests were performed to determine significance for each strain relative to survival on *vector RNAi* (**P* < 0.05, ***P* < 0.01, ****P* < 0.001). Error bars represent SEM.

3.5 Discussion

Here we demonstrate a novel role for the microRNA pathway in heat shock recovery in *Caenorhabditis elegans.* We identified a specific miRNA, miR-85, that represses the expression of stress response genes, including a HS-induced chaperone, *hsp-70*. We show that animals lacking miR-85 or miR-85 target sites within the 3'UTR of *hsp-70* overexpress *hsp-70* under optimal temperature (20°C) conditions. Upon HS, though, this difference vanishes as the expression of *hsp-70* and other HS-induced genes is robustly activated at the transcriptional level. However, during recovery from HS, the normally rapid decline in *hsp-70* expression falters in the absence of miR-85 or its target sites in the *hsp-70* 3'UTR. Moreover, we present evidence that this over-expression of *hsp-70* during HS recovery reduces organismal viability. Our study reveals the importance of downregulating *hsp-70* levels after HS in an intact animal and highlights the role of miR-85 in facilitating this process.

3.5.1 Role of the miRNA pathway in stress responses

Across animal species, disruption of a single miRNA rarely results in overt phenotypes (Leung and Sharp 2010; Bartel 2018). However, the majority of studies have been performed under well-controlled laboratory conditions that seldom reflect stressful events encountered in the wild. When animals are challenged by bouts of stress, specific miRNAs have emerged as important regulatory tools that add robustness to developmental pathways and promote

survival programs (Emde and Hornstein 2014; Leung and Sharp 2010). One striking example is illustrated by the role of miR-7 in *Drosophila*. Flies lacking this highly conserved miRNA exhibit no apparent abnormalities under controlled laboratory conditions (X. Li and Carthew 2005). However, when miR-7 mutants were subjected to a series of temperature fluctuations eye development failed (X. Li et al. 2009). Examples can also be found in tumor microenvironments, where localized cellular stress can reveal roles for specific miRNAs (Mendell and Olson 2012). In mice, deletion of miR-10a caused no obvious defects until the mutant strain was challenged with a model of intestinal neoplasia (Stadthagen et al. 2013). In this background, the lack of miR-10a was associated with a dramatic increase in the development of adenomas in female mice (Stadthagen et al. 2013). There is also extensive evidence of miRNAs acting as key regulators in plants responding to various forms of stress, again unveiling previously unappreciated functions that are not evident under optimal growth conditions (Song et al. 2019).

In *C. elegans,* few miRNA-associated phenotypes have been identified under normal laboratory conditions (Miska et al. 2007). However, stress-responsive miRNAs have been reported (Schreiner et al. 2019; Brunquell et al. 2017; Nehammer et al. 2015) and a few miRNAs, such as miR-71 and miR-246, have been shown to influence viability after a bout of stress (De Lencastre et al. 2010). Nonetheless, there remains much to learn about the precise roles and targets of miRNAs in *C. elegans*, underscoring the importance of investigating miRNA function under conditions that mimic challenges faced in the natural world.

Here, we demonstrate a role for the miRNA pathway in regulating heat shock survival in *C. elegans.* The auxin-inducible degron system allowed us to deplete ALG-1 during the HS period or immediately following it. This method enabled us to conclude that ALG-1 functions in the HSR, but it is unclear if this role is restricted to the recovery phase or also engaged during high temperatures. The ability of the miRNA complex to regulate gene expression during HS is an open question. In mammalian cell culture, stress can result in modification of Argonaute proteins with poly(ADP-ribose) moieties and accumulation in Stress Granules (SGs), which store translationally inert mRNAs (Leung, Calabrese, and Sharp 2006; Leung et al. 2011). These events are associated with relief of miRNA-mediated repression (Leung et al. 2011). There is also evidence that arsenite stress induces changes in target interactions by Argonaute that, instead, might enhance translational repression (Karginov and Hannon 2013). A further consideration is the general stabilization and translational repression of pre-existing mRNAs upon heat shock, which is a conserved response across organisms including those that lack the miRNA pathway, such as *S. cerevisiae* and *E. coli* (de Nadal and Posas 2011; Morano, Grant, and Moye-Rowley 2012; Henry, Yancey, and Kushner 1992). Thus, the importance of miRNA-mediated gene regulation during elevated temperatures remains an open question.

3.5.2 Post-transcriptional regulation of *hsp-70*

From bacteria to humans, dramatic up-regulation of *hsp-70* is a conserved feature of the heat shock response (Susan Lindquist 1986). While many

organisms also have constitutively expressed *hsp-70* homologs, the stress induced members are distinguished by their rapid induction in response to increased temperature (Bettencourt et al. 2008). The rapid accumulation of *hsp-70* triggered by HS and other stresses is regulated at the transcriptional level by Heat Shock Factor 1 (HSF1) (Vihervaara, Duarte, and Lis 2018). Upon return to non-stress conditions, this transcriptional program ceases and, at least in some organisms, a post-transcriptional mechanism acts to reduce *hsp-70* to near basal levels (Yost, Petersen, and Linquist 1990b). Studies in human and *Drosophila* cells have shown that under control temperatures, *Hsp70* mRNA is relatively unstable (Petersen and Lindquist 1988; N G Theodorakis and Morimoto 1987). During HS though, accelerated decay of *Hsp70* mRNA appears to be suspended, allowing high levels of the transcripts to accumulate for translation. Then, during recovery rapid degradation of *Hsp70* mRNA resumes (Petersen and Lindquist 1988; N G Theodorakis and Morimoto 1987). It has been shown in *Drosophila* cells that degradation of *Hsp70* mRNA is achieved through efficient deadenylation and subsequent 5'-decapping and decay, and, consistent with previous studies, this process appears to arrest during stress and resume during recovery (Bönisch, Temme, and Moritz 2007; Temme et al. 2004). Regulation of *Drosophila Hsp70* mRNA stability is mediated by its 3'UTR (Simcox et al. 1985; Yost, et al. 1990; Bönisch et al. 2007). Inspection of the *hsp-70* 3'UTR sequence revealed well-conserved "instability motifs" (AUUUA), which were hypothesized to contribute to the targeted deadenylation of *Hsp70* (Yost, et al. 1990). However, disruption of these elements in reporters fused to the *Hsp70* 3'UTR did not affect mRNA decay

rates in *Drosophila* cells, leaving the precise destabilizing elements within the 3'UTR yet to be defined (Bönisch et al. 2007).

Our study establishes a regulatory role for the 3'UTR of *hsp-70* in *C. elegans.* Our results are consistent with a model whereby miR-85 binds target sites in the *hsp-70* 3'UTR to promote mRNA degradation (Figure 4E). Despite multiple attempts, we failed to generate precise mutations that only changed the miR-85 target sites in the 3'UTR of *hsp-70,* so it remains possible that additional 3'UTR regulatory elements are disrupted in *hsp-70*Δ*TS* animals. While *hsp-70* mRNA levels were elevated to similar extents in *miR-85* or *hsp-70*Δ*TS* mutants under control temperatures and up to 4 hours into HS recovery, levels were markedly higher at the later recovery time points in animals lacking miR-85 (Figure 4A and E, 16, 24 hr time points). This difference may stem from mis-regulation of other miR-85 targets in the *miR-85* mutant, which could indirectly affect *hsp-70* expression during recovery.

The apparent pause in miRNA-mediated regulation of *hsp-70* during HS in *C. elegans* is reminiscent of the halt in *Hsp70* mRNA decay observed in *Drosophila* and human cells (Petersen and Lindquist 1988; N G Theodorakis and Morimoto 1987). Given the parallels, it is tempting to speculate that the miRNA pathway may also contribute to the post-transcriptional regulation of *Hsp70* in other organisms. As described above, modification and re-localization of

Argonaute during stress could limit the repressive ability of the miRNA complex and potentially release *hsp-70* mRNA for maximal expression. Another mechanism that can override effective regulation by the miRNA pathway is an over-production of target transcripts (Leung and Sharp 2010). As miR-85 levels remain constant during HS, it is possible that the bolus of *hsp-70* mRNA and possibly other miR-85 targets induced by transcription exceeds a threshold where miRNA regulation contributes little to overall target levels.

3.5.3 Down-regulation of *hsp-70* after heat shock is important for survival

We found that maintenance of high *hsp-70* levels after HS negatively impacts survival in *C. elegans.* Since depletion of *hsp-70* mRNA by RNAi could compensate for the lack of miR-85 mediated regulation, overexpression of *hsp-70* is likely the primary cause of reduced viability in *miR-85* or *hsp-70*Δ*TS* mutants subjected to HS. HSP-70 is a molecular chaperone that can promote protein folding, refolding, disaggregation or degradation, activities under high demand in elevated temperatures (Rosenzweig et al. 2019). Given these protective roles, why then would excess HSP-70 be detrimental? This is a longstanding question as previous studies have shown that constitutive overexpression of *Hsp70* in *Drosophila* cells reduced growth rates (Feder et al. 1992) and in mice negatively impacted development and lifespan (Vanhooren et al. 2008). Given its many roles in the cell under normal growth conditions, which include facilitation of protein folding, translocation across membranes, complex assembly and disassembly, and regulation of protein activity and stability (Rosenzweig et al. 2019), there are

several possibilities that could explain the danger of excess Hsp70. The ability of Hsp70 to interact promiscuously with a variety of clients could result in a general disruption in protein homeostasis where deviations in folding or complex formation dynamics lead to aberrant or unstable complexes. High levels of *Hsp70* could also change the stoichiometry with binding partners, such as the transcription factor HSF1 or the endoribonuclease Ire that mediates ER stress, reducing their active concentrations (Masser et al. 2019). There is also evidence that Hsp70 interacts with lipids in mammalian culture cells, and it has been speculated that aberrant membrane interactions could contribute to the cellular toxicity caused by excess Hsp70 (Arispe et al. 2004). While it is yet to be determined how high levels of HSP-70 hinder the ability of *C. elegans* to recover from HS, this finding highlights the importance of down-regulating *hsp-70* expression post stress.

Curiously, the overexpression of *hsp-70* observed in *miR-85* or *hsp-70*Δ*TS* mutants was not associated with developmental abnormalities in animals cultured at ambient temperatures. Despite the up-regulation of several stress-related genes in the *miR-85* mutants, the development of these animals is indistinguishable from wildtype. These observations suggest that under ideal conditions the robustness of developmental programs is not dependent on regulation by miR-85. However, this miRNA was found to be required for recovery from HS. Thus, environmental perturbations likely to be encountered in the wild, such as elevated temperatures, can reveal roles for miRNA genes previously considered to be dispensable. Furthermore, our study demonstrates that repression of *hsp-70* by miR-85

promotes survival after HS in *C. elegans,* establishing an important role for the miRNA pathway in post stress gene regulation.

3.6 Materials and Methods

3.6.1 Nematode culture and heat shock viability assays

C. elegans strains were cultured under standard conditions and synchronized by hypochlorite treatment (Wood 1988). Heat shock viability assays were performed by plating synchronized L1 worms on NGM plates seeded with OP50 that were grown for 44 hrs at 20°C before raising the temperature to 35°C for 4 hrs of heat shock. Worms were recovered for 24hrs at 20°C before scoring. When possible, assays were blinded before synchronization and were unblinded only after scoring viability. *Alg-1(gk214)* worms were grown for 47 hrs before HS treatment as they are slightly developmentally delayed (Zisoulis et al. 2012). The auxin-induced degradation was performed by moving worms to NGM plates supplemented with 1% auxin (indole-3-acetic acid) during either 4 hrs of HS or 24 hrs of recovery.

3.6.2 Western blotting

Western blotting was performed as previously described using mouse monoclonal antibodies against Tubulin or FLAG (Sigma), and rabbit polyclonal antibodies against Hsp70 (Proteintech) (Zisoulis et al. 2010; Van Wynsberghe et al. 2011).

3.6.3 RT-qPCR

Quantitative RT-PCR analyses of mRNA (SYBR Green) levels were performed according to manufacturer's instructions using the QuantSudio machine (ABI Biosystems). The housekeeping gene, *ama-1*, was used for normalization of experimental Ct values. Three biological replicates were performed with three technical replicates for each target gene.

Primer sequences:

ama-1 Forward*: 5'* – CACTGGAGTTGTCCCAATTCTTG – 3'

ama-1 Reverse*: 5'* – TGGAACTCTGGAGTCACACC – 3'

hsp-70 Forward*: 5'* – CCGCTCGTAATCCGGAGAATACAG – 3'

hsp-70 Reverse*: 5'* – CAACCTCAACAACGGGCTTTCC – 3'

3.6.4 Northern blotting

PAGE northern blotting was performed as previously described using IDT StarFire probes for cel-mir-85-3p and let-7 (Van Wynsberghe et al. 2011).

let-7: AACTATACAACCTACTACCTCA/3StarFire/

mir-85-3p: GCACGACTTTTCAAATACTTTGTA/3StarFire/

3.6.5 RNA Sequencing

N2 wildtype (WT) and *mir-85(n4117)* worms were grown to L4 stage (47 hrs at 20°C) under standard growth conditions (Wood 1988).The experimental group was subjected to heat stress by raising the temperature to 35°C for 3 hrs after

developing for 44 hrs at 20°C. Animals from the control (20°C) and experimental group (35°C) were collected, snap-frozen and total RNA was isolated using a standard TRIzol (Life Technologies) RNA extraction. cDNA sequencing libraries from three independent biological replicates were prepared from 1ug of total RNA using the standard protocol from the Illumina Stranded TruSeq RNA library prep kit. Ribosomal RNA was removed prior to library preparation using RiboZero Gold (Illumina). cDNA libraries were sequenced on the Illumina High Seq 4000 (SR75). Reads were aligned to the *C. elegans* genome WS235 using STAR (Dobin et al. 2013). Aligned reads were sorted using Samtools (H. Li et al. 2020). Reads were counted using FeatureCounts and Ensembl 88 gene annotations (Liao et al. 2014). Differential gene expression was determined using DESeq2 with default parameters (Love, Huber, and Anders 2014). Classes of genes were filtered, and protein-coding genes (PCGs) were used for downstream analysis. PCGs with +/- 1 Log2FC, a basemean of at least 10, and adjusted p-value of < 0.05 were considered significantly mis-regulated. Volcano plots were generated using EnhancedVolcano (Blighe, Rana, and Lewis 2020).

3.6.6 RNAi

Feeding RNAi was performed as previously described (De-Souza et al. 2019). Briefly, animals were moved to either empty vector or *hsp-70* RNAi plates during a 24 hr recovery period at 20°C after a 3 hr HS for RT-qPCR analysis or after 4 hr HS for viability experiments. RNAi plates were supplemented with 1mM

of IPTG, tetracycline (12.5ug/ml), and ampicillin (100ug/ml). RNAi bacteria was grown for 16 hrs at 37°C and concentrated 10x before adding to plates.

3.6.7 Strains

The following strains were used in this study: wild type (WT) N2 Bristol, QK155 (AID::ALG-1): *alg-1(xk20 | 4xflag::degron::alg-1) X; ieSi57 II* (a gift from the John Kim Lab), VC446 *alg-1(gk214),* RB574 *alg-2(ok304),* PQ629 *mir-85(n4117)*. PQ610 *mir-85(ap437)* and PQ659 *hsp-70ΔTS(ap443)* strains were generated using CRISPR/Cas9 with a single guide RNA and back-crossed to N2 three times. The miR-85 rescue strain PQ602 was made by crossing *mir-85(n4117)* to COP2068: knuSi832[pNU2156 (mir-85 in cxTi10882, unc-119(+))] IV; unc-119(ed3) III), using a MosSCi integration system (Nemametrix) (Philip et al. 2019).

3.7 Acknowledgements

We thank Dr. Cindy Voisine and members of the Pasquinelli Lab for helpful discussions and critical reading of the manuscript. This work was supported by the National Institutes of Health [R35 GM127012 to A.E.P, T32 GM007240 to D.C.P.] and a University of California, San Diego, Eureka Scholarship to D.M.B. Sequencing of libraries used for RNA-seq analyses was conducted at the IGM Genomics Center, University of California, San Diego, CA. Some strains were provided by the CGC, which is funded by the NIH Office of Research Infrastructure Programs (P40 OD010440).

Chapter 3, in full, is a reprint of material as it occurs in *PLoS Genetics*, "Recovery After Heat Shock Requires the microRNA Pathway in *Caenorhabditis elegans.*" Pagliuso, D.P., Bodas, D.M., and Pasquinelli A.E., PLOS, 2021. I was the primary author.

Chapter 4

Investigating the Role of miR-85 in *Caenorhabditis elegans* Fertility

4.1 Introduction

MicroRNAs have been predicted to target over 50% of the mammalian transcriptome (Friedman et al. 2009). However, most miRNAs have been found to be individually dispensable. In *C. elegans,* there are only a few miRNAs that have been assigned specific targets and functions (Miska et al. 2007). Looking for clues during critical transitions through development and in the context of stress has helped reveal previously unappreciated roles for miRNAs. One explanation for this could lie in the rapid and precise changes in gene expression that control developmental transitions or are stimulated in response to stress. Let-7, for example, has been characterized as a regulator of developmental timing of *C. elegans* and miR-85, as discussed in Chapter 3, has been identified to regulate the heat shock response (Reinhart et al., Pagliuso).

In addition to the decreased heat shock viability in loss of function *miR-85* animals, we also identified that these animals have a significant reduction in brood size. In this chapter, I will discuss ongoing work to identify how miR-85 regulates fertility in *C. elegans*. In conjunction, I will present evidence suggesting heterochronic regulation of miR-85 and speculate about how this might connect to the fertility defect we observe in loss of function *miR-85* animals. Furthermore,

I will highlight efforts to distinguish between oocyte and sperm defects as they pertain to fertility. Then, I will address and how our attempts to identify roles for specific miR-85 targets in regulation of brood size through RNAi experiments revealed new avenues in potential cross talk between small RNA pathways. Finally, I will report on our attempts to connect heat shock and fertility phenotypes in *miR-85* mutants and present data to suggest that unlike HS, miR-85 is likely operating independently of *hsp-70* in the context of fertility.

4.2 miR-85 Is Developmentally Regulated

Our original interest in miR-85 stems from an intriguing finding from our small RNA Sequencing during heat shock as reported in Schreiner et al. In that study, early stage L4 staged animals were either grown at control temperatures of 20°C or subjected to a 6 hr heat shock at 35°C and immediately collected for RNA expression analysis. miR-85 was the most downregulated miRNA during heat shock compared to control temperatures and this potential thermoregulation prompted our further investigation (Schreiner et al. 2019). However, when I used TaqMan RT-qPCR, Northern Blots, and small RNA sequencing after 4 hrs of heat shock in L4 animals, I observed no change in expression in control versus heat shock, suggesting this miRNA was not thermoregulated. Instead, it seemed that the most likely explanation could be developmental regulation due to the differences in protocols. Schreiner and colleagues grew animals for 40 hours before performing HS and I grew animals for 44 hours before HS. Heat shock is

known to substantially disrupt major transcriptional changes that occur during L4 development, inducing developmental delay as the animal shifts almost solely to responding to heat stress. In fact, HS of early versus late L4 animals can cause up to a 3-fold difference in viability (Zevian and Yanowitz 2014). Furthermore, the transcriptional program of the HSR during HS is shown to be very dynamic until 3-4 hours of constant elevated temperatures where changes then plateau and are sustained after this time (Jovic et al. 2017). Given this, I began to carefully assess the possibility of developmental regulation of miR-85.

Previously, researchers reported that miR-85 may be regulated by a heterochronic protein, LIN-28 (Tsialikas et al. 2017). LIN-28 is a conserved RNA binding protein that controls stem cell lineages and inhibits *let-7* miRNA processing in mammals (Rybak et al. 2008). In *C. elegans,* LIN-28 expression is controlled through negative regulation by miRNAs including lin-4 and let-7 family members and activation by the transcription factor, LIN-14. The careful orchestration of its expression is imperative to timing the L2 cell fate progression within the heterochronic pathway of *C. elegans* development (Tsialikas et al. 2017). Loss of *lin-28* results in misexpression of *let-7,* a developmentally regulated miRNA, which is typically expressed in late larval stages. However, *lin-28* mutants start expressing *let-7* in L2, which promotes precocious expression of adult fates (Reinhart et al. 2000). Additionally, *lin-28* mutants expressed miR-85 in the L2 stage whereas WT animals did not express miR-85 until late L3 (Lehrbach et al. 2009). In WT L2 worms, the pre-miR-85 sequence is detectable by Northern blot but the mature form is not detectable until late L3. However, L2

stage *lin-28* mutants produced both the pre-miR-85 and mature miR-85 at detectible levels. While it remains to be directly tested, these findings suggest that miR-85 may be developmentally regulated post-transcriptionally by LIN-28. Though, researchers speculated that miR-85 regulation by LIN-28 is likely indirect, and is perhaps an outcome of alternative temporal cues (Lehrbach et al. 2009). This hypothesis is supported by the reciprocal phenotype of increased heat shock tolerance observed in *lin-28* mutants (V Ambros and Horvitz 1984) . While the connection between developmental timing and regulation by LIN-28 is not clear, the fact that miR-85 may be developmentally regulated could explain the differential expression patterns captured in our heat shock experiments. Delayed development in heat shock could slow the upregulation of miR-85 expression, making it appear down-regulated in heat shock samples.

I analyzed the expression of miR-85 through a developmental time course and observed that only after 40 hours of growth at 20°C (L4) could I detect the mature miR-85 sequence by Northern Blot, with the greatest accumulation of miR-85 detected at 47 hours (Figure 4.1A). The expression persisted into early adulthood at 72 hrs of development. We also used a miR-85 promoter driven GFP strain [*mir-85p::GFP + unc-119(+)*] to track expression of miR-85 in CTL and HS conditions. The reporter expressed GFP throughout the uterus, spermatheca, and gonad, consistent with previous reports (Figure 4.2B). However, these findings should be investigated further, as the reporter only includes 2kb of the promoter region upstream of miR-85 and could be missing additional regulatory elements. Despite this, the same promoter sequence was

used to generate the MoSCi rescue construct which successfully restored heat shock viability of the *miR-85(n4117)* mutant. It's difficult to imagine that miR-85 only acts on genes in gonad, spermatheca, and uterus due to its important regulation of the HSR through *hsp-70* which is expressed ubiquitously (Das et al. 2020; Zeng et al. 2004). It's possible that heat shock re-routes miR-85 expression to other tissues. However, the promoter fused GFP reporter did not reveal changes in tissue expression upon HS.

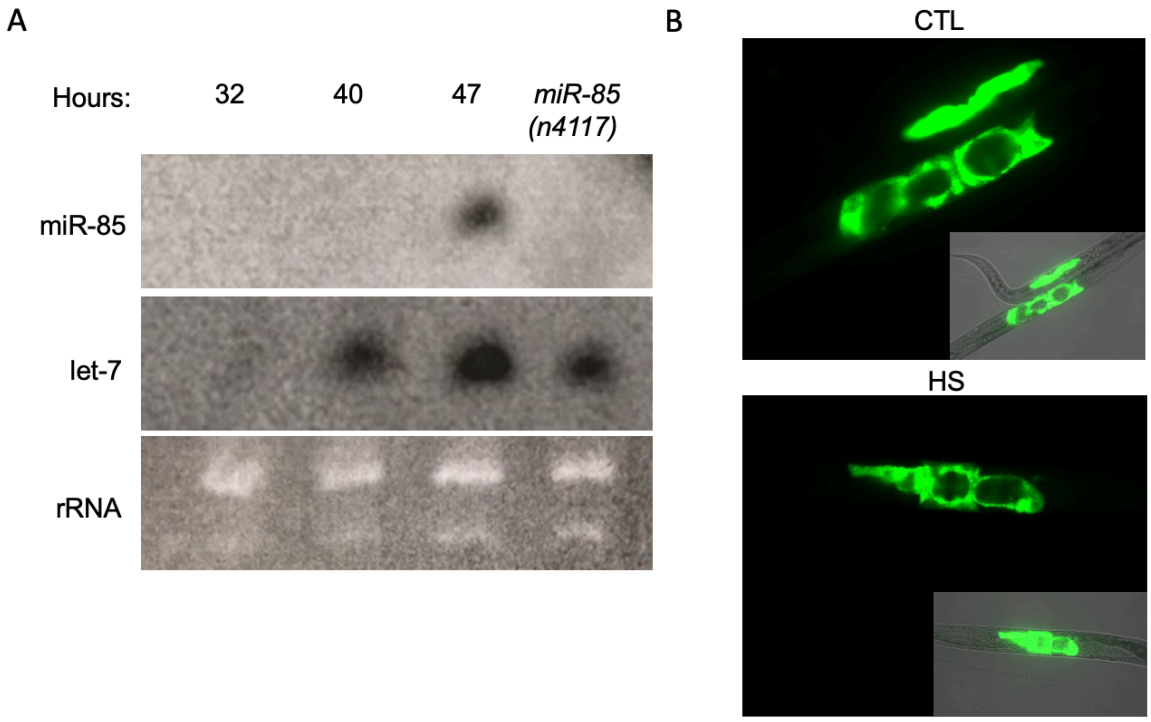


Figure 4.1: Temporal and Spatial Expression of miR-85. (A) Northern blot of miR-85 and let-7 after 32, 40, and 47 hrs of development at 20°C from L1. RNA from *miR-85(n4117)* animals collected at 47hrs loaded into the last lane. Ribosomal RNA was used as a loading control. (B) Microscopy of developmentally synchronized *mir-85p::GFP* + *unc-119(+)* animals in CTL (20°C) and after 3 hrs HS (35°C) imaged at 40X.

4.3 miR-85 Regulates Fertility in *C. elegans*

After identifying the reduced viability after heat shock, we asked if *miR-85* animals had any other phenotypes. We tested for brood size using *miR-85(n4117)* and WT animals at 20°C and observed a reproducible decrease in fertility. However, given the nature of the deletion in the *mir-85(n4117)* animals, we questioned if the phenotype was due to loss of *miR-85* or potentially influenced by disruption to the host PCG (F49E12.14). As described in Chapter 3, we also tested *miR-85(n4117);miR-85+,* a strain that utilizes a miR-85 transgene to add back a functional copy of *miR-85* to the original mutant. When we tested all three strains, we saw that *miR-85(n4117);miR-85+* rescued the brood size phenotype to WT levels (Figure 4.2A). These findings provided strong evidence to support a role for miR-85 in the regulation of *C. elegans'* brood size.

Upon determining that *miR-85* animals have low brood sizes, we set out to identify additional clues that might help us discern miR-85's role in fertility. First, we wondered if the timing of germline development differed from WT, perhaps a related phenotype to the developmental regulation of *miR-85.* In *C.* elegans, the germline develops in an assembly line fashion where the most distal germ cells remain in mitosis, the middle enter meiosis, and proximal cells begin to differentiate into sperm and finally oocytes (Ellis and Kimble 2007). We tracked the progression of cells through these phases compared to WT and did not see any differences in the migration of distal tip cells (DTC) or gonadal outgrowth. Overall, the gonad appeared to be of similar size to WT and did not

present overt defects in germline development. However, closer analysis of the timing to oogenesis revealed that *miR-85(n4117)* developed oocytes slightly earlier than WT (~ 3hrs earlier). MicroRNA Argonautes ALG-1 and ALG-2 have been attributed to the maintenance of germ cell progression from spermatogenesis to oogenesis, consistent with miRNA mediated regulation of this timing (Bukhari et al. 2012). Additionally, the overall fecundity of hermaphroditic *C. elegans* is limited by the number of sperm produced prior to the transition to oocyte production. It's possible that *miR-85(n4117)* produce fewer sperm because of the accelerated transition to oocyte development and that this might explain the reduced brood size (Ellis and Stanfield 2014). Nevertheless, these observations are preliminary, and more work should be done to assess the contributions of miR-85 to germline development and timing, perhaps in conjunction with the *miR-85(n4117);miR-85*+ strain.

In addition to the accelerated transition from spermatogenesis to oogenesis, we also observed that *miR-85(n4117)* laid eggs about 4-5 hours earlier than WT. They were also more prone to laying unfertilized oocytes. As the appearance and number of oocytes appeared similar to WT, this could suggest a role for miR-85 in fertilization. We were eager to discern whether miR-85 regulated proper fertilization through spermatogenesis and how defects in this process might explain the brood size phenotype. To test if the loss of function *miR-85(n4117)* animals had a sperm defect, we outcrossed *miR-85(n4117)* males to WT hermaphrodites and then compared the outcrossed progeny to the brood size of WT males mated to WT hermaphrodites. We classified successful

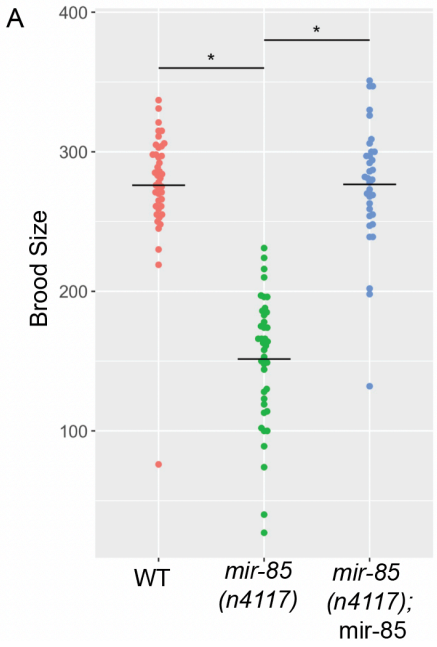
matings as those producing at least 50% male progeny. If progeny sizes were equal in animals mated with WT males or *miR-85(n4117)* males, it would suggest that *miR-85(n4117)* sperm is not contributing to the reduced brood size. However, WT hermaphrodites mated to *miR-85(n4117)* males had fewer progeny than animals mated to WT males (Figure 4.2B). These findings support a model where miR-85 helps maintain spermatogenesis and that loss of this miRNA either reduces the number of sperm available or the sperm are defective in some way that makes them unable to efficiently fertilize oocytes.

A surprising discovery, however, was in a complementary study where *miR-85(n4117)* hermaphrodites were crossed to WT males or *miR-85(n4117)* males. In this scenario, we can assess the contribution of oocytes to fertility. We also observed that WT males crossed to *miR-85(n4117)* hermaphrodites had a lower brood size, suggesting that miR-85 is also affecting fertility through regulation of oocytes. If miR-85 was solely acting through spermatogenesis, we would have expected to see a rescue of the brood size when crossed to WT males. Overall, our findings from these outcrosses indicate that miR-85 may be necessary for the formation and maintenance of gametes and that both the sperm and oocytes of *miR-85(n4117)* animals affect brood size.

Finally, we were intrigued by the possibility that miR-85 targeting of *hsp-70* regulated brood size as well as heat shock recovery. We tested the brood size of the *hsp-70*Δ*TS* mutant at 20°C which removes miR-85 target sites within the 3'UTR of *hsp-70.* However, we observed no defect in brood size suggesting that *hsp-70* misregulation in *miR-85(n4117)* is unlikely to explain the brood size

phenotype. miRNAs can target and repress hundreds of mRNAs and it is likely that miR-85 is acting through other targets to affect fertility.

A



B

		Male		
		Self	N2	mir85
Hermaphrodite	N2	262.9 ± 17.20	918.82 ± 27.80	552.29 ± 32.37
	mir85	148.0 ± 19.93	71.93 ± 16.62	107.33 ± 26.21

Mann-Whitney U test p values	Group 1	Group 2	p-value
	N2	N2 x N2	5.67E-06
	N2	N2 x mir85	1.02E-06
	mir85	mir85 x N2	0.01069
	mir85	mir85 x mir85	0.1285

Figure 4.2: Fertility Defects of *miR-85(n4117)* Animals. (A) Total brood size of animals at 20°C of three independent biological replicates (n=10). *miR-85(n4117)* had a significantly reduced brood size compared to WT of *miR-85(n4117);miR-85* animals (*P < 0.05, student's t-test, two-tailed). (B) Average brood size of outcrossed *miR-85(n4117)* and WT animals. Three independent biological replicates were performed (n=5, per replicate).

4.4 miR-85 May Regulate the RNAi Pathway

Our next aim was to identify a specific miR-85 target that could explain the decreased brood size phenotype. We screened through predicted targets of miR-85 from TargetScanWorm, prioritizing the 133 well-conserved targets, and further narrowing this list by ranking genes with known functions in development or fertility. We tested if RNAi knock down of any of these targets could rescue

the *miR-85(n4117)* brood size phenotype back to WT levels. First, we asked if *hsp-70(RNAi)* could rescue the brood size phenotype as we had previously established this gene to be regulated by mir-85 and knock down of *hsp-70* during recovery after HS rescued viability. While we did observe a partial rescue of brood size by *hsp-70(RNAi)*, this appeared to be non-specific. Control *RNAi* treatments such as *GFP(RNAi)* also rescued brood size in *miR-85(n4117)* animals. In addition, almost all other target genes tested, including *lin-29* and *cog-1* showed a full or partial rescue of brood size in *miR-85(n4117)* animals (Figure 4.3). We also tested *xrn-2(RNAi),* a 5' to 3' exonuclease known to target miR-85 for degradation, which partially rescued brood size (Chatterjee and Großhans 2009). Given the non-specific RNAi rescue, we did not feel confident this method would help us determine a specific gene miR-85 acts through to regulate fertility.

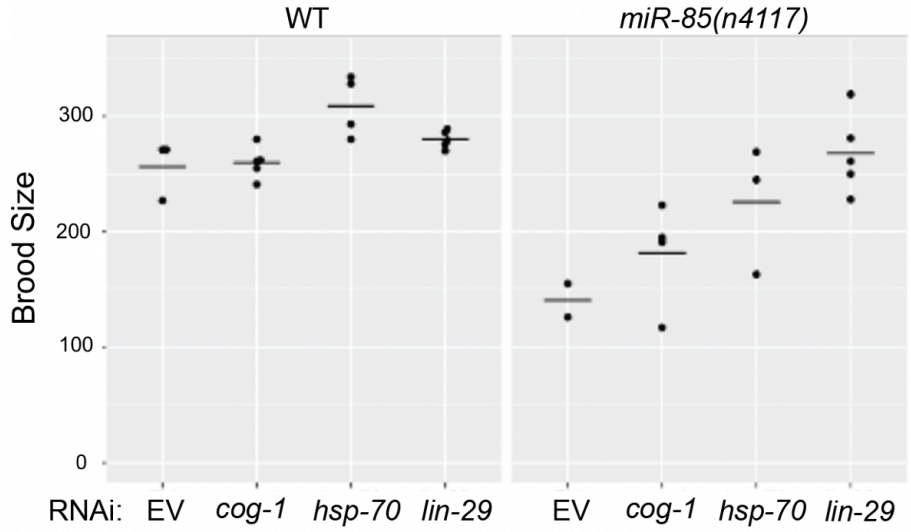


Figure 4.3: Non-specific Rescue of *miR-85(n4117)* Fertility by RNAi. Brood size of WT and *miR-85(n4117)* animals moved to *vector (EV), cog-1, hsp-70,* and *lin-29* RNAi. Representative image of one biological replicate (n=5) at 20°C.

The non-specific RNAi rescue of brood size elicited further questions regarding miR-85 and fertility. In *C. elegans,* RNAi utilizes small interfering RNAs (siRNAs) to silence specific genes. siRNAs can originate from exogenous (exo-RNAi) or endogenous (endo-RNAi) sources of double stranded RNA (dsRNA). Since RNAs from exogenous and endogenous sources require overlapping machinery to exert their regulatory roles, there can be competition between these pathways (Ketting et al. 2001). As such, repression of one source can enhance the silencing directed by the other (De-Souza et al. 2019). Recently, miRNAs have been identified to regulate the RNAi pathway in *C. elegans.* When the miRNA family miR-35-41 is deleted, there is enhanced RNAi by exogenous RNAs and reduced silencing of endogenous RNAi targets (Massirer et al. 2012). It's possible that miR-85 exerts similar regulation on the RNAi pathway as miR-35-41 which showed non-specific exo-RNAi reduced endo-RNAi activity in the mutant background (Massirer et al. 2012). In turn, targets normally repressed by endo-siRNA could be upregulated and contribute to the non-specific rescue of brood size. To further assess this possibility, it would be interesting to cross *miR-85(n4117)* to mutants of core RNAi factors such as *rde-1*, *rde-4*, and *rrf-1* and assess RNAi sensitivity. Double mutants could be evaluated for brood size or sensitivity to exo-RNAi such as *unc-22* which leads to varying degrees of twitching phenotypes depending on the penetrance (Massirer et al. 2012).

Finally, we wondered if the potential defect in RNAi could be inherited in *miR-85* animals. Double stranded RNA-mediated gene silencing can be epigenetically inherited for more than five generations (Fire et al. 1998). Defects

in heritable RNAi machinery, including HRDE-1, an Argonaute protein that associates with small interfering RNAs in the germ cells of the progeny of animals exposed to dsRNA to promote RNAi inheritance, can lead to loss of heritable gene silencing over generations (Spracklin et al. 2017). This causes abnormal gamete formation and ultimately, sterility. Multigenerational RNAi inheritance is required to transmit epigenetic information through generations and promote germline immortality of the germ-cell lineage. At 25°C, *hrde-1* mutants produce progressively fewer offspring until they reach sterility as an outcome of germline mortality (Buckley et al. 2012). We asked if *miR-85(n4117)* mutants exhibited a similar mortal germline (*Mrt*) phenotype.

WT and *miR-85(n4117)* animals were moved from 20°C to 25°C and then maintained for seven subsequent generations at 25°C. If the *miR-85(n4117)* animals had a mortal germline (*Mrt)*, we would expect animals to go sterile (<50 progeny) within seven generations while WT would produce a consistent number of progeny over time. Initial studies suggested *miR-85(n4117)* animals were *Mrt* as they appeared to go sterile after three generations. However, this seemed to reflect variability in these mutants and was likely due to the small sample size originally assessed. Subsequent studies with larger sample sizes (n=10) did not consistently result in *Mrt* phenotypes. While deficient heritable RNAi does not appear to cause sterility in *miR-85(n4117),* it does not rule out possible defects in RNAi pathways or hypersensitivity to RNAi. Since *GFP(RNAi)* partially rescued *mir-85(n4117)* brood size, we speculate that dsRNA may be redirecting or titrating shared endo- and exo-RNAi factors, leading to misregulation of endo-

siRNA pathways. Testing whether RNAi sensitivity is specific to the germline or soma may provide further insights into aberrant behavior of endo-siRNA pathways. Crossing *miR-85(n4117)* to *rrf-1,* a germline RNAi sensitive strain, and *pww-1*, a soma sensitive RNAi strain, could be a valuable method to assess if RNAi sensitivity is germline specific. If so, we would expect no defects with *GFP(RNAi)* in the *miR-85(n4117); ppw-1* cross but see a similar non-specific RNAi rescue of brood size in *miR-85(n4117);rrf-1*. Ultimately, performing small RNA sequencing using a protocol adapted to capture total small RNAs may provide further insight about specific classes of small RNAs that are misregulated in the *miR-85* background and suggest further functional follow up (Reed et al. 2020).

4.5 Discussion

We identified that *miR-85* is developmentally expressed and regulates fertility in *C. elegans.* The mechanism as to how miR-85 exerts its regulatory roles to affect fertility remain unclear. However, the broad roles in fertility and heat shock recovery suggest that, although miR-85 is not conserved beyond *Caenorhabditis,* higher order eukaryotes may have miRNAs with similar functions to that of miR-85 in *C. elegans.* Long considered transcriptional noise or part of junk DNA, miRNAs are clearly responsible for important functions and continue to deepen our understanding of molecular biology.

Given the temporal regulation of miR-85 expression during larval development, we hypothesized that miR-85 may control temporal gene expression, contributing to the predictable pattern of germline development, gametogenesis and consequently, fertility. However, our phenotypic analysis has yet to unveil specific molecular targets of miR-85 and more work needs to be done to understand how exactly miR-85 regulates brood size. Total RNA sequencing at 20°C in *miR-85(n4117)* and WT (N2) animals revealed very few changes in gene expression, as described in Chapter 3. *miR-85(n4117)* mutants do not have overt developmental differences compared to WT animals. Despite extensive efforts to look for clues in the sets of up and down-regulated genes in our RNA sequencing results, we did not observe a significant enrichment for genes involved in germline development or small RNA pathways. However, the small number of differentially expressed genes appears to be somewhat limiting for this analysis. Moreover, assessment of conserved (n=133) and non-conserved (n=4,329) targets of miR-85 from TargetScanWorm did not reveal any enrichment for genes involved in fertility phenotypes (Jan et al. 2011). Careful consideration of genes that are repressed at the time miR-85 is upregulated in the early L4 stage of development may be worth investigating as potential targets of miR-85.

Determining how miR-85 is regulated and where it is expressed could also inform future directions. Mature miR-85 was expressed earlier in development in *lin-28* animals compared to WT, suggesting that *lin-28* may directly or indirectly regulate miR-85 expression (Lehrbach et al. 2009). However, it's possible that the

pre-miRNA sequence is post-transcriptionally modified or bound by another factor that is expressed at least during L3 and early L4, perhaps blocking processing by Dicer. Although, we determined that miR-85 is unlikely to have modifications to its 3' or 5' ends through beta-elimination assays and poly A polymerase (PAP) treatments which can be resolved by PAGE Northern. Another possibility is that miR-85 could be expressed in a tissue specific manner and the spatial expression could change during development of heat shock. Examining the cellular and tissue-specific localization may help address many unanswered questions about miR-85 and developing new tools to do so would be of great value. While it's entirely possible that the promoter-fused GFP strain accurately depicts miR-85 expression, introducing a GFP sequence at the endogenous mir-85 locus may reveal additional tissues in which miR-85 is expressed and would likely provide the clearest read out of miR-85 expression.

Loss of *miR-85* results in slightly accelerated timing of gamete production which may explain, in part, the defects in fertility. The germline develops in a predictable pattern where meiosis begins 33 hrs after hatching, spermatogenesis commences after 42 hrs and the transition to oogenesis begins after 48 hrs (Ellis and Kimble 2007). Mature miR-85 is most abundant at 44-47 hrs after hatching, coinciding with the transition to oogenesis. Loss of *miR-85* results in an accelerated transition to oocyte production, perhaps truncating the typical window dedicated to sperm production. The transition from sperm to oocyte production is governed by the somatic sex differentiation pathway and genes involved in this pathway such as *sdc-1,* which controls X-chromosome dosage, and *fem-3,* which

is required for male development, merit further investigation in the *mir-85* background. Furthermore, crossing *miR-85(n4117)* to germline development reporters may clarify the specific differences in developmental timing.

Finally, assessing the contributions of tissue specific RNAi sensitivity may provide insight as to why non-specific RNAi rescues brood size but does not rescue heat shock viability. Only introduction of *hsp-70(RNAi)* during recovery after heat stress rescued viability in *miR-85* mutants while *vector, GFP,* and *dpy-10(RNAi)* failed to rescue viability. Still, it is possible the fertility and heat shock phenotypes are related. The *C. elegans* germline is inherently sensitive to elevated temperatures (Spike et al. 2008). Arrested ovulation and damage to sperm can affect fertility after exposure to high temperatures (Aprison and Ruvinsky 2014). Given the widespread misregulation of stress-response genes in *miR-85(n4117)* under controlled temperatures (20°C), it's possible that, at a molecular level, these mutants are under chronic stress, and this could negatively impact processes that regulate fertility. However, our preliminary analysis suggests that this connection is unlikely to be solely mediated by *hsp-70* misregulation. Further investigation of germline morphology and potential involvement of RNAi factors is warranted in the search for greater mechanistic insight into miR-85 regulation of *C. elegans*' fertility.

4.6 Acknowledgements

Chapter 4 contains unpublished material coauthored with Bodas, D.M. and Pasquinelli, A.E. I was the primary researcher and author of this material.

Chapter 5

Conclusions

Since the discovery of the heat shock response, over fifty years ago, the transcriptional induction of protein coding genes has been of great interest. However, work in our lab and others now provide evidence to suggest that non-coding RNAs are also important for the HSR and may have essential roles in promoting survival upon a stressful encounter. In addition, we distinguish a subset of heat shock induced non-coding RNAs that are under the direct transcriptional regulation as HSF-1, the canonical coordinator of the HSR. In addition to lincRNAs, repeat-derived RNAs, and pseudogene-derived RNAs, we also show that a subset of miRNAs are differentially expressed in response to stress and that miR-239 is directly regulated by HSF-1, broadening the repertoire of HSF-1 targets to include non-coding RNAs. Nevertheless, despite the dramatic upregulation of miR-239, it remains unclear as to how, or if, this miRNA contributes to the HSR.

The HSR is a transient reprogramming of cellular processes that shifts the bulk of cellular energy towards producing heat shock proteins. Despite the short-lived change in response to stress, little is known as to how an organism resets its cellular processes after the stress is ameliorated. Here we identify a role for a microRNA, miR-85, in resetting of the molecular landscape after stress in *C.*

elegans. We identified that animals lacking *miR-85* maintain high levels of *hsp-70* after heat shock which is detrimental to their viability. However, knock down of *hsp-70* restores heat shock viability back to WT levels. These results elicit additional questions for future investigation. Here I will speculate as to why high levels of *hsp-70* after heat shock may be detrimental to an organism, discuss why animals might fail to survive stress, and suggest future directions for this field including the exploration of stress as it relates to fecundity.

5.1 Hsp70 Regulation

Hsp70 is quickly upregulated in response to stress across all organisms in which it has been studied. In *Drosophila* and now*,* in *C. elegans,* we show that Hsp-70 is subject to rapid repression after stress. This regulation after stress is critical for viability in *C. elegans* yet the mechanisms for posttranscriptional regulation remain unclear. Studies in mammalian and *Drosophila* cells suggest that a mechanism for selective degradation of *hsp70* mRNA limits the translation of HSP70 protein and control of *hsp70* mRNA stability is the most likely the defining factor of cellular HSP70 levels (Balakrishnan and De Maio 2006). Additionally, previous studies suggest that HSP70 protein levels influence mRNA levels through a feedback loop (DiDomenico et al.). In *Drosophila,* inhibition of HSP70 protein synthesis resulted in the stabilization and accumulation of *hsp70* messages. Only when a threshold level of HSP70 was restored after the inhibition were heat shock transcripts destabilized (DiDomenico et al.). This is corroborated

by the fact that *hsp70* mRNA has a relatively short half-life in cells recovering after stress and that the half-life is further reduced in cells already containing HSP70 protein (N G Theodorakis and Morimoto 1987; Nicholas G Theodorakis, Drujan, and De Maio 1999). In *C. elegans,* our work suggests that miR-85 targets and represses *hsp-70* mRNA, however, it would be interesting to assess how HSP-70 protein accumulation influences *hsp-70* mRNA levels after heat stress. For example, HSP-70 could be tagged with an Auxin-Inducible Degron (AID) and be degraded after heat shock (L. Zhang et al. 2015). The levels of *hsp-70* mRNA could be quantified with and without HSP-70 protein by RT-qPCR.

Generating an AID::HSP-70 strain would also be helpful for understanding the broader influence of HSP-70 levels on heat shock recovery. Degradation of HSP-70 after HS would allow further analysis of its contributions to an animal's viability, and processes of transcription and translation which are known to be globally repressed during HS (Lindquist). Based on our findings that RNAi knockdown of *hsp-70* during HS recovery rescues the viability of *miR-85* animals, we might anticipate that degradation of HSP-70 using the AID system would have a similar outcome. However, it's also possible that degradation of HSP-70 protein may not rescue the viability phenotype, suggesting that high levels of *hsp-70* mRNA are problematic for the organism after stress. High levels of *hsp-70* mRNA could monopolize available translational machinery, making it difficult to restore translation of other existing transcripts (Shalgi et al. 2013). Previous studies support a model in which HSP70 levels in flies dictates the restoration of normal transcription and translation after heat stress and using this tool, we could assess

this model in *C. elegans* (DiDomenico et al.). Through polysome fractionation, we could also study the distribution of ribosomes and assess global changes in translation with and without HSP-70 after heat shock in WT and *miR-85* animals. Similarly, we could assess the contributions of HSP-70 overexpression in *miR-85* and the *hsp-70ΔTS* mutant during HS recovery on the transcriptome through RNA sequencing. One possibility is that high levels of HSP-70 may inhibit HSF-1 post HS, leading to a global decline in new heat shock transcripts (Abravaya et al. 1992). By degrading HSP-70 with the AID system after HS, we could determine how heat shock transcripts change in response to HSP-70 and begin to untangle the web of interactions between chaperones.

In animals lacking miR-85, prolonged upregulation of *hsp-70* mRNA and protein were observed after HS. However, it remains unclear if transcription of new *hsp-70* messages continues through recovery to contribute to these high mRNA and protein levels or if the failure to degrade existing transcripts in the *miR-85* mutant is sufficient to generate such high levels of protein. One experiment to begin to test this could involve knocking down *hsf-1* during HS recovery, which would likely reduce additional *hsp-70* transcription. If no change in HSP-70 protein is observed, this might suggest that existing transcripts are the predominant contributors to sustained HSP-70 levels. Testing for changes in pre-mRNA and mature mRNA levels of *hsp-70* by RT-qPCR may also be informative.

Development of additional tools to study the regulation of HSP-70 after heat shock may help address questions around the spatial and temporal regulation of this chaperone. Generating an endogenously GFP-tagged HSP-70 strain will

allow for improved quantification of HSP-70 protein levels after HS. Given that the HSP-70 antibody likely detects multiple isoforms, it is difficult to distinguish between constitutively expressed isoforms and the heat-shock induced form through western blotting. Using a GFP antibody could resolve this limitation. Additionally, this would provide information about the tissue-specific regulation *of hsp-70* by miR-85 if the GFP-tagged HSP-70 strain were crossed to *miR-85(n4117).* Finally, this strain could be used for microscopy after HS to assess the distribution of HSP-70. One possibility is that excess HSP-70 forms harmful self-aggregates which may be unable to perform usual chaperone activity. Furthermore, a GFP antibody could be utilized for capturing HSP-70 in complex with client proteins. Mass spectrometry analysis of proteins associated with HSP-70 after HS in the *miR-85(n4117)* background could inform next steps and provide insight into why an abundance of HSP-70 is so detrimental.

While our model of post-transcriptional regulation of *hsp-70* agrees with previously proposed models in *Drosophila* cells (Temme et al. 2007; Yost et al. 1990), alternative or additional modes of *hsp-70* regulation are possible. For example, in mammalian cells, HSP70 contributes to a feedback mechanism in which it binds to its own mRNA transcripts, reducing the amount of available messages for translation (Balakrishnan and De Maio 2006). This process may aid in the restoration of HSP-70 levels after stress as part of a self-limiting mechanism of expression. In addition, HSP70 may regulate its abundance through interaction with HSF-1. HSP70 can associate with the transactivation domain of HSF-1, limiting its activity in the presence of high levels of HSP70 (Abravaya et al. 1992).

During HS, HSP70 is titrated away to misfolded proteins, allowing for the activation of HSF-1. In this model, HSP70 self regulates indirectly, through interaction with HSF-1. To date, our understanding of post-transcriptional regulation of HSP70 is limited but further work to study the role of miRNAs in other species is certainly warranted, especially in the context of cancer where HSP70 is frequently overexpressed (Murphy 2013).

5.2 Why Do Animals Die After Heat Shock?

Wildtype *C. elegans* subjected to heat shock had a 50-60% survival rate, meaning that 40-50% of animals died. In *miR-85* animals, we see this survival decline to 30%. We determined that high levels of *hsp-70* after HS in *mir-85(n4117)* animals was responsible for the lower viability. However, it remains unclear as to why these animals die and why high levels of HSP-70 are so harmful. HSP-70 is a chaperone, known to help ameliorate the toxic effects of stress-induced protein misfolding. Originally, we predicted that higher basal levels of *hsp-70* in *miR-85(n4117)* animals would 'prime' them for exposure to heat stress, perhaps promoting their survival. In a way, this model resembles a mild or hormetic stress which can actually prove beneficial for an organism's growth and survival (Kumsta et al. 2017). However, our viability assays did not agree with this model and instead, lead us to consider how Hsp70 misregulation could actually be harmful. Despite this, it's possible that pre-conditioning *miR-85(n4117)* animals with short bouts of stress may have different consequences than observed in WT,

and even 15 to 30 minutes of heat stress could generate chronic misregulation of *hsp70*.

There is mounting evidence to suggest that chronic overexpression of Hsp70 can be detrimental to an organism. In mice, overexpression of Hsp70 leads to slowed growth and makes animals more prone to developing tumors (Vanhooren et al. 2008). In flies, extra copies of Hsp70 may prove initially beneficial for survival after heat shock but chronic overexpression can slow development and ultimately proves detrimental for survival to adulthood (Feder et al. 1992). These findings suggest an evolutionary trade-off, or antagonistic pleiotropy, that is defined by the balance of negative and beneficial effects of Hsp70 expression. However, the extent of cellular consequences from Hsp70 overexpression are not clear and how this reduces survival in *C. elegans* is unknown.

One enticing hypothesis proposed by Hoekstra and Montooth relies on our understanding that inducing Hsp70 requires an immense energetic cost to an organism (Hoekstra and Montooth 2013). In flies, they found a transient yet significant increase in metabolic rate associated with increased *Hsp70* gene copy number. They argue that the upper bounds of beneficial Hsp70 copy number may be set by the energetic cost (Hoekstra and Montooth 2013). If an organism is overexpressing Hsp70 long term, there may not be enough cellular energy available to accommodate the normal cellular requirements for growth and development. While we do not see that *miR-85(n4117)* animals develop any different than WT, it would be interesting to carefully assess lifespan and

healthspan phenotypes in these animals. Preliminary analysis of *miR-85(n4117)* lifespan revealed a slightly shortened lifespan compared to WT but additional replicates need to be performed. Furthermore, the influence of *hsp-70* on lifespan could be studied by growing these mutants on *hsp-70(RNAi).* It would be particularly intriguing if knock down of *hsp-70* increased the lifespan of *miR-85* mutants.

Hsp70 also requires energy from ATP hydrolysis and the aid of a cochaperone, Hsp40, to dissociate from client proteins. If Hsp40, ATP, or nucleotide exchange factors are limiting, Hsp70 may remain is its high affinity ADP-bound state, stuck on client proteins (Mayer and Bukau 2005). In *miR-85(n4117)* animals, high amount of HSP-70 could remain associate with client proteins long after normal features of translation are restored after heat stress in WT animals. If HSP-70 is still bound to clients, this may slow the restoration of cellular processes. By mutating the ATP-ase domain of *hsp-70,* we could assess the possibility that ATP exchange is limiting the release of client proteins after heat shock in *miR-85(n4117)* animals, inhibiting the return to normal activities of translation and protein folding. In addition, analysis of cochaperone expression and activity would be warranted during HS recovery to assess the potential stoichiometric imbalances of HSP-40 and HSP-70 in *miR-85(n4117)* animals. Finally, polysome fractionation through HS recovery would be invaluable for assessing the global effects on translation in *miR-85(n4117)* and *hsp-70ΔTS* animals.

Lastly, careful assessments of phenotypes associated with Hsp70 overexpression may help define the pleiotropic nature of this heat shock chaperone. Parallel studies of WT, *miR-85(n4117),* and *hsp-70ΔTS* animals may help distinguish new roles for Hsp70 and relate its overexpression to decreased viability. Using microscopy, we can image whole animals throughout a recovery time course, following each strain until death and looking for morphological clues that may explain differences in survival. For example, we may identify features resembling necrosis which have been characterized in *C. elegans* subjected to heat stroke which include apoptotic cells and disrupted germline development (Kourtis, Nikoletopoulou, and Tavernarakis 2012). We could also assess HSP-70 localization after HS by generating and monitoring expression with a GFP-tagged strain. Hsp70 and Hsp40 aid in the translational recovery from stress and increasing evidence points to the clearance of stress granules as the mechanism in which this is accomplished. However, the precise relationship between stress granule dissipation and translational recovery is not clear (Walters and Parker 2015). By comparing HSP-70 localization in WT and *miR-85(n4117)* animals after stress, we could begin to understand the contributions of HSP-70 to granule formation and granule disaggregation and how it might influence the restoration of translation after HS. Overall, further assessment of phenotypes may elucidate the molecular consequences of *hsp-70* overexpression after HS. Ultimately, rigorous comparing and contrasting of morphological and molecular differences in animals from isogenic populations that either perish or prevail after stress presents a

unique opportunity to help answer the question as to why a subset of animals die after stress.

5.3 What is the relationship between heat shock and fertility?

miR-85 mutants are both sensitive to heat stress and have defective fertility. It remains unclear as to how or if these phenotypes are related. The only transcript that is both upregulated in RNA sequencing analysis and contains a miR-85 target site is *hsp-70.* However, disruption to the miR-85 target sites within the 3'UTR of *hsp-70* fails to rescue the reduced brood size of *miR-85(n4117)*, suggesting that unlike the heat shock viability phenotype we observed, brood size is not regulated through *hsp-70*. If other stress response genes are upregulated in the *miR-85* background independently of *hsp-70* regulation, they could be stimulating a pseudo HSR in the cell and be contributing to the fertility defects. One possible connection between heat shock and fertility could be through misregulation of RNAi pathways. If loss of *miR-85* is sufficient to trigger a heat-shock-like molecular phenotype, RNAi pathways that normally repress cellular reprogramming in germ cells could be misregulated, which is a reported outcome of heat shock (Rogers and Phillips 2020). The misregulation of germ cell fates therefore, could explain the decreased fertility. RNA silencing of somatic genes and repetitive elements is important for the maintenance of reproductive potential

across generations. If RNAi is misregulated in the germline due to the stress induced by loss of *miR-85*, there could be reason to believe sensitivity to heat stress and fertility may be connected.

Another critical consideration is the inability of RNA sequencing methods to discern translational inhibition of mRNAs. miRNAs are thought to promote mRNA decay in the soma but favor translational repression and mRNA stabilization in the germline (Dallaire, Frédérick, and Simard 2018). We do not yet have a complete picture as to how miR-85 regulates target transcripts in the germline as they may not be subject to decay and therefore not be differentially expressed when analyzed by traditional RNA sequencing methods. One approach to more completely understand miR-85 mediated regulation would be performing germline and somatic specific RNA sequencing alongside Ribosome Profiling. From this, we would have greater insight into tissue specific regulatory roles of miR-85 as well as broaden our list of candidate miR-85 targets that could explain the fertility defect.

Studying the expression of hsp-70 in early stages of development, before miR-85 is expressed, would also be valuable for understanding how *hsp-70* is regulated in other contexts. For instance, if an animal is subjected to heat shock before miR-85 is developmentally upregulated, what happens to *hsp-70* expression? While it's possible that miR-85 plays its most critical roles during development, helping regulate specific, temporal expression of genes in early L4 stages, we also know that loss of miR-85 leads to detrimental effects on heat shock recovery in L4 animals. These events may not be entirely mutually exclusive

and there are a multitude of hypotheses that deserve further investigation. One possibility is that miR-85 is induced upon stress, independent of developmental stage, to ensure proper degradation of *hsp-70* after stress. A second possibility is that *hsp-70* is targeted by a different miRNA or mechanism of regulation at early life stages. However, our HS conditions of 4 hours at 35°C do not induce death in early larval (L2) staged WT or *miR-85(n4117)* animals, suggesting that these animals are more resilient to stress. It's possible that L2 heat shocked *miR-85* animals do not overexpress *hsp-70* after exposure to stress in early larval stages or that *hsp-70* misregulation in early larval stages is not detrimental to viability as we observe in L4 animals. All these possibilities merit further consideration. However, more robust heat shock treatments for early larval stages animals will likely be required to discern differences in phenotypes.

It's also intriguing to consider the potential trade-offs that might influence an organism's ability to respond to stress while allocating resources towards maintaining fecundity. In the natural world, animals must maintain long-term reproductive success while coping with environmental extremes. It's unlikely that a reproductive strategy that only aims to maximize offspring production would be favorable as fecundity could be lost in a single stressful encounter. In mosquitos, eating a hot blood meal can trigger a heat shock response, producing elevated levels of *hsp70* to protect the midgut and promote protein digestion (Benoit et al. 2011). However, *hsp70* plays dual roles in this model as it also helps maintain egg laying abilities after a hot blood meal. RNAi against *hsp-70* after feeding reduced the number of eggs laid and suggests a direct connection between the HSR and

fecundity (Benoit et al. 2011; 2019). In *C. elegans* it appears that trade-offs exist as part of a dynamic response that is carefully tuned based on the severity and length of a stressful encounter (Aprison and Ruvinsky 2014). After chronic, mild stress, *C. elegans* are able to recover fecundity but the overall number of eggs laid is lower than animals grown at constant, permissive temperatures. At temperatures greater than 29°C, there is irreversible damage to the sperm that can limited brood size yet ovulation can persist as somewhat of a wishful thinking approach. If the stress might soon cease, the animals can be poised to rapidly recover (Aprison and Ruvinsky 2014).

Heat stress has been documented to reduce fertility across a variety of species (Takahashi 2011). In *C. elegans* and humans, small changes in temperature can affect spermatogenesis (Kurhanewicz et al. 2020). While some of the defects are a result of physical damage from high temperatures, including DNA breaks, other aspects of fertility may be attributable to changes in the molecular landscape that shunt resources towards survival and limit fecundity. It's possible that stress-induced genes in *miR-85(n4117)* under controlled temperatures trip similar cell signaling pathways that reduce brood size induced by heat stress. Still, the question remains: is the physical change in temperature causing the stress or is it the cellular response to that stress that is ultimately detrimental?

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