UNIVERSITY OF CALIFORNIA SAN DIEGO

Uncovering the role of non-coding RNAs in the *Caenorhabditis elegans* Heat Shock Response

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

in

Biology

by

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2019

The Dissertation of William Philip Schreiner is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California San Diego

2019

DEDICATION

Jenny Xiao and my Family.

EPIGRAPH

Que es mi barco mi tesoro,

que es mi Dios la libertad;

mi ley, la fuerza y el viento;

mi ùnica patria, la mar.

—Josè de Espronceda

Canciòn del pirata

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ABSTRACT OF THE DISSERTATION

Uncovering the role of non-coding RNAs in the *Caenorhabditis elegans* Heat Shock Response

by

William Philip Schreiner

Doctor of Philosophy in Biology

University of California San Diego, 2019

Professor Amy Pasquinelli, Chair

Only ~one percent of the genome is protein coding. This finding led to the belief that much of the non-coding genome is junk DNA. However, we now understand that much of the non-coding genome is transcribed to some extent. This knowledge coupled with the fact that some RNAs transcribed from non-coding regions of the genome (ncRNA) are involved in important biological processes, has shattered the notion that the non-coding portion of the genome is simply detritus. In my thesis, I explore the role of various classes of non-coding RNAs in the response to Heat Stress.

In Chapter 2, I review miRNA biogenesis. MicroRNAs undergo a complex biogenesis

process in order to be processed into mature miRNAs capable of gene regulation. The steps of miRNA biogenesis are tightly regulated, and perturbations in them are associated with disease. MicroRNAs appear to play especially critical roles in the nervous system, and in Chapter 2 I focus on examples of how differentially regulated or misregulated miRNA biogenesis can affect the function of the nervous system. Chapter 3 focuses on my analysis of miRNAs and additional ncRNAs in the *C. elegans* Heat Shock Response (HSR). The HSR has been studied for over fifty years, yet there is a dearth of information considering the roles of ncRNAs in the HSR. I explore how different classes of ncRNAs, including miRNAs, are differentially regulated in HS in *C. elegans*. Interestingly, I show that some specific ncRNAs are regulated by Heat Shock Factor 1 (HSF-1), which is a conserved transcription factor that is responsible for the upregulation of specific genes in HS. These results illustrate that ncRNAs likely play important roles in the HSR.

MicroRNAs are also implicated in *C. elegans* aging. The *C. elegans* Argonaute proteins, ALG-1 and ALG-2, which are guided by miRNAs to mRNAs to repress gene expression are also implicated in aging. In Chapter 4, I detail my analysis on identifying targets of miRNAs that are associated with either ALG-1 or ALG-2 miRNA mediated regulation in aging.

Chapter 1

Introduction

1.1 Non-Coding RNAs and the Heat Shock Response

The Heat Shock Response (HSR), discussed below, has been studied for over fifty years. Virtually every level of gene expression participates in the regulation of this pathway. However, there exists a real knowledge gap with respect to the function of non-coding RNAs in the HSR. Of course, there is always more to learn about how a pathway is regulated at every level. Yet, this knowledge gap looks more like a chasm when compared to all of the ways in which we know the HSR can be regulated. The absence of information with respect to non-coding RNAs is likely due to the fact that much of the non-coding genome was, until quite recently, thought to be junk (Hangauer et al., 2013). One class of non-coding RNA, microRNAs (miRNAs), has members known to regulate many biological processes yet, these small RNAs were only discovered 25 years ago (Ambros and Ruvkun, 2018). The abundance of long non-coding RNAs (lincRNAs) and other types of ncRNAs was only realized recently (Hangauer et al., 2013). In this introduction, I will provide background on different classes of non-coding RNAs, the Heat Shock Response, and how non-coding RNAs may be leveraged to respond to Heat Stress.

1.2 MicroRNAs are Non-Coding RNAs that Regulate Gene Expression

MicroRNAs (miRNA) are small 22 nucleotide (nt) RNAs that guide Argonaute (AGO) proteins to their targets and repress their expression (Figure1.1). The first microRNA discovered, lin-4, was identified in 1993 (Lee et al., 1993; Wightman et al., 1993). Forward genetic screens found that *lin-4* mutants developed abnormally, and genetic epistasis experiments showed that lin-4 inhibited *lin-14* (Ambros, 1989). lin-4 distinguished itself from the many protein coding genes involved in *C. elegans* development when researchers found that the *lin-4* gene gave rise not to a messenger RNA, but rather to a small RNA of approximately 22 nt (Lee et al., 1993; Wightman et al., 1993). Another clue to the function of lin-4 was uncovered when researchers found that the lin-4 RNA could partially base pair with the 3' Untranslated Region (UTR) of *lin-14* mRNA. This, coupled with the fact that downregulation of LIN-14 protein levels requires lin-4, led to the idea that lin-4 may be inhibiting the translation of *lin-14* through base pairing to its 3'UTR. Researchers initially thought this miRNA was a nematode specific phenomenon. The discovery of let-7 as a microRNA a few years later disabused people of this notion. Like, *lin-4*, *let-7* was found through a genetic screen and is involved in the heterochronic pathway (Reinhart et al., 2000). *Let-7* mutants exhibit delayed developmental phenotypes, which in turn leads to vulva bursting as key cells fail to develop properly (Reinhart et al., 2000). Interestingly, these phenotypes can be suppressed by mutations in *lin-41* (Reinhart et al., 2000; Slack et al., 2000). Like lin-4, let-7 was predicted to bind to the 3'UTR of its target. These results indicated the presence of a second *C. elegans* miRNA. Strikingly, Pasquinelli and colleagues found that let-7 is conserved widely in metazoans including, Drosophila, mice, and humans (Pasquinelli et al., 2000).

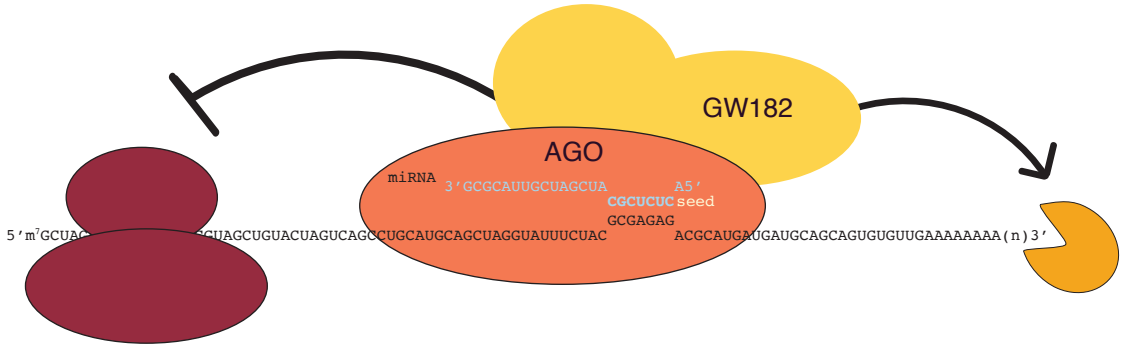

Figure 1.1: MicroRNA Targeting. Argonaute proteins (AGO) are guided to their targets by miRNAs. The seed (nt 2-8) are most important for target recognition. GW182 and other AGO associated proteins promote RNA degradation and/or translational inhibition.

This discovery heralded a boom in miRNA research, and hundreds of miRNAs were discovered in many model species. As of 2019, the *C. elegans* genome is predicted to encode over 400 microRNAs (Kozomara et al., 2019). Strikingly, unlike lin-4 and let-7, the majority of *C. elegans* microRNAs appear individually dispensable for viability (Miska et al., 2007). That is, single miRNA mutants for most *C. elegans* miRNAs do not exhibit easily observable phenotypes, such as abnormal development or reduced brood size. One possibility for this observation is biological redundancy. For instance, microRNAs may function in parallel, and many microRNAs are grouped into families, determined by sequence similarity. For example, members of the miR-35 family do not exhibit defects when individually deleted; however, removal of the entire family is lethal (McJunkin and Ambros, 2014). Still the majority of microRNA families display no obvious phenotypes when every member is removed (Alvarez-Saavedra and Horvitz, 2010).

One longstanding idea suggests that some of these seemingly inert miRNAs have roles in responding to stress or non-ideal conditions (Ambros and Ruvkun, 2018). It is important to note that previous phenotypic screens of miRNA mutants were conducted at optimal growth temperatures, on plates replete with food —conditions that *C. elegans* do

not seem likely to encounter in the wild. In recent years, this idea has borne some fruit (Figure1.2). For example, miRNAs have been implicated in the response to heat stress, as miR-71 and miR-239 levels increase in response to Heat Shock, and *C. elegans* lacking miR-71 are less viable than WT animals during heat stress (de Lencastre et al., 2010; Nehammer et al., 2015). Interestingly, *miR-239* mutants fare better when subjected to HS than their wildtype counterparts. Previously uncharacterized miRNAs also respond to graphene oxide and bacterial infection stressors (Bartel, 2018). One particularly fascinating example of the nuanced yet crucial role miRNAs can exhibit is that of the lowly expressed miR-797. Drexel and colleagues noticed that this miRNA is specifically expressed in carbon dioxide sensing neurons (Drexel et al., 2016). In these neurons, miR-791 represses two genes that are expressed in most other cells, and this repression is necessary for worms to respond to carbon dioxide (Drexel et al., 2016). In mammalian cells specific miRNAs are also involved in stress pathways. In cells subjected to UV stress, the genome guardian protein p53 is activated, and p53 promotes the transcription of the miR-34 family of miRNAs (Leung and Sharp, 2010). Members of the miR-34 family then promote cell growth arrest (Leung and Sharp, 2010). In Chapter 4, I will discuss the role of specific miRNAs that respond to and potentially regulate the HSR in *C. elegans*.

Mature 22 nt miRNAs are responsible for target recognition and repression. However, they begin their lives as much longer primary transcripts (pri-miRNA) that are transcribed by RNA Polymerase II, and, thus, capped, and polyadenylated (Ha and Kim, 2014). The microprocessor, consisting of Drosha and DGCR8 (also known as Pasha), is responsible for cleaving away the 5' and 3' pri-miRNA flanking regions to produce the ~65 nucleotide pre-miRNA stem loop (Ha and Kim, 2014). The pre-miRNA is then exported into the cytoplasm and processed into a mature miRNA duplex by the enzyme Dicer (Ha and Kim, 2014). Typically, only one strand of the duplex is loaded into Argonaute (AGO), the other

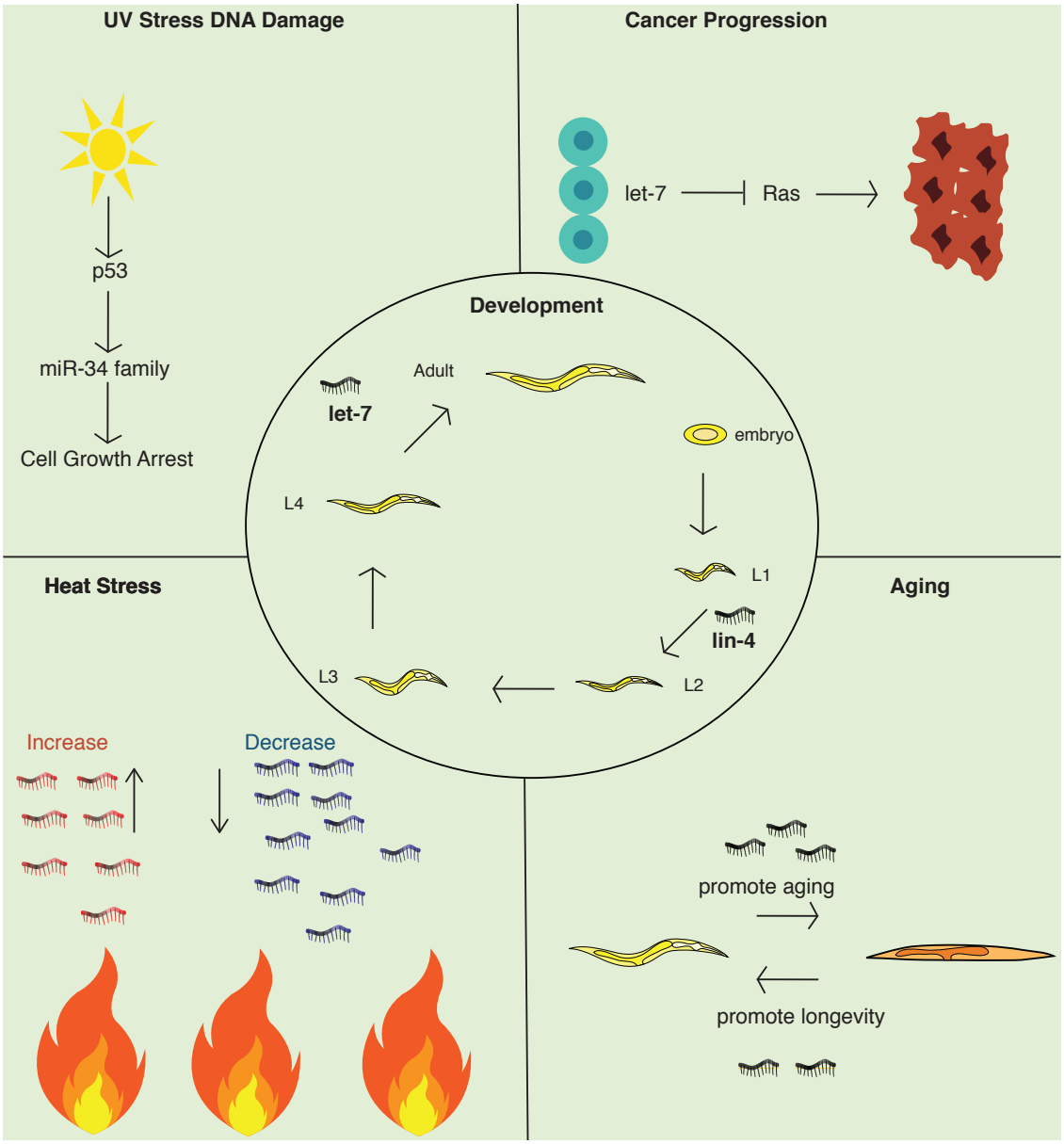

Figure 1.2: The Role of MicroRNAs. MicroRNAs are involved in the regulation of many biological processes. **Development** MicroRNAs like lin-4 and let-7 are involved in *C. elegans* larval development. Mutations in these miRNAs are associated with severe developmental phenotypes and lethality. **Cancer Progression** In humans the let-7 microRNA targets the oncogene RAS and mutation of let-7 is associated with specific cancers. **Aging** In *C. elegans* specific microRNAs promote longevity, whereas others can decrease longevity. **Heat Stress** The levels of specific miRNAs change in Heat Shock. Loss of some of these miRNAs leads to viability phenotypes. **UV Stress/DNA Damage** DNA damage causes p53 to promote the transcriptional upregulation of the miR-34 family, which in turn promotes Cell Growth Arrest and Apoptosis.

strand, termed passenger, is often quickly degraded. Once, the guide strand microRNA is loaded into Argonaute the resulting miRNA silencing complex, termed miRISC, can bind to and repress its targets. Many organisms have multiple AGO proteins capable of binding miRNAs (Meister, 2013). For instance, humans have four AGO proteins (Meister, 2013). *C. elegans* miRNAs function with two specific AGOs, ALG-1 and ALG-2 (Grishok et al., 2001). Deletion of both of these AGOs is lethal, while deletion of *alg-1* individually produces more severe phenotypes than deletion of *alg-2* alone (Grishok et al., 2001; Vasquez-Rifo et al., 2012). Thus, *alg-1* is thought of as the primary AGO protein in *C. elegans*. Interestingly, loss of *alg-1* or *alg-2* results in opposite aging phenotypes, correlating to shorter and longer lifespans respectively. I will explore the role of these AGOs in aging in more detail in Chapter 4. In *C. elegans* and most animals, AGOs recruit additional proteins to silence their targets (Bartel, 2018; Meister, 2013). MicroRNA mediated silencing is achieved with the help of GW182 proteins which interact with AGO proteins and recruit deadenylase complexes which degrade the poly(A) tail (Bartel, 2018). This in turn leads to degradation of the target through decapping and 5' to 3' mRNA degradation (Bartel, 2018). MiRISC targeting can also lead to translational repression of the target through the recruitment of the translation repressor DDX6 (Izaurralde, 2015). In a variety of post-embryonic cultured mammalian cells, mRNA destabilization appears to be the primary factor contributing to gene silencing (Eichhorn et al., 2014). However, in other contexts such as the zebrafish embryo, translational repression appears to be the primary effect of miRNA targeting (Bazzini et al., 2012). Chapter 2 provides more extensive information concerning miRNA biogenesis and miRNA mediated regulation of targets.

The way in which miRNAs bind to their targets can also affect silencing. In plants, where miRNAs perfectly base pair with their targets, Argonaute can endonucleolytically cleave the target leading to degradation (Bartel, 2018). However, in most animals, including

C. elegans, perfect base pairing is rare, and miRNAs usually bind imperfectly to their target mRNAs. Typically, the miRNA seed region, nt 2-8, initiates target binding and miRNA 3' sequences can confer additional specificity to target engagement (Figure 1.1) (Bartel, 2018). Since the seed region is the most important determinant of microRNA specificity, it can be used to help predict miRNA targets by searching for possible seed matches in 3'UTRs. While these predictions are no substitute for experimental evidence, as they do not definitely indicate miRNA targeting, they represent a way to help winnow the possible targets of a miRNA. I will provide evidence for the utility of miRNA-target prediction methods in Chapters 3 and 4.

1.3 Long non-coding RNAs

The discovery of microRNAs heralded a renewed interest in the exploration of non-coding RNA (ncRNA) biology. Fortunately, the advent of next-generation sequencing provided thousands of new ncRNAs for researchers to explore. In fact, it soon became clear that the majority of the human genome is transcribed to some extent (Hangauer et al., 2013). Of particular interest are Long Intergenic Non-Coding RNAs (lincRNAs) and Long Non-Coding RNAs (lncRNAs). LincRNAs, are non-coding RNAs longer than 200 nucleotides that do not overlap protein coding genes. LncRNAs are longer than 200 nucleotides, and can overlap protein coding genes (Table1.1). Of note, these cutoffs are somewhat arbitrary as there are also ncRNAs less than 200 nucleotides that remain uncharacterized. In *C. elegans*, hundreds of lincRNAs and additional unclassified ncRNAs have been identified (Nam and Bartel, 2012).

Although these RNA-seq studies illustrated the pervasive transcription of the non-coding genome, specific noncoding RNAs were already known to fulfill critical biological

functions. Transfer RNAs (tRNAs) were discovered in 1958 —five years after the discovery of the structure of DNA and almost fifty years before the advent of next generation sequencing (Hoagland et al., 1958). Ribosomal RNAs, small nucleolar RNAs and small nuclear RNAs were all discovered around the same time (Hadjiolov et al., 1966; Palade, 1955). In addition, in the last few decades researchers uncovered specialized roles for specific lncRNAs. For instance, in female mammals one of the X chromosomes is randomly inactivated by the non-coding RNA Xist which is expressed from the inactive X chromosome and coats the inactive chromosome, thereby, inactivating it (Sahakyan et al., 2018). In mammalian cell culture the HOTAIR ncRNA, which is transcribed from the HOX gene locus, was found to be important for the repression of HOX genes (Rinn et al., 2007).

While careful studies that explore the role of a specific non-coding RNA or group of non-coding RNAs are indispensable, Next-Gen sequencing technologies have provided clues concerning the function of the multitude of newly identified ncRNAs, especially lincRNAs. Cell fractionation experiments followed by transcriptome profiling show that lincRNAs are found in virtually all cellular compartments (Quinn and Chang, 2016). This specific sub-cellular enrichment data can provide clues as to the function of lincRNAs. For instance, nuclear lincRNAs appear to be enriched in chromatin relative to nucleoplasm, and this chromatin association fits with the role of some characterized lincRNAs as chromatin modifiers (Quinn and Chang, 2016). Although, some lincRNAs localize to the nucleus, and many characterized lincRNAs such as NEAT1, HOTAIR, and MALAT1 are nuclear, lincRNAs in general are more abundant in the cytoplasm than the nucleus (Carlevaro-Fita and Johnson, 2019). Interestingly, three characterized *C. elegans* lincRNAs, *lep-5*, *rncs-1*, and *tts-1*, function in the cytoplasm (Hellwig and Bass, 2008; Essers et al., 2015; Kiontke et al., 2019). In mammalian cell culture, a subset of cytoplasmic lincRNAs associate with ribosomes, which would at first glance appear to invalidate their status as non-coding;

however, Mass Spectrometry experiments do not detect peptides for most of these lincRNAs (Quinn and Chang, 2016). High-throughput methods have also been useful in identifying potentially physiologically relevant lincRNAs. In 2017, Liu and colleagues undertook a massive CRISPRi screen and targeted over 16000 lincRNAs in multiple cell types and found almost 500 lincRNAs that had modified growth phenotypes when disrupted (Liu et al., 2017). Strikingly, 89% of lincRNAs only exhibited a phenotype in one cell type —painting a nuanced role for lincRNA function. While these results demonstrate the power of high throughput screens, follow-up studies are required to determine the functional role of these non-coding RNAs in a native cellular environment. For instance, depletion or deletion of HOTAIR in cell culture has known molecular phenotypes, and the HOTAIR RNA is highly expressed in breast cancers (Gupta et al., 2010). However, deletion of this RNA in mouse models does not appear to lead to any physiological effects, and the animals appear to be phenotypically normal (Amândio et al., 2016).

C. elegans represents a powerful model to study lincRNA biology in an intact organism. Worms are transparent, consist of different tissues, and are amenable to genetic manipulation. While most *C. elegans* non-coding RNAs have been identified by high throughput methods (Nam and Bartel, 2012; Akay et al., 2019), careful studies of individual lincRNAs have been crucial for uncovering physiological roles for lincRNAs. Interestingly, some of these studies have implicated *C. elegans* lincRNAs in the response to stress. Hellwig and Bass found that *rncs-1* increases in starved worms and titrates away Dicer activity, which in turn affects the expression of Dicer regulated genes during starvation (Hellwig and Bass, 2008). The lincRNA *tts-1* is found on ribosomes in the long lived *daf-2* mutant and is required for *daf-2* mediated lifespan extension (Essers et al., 2015). Whether additional lincRNAs function in stress responses has yet to be determined. In Chapter 4, I will present evidence that additional lincRNAs respond to heat stress and could play a role

in the Heat Shock Response.

In addition to lncRNAs, pseudogenes also represent a large class of non-coding genes in *C. elegans* with over 1900 annotated in the *C. elegans* genome (Lee et al., 2018). Pseudogenes are non-coding genes that have homology to protein coding genes but no longer have the capacity to be translated into a functional protein (Pink et al., 2011). Some of these pseudogenes are transcribed into a ncRNA. In *C. elegans* the Nonsense Mediated Decay (NMD) pathway, which recognizes premature stop codons, degrades a subset of the transcribed pseudogenes (Muir et al., 2018). Although pseudogenes by definition are not translated, they should not be discounted as nonfunctional. For instance, the pseudogene of the tumor suppressor PTEN, PTENP1 acts as decoy for miRNAs that would normally target PTEN, allowing the functional PTEN mRNA to escape miRNA regulation (Johnsson et al., 2013). In *C. elegans* the pseudogene *dct-10* promotes cell proliferation through an unknown mechanism (Pinkston-Gosse and Kenyon, 2007). In Chapter 3, I will present evidence that this pseudogene is directly regulated by the canonical Heat Shock Factor 1 (HSF-1) transcription factor and show that the expression of many pseudogenes is regulated by heat stress.

Table 1.1: Comparison of different classes of non-coding RNAs

Non-Coding RNA	Size (Nucleotides)	Description	Role / Possible Roles
tRNA	75-90	Transfer RNA	Translation
snoRNA	60-170	Small Nucleolar RNA	rRNA Modification
rRNA	1.5 kb - 5kb	Ribosomal RNA	Translation
microRNA	~22	Guides Argonaute proteins to mRNA causing target repression	Development, Aging, Response to stress, etc see Figure1.1
lncRNA	Greater than 200	Long-Non Coding RNA can overlap protein coding gene	Chromatin modifcation, scaffolding, decoy, etc.
lincRNA	Greater than 200	Subset of lncRNA, does not overlap protein coding gene	Chromatin modifcation, scaffolding, decoy, etc.
Pseudogene	Variable	Derived from gene; no longer translated into functional protein	microRNA Sponge, generation of siRNAs, non-functional etc
Repeat RNA	Variable	RNA transcribed from Transposable element	enhancer RNAs, formation of nSBs in HS, inhibition of transcription in HS, etc.

1.4 Transposable Elements

Transposable Elements (TEs) are another type of overlooked non-coding RNA that can respond to stress. TEs were first discovered by Barbara McClintock over half a century ago (McClintock, 1984). McClintock noticed that some genes appeared to “jump” or change location in the genome. There are two main types of transposable elements: DNA transposons, and retrotransposons. DNA elements encode a transposase and

transpose through a cut and paste mechanism, meanwhile retrotransposons use an RNA intermediate and often encode their own reverse transcriptase (Sotero-Caio et al., 2017). TEs can be further demarcated into autonomous or non-autonomous, with non-autonomous transposons unable to transpose as they no longer encode the factors necessary for transposition. The amount of DNA derived from repetitive elements varies by species and there is a linear correlation between TE content and genome size in eukaryotic organisms (Sotero-Caio et al., 2017). In *C. elegans* ~12 % of the genome is TE derived (Bessereau, 2006). In humans fully 40% is thought to be TE in origin and astoundingly in Maize the number is 85% (Biémont, 2010). In the *C. elegans* genome much of the repeat DNA is derived from the Helitron TE, as Helitron sequences make up ~2% of the total genome (Kapitonov and Jurka, 2001). These unusual repetitive elements were initially identified in *C. elegans*, *Arabidopsis*, and Rice (Kapitonov and Jurka, 2001). Unlike other DNA transposons, which use a "cut and paste" mechanism, Helitrons use a DNA Helicase for their transposition—hence their interesting name—and copy their DNA sequence into a form that can be inserted elsewhere in the genome (Kapitonov and Jurka, 2001). These elements are also unique in that they seem to be particularly adept at capturing host genes thereby potentially altering host genomes (Kapitonov and Jurka, 2007). Some of the first evidence for this comes from Maize where researchers found that many inbred lines diverge in their gene content. This divergence appears to be driven by Helitrons as many of the differences amongst the lines result from partial gene insertions which are driven by Helitrons (Kapitonov and Jurka, 2007).

It is easy to imagine how active transposition events could wreak havoc on the genome. Indeed, transposition events can lead to deleterious mutations. For instance, Alu elements can become inserted in the BRACA2 breast cancer associated gene, and LINE-1 activity is associated with gastrointestinal cancers. (Anwar et al., 2017; Burns,

2017). Organisms quell the potentially damaging effects of transposons through chromatin silencing. Repetitive regions of the genome are often methylated and decorated with repressive chromatin marks like H3K9me3 (Anwar et al., 2017). In *C. elegans*, repetitive regions are enriched in the distal arms of chromosomes and are marked by H3K9me2/3 (Ahringer and Gasser, 2018). In the germline, where these epigenetic controls are relaxed small RNA pathways are leveraged to silence active transposons (Hoogstrate et al., 2014). In mammals and *Drosophila*, piRNAs repress transposons by guiding piRNA specific Argonautes to transposons (Czech et al., 2018). In *C. elegans* 22G RNAs and piRNAs are responsible for silencing transposons, and defects in these pathways can lead to mutations and/or sterility (Bagijn et al., 2012; Gu et al., 2009).

Given that transposons are foreign in origin and their transposition can lead to mutations in the host genome, they were initially termed selfish elements (Orgel and Crick, 1980). But it is also important to note that by altering the genome, transposons are potent shapers of evolution. In some instances, transposition events can occur that are favorable not only to the continued replication of the transposon but also to the fitness its host. In *Drosophila* telomeres are extended not by telomerase but rather by specialized retrotransposons, and V(D)J recombination is hypothesized to have evolved from a primordial transposon. (Jones and Gellert, 2004; Pardue et al., 1996). Certain TEs also appear to be upregulated in response to HS, and in some instances aid in the cellular response to excessive temperature. In Humans and *Drosophila*, SAT III repeats and omega repeats respectively form nuclear stress bodies (nSBs) and associate with HSF-1 in HS (Hogan et al., 1995; Jolly et al., 2004; Jolly and Lakhotia, 2006; Goenka et al., 2016). While the role of these nSBs is not yet fully clear, there is evidence that Sat III repeat mediated nSBs contribute to the general downregulation of transcription seen in Heat Stress by sequestering RNA processing factors and transcription associated factors (Goenka et al., 2016). Furthermore,

knockdown of these repeats leads to an increase in transcription, whereas over expression of these repeats leads to a decrease in transcription (Goenka et al., 2016). In addition the B2 SINE RNA, which is transcribed by pol III, acts to inhibit pol II transcription in heat stress —which may explain the general repression of transcription in Heat Stress (Mariner et al., 2008; Yakovchuk et al., 2009). In *C. elegans*, repetitive RNAs have also been shown to increase at elevated temperatures (**?**). In Chapter 3 I show that the expression of many repetitive elements changes in HS and that Helitrons are actively regulated by the canonical Heat Shock Factor (HSF-1).

1.5 Heat Stress and the Heat Shock Response

In order to survive in the wild, organisms have to cope with potentially fatal insults. Organisms are especially sensitive to severe changes in temperature, and if not properly addressed excessive thermal stress can prove fatal. At the cellular level Heat Stress (HS) can disrupt cellular membranes, depolymerize the cytoskeleton, and denature proteins among many other potentially deleterious effects (Richter et al., 2010). Many molecular processes are also inhibited in heat shock such as splicing, translation, and transcription (Shalgi et al., 2014, 2013; Teves and Henikoff, 2011). Interestingly, the first evidence for a molecular response to HS came from Ferruccio Ritossa over fifty years ago in a serendipitous discovery (Ritossa, 1962). When a laboratory technician inadvertently placed a *Drosophila* polytene chromosome preparation in a 30°C incubator instead of the standard 25°C, Ritossa noticed a puffing or opening pattern at distinct loci on the slides. Ten years later researchers found that these chromosomal puffs correspond to transcription of the aptly named Heat Shock Proteins (HSPs) (Tissiéres et al., 1974; McKenzie et al., 1975; Spradling et al., 1975). HSPs act as chaperones and help proteins fold in the denaturing

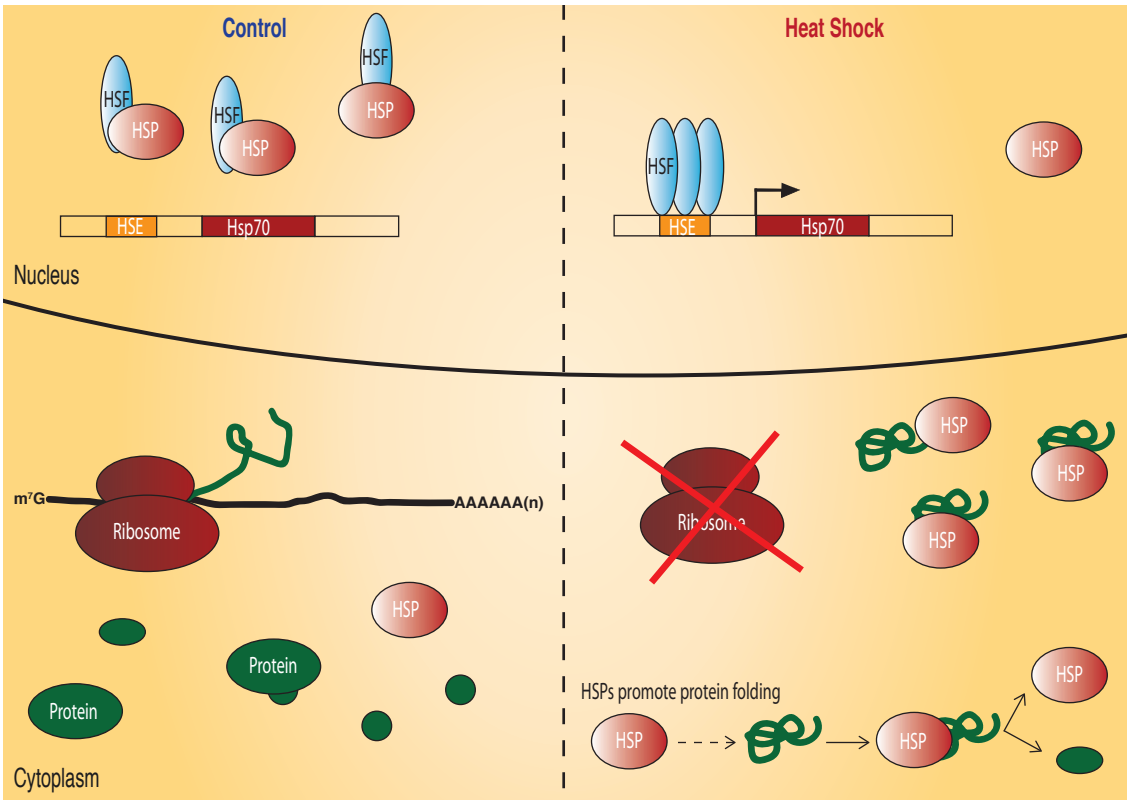

Figure 1.3: The Heat Shock Response. Under control conditions HSF-1 is complexed with chaperones (Panel 1), and cellular processes like translation proceed normally. Heat Stress leads to protein denaturation and inhibition of cellular processes like translation (Panel 2). HSPs complexed with HSF-1 are titrated away by misfolding proteins; HSF-1 is able to bind to Heat Shock Elements (HSEs) upstream of HSPs and promote the expression of Chaperones.

conditions caused by excessive cellular heat. HSPs can also help target damaged proteins for destruction, thus, cleaning the proteome. Scientists found Heat Shock Elements (HSEs) up stream of HSP genes, and that a transcription factor, Heat Shock Factor 1 (HSF-1) binds to these regions to promote the expression of HSPs (Richter et al., 2010). HSF-1 is a widely conserved regulator of the Heat Shock Response (HSR) and is found in many eukaryotic organisms including Yeast, *C. elegans*, *Arabidopsis*, and Humans.

Although HSF-1 and HSPs are named for their role in the HSR, various types of stress activate HSF-1 and the production of HSPs, including ER stress, heavy metal stress, and oxidative stress (Gomez-Pastor et al., 2018). In *C. elegans*, HSF-1 also plays

crucial roles in development (Li et al., 2016). Aging is thought of as, in part, a protein misfolding pathophysiology, and HSF-1 has also been implicated in *C. elegans* longevity as the overexpression of HSF-1 extends lifespan whereas knockdown shortens it (Hsu et al., 2003; Morley, 2003). Misregulation of the HSR in humans is associated with a variety of pathophysiologies, including cancer and protein misfolding diseases. On one hand, the ability of HSF-1 and HSPs to help proteins fold is thought to help otherwise toxic cancer cells survive (Mendillo et al., 2012). On the other, there is interest in leveraging the ability of HSF-1 to promote protein folding to ameliorate protein folding diseases such as Huntington's disease and Parkinson's disease (Gomez-Pastor et al., 2018).

Given its crucial role, it should come as no surprise that HSF-1 activity is tightly regulated. While the levels of HSF-1 remain constant, its localization and activity change upon heat stress. In unstressed animals, HSF-1 is thought to be complexed with chaperones (Figure1.3; panel 1). Heat stress titrates away these chaperones leaving HSF-1 able to be activated, polymerize, and bind to HSEs in the nucleus (Figure1.3; panel 2) (Li et al., 2017). Further, HSF-1 has been shown to undergo a variety of post-translational modifications that modify its activity, including phosphorylation (Li et al., 2017). In *C. elegans*, specific negative regulators of HSF-1 exist to modulate its activity. In control conditions, Heat Shock factor Binding protein (HSB-1) negatively regulates HSF-1 activity (Morimoto, 1998; Satyal et al., 1998). *C. elegans* HSF-1 activity is also stymied by the insulin signaling pathway through Daf-16-Dependent Longevity 1 and 2 (DDL-1/2) (Chiang et al., 2012). In addition to transcriptional regulation by HSF-1, organisms take advantage of additional mechanisms to respond to excessive temperature. Indeed, virtually every level of gene expression has been implicated in the response to HS. For instance, the Hikeshi protein has been shown to import Hsp70 into the nucleus following Heat Stress and is important for the survival of cells following HS (Kose et al., 2012). Regulation of Polyadenylation has also been found to

be important for the HSR as Poly A Polymerase (PAP) is inhibited in HS by PARP1 through ADP-ribosylation (Di Giammartino et al., 2013). Interestingly, PAP is still able to associate with and polyadenylate HSP mRNAs (Di Giammartino et al., 2013). Furthermore, additional stress responsive transcription factors drive the HS induced transcriptional program. In murine and human cell culture Serum Response Factor promotes the upregulation of cytoskeletal gene expression in Heat Stress, whereas HSF-1 exclusively promotes the expression of HSPs and other chaperones (Mahat et al., 2016). Similarly, work in Yeast has shown that the majority of transcriptional changes that take place in HS are caused by msn2/4 not HSF-1 (Solís et al., 2016).

In *C. elegans* the Heat Shock Response is partly cell non-autonomous. The AFD neurons sense elevated temperature and promote the HSR in distal cells through a serotonin signaling mechanism (Prahlad et al., 2008; Tatum et al., 2015). After the discovery that the *C. elegans* HSR is cell non-autonomous, researchers also uncovered evidence that the Mitochondrial Unfolded Protein Response, and the ER Stress Response are also regulated in a cell non-autonomous manners (Taylor and Dillin, 2013; Zhang et al., 2018).

1.6 NcRNAs as new players in the HSR

Clearly, regulation of the HSR is multifaceted. Yet, there is a considerable knowledge gap concerning the role of non-coding RNAs in the HSR. Perhaps, the best evidence we have is concerning miRNAs and the HSR. Studies in *C. elegans* have shown that HS can elicit changes in microRNA expression and that *C. elegans* lacking some of these miRNAs have HS viability phenotypes (de Lencastre et al., 2010; Nehammer et al., 2015). Still the majority of microRNAs that change in HS in *C. elegans* have been uncharacterized. For instance, miR-4936 (discussed further in Chapter 4) increases over 100-fold in HS; yet,

owing to the fact that no available mutants exist for this miRNA, there have not been any studies with respect to the role of this miRNA in HS (Schreiner et al., 2019). There is also a dearth of information concerning the function of less well-studied classes of non-coding RNAs, such as lincRNAs, pseudogenes, and Repeat RNAs in general, and the function of these ncRNA species in HS remains even more elusive. As mentioned in section 1.4 and further expanded upon in Chapter 3, certain repeat RNAs increase in HS and in mammalian cells, and some of these RNAs may be responsible for the general repression of transcription and RNA processing seen in HS.

Interestingly, there are indications that RNA itself may be a malleable substrate used by organisms to survive thermal stress. In Prokaryotes, certain RNAs act as “RNA thermometers”. For instance, Salmonella has a class of RNA structures known as FourU thermometers. These RNA structures are found in the 5' UTR of heat shock responsive genes. Increasing temperature relaxes the structure and allows for translation of the downstream gene (Waldminghaus et al., 2007). These results demonstrate that organisms can leverage the ability of RNA to change conformation upon changes in temperature to modulate gene expression. Whether RNA thermometers exist in Eukaryotes is, at present, unknown. It is possible to imagine that this ability could be leveraged to provide quick regulation of gene expression in Heat Stress conditions. Furthermore, there is some evidence that RNA itself can act as a chaperone (Docter et al., 2016; Choi et al., 2008). In a 2016 study, Docter *et al* found that RNA can function just as well as a canonical protein chaperone in helping misfolded proteins regain their confirmation (Docter et al., 2016).

The extent of non-coding RNA expression in the genome was previously underestimated(Hangauer et al., 2013). Now that we are aware of their presence, we can carefully examine the role of these RNAs in more depth. The HSR represents an excellent paradigm to study non-coding RNA biology, as much has been learned about the transcriptional and

post-translational mechanisms employed during this stress. By studying the HSR with a focus on ncRNAs, researchers can fill knowledge gaps and determine the function of regulatory RNAs in the HSR. I will present evidence for broad changes in ncRNA expression in HS and provide examples of specific ncRNAs being regulated by HSF-1, the master regulator of the HSR. Further insights into the regulation of the HSR could also be useful at the clinical level, as genes involved in the regulation of the HSR are indicated in numerous maladies.

Chapter 2

Making and Maintaining MicroRNAs in Animals

2.1 Introduction

It has now been over 20 years since the first microRNA (miRNA) genes were discovered as critical regulators of developmental transitions in *Caenorhabditis elegans* worms (Lee et al., 1993; Wightman et al., 1993). Since then, thousands of miRNA genes have been documented in plants and animals (Griffiths-Jones, 2004; Kozomara and Griffiths-Jones, 2014). As a class, miRNAs are essential regulators of gene expression in multicellular organisms, and mis-regulation of specific miRNAs can result in abnormal phenotypes in model organisms and disease in humans (Hammond, 2015; Lin and Gregory, 2015; Tuna et al., 2016). Thus understanding how miRNAs are produced and maintained in the endogenous context is paramount for realizing their full biological functions.

MiRNAs are approximately 22 nucleotides (nt) in length and function by guiding Argonaute (AGO) and associated proteins, called the miRNA-induced silencing complex

(miRISC), to their messenger RNA (mRNA) targets. Once there, miRISC generally represses gene expression through translational inhibition and mRNA degradation (Jonas and Izaurralde, 2015). In animals, miRNAs use partial base-pairing to recognize their targets (Ha and Kim, 2014). This property allows one miRNA to bind a variety of target site sequences. In fact, it has been estimated that over 50% of the human genome is regulated by miRNAs (Friedman et al., 2009).

The mature, 22 nt form of the miRNA is a product of multiple processing steps (Fig. 1.1). Most animal miRNAs share a common, well-conserved biogenesis pathway, although there are also noncanonical routes for specific miRNAs (Finnegan and Pasquinelli, 2013; Ha and Kim, 2014; Xie and Steitz, 2014). In the most common pathway, RNA polymerase II transcribes much longer primary miRNAs (pri-miRNAs) that contain 5'm7G caps and 3'polyadenosine (polyA) tails. The microprocessor, consisting of Drosha and Digeorge syndrome critical region gene 8 (DGCR8; also known as Pasha), then cleaves the pri-miRNA into a 70 nt precursor miRNA (pre-miRNA). This pre-miRNA is exported from the nucleus to the cytoplasm by Exportin 5. In the cytoplasm, Dicer cleaves the terminal loop generating a 22 nt double stranded miRNA. Only one strand of the duplex is used for targeting (guide) while the other strand (passenger) is discarded.

While each tissue of a multicellular animal expresses its own set of miRNAs, neuronal cells seem particularly reliant on the miRNA pathway. Of the hundreds of miRNAs discovered, nearly half are expressed to some degree in the mammalian brain (Shao et al., 2010). Early miRNA studies in model systems pointed to the functional importance of this pathway in neuronal development and function. For example, brain morphogenesis fails in Zebrafish defective for general miRNA biogenesis (Giraldez et al., 2005). In *C. elegans*, specific miRNAs were found to be essential for controlling the fate of two asymmetric chemosensory neurons (Chang et al., 2004; Johnston and Hobert, 2003). A wide body of

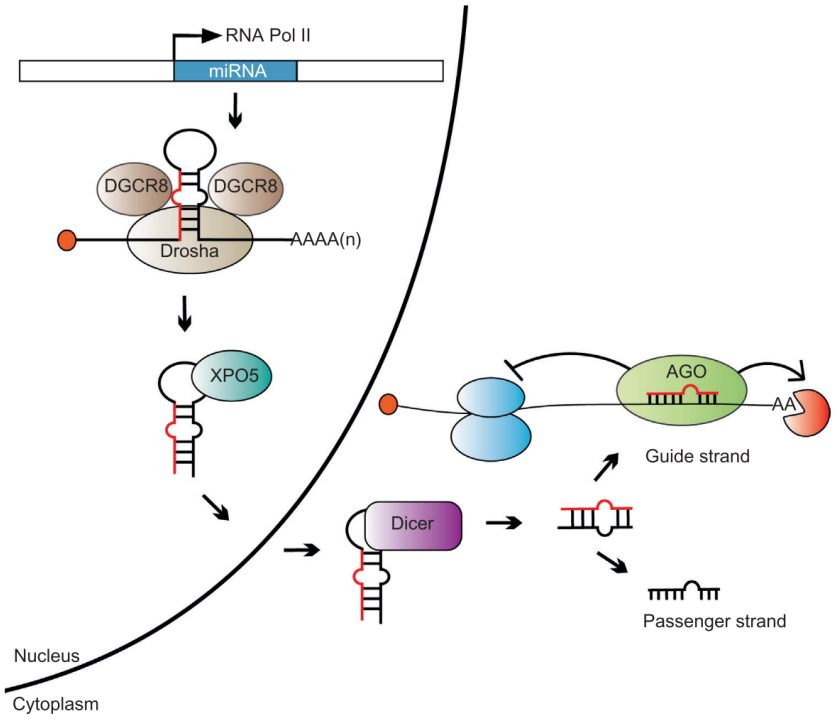

Figure 2.1: MicroRNA Biogenesis. MiRNAs are transcribed in the nucleus by RNA polymerase II. The resulting primary transcript (pri-miRNA) is processed by the microprocessor complex comprised of Drosha and DGCR8. The excised precursor miRNA (pre- miRNA) is then exported from the nucleus by Exportin 5. Once in the cytoplasm the pre-miRNA is cleaved by Dicer. The guide strand of the transient 22 nt double stranded Dicer product is selected for incorporation into AGO, forming the miRISC. Typically miRISC binds the 3'UTR of a target mRNA and triggers translational inhibition and mRNA destabilization.

research has now shown that miRNAs are important for a variety of neuronal processes, such as neurogenesis, axon guidance, and mature neuron functioning (Schratt, 2009; Shi et al., 2010).

MiRNAs biogenesis is an essential process for effective miRNA-mediated gene silencing. Small perturbations in miRNA biogenesis can alter the mature miRNA sequence or levels and, thus, affect gene expression. Here we provide an overview of the miRNA biogenesis pathway in animals, with an emphasis on examples relevant to neurogenesis.

2.2 Genomic Organization

MiRNAs genes reside in a variety of genomic arrangements that differ in frequency across organisms. In mammals the majority of miRNAs are located within the intronic regions of genes (Chang et al., 2015; Kim and Kim, 2007; Monteys et al., 2010; Rodriguez et al., 2004; Saini et al., 2008). There are also rare examples of exonic sequences, usually 3'untranslated regions (UTRs), within mRNAs that encode miRNAs (Chang et al., 2015; Kim and Kim, 2007; Rodriguez et al., 2004). MiRNAs that are found in host genes, whether they are exonic or intronic, are often cotranscribed with their protein-coding counterpart (Baskerville and Bartel, 2005; Rodriguez et al., 2004). However, this is not always the case, and some miRNAs that are located within host genes have their own transcriptional regulatory elements (Corcoran et al., 2009; Monteys et al., 2010; Ozsolak et al., 2008). In *C. elegans*, most miRNA genes are intragenic but even the ones located within protein-coding genes typically have their own promoters (Martinez et al., 2008).

MiRNAs are often arranged in clusters, where closely spaced miRNAs are cotranscribed as part of a common primary transcript (Bartel, 2009; Lau et al., 2001; Lee et al., 2002). miRNA clusters sometimes encode members of the same family of miRNAs, where

each miRNA has identical 5' end sequences (Bartel, 2009). This arrangement is thought to facilitate the expression of related miRNAs that are capable of regulating shared targets. However, not all clusters include related miRNAs and some contain multiple family members. For example, the miR-17-92 cluster in humans consists of six miRNAs that belong to four different miRNA families: miR-17 and miR-20a are part of the miR-17 family, miR-19a and miR-19b-1 are part of the miR-19 family, and miR-18 and miR-92a-1 are each part of their own distinct families (Ota et al., 2004). This cluster is often perturbed in cancers, and may also be important for regulating the expression of genes involved in neurodegenerative diseases (He et al., 2005; Mogilyansky and Rigoutsos, 2013).

2.3 MicroRNA Transcription

The biogenesis of most miRNAs initiates with transcription by RNA polymerase II (Bracht et al., 2004; Cai et al., 2004; Lee et al., 2004). The resulting pri-miRNAs vary widely in size from hundreds to thousands of nts long (Chang et al., 2015; Saini et al., 2008). For details on the identification of miRNA transcription start sites see Chapter 3, Computational and -Omics Approaches for the Identification of miRNAs and Targets, of this book by Hatzigeorgiou and colleagues. In general the transcription of miRNAs is controlled by the same mechanisms that govern the synthesis of protein coding mRNAs. In addition to regulation by chromatin and DNA modifications, specific transcription factors have been shown to control the expression of particular miRNAs during development and in response to various extrinsic conditions (Liu et al., 2013; Marson et al., 2008; Schanen and Li, 2011). For instance, the well-studied p53 transcriptional regulator not only controls the expression of numerous protein coding genes but also stimulates the transcription of specific miRNAs, including members of the miR-34 family, upon DNA damage (Bommer

et al., 2007; Chang et al., 2007; Corney et al., 2007; He et al., 2007; Raver-Shapira et al., 2007; Tarasov et al., 2007; Tazawa et al., 2007). Up-regulation of miR-34 family miRNAs is critical for the tumor suppressive function of p53 because these miRNAs repress the expression of cell proliferation genes and promote apoptosis (Bommer et al., 2007; Chang et al., 2007; Corney et al., 2007; He et al., 2007; Raver-Shapira et al., 2007; Tarasov et al., 2007; Tazawa et al., 2007). Interestingly, miR-34 levels also increase during adulthood in mammals, flies, and worms (Cao et al., 2010; de Lencastre et al., 2010; Ibáñez-Ventoso et al., 2006; Li et al., 2011a,b; Liu et al., 2012), and in Drosophila this has been shown to have a neuroprotective role (Liu et al., 2012). Whether this induction is also mediated by p53 or other transcription factors is yet to be determined.

A recurring motif in the miRNA pathway is transcriptional regulation through negative feedback loops. One striking example of such regulation involves miR-133b and pituitary homeobox 3 (PITX3) in mouse dopaminergic neurons (Kim et al., 2007). PITX3, a homeodomain transcription factor, promotes the expression of miR-133b. However, PITX3 contains miR-133b binding sites in its 3'UTR. Thus rising levels of miR-133b lead to repression of PITX3, halting its own transcription (Kim et al., 2007). An even more intricate loop exists for miR-273 and lsy-6 in a pair of worm chemosensory neurons. These miRNAs, along with the transcription factors DIE-1 and COG-1, form a double negative feedback loop that is important for determining the left/right fate of two *C. elegans* head neurons (Johnston et al., 2005). In this example the left neuron expresses die-1, which induces transcription of lsy-6 miRNA, a repressor of cog-1; in the right neuron, this pathway is inhibited through the expression of miR-273, which targets die-1 for down-regulation (Johnston et al., 2005).

2.4 Primary MicroRNA Processing

Pri-miRNA processing occurs in the nucleus and is often cotranscriptional (Ballarino et al., 2009; Morlando et al., 2008; Pawlicki and Steitz, 2008). In most cases an enzyme complex termed the microprocessor recognizes and excises the approximately 70 nt stem loop pre-miRNA (Denli et al., 2004; Gregory et al., 2004; Han et al., 2004; Landthaler et al., 2004). Typically, the miRNA sequence is embedded in a base-paired stem that is flanked by a basal region that consists of an 11 base pair (bp) lower stem that is preceded by unpaired sequences and a terminal loop of 20 nt (Han et al., 2006; Zeng et al., 2005). A set of primary and secondary sequence motifs have been shown to regulate pri-miRNA processing in human cells. First the distance from the junction between the basal single-stranded and double stranded region to the cut site appears to be critical for accurate cleavage, with an 11 bp distance being ideal (Auyeung et al., 2013; Han et al., 2006; Zeng et al., 2005). Second, three important motifs reside in efficiently cleaved miRNAs: a UG at the basal junction, a UGUG in the apical loop, and a CNNC motif downstream of the stem loop (Auyeung et al., 2013). Additionally, base-pairing at all nts except nt 8, as measured from the basal junction, is a feature in the stem structure of efficiently processed primary miRNAs (Fang and Bartel, 2015). While these elements have been proposed to distinguish microprocessor substrates from other structured RNAs in the cell, they are not universally shared by all miRNAs or across species. The consensus seems to be that pri-miRNA processing is modular, with different elements contributing to recognition and cleavage by the microprocessor (Auyeung et al., 2013; Nguyen et al., 2015).

Recent studies have demonstrated that structural remodeling of some miRNA primary transcripts is necessary for efficient recognition by the microprocessor. In *C. elegans* the let-7 gene encodes primary transcripts that undergo a form of splicing where the 5'

region is removed and replaced with splice leader sequence (Bracht et al., 2004; Mondol et al., 2015). This processing event enables the pri-miRNA to adopt a conformation that makes it a more favorable substrate for Drosha processing (Mondol et al., 2015). The mammalian miR-17-92 clustered miRNA gene is another example where restructuring of the primary transcript is important for microprocessor recognition (Du et al., 2015). The nascent pri-miR-17-92 transcript folds into a structure that blocks processing of all but one of the six miRNAs in this cluster. Cleavage of this transcript to form a progenitor-miRNA removes the sequences that create the inhibitory structure, allowing for efficient processing of all miRNAs in the cluster. Thus upstream processing events that regulate pri-miRNA structure can control their entrance into the miRNA biogenesis pathway.

The microprocessor consists of Drosha and DGCR8 proteins and is essential for the production of most miRNAs. Mice lacking either DGCR8 or Drosha are not viable (Chong et al., 2010; Wang et al., 2007). Monoallelic loss of DGCR8 and other genes on chromosome 22 is associated with Digeorge syndrome (de la Chapelle et al., 1981). Patients with Digeorge syndrome display cognitive defects and have a higher than normal chance of developing schizophrenia (Karayiorgou et al., 1995, 2010). In a mouse model of Digeorge syndrome, haploinsufficiency of Dgcr8 alone led to altered biogenesis of specific miRNAs and various cognitive abnormalities in the animals (Fénelon et al., 2011; Stark et al., 2008). These findings indicate that neuronal function may be particularly sensitive to changes in miRNA dose.

As the catalytic subunit of the microprocessor, Drosha is a ribonuclease (RNase) type III enzyme with two RNase III domains: RIIIDa and RIIIDb (Denli et al., 2004; Gregory et al., 2004; Han et al., 2004; Landthaler et al., 2004). Each of these domains cuts on either side of the RNA stem at 11 bp above the basal junction. This cleavage event usually results in a 2 nt overhang on the 3'end of the product, which is important for subsequent

Dicer processing. DGCR8 contains two RNA binding domains and assists in pri-miRNA processing by binding the pri-miRNA near the terminal loop structure. Recent studies have shown that one Drosha and two DGCR8 proteins interact with pri-miRNAs in a heterotrimeric complex with Drosha positioned at the cleavage sites and DGCR8 proteins bound to each RNase III domain (Herbert et al., 2016; Kwon et al., 2016; Nguyen et al., 2015). This arrangement enables the microprocessor to evaluate multiple elements in the pri-miRNA that contribute to accurate and efficient cleavage.

While the microprocessor can cleave pri-miRNAs in vitro a variety of cofactors regulate this step in vivo (Finnegan and Pasquinelli, 2013). An example pertinent to neuronal function is the involvement of several amyotrophic lateral sclerosis (ALS) associated genes in miRNA biogenesis (Eitan and Hornstein, 2016). The fused in sarcoma/translocated in liposarcoma (FUS/TLS) and TDP-43 TAR DNA-binding protein 43) proteins have been shown to promote the expression of several miRNAs important for neuronal function (Buratti et al., 2010; Gregory et al., 2004; Kawahara and Mieda-Sato, 2012; Ling et al., 2013; Morlando et al., 2012). These proteins interact with the microprocessor and seem to stabilize its recruitment to particular miRNA genes. ALS is a neurodegenerative disease where progressive loss of motor neurons leads to muscle wasting and death. A molecular signature of ALS is the accumulation of pathological aggregates that often include FUS/TLS and TDP-53 (Arai et al., 2006; Deng et al., 2010; Neumann et al., 2006). Thus depletion of functional FUS/TLS and TDP-53 may result in inadequate processing of miRNAs required for neuronal maintenance; for more details on noncanonical functions of the microprocessor, see Chapter 6, MiRNA-Dependent and Independent Functions of the Microprocessor in the Regulation of Neural Stem Cell Biology, of this book.

2.5 Export From the Nucleus

The newly processed pre-miRNA must be exported from the nucleus to the cytoplasm for further maturation steps. This is accomplished by the nuclear transport factor Exportin 5 and its GTP bound Ran cofactor (Bohnsack et al., 2004; Lund et al., 2004; Yi et al., 2003). Exportin 5 preferentially binds pre-miRNAs with canonical structures, including the 2 nt 3'overhang and a paired stem (Okada et al., 2009). Upon nuclear exit, GTP (guanosine triphosphate) hydrolysis by Ran triggers release of the pre-miRNA into the cytoplasm. Depletion of Exportin 5 leads to decreased mature and pre-miRNA levels, indicating that pre-miRNAs trapped in the nucleus may be subject to degradation (Lund et al., 2004; Yi et al., 2003). Inactivating mutations in Exportin 5 have been detected in human colon, stomach, and endometrial tumors (Melo et al., 2010). In cancer cells lines expressing mutant Exportin 5 a subset of miRNAs were significantly down-regulated compared to cells supplemented with wildtype Exportin 5. Since many of these Exportin 5 dependent miRNAs have potential tumor suppressive functions, their depletion in tumor cells with defective Exportin 5 activity may contribute to the oncogenic phenotype. Consistent with the observation that some miRNAs may be more sensitive to the loss of Exportin 5, a recent study that examined the effect of Exportin 5 deletion in human cells found only a modest decrease in the abundance of most miRNAs (Kim et al., 2016). Thus alternative pathways must exist for the delivery of precursor miRNAs to the cytoplasm for final maturation (Xie et al., 2013). The nonessential nature of Exportin 5 for miRNA biogenesis is also consistent with the lack an obvious homolog of this factor in organisms such as *C. elegans* (Büssing et al., 2010).

2.6 Pre-MicroRNA Processing

After exiting the nucleus the pre-miRNA needs to be processed further to produce the mature miRNA. Dicer, an RNase III enzyme, catalyzes this step by excising the terminal loop, which results in a ~22 nt dsRNA that contains the future mature miRNA. Dicer was initially discovered in Drosophila as the enzyme responsible for generating small interfering RNA (siRNA) guides from longer dsRNAs used to induce RNA interference (RNAi) (Bernstein2001). Subsequent work by many labs determined that an essential function for Dicer is the processing of endogenous miRNA precursors (Grishok et al., 2001; Hutvágner et al., 2001; Ketting et al., 2001; Knight and Bass, 2001). Since Dicer is required for the maturation of almost all miRNAs (Kim et al., 2016), it is not surprising that global loss of this factor is associated with severe pleiotropic phenotypes and eventual lethality (Bernstein et al., 2001; Giraldez et al., 2005; Grishok et al., 2001; Ketting et al., 2001; Knight and Bass, 2001). Directed knock out of Dicer in specific types of neurons has demonstrated the importance of this factor in the differentiation, function, and survival of these cells (Cuellar et al., 2008; Damiani et al., 2008; Davis et al., 2008; Haramati et al., 2010; Schaefer et al., 2007; Shin et al., 2009; Tao et al., 2011). Tissue specific loss of Dicer, and hence miRNA maturation, has also been used to reproduce disease phenotypes in model systems. For example, ablation of Dicer in mouse postmitotic motor neurons results in defects characteristic of spinal muscular atrophy, including neurodegeneration and muscle wasting (Haramati et al., 2010).

Similar to Drosha, Dicer contains two RNase III domains, and recent structural work suggests an evolutionary connection between these two factors (Kwon et al., 2016). The RIIIDb domain and the RIIIDa domain cleave the miRNA near the terminal loop at the 5' and 3'ends, respectively (Lau et al., 2012; Zhang et al., 2004). To position the cleavage

sites, it has been postulated that Dicer acts as a molecular ruler, binding to the free 5' and 3'ends at the base of the stem and measuring 22 nts toward the loop region (MacRae et al., 2006; Park et al., 2011).

Like the microprocessor, Dicer function is also regulated by a variety of cofactors. In mammals the HIV-1 TAR RNA binding protein (TRBP) interacts with Dicer (Chendrimada et al., 2005; Haase et al., 2005). In addition to stabilizing Dicer protein, TRBP also influences the cleavage size and miRNA strand selected for loading into AGO (Fukunaga et al., 2012; Lee et al., 2013; Paroo et al., 2009; Wilson et al., 2015). Protein activator of PKR (PACT) is another Dicer partner that regulates the fidelity of pre-miRNA maturation (Lee et al., 2006). Structural studies have revealed that PACT and TRBP bind to the same amino acid stretch in Dicer (Wilson et al., 2015). Mutation of this surface in Dicer abolishes interaction with either cofactor or results in altered cleavage sizes and defective strand selection for a subset of miRNAs (Wilson et al., 2015). It is presently unclear why certain miRNAs are more dependent on TRBP or PACT for accurate maturation.

Considering its essential role in the biogenesis of almost all miRNAs, it is intriguing that the expression and function of Dicer is repressed at multiple levels. Through a negative feedback loop, Dicer itself is repressed by the miRNA pathway. In humans the Dicer mRNA contains several let-7 complementary sites that mark it for destabilization and translational inhibition by miRISC (Forman et al., 2008; Tokumaru et al., 2008). During zebrafish hindbrain development, the expression of miR-107 targets Dicer for down-regulation, which results in decreased biogenesis of miR-9 (Ristori et al., 2015). Reduced accumulation of miR-9 was shown to be important for preventing over-proliferation of neurons at the expense of differentiation. The activity of human Dicer protein has been shown to be regulated by its N-terminal helicase domain (Ma et al., 2008). Deletion of this domain greatly increases its catalytic efficiency. Autoregulation of Dicer activity in vivo is presumably

mediated by structural rearrangement of the helicase domain, possibly by cofactor binding or modifications.

The best characterized inhibitors of Dicer function act on its pre-miRNA substrates. In one example, general repression of pre-miRNA processing occurs during the macrophage inflammatory response through induction of monocyte chemoattractant protein 1-induced protein (MCPIP1) (Suzuki et al., 2011). MCPIP1 cleaves the loop regions of pre-miRNAs, which reduces their recognition by Dicer and makes them vulnerable to rapid degradation. In contrast to global miRNA down-regulation, processing of specific miRNA precursors by Dicer is regulated by the RNA binding protein LIN28 (Heo et al., 2008; Newman et al., 2008; Rybak et al., 2008; Viswanathan et al., 2008). In mammalian cells, LIN28 recognizes sequences in the loop region of let-7 family miRNAs (Heo et al., 2008; Loughlin et al., 2012; Nam et al., 2011; Newman et al., 2008; Piskounova et al., 2008). While binding of LIN28 to let-7 precursor miRNAs is sufficient to block Dicer, this interaction also recruits RNA uridylyltransferase enzymes that add U tails to the pre-miRNA 3'ends (Heo2009, Thornton2012). The uridylated RNA is then a substrate for rapid decay by Dis3l2 (Chang et al., 2013; Ustianenko et al., 2013). This regulatory pathway may be integral to the effects of brain-derived neurotrophic factor (BDNF) on neuronal survival, structure, and synapse function in the mammalian brain (Huang et al., 2012). In cultured mammalian neurons, BDNF was found to induce the expression of LIN28, which led to the down-regulation of let-7 miRNAs. The resulting de-repression of let-7 targets proved necessary for the ability of BDNF to stimulate the outgrowth of neuronal dendrites in culture.

2.7 Mature MicroRNA Complex Formation

The final step of miRNA maturation involves selection of the single stranded miRNA for loading onto AGO (Dueck and Meister, 2014). During this process, one half of the 22 nt duplex is chosen as the guide sequence and stably bound by AGO. The other strand, called the passenger or star, is discarded and presumably degraded through poorly defined mechanisms. MiRNA strand selection is not a random process as only one half of the Dicer product accumulates as the mature miRNA in most cases. Although the choice of one strand over the other is not always predictable, the favored strand often contains certain structural and sequence features. The guide strand typically exhibits looser 5' base pairing to its partner, which may facilitate its incorporation into AGO (Khvorova et al., 2003; Schwarz et al., 2003). Additionally, miRNA guide strands usually have a 5' uracil and are purine rich (Hu et al., 2009). The Dicer interacting proteins, TRBP and PACT, also influence strand selection (Lee et al., 2013). The current model suggests that the association of Dicer and TRBP/PACT with AGO aids in the selection of the guide strand from the newly processed miRNA duplex. Occasionally, passenger strands are detected in miRISC and, thus, have the potential to regulate distinct sets of genes (Okamura et al., 2008).

The fidelity of strand selection can have major implications for gene expression since target regulation by miRISC is dependent on the miRNA sequence. Upon binding to AGO the mature miRNA is positioned to search for targets with complementary sequences. Structural studies have shown that initially nts 2-5 in the miRNA, called the seed region, are available for pairing (Elkayam et al., 2012; Nakanishi et al., 2012; Schirle and MacRae, 2012). Binding to a target sequence then induces a structural rearrangement that exposes nts 6-8 for additional interactions with the target (Schirle et al., 2014). Additionally, this conformational change-induced by seed pairing positions sequences in the miRNA 3'end

for possible pairing interactions (Schirle et al., 2014). These studies have reinforced the importance of 5' seed pairing interactions and raised the possibility of additional sequences contributing to the recognition of specific target sites.

While AGO and its miRNA guide are responsible for binding specific targets, additional factors are required to regulate the expression of the bound mRNA. GW182 proteins bind directly to AGO and recruit factors that trigger translational inhibition and decay of the target mRNA (Pfaff and Meister, 2013). The mechanism of translational repression by miRISC is not well understood but, in most cases, it is also coupled with mRNA destabilization (Jonas and Izaurralde, 2015). In animals, GW182 proteins associated with miRISC recruit deadenylation factors to initiate decay of the mRNA (Behm-Ansmant et al., 2006; Jakymiw et al., 2005; Liu et al., 2005; Meister et al., 2005; Rehwinkel et al., 2005). Shortening of the polyA tail is thought to weaken interactions between the mRNA termini, which exposes the 5' end for decapping. Upon removal of the 5'm7G cap the mRNA is rapidly degraded by 5' to 3' exonucleases. For a detailed examination of how miRISC regulates target gene expression, see Chapter 2, Essentials of MicroRNA-Dependent Control of mRNA Translation and Decay, MicroRNA Targeting Principles and Methods for Target Identification, of this book.

2.8 MicroRNA Stability

Compared to mRNAs, miRNAs as a class are exceptionally stable molecules. Half-life studies in mouse fibroblast cells have shown that the average miRNA can persist for several days (Gantier et al., 2011). A general stabilizing factor for miRNAs is their association with AGO. The cellular abundance of mature miRNAs can be directly regulated by the availability of AGO (Diederichs and Haber, 2007; Winter and Diederichs, 2011). When

bound by AGO the miRNA termini are likely less accessible to exonucleases. Curiously though, target engagement by miRISC has been found to increase or decrease the stability of the bound miRNA depending on the circumstances. In *C. elegans*, target interactions protect at least some miRNAs from decay (Chatterjee et al., 2011; Chatterjee and Großhans, 2009). This target-mediated miRNA protection is thought to prevent release of the miRNA from AGO, safeguarding it from the 5' to 3'exonucleases XRN-1/-2. Conversely, pairing of miRNAs to target sites in mammalian and Drosophila cells has been reported to trigger their destabilization (Cazalla et al., 2010; de la Mata et al., 2015). Extensive base pair interactions were found to induce the addition of nts to the 3'end of the miRNA, which then serve as a signal for 3'–5' degradation (Ameres et al., 2010). Recent work in rodent primary neurons has revealed a setting where this pathway is particularly robust (de la Mata et al., 2015). Even in these cells, though, extensive pairing, beyond what is possible with most natural miRNA targets sites, was required for target RNA-directed miRNA degradation. Thus it remains to be determined if target engagement has a prominent role in regulating miRNA stability in vivo.

Several nucleases have been implicated in miRNA degradation. As mentioned above the 5' to 3'exonucleases XRN-1/-2 can digest miRNAs released from AGO when target sites are scarce (Chatterjee et al., 2011; Chatterjee and Großhans, 2009). In worms the decapping scavenger protein stimulates miRNA decay by helping to recruit XRN-1 to these substrates (Bossé et al., 2013). The 3'to 5' exoribonuclease, ERI1 appears to be a general regulator of miRNA stability in mammalian cells, as loss of this factor resulted in global up-regulation of mature miRNA levels (Thomas et al., 2014). Other decay enzymes seem to be more selective in their miRNA targets. For example, polynucleotide phosphorylase, an interferon inducible 3'to 5' exoribonuclease, specifically targets miR-221 for rapid degradation in human melanoma cells (Das et al., 2010).

While the mechanisms responsible for controlling the turnover of global or select miRNAs are still being elucidated the regulation of miRNA stability seems especially active in neuronal cells. Dynamic miRNA stability has been proposed to be important for learning. In the snail Aplysia, exposure to serotonin resulted in down-regulation of the mature levels of several brain-enriched miRNAs (Rajasethupathy et al., 2009). Serotonin is a neurotransmitter released during learning that promotes the activation of CREB (cAMP Response Element Binding), a transcription factor important for pathways involved in long-term memory. One output of serotonin signaling is the rapid decay of miR-124, which relieves CREB from repression by this miRNA.

In another example of induced miRNA decay a specific set of miRNAs become destabilized in response to dark adaptation in mouse retinal cells (Krol et al., 2010). This change in miRNA levels was shown to be relevant for derepression of a target important for photoreceptor function. Interestingly, this study also demonstrated that miRNAs in general have decreased stability in neuronal cells (Krol et al., 2010).

2.9 Concluding Remarks

In the relatively short amount of time since their discovery, miRNAs have risen to prominence as essential regulators of diverse biological pathways. A key to realizing their roles in cell specification and function is an understanding of how miRNAs are produced and maintained. Neuronal cells in particular seem to engage a myriad of factors and mechanisms to regulate the expression of miRNAs required for their various developmental pathways and activities. The basic steps involved in miRNA biogenesis have been defined. The current challenge is to discover how this process is regulated in different cell types, in response to various external stimuli, and in disease situations.

2.10 Acknowledgements

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Chapter 2, in full, is a reprint of the material as it appears in Essentials of Noncoding RNA in Neuroscience: Ontogenetics, Plasticity of the Vertebrate Brain. William P. Schreiner and Amy E. Pasquinelli, Elsevier, 2017. I was the primary author.

Chapter 3

Remodeling of the *Caenorhabditis elegans* non-coding RNA transcriptome by heat shock

3.1 Abstract

Elevated temperatures activate a heat shock response (HSR) to protect cells from the pathological effects of protein mis-folding, cellular mis-organization, organelle dysfunction and altered membrane fluidity. This response includes activation of the conserved transcription factor heat shock factor 1 (HSF-1), which binds heat shock elements (HSEs) in the promoters of genes induced by heat shock (HS). The upregulation of protein-coding genes (PCGs), such as heat shock proteins and cytoskeletal regulators, is critical for cellular survival during elevated temperatures. While the transcriptional response of PCGs to HS has been comprehensively analyzed in a variety of organisms, the effect of this stress on the expression of non-coding RNAs (ncRNAs) has not been systematically examined. Here

we show that in *Caenorhabditis elegans* HS induces up- and downregulation of specific ncRNAs from multiple classes, including miRNA, piRNA, lincRNA, pseudogene and repeat elements. Moreover, some ncRNA genes appear to be direct targets of the HSR, as they contain HSF-1 bound HSEs in their promoters and their expression is regulated by this factor during HS. These results demonstrate that multiple ncRNA genes respond to HS, some as direct HSF-1 targets, providing new candidates that may contribute to organismal survival during this stress.

3.2 Introduction

In the natural world, animals experience a variety of potentially lethal environmental challenges. Heat stress is one of the most recognizable and can be fatal, if not properly addressed. Organisms have evolved an ancient response to cope with this dangerous insult: the heat shock response (HSR) (Richter et al., 2010). Heat Shock Factor 1 (HSF-1), serves as a master transcriptional regulator of the HSR in eukaryotes (Åkerfelt et al., 2010). Elevated temperatures trigger activation and binding of HSF-1 to heat shock elements (HSEs) in the promoters of genes encoding heat shock proteins (HSPs) and other factors that protect the cell from heat-induced damage. For example, HSPs act as molecular chaperones to deal with the rampant protein mis-folding associated with heat stress. While heat shock (HS) has been shown to induce widespread changes in gene expression in cell as well as animal models, a limited set of genes appears to be direct targets of HSF-1 activation (Duarte et al., 2016; Mahat et al., 2016; Solís et al., 2016). Instead, downstream transcriptional effectors mediate many of the other HS-induced changes in gene expression (Duarte et al., 2016; Mahat et al., 2016; Solís et al., 2016).

There is also potential for post-transcriptional mechanisms to regulate the levels of

protein-coding mRNAs during the HSR (Leung and Sharp, 2010; Shalgi et al., 2014; Zhou et al., 2015; Di Giammartino et al., 2013). In *Caenorhabditis elegans*, specific microRNAs (miRNAs) have been found to be up- or downregulated during HS (Brunquell et al., 2017; Nehammer et al., 2015). MiRNAs function as small, ~22 nt, guide RNAs that use partial base-pairing to recruit Argonaute (AGO) proteins to specific mRNAs, triggering mRNA destabilization and translational repression (Bartel, 2018). While direct mRNA targets of HS-regulated miRNAs are yet to be determined, loss of some of these miRNAs has been shown to affect the viability of *C. elegans* subjected to HS (Nehammer et al., 2015; de Lencastre et al., 2010). For example, deletion of *miR-71* or *miR-239* results in reduced or enhanced survival at elevated temperatures, respectively.

In addition to miRNAs, the expression of other types of non-coding RNAs (ncRNAs) can respond to fluctuations in environmental conditions (Essers et al., 2015; Hellwig and Bass, 2008). The *C. elegans* long ncRNA, *rncs-1* (RNA non-coding starvation upregulated) is induced by food deprivation and potentially regulates other small RNA pathways (Hellwig and Bass, 2008). In a variety of organisms, HS and other stress conditions have been shown to cause accumulation of RNAs from transposon and other types of repetitive sequences (Horváth et al., 2017). Currently, it is unclear if the HS-induced changes in ncRNA expression are a direct result of the HSR or the aftermath of defective transcriptional or post-transcriptional silencing mechanisms under these conditions. Additionally, whether specific ncRNAs or generally all members of a certain class respond to HS has not been systematically investigated.

To examine the organismal response of different classes of ncRNAs to HS, we surveyed the expression of short ncRNAs (<30 nt), including miRNAs and Piwi RNAs (piRNAs), and longer ncRNAs (>100 nt), including long intergenic ncRNAs (lincRNAs), unclassified ncRNAs, pseudogene- and repeat-derived RNAs, in *C. elegans* under HS versus control

temperature conditions. Within each class, we observed HS-induced changes for specific transcripts that included rapid and dramatic upregulation of some ncRNAs. Additionally, we observed an accumulation of extended protein-coding gene (PCG) transcripts in HS conditions and developed filters to identify these aberrant readthrough RNAs. Similar to canonical HS-responsive PCGs, we found that the promoter sequences of the miRNA *miR-239*, *Helitron1_CE* transposons and the pseudogene *dct-10* contain Heat Shock Elements (HSEs) that are bound by HSF-1 in response to HS. Furthermore, we show that upregulation of these ncRNAs in HS is regulated by HSF-1, suggesting that they are direct transcriptional targets of the HSR. Overall, our comprehensive analysis of ncRNA expression in response to HS revealed new types of molecules that are regulated by and, in turn, may contribute to organismal survival in the face of this stress.

3.3 Materials and Methods

3.3.1 Sequencing and analysis of mRNAs and long ncRNAs

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 h. Animals were then collected, snap-frozen and total RNA 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 sequence 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 (Li et al., 2009). Reads were counted using FeatureCounts and Ensembl 88 gene annotations (Liao et al., 2014). Differential expression of gene expression was determined using DESeq2 (Love et al., 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 version of the Wormbase ftp site (Lee et al., 2018). 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 the

list of upregulated PCGs. In addition, PCGs with an IR score >1 were also filtered out as these 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 et al., 2015) Primary aligned reads were obtained using the following command: 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.

3.3.2 Small RNA sequencing and analysis

Caenorhabditis elegans were grown to the L4 stage at 20°C then shifted to 35°C for 6 h. 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 from two independent biological replicates. piRNAs were analyzed by mapping small RNA sequencing data to a database of *C. elegans* piRNAs obtained from Wormbase using the STAR 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).

3.3.3 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 the MACS2 command macs2 callpeak -g ce —keep-dup auto —call-summits -q 1e-6 using combined biological replicates and the single input replicate available (Zhang et al., 2008). To normalize ChIP-seq data for display purposes, Bowtie2-mapped reads from combined biological 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.

3.3.4 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., 2009). The most significant motif identified in HSF-1 peak summits closely resembles the previously-identified HSE motif using the same dataset (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-bp regions centered on HSF-1 summits (Grant et al., 2011).

HSF-1 RNAi and overexpression

RNAi was performed by feeding animals either empty vector or hsf-1(RNAi). RNAi experiments 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 (Chiang et al., 2012; Kumsta et al., 2017).

qRT-PCR

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 are 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: GTTGATGACGAGACGAGTGTGAG

3.4 Results

Multiple classes of RNA genes respond to heat shock in *C. elegans*

The transcriptional response of PCGs to HS has been intensively studied, yet the effect of this stress on non-coding RNA (ncRNA) expression has not been examined systematically (Richter et al., 2010; Åkerfelt et al., 2010; Vihervaara et al., 2018). Here we used RNA sequencing to compare the levels of various types of RNA from last larval stage (L4) *C. elegans* maintained at 20°C (control, CTRL) or shifted to 35°C (HS) for 4 (RNA-seq) or 6 h (smRNA-seq). Along with the expected HS-induced changes in mRNA expression (Supplementary Table S1), this profiling also revealed specific RNA genes belonging to the miRNA, piRNA, lincRNA, unclassified ncRNA, pseudogene and repeat families that changed at least two-fold in response to heat stress (Figure 3.1A).

While our conditions elicited changes in protein-coding mRNA expression comparable to previously published studies of the transcriptional response to HS in *C. elegans*, we noticed some peculiarities in the upregulated gene set (Brunquell et al., 2016; Jovic et al.,

2017). Visual inspection of the sequencing reads mapped to the UCSC Genome Browser indicated several false positive gene calls present in the list of PCGs upregulated by HS. For example, *srt-42* was originally listed as the second most highly upregulated gene in HS but reads associated with this gene did not conform to the expected splicing pattern and included sequences stretching upstream into the neighboring HSP gene, *hsp-16.41* (Figure 3.1B). Thus, the reads covering *srt-42* likely emanate from transcripts that failed to be properly terminated from the highly HS-induced *hsp-16.41* gene. These types of aberrant transcripts, known as DoGs for Downstream of Gene containing transcripts, have been previously detected in various stress conditions (Vilborg et al., 2017, 2015). While a functional role for DoGs is yet to be demonstrated, incomplete transcriptional termination of a highly induced upstream gene can provide RNA-seq reads that falsely assign a downstream gene on the same strand as also upregulated (Vilborg et al., 2015, 2017). Since these false positives are unlikely to maintain coding potential, we filtered them from our original list of HS upregulated PCGs by removing 49 genes with reads in the upstream Intergenic Junction that were 2-fold higher in HS and that had a ratio of intronic to exonic reads of 0.4 or greater (Figure 3.1B and Supplementary Table S1).

Another source of mistakenly called upregulated PCGs was the intronic residence of tRNAs and snoRNAs that apparently failed to be properly terminated in HS (Figure 3.1B). These longer versions of tRNAs and snoRNAs enabled them to be detected in standard RNA-seq, whereas the normally shorter forms are not captured in this procedure. By filtering out genes with an intron to exon ratio (IR) >1 due to accumulation of an intronic ncRNA in HS, we removed another 14 PCGs from the upregulated list (Figure 3.1B and Supplementary Table S1). Finally, PCGs that overlapped a repetitive element that was likely responsible for the increased sequencing reads in HS were also filtered out (Figure 3.1B and Supplementary Table S1). The bioinformatic steps used for filtering are detailed

in the 'Materials and Methods' section and available online (https://github.com/wschrein).

Figure 3.1: HS alters the expression of coding and non-coding RNAs. (A) Small RNA-seq and stranded paired-end RNA-seq were used to analyze changes in RNA expression in Caenorhabditis elegans shifted from 20°C (control, CTRL) to 35°C (HS) for 4 (RNA-seq) or 6 h (smRNA-seq). The numbers of differentially expressed genes in each RNA category are indicated. 'ALL' refers to the total number of annotated genes in each class. (B) Strategy used to filter out false positive upregulated mRNAs. See 'Materials and Methods' section for further details; codes used for filtering are available at github.com/wschrein. (C) DAVID Functional Annotation Clustering of Genes up- and downregulated in response to HS (Huang et al., 2009b). Representative members of each cluster with an enrichment score > 2 are shown. Size of dot corresponds to number of genes in each cluster. (D) TEA for genes up- and downregulated in response to HS. TEA was performed using the Wormbase TEA tool (Angeles-Albores et al., 2016). Abbreviations: PVD-Sensory neuron (polymodal nociceptive for mechanosensation and thermosensation), Hyp7-entire syncytium of hyp7, Hyp6-Cylindrical hypodermal syncytium in head, Psub1-Embryonic founder cell, AB-Embryonic founder cell, Psub3-Embryonic founder cell, EMS-Embryonic Cell, Z2-Germ line precursor cell, Z3-Germ line precursor cell.

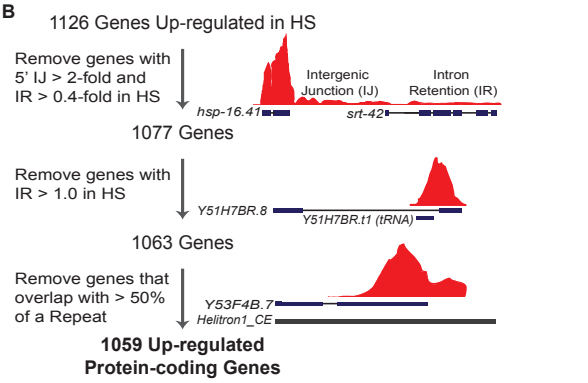
Figure 1

A

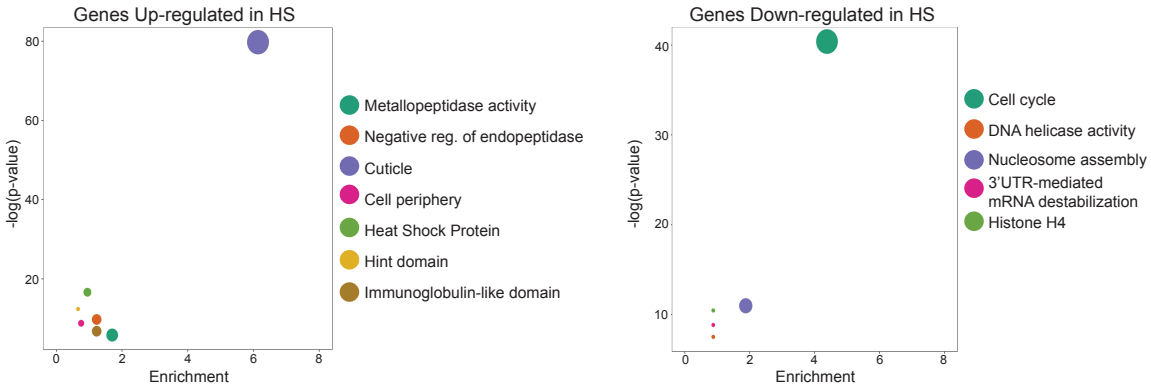
Number of RNA species with ≥ 2-fold change in expression after heat shock

	UP	DOWN	ALL
miRNA	8	15	437
piRNA	15	63	15363
lincRNA	9	1	176
ncRNA	71	1	8037
pseudogene	94	23	1922
repeat	21	23	181
mRNA	1059	801	20203

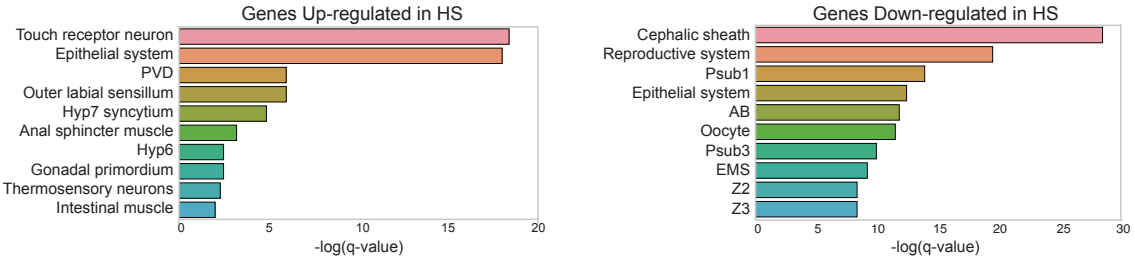
B



C



D



With a higher confidence set of PCGs differentially regulated by HS, we confirmed that our conditions elicited changes in mRNA expression comparable to previously published studies of the transcriptional response to HS in *C. elegans* (Brunquell et al., 2016; Jovic et al., 2017; Melnick et al., 2019). The extensive changes in PCG expression induced by HS aligned with molecular pathways expected to be regulated by this stress. DAVID Gene Ontology (GO) analyses of genes upregulated by HS indicated strong enrichment for those associated with cuticle maintenance, HSPs and enzymatic factors (Figure 3.1C and Supplementary Table S1) (Huang et al., 2009a,b). Genes in these categories have well-established roles in stress protection and have been previously found to be upregulated in HS (Figure 3.1C and Supplementary Table S1) (Li et al., 2016; Brunquell et al., 2016; Jovic et al., 2017). Distinct functional annotations, largely involved in nucleic acid binding, were enriched in the genes downregulated by HS (Figure 3.1C and Supplementary Table S1). Decreased expression of genes associated with DNA replication is consistent with halted growth and cell division triggered by heat stress (Richter et al., 2010).

The up- and downregulated gene sets also pointed to distinct tissue responses to HS, consistent with cell non-autonomous regulation of the HSR in C. elegans (Prahlad et al., 2008; O'Brien and van Oosten-Hawle, 2016). Using tissue enrichment analysis (TEA), we observed that upregulated genes were associated with neuronal and epithelial cells (Figure 3.1D) (Angeles-Albores et al., 2016). In particular, enrichment in thermosensory neurons may reflect the role of these neurons in activating the HSR in a cell non-autonomous manner (Figure 3.1D) (Prahlad et al., 2008). Downregulated PCGs were associated with reproductive tissues, which likely corresponds to the delay of development in stress conditions.

Having confirmed that the HS conditions used in these studies elicited the expected changes in PCG expression, we next examined the effect of HS on major classes of ncRNA

genes. The finding that particular members of each ncRNA class were up- or downregulated in response to HS suggests that the HSR controls the transcription or stability of specific ncRNAs.

3.4.1 Specific miRNAs respond to thermal stress

Of the 205 miRNAs we detected by small RNA sequencing, 8 were upregulated at least 2-fold in *C. elegans* subjected to HS compared to CTRL conditions (Figure 3.2A and B; Supplementary Table S2). Consistent with a previous study, miR-239b was one of the most highly upregulated miRNAs following HS (Figure 3.2A and B) (Nehammer et al., 2015). The largest fold change was observed for miR-4936, which increased by over 200-fold in HS (Figure 3.2A-C). This miRNA was virtually undetectable at the CTRL temperature but accumulated to high levels in HS (Figure 3.2C). Furthermore, the predominant isoform we detected for miR-4936 in HS (5' AUUGCUUUGUGGCUUUGCUGGUAAC 3') differs from the reference sequence listed at miRBase (5' UGCUUUGUGGCUUUGCUGGUA 3') (Kozomara et al., 2019). This difference in 5'-end sequence is expected to affect target recognition, which is largely driven through initial pairing of miRNA nucleotides 2-7 (seed sequence) (Bartel, 2018). Since target mRNA degradation is often the outcome of miRNA regulation, we searched the set of downregulated PCGs for complementary sites in their 3'UTRs to the seed sequence (nt 2-7) of upregulated miRNAs (Supplementary Table S2). The predicted targets of these miRNAs reflect GO terms associated with genes whose levels decrease in heat stress, such as 3'UTR binding (Figure 3.2B). Furthermore, some of these downregulated genes are potential targets of multiple upregulated miRNAs, suggesting cooperativity (Figure 3.2D). These observations are consistent with a role for the upregulated miRNAs in contributing to the repression of some PCGs in HS.

Whereas 52 miRNAs were found to be downregulated in HS, most of these were the

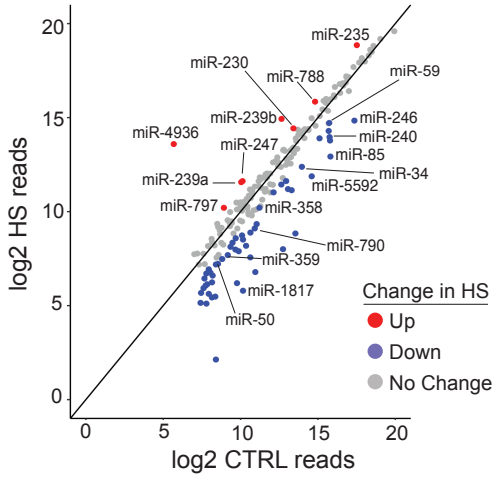
lowly expressed passenger strands of the initially processed miRNA duplex (Supplementary Table S2). The levels of only 15 guide strand miRNAs were reduced more than 2-fold after HS (Figure 3.2A and B; Supplementary Table S2). Target predictions for the 11 downregulated miRNAs that were among the 100 most abundant miRNAs revealed top enriched GO terms associated with molting and movement (Figure 3.2B). Twenty-one of these target genes have the potential for recognition by three or more miRNAs downregulated by HS, raising the possibility that increased expression of these genes is facilitated by alleviated miRNA repression (Figure 3.2E). Interestingly, the Period protein homolog lin-42 is strongly upregulated in HS and a predicted target of four different miRNAs with reduced expression in HS (Figure 3.2E and Supplementary Table S2). This gene regulates molting cycles and acts as a general transcriptional repressor of miRNA genes (McCulloch and Rougvie, 2014; Monsalve et al., 2011; Van Wynsberghe et al., 2014; Perales et al., 2014). Thus, increased expression of lin-42 in HS could contribute to transcriptional repression of many miRNA genes. This would be consistent with the decreased levels of passenger strand miRNAs, which are more sensitive to changes in transcription than the stable guide strands (Marzi et al., 2016).

In addition to miRNAs, we also identified 11,979 different piRNAs in our small RNA sequencing datasets. Of these, 15 increased and 63 decreased by at least 2-fold in HS compared to control conditions (Supplementary Table S2). Using the piRTarBase piRNA target prediction tool (Wu et al., 2019), we found that very few differentially regulated PCGs have potential for regulation by these piRNAs (Supplementary Table S2). Thus, the limited differences in piRNA levels are unlikely to contribute much to the extensive changes in PCG expression observed in HS.

Figure 3.2: The expression of specific miRNAs is regulated by HS. (A) Expression of miRNAs in CTRL versus HS detected by small RNA-sequencing. Results represent the average of two independent biological replicates and miRNAs reproducibly up- (red) and down- (blue) regulated by $\geq$ 2-fold are indicated. (B) List of guide strand miRNAs within the top 100 expressed miRNAs that are reproducibly up- or downregulated in response to HS. The final column shows the most highly enriched biological process GO term of potential targets for each miRNA (see Supplementary Table S2). GO analysis was performed using DAVID (Huang et al., 2009b). (C) Analysis of miR-4936 expression in CTRL versus HS conditions by northern blotting. 5S rRNA serves as a loading control. (D and E) Network analysis of differentially regulated mRNAs targeted by at least three up- or downregulated miRNAs. Cytoscape was used to draw networks (https://cytoscape.org) (Shannon et al., 2003).

Figure 2. Heat shock alters the expression of miRNAs

A HS-induced changes in miRNA expression



B

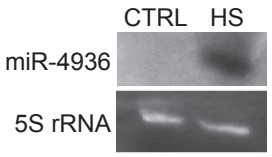
miRNAs up-regulated in HS

miRNA	Fold Change	Top GO of targets
miR-4936	224.3	Cell Division
miR-239b	4.9	Reg of Translation
miR-239a	2.9	Oogenesis
miR-247	2.8	mRNA 3'-UTR binding
miR-235	2.5	Embryo development
miR-797	2.4	Embryo development
miR-788	2.0	None
miR-230	2.0	mRNA 3'-UTR binding

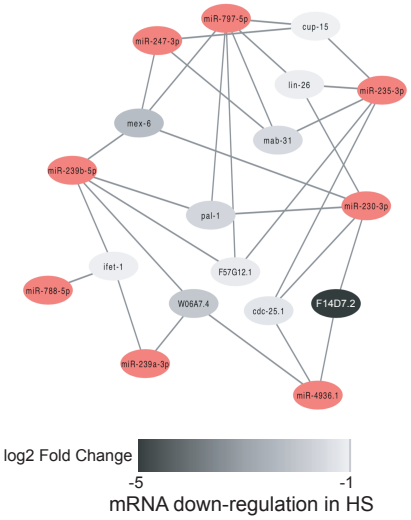
miRNAs down-regulated in HS

miRNA	Fold Change	Top GO of Targets
miR-1817	-20.3	Molting cycle
miR-85	-7.4	Muscle contraction
miR-5592	-6.6	Locomotion
miR-246	-5.7	Neg reg of endopeptidase
miR-240	-3.5	Neuron projection dev
miR-790	-3.1	None
miR-34	-3.0	Body morphogenesis
miR-359	-2.8	Axon regeneration
miR-50	-2.3	Molting cycle
miR-59	-2.0	Locomotion
miR-358	-2.0	Body motion

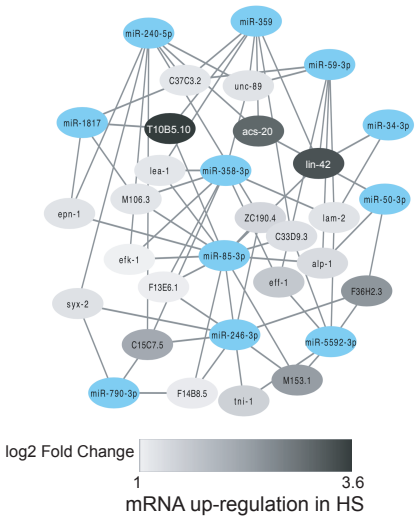
C



D Overlapping targets of up-regulated miRNAs



E Overlapping targets of down-regulated miRNAs

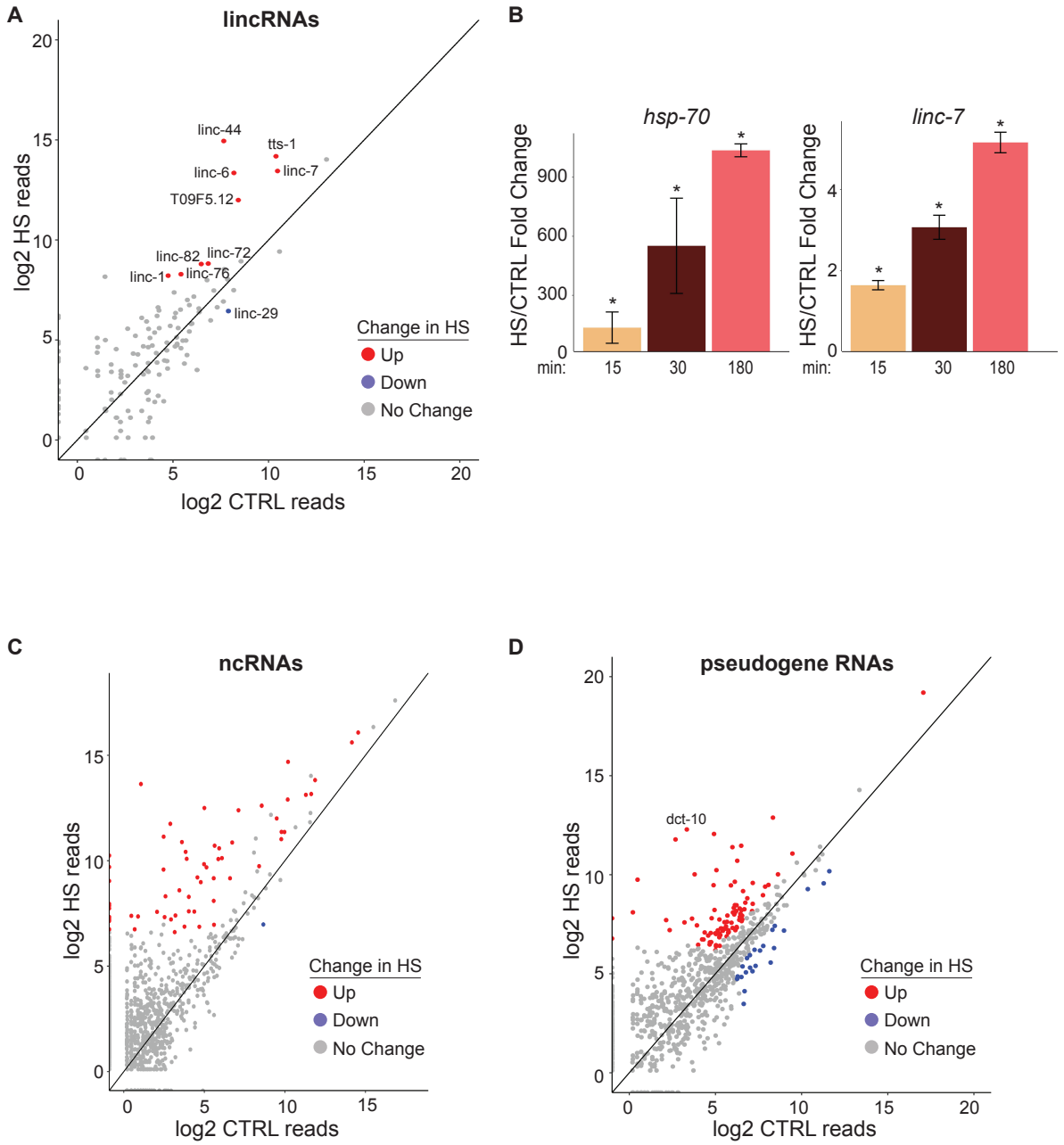


3.4.2 Specific long non-coding RNAs accumulate in HS

In contrast to miRNAs, where similar numbers of the more abundant species were up- and downregulated in HS, longer ncRNAs primarily increased in levels in response to heat stress. Approximately 150 genes are annotated as long intergenic non-coding RNAs (lincRNAs) in *C. elegans* (Nam and Bartel, 2012). While functional roles for the lincRNAs are largely unknown, the lincRNA genes *tts-1* and rncs-1 have been associated with longevity and stress pathways, respectively (Essers et al., 2015; Hellwig and Bass, 2008). Through our RNA profiling, we found that nine relatively abundant lincRNAs increased and only one lincRNA decreased by more than two-fold in HS compared to CTRL conditions (Figure 3.3A and Supplementary Table S3). Interestingly, *tts-1* was among the highly upregulated lincRNAs, suggesting that its role in longevity may be linked to a stress pathway (Essers et al., 2015). At least one of the lincRNAs, linc-7, appears to be rapidly upregulated by HS (Figure 3.3B). Interestingly, this lincRNA contains five sites with complementarity to the seed region of the rapidly upregulated miRNA, miR-239b-5p. Additionally, linc-82 contains eight seed matches for miR-239a-3p and miR-230-3p, both of which accumulate in HS (Figure 3.2A and B; Supplementary Table S2). These potential lincRNA-miRNA interactions may regulate the stability, availability or function of these specific RNAs during HS.

Figure 3.3: HS alters the expression of long non-coding RNAs. (A) Expression of long intergenic non-coding RNAs (lincRNAs) in CTRL versus HS detected by stranded paired-end sequencing. Significantly up- (red) and downregulated (blue) lincRNAs ($\geq$2-fold change with baseMean $\geq$ 50 and padj < 0.01 from three independent replicates) are indicated. (B) qRT-PCR analysis of *hsp-70* and *linc-7* 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). (C) Expression of ncRNAs in CTRL versus HS detected by stranded paired-end sequencing. Significantly up- (red) and downregulated (blue) ncRNAs ($\geq$2-fold change with baseMean $\geq$ 50 and padj < 0.01 from three independent replicates) are indicated. (D) Expression of pseudogene RNAs in CTRL versus HS detected by stranded paired-end sequencing. Significantly up- (red) and downregulated (blue) pseudogene RNAs ($\geq$2-fold change with baseMean $\geq$ 50 and padj < 0.05 from three independent replicates) are indicated.

Figure 3


A
lincRNAs
log2 HS reads
log2 CTRL reads
linc-44
tts-1
linc-6
linc-7
T09F5.12
linc-82
linc-72
linc-1
linc-76
linc-29
Change in HS
Up
Down
No Change
B
hsp-70
linc-7
HS/CTRL Fold Change
min:
15
30
180
C
ncRNAs
log2 HS reads
log2 CTRL reads
Change in HS
Up
Down
No Change
D
pseudogene RNAs
dct-10
log2 HS reads
log2 CTRL reads
Change in HS
Up
Down
No Change

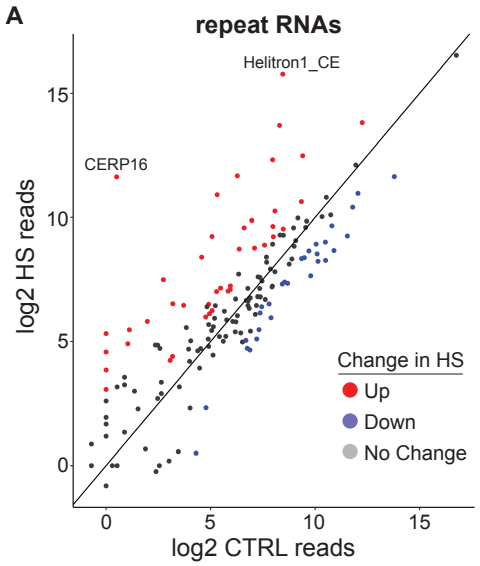
In addition to lincRNAs, over 7000 unclassified ncRNAs that do not belong to a previously characterized class of non-coding RNA are annotated in the *C. elegans* genome (Lee et al., 2018). Of these, 71 increased and 1 decreased at least 2-fold in response to HS (Figure 3.3C and Supplementary Table S3). We also detected expression for 1455 pseudogenes and found that 94 were up- and 23 were downregulated in HS (Figure 3.3D and Supplementary Table S4). The relatively large number of pseudogene RNAs that accumulated to high levels in HS suggests that elevated temperatures disrupt the surveillance pathways that normally suppress pseudogene expression.

3.4.3 Repetitive element-derived RNAs accumulate in heat shock

By re-mapping our RNA-sequencing data to a list of consensus *C. elegans* repetitive elements obtained from repbase, we identified reads for 165 types of repetitive elements in the *C. elegans* genome (Bao et al., 2015). Similar numbers of repeats (~20) were found to increase or decrease in HS; however, the magnitude of change was greatly amplified in the upregulated set (Figure 3.4A and B; Supplementary Table S4). The apparent strong upregulation of CERP16 is likely due, at least in part, to the position of this element immediately downstream of *hsp-16.2* and *hsp-16.41*. The DoGs produced by each of these highly induced HSP genes are likely a predominant source of reads for this repeat element.

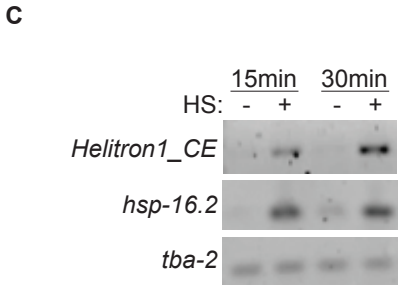
Figure 3.4: Upregulation of multiple repetitive element RNAs during HS. (A) Expression of repetitive element RNAs in CTRL versus HS detected by stranded paired-end sequencing and mapped to a database of repetitive elements (Bao et al., 2015). Significantly up- (red) and downregulated (blue) repeat RNAs ($\geq$2-fold change with baseMean $\geq$ 100 and padj < 0.05 from three independent replicates) are indicated. (B) List of repetitive element RNAs upregulated at least 10-fold in HS. (C) Semi-quantitative RT-PCR detection of the indicated RNAs in CTRL and after 15 or 30 min of HS. (D) Quantitative RT-PCR analysis of *hsp-16.2* and *Helitron1_CE* RNA expression during a time course of HS. Mean fold changes and SEM from three independent replicates are shown. *P < 0.05, **P < 0.01, ***P < 0.001 (t-test, two-sided).

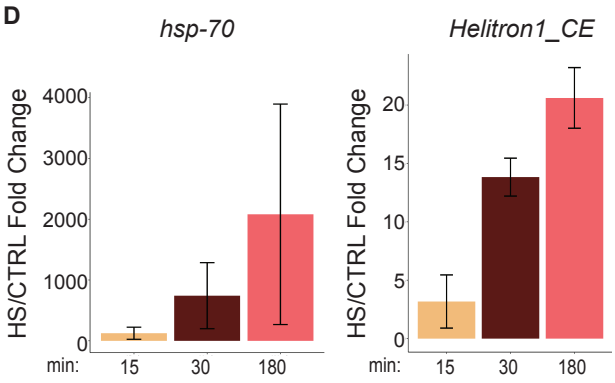
Figure 4



B

Repeat	Fold Up
CERP16	1698.5
Helitron1_CE	156.5
Merlin1m_CE	47.6
TIR43YW1_CE	41.6
HelitronY1_CE	41.0
HelitronY1A_CE	20.2
CER7-LTR_CE	17.3
RCD1	13.6





In contrast, at least some members of the rolling circle DNA transposons known as Helitrons appeared to be strongly induced independently of neighboring PCGs (Figure 3.4B and see below). RNA from *Helitron1_CE* rapidly accumulated to high levels upon HS (Figure 3.4C and D). Our reanalysis of previously published *C. elegans* HS RNA-seq data also confirmed fast induction of Helitron repetitive element RNA (Brunquell et al., 2016).

3.4.4 HSF-1 controls the expression of specific ncRNAs

While defective small RNA silencing pathways may explain some of the increased repeat RNA expression during HS, the rapid and massive accumulation of Helitron RNAs suggests that transcriptional induction is also involved (Weiser and Kim, 2019). We noted that *Helitron1_CE* exhibited an HSR comparable to that of canonical HS-induced genes, such as *hsp-16.42*, with barely detectable RNA levels that quickly rise upon HS (Figure 3.4C). Thus, we predicted that, like HSP genes, Helitrons could be direct transcriptional targets of HSF-1. To investigate this possibility, we remapped *C. elegans* HSF-1 and Pol II ChIP-seq data from Li and colleagues to include repeat and other ncRNA genomic loci (Li et al., 2016). Similar to the promoter region for *hsp-16.2* and *hsp-16.41*, we found evidence for HSF-1 and Pol II association with *Helitron1_CE*, and other Helitron family members, in response to HS (Figure 3.5A; Supplementary Tables S1 and 4). Furthermore, the HSF-1 peaks overlapped with multiple copies of heat shock response elements (HSEs) present in the Helitron genes (Figure 3.5A and Supplementary Table S4).

The interaction of HSF-1 with Helitron regions suggests that HSF-1 could directly contribute to the upregulation of Helitron RNA in HS. Using conditions that decrease (hsf-1(RNAi)) or increase (transgenic overexpression; hsf-1(OEX)) the levels of HSF-1, we observed the expected opposite effects on the expression of *hsp-16.2*, an established HSF-1 target, in response to HS (Figure 3.5B). Likewise, the induction of *Helitron1_CE*

expression by HS was significantly reduced in hsf-1(RNAi) and enhanced in hsf-1(OEX) conditions (Figure 3.5B). Given the abundance of HSEs present in some Helitrons and the sensitivity of *Helitron1_CE* to HSF-1 levels, upregulation of these repeat genes during HS may be largely regulated at the transcriptional level.

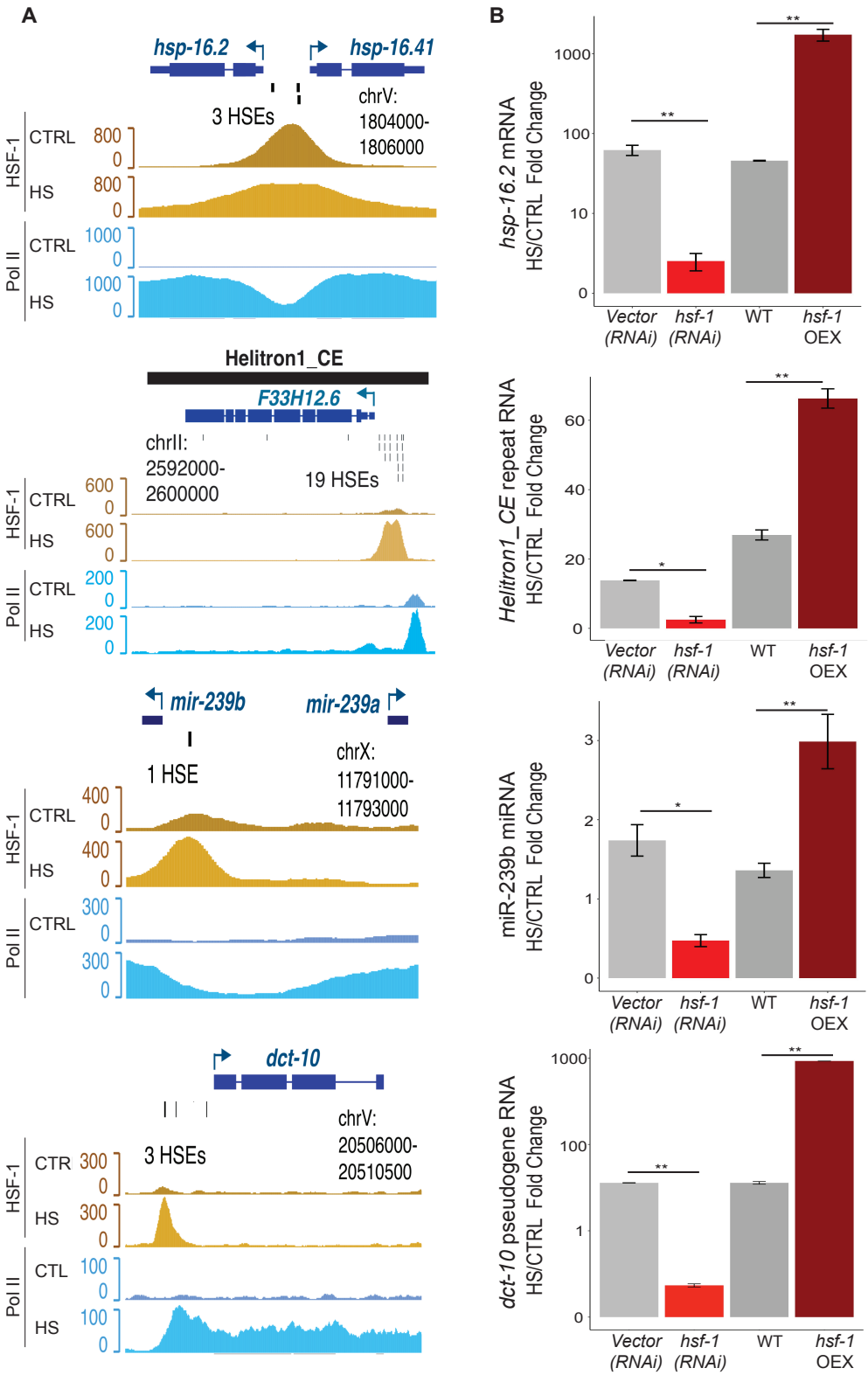
To explore the possibility that other ncRNAs may be part of the direct HSF-1 transcriptional program in HS, we analyzed their putative promoter regions (1 kb upstream of the annotated start site) for HSEs and HSF-1 peaks in the ChIP-seq data from Li et al., (Li et al., 2016). Of the upregulated miRNAs, only the miR-239 locus fit these criteria (Supplementary Table S2). This region is situated between miR-239b and miR-239a, which are transcribed in opposite directions. The single HSE and greater level of HSF-1 ChIP-seq reads are closer to the start of miR-239b, but enhanced Pol II occupancy in HS is observed over both miRNAs, consistent with their mutual upregulation in HS (Figures 3.2A and B and 5A; Supplementary Table S2). Furthermore, we found that reducing or increasing HSF-1 levels resulted in lower or higher miR-239b levels, respectively, in response to HS (Figure 3.5B). For the upregulated longer ncRNA genes, HSEs and evidence of HSF-1 binding in HS were detected in the promoter regions of 0 lincRNAs, 11 unclassified ncRNAs and 5 pseudogenes (Supplementary Table S4). Consistent with the DAF-16/FOXO-controlled tumor gene, *dct-10* , being one of the most robustly induced pseudogenes, we found that its promoter contains multiple HSEs and its expression in HS is regulated by hsf-1 (Figure 3.5 and Supplementary Table S4). Altogether, these findings point to an expanded role for HSF-1 in directing the transcription of specific ncRNA genes as part of the HSR in *C. elegans*.

3.5 Discussion

Here we surveyed the response of multiple classes of ncRNA, as well as protein-coding, genes to an episode of HS in *C. elegans* . Our analysis shows that, of the currently annotated genes, HS induced at least a 2-fold change in the expression of ~9% PCGs, 5% miRNAs, 0.5% piRNAs, 6% lincRNAs, 0.1% unclassified ncRNAs, 6% pseudogenes and 24% of the repeat families. Furthermore, some of the most upregulated ncRNAs, such as miR-4936, were barely detectable under control temperature conditions, demonstrating that often ignored, lowly expressed genes should be reconsidered in different contexts. Our finding that several ncRNA genes parallel canonical HSR genes, such as HSPs, in their dependence on HSF-1 for rapid induction suggests that regulatory RNAs may also have important roles in mitigating the damage caused by excessive heat.

Figure 3.5: 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).

Figure 5


A
hsp-16.2
hsp-16.41
3 HSEs
chrV:
1804000-
1806000
HSF-1
CTRL
HS
Pol II
CTRL
HS
Helitron1_CE
F33H12.6
chrII:
2592000-
2600000
19 HSEs
mir-239b
mir-239a
1 HSE
chrX:
11791000-
11793000
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

3.5.1 Aberrant 3'-extended transcripts accumulate in heat shock

During our analysis of differentially regulated PCGs in control versus HS conditions, we noticed a previously described phenomenon known as DoGs for Downstream of Genes (Vilborg et al., 2015, 2017; Melnick et al., 2019). DoGs result from transcriptional readthrough, leading to mRNAs with long 3' extensions that include normally intergenic sequence (Vilborg et al., 2015, 2017). Sometimes DoGs read into adjacent downstream PCGs, which can result in aberrant gene calls (Vilborg et al., 2017). Thus, the reads assigned to the downstream gene actually belong to transcripts that are chimeric with the upstream gene and are unlikely to retain coding potential. Such false positives are particularly problematic for compact genomes where closely spaced genes reside in the same orientation. To deal with this issue in our set of PCGs upregulated in HS, we applied two filters to remove candidates likely emanating from DoGs. We found it necessary to combine the criteria of increased intergenic junction and intron reads because *C. elegans* 5'UTRs are incompletely annotated and splicing is generally less efficient in HS conditions (Shalgi et al., 2014; Yost and Lindquist, 1986) This filtering pipeline may be useful for analyzing other *C. elegans* RNA-seq datasets, since DoGs seem to be generated by a variety of stress conditions (Vilborg et al., 2015, 2017; Melnick et al., 2019).

The reason for transcriptional readthrough and DoGs generation during stress is currently unclear (Vilborg et al., 2017). Since transcriptional termination of most PCGs by RNA Pol II involves the cleavage and polyadenylation machinery, it is conceivable that this process is generally less efficient at elevated temperatures (Proudfoot, 2016). The termination step can also be influenced by chromatin architecture and structural context of the poly(A) signal, which may be sensitive to temperature changes (Proudfoot, 2016). Furthermore, transcriptional termination efficiency by RNA Pol III also seems reduced in HS. We detected 3'-extended tRNA and snoRNA transcripts in the HS, but not control,

RNA samples; whereas the mature forms of these Pol III RNAs are too short for capture in standard RNA-seq library preparations. Since tRNA and snoRNA genes are commonly embedded in introns of PCGs in *C. elegans* , the extended forms observed in HS sometimes overlapped exons. We were able to filter out these falsely called upregulated PCGs by removing genes with intron retention scores >1. While the cause of 3'-extended Pol II and III transcripts in heat shocked *C. elegans* is yet to be determined, an awareness that these aberrant transcripts can accumulate is important for understanding changes in coding potential induced by stress.

3.5.2 Specific ncRNAs from multiple classes respond to heat shock

Although the majority of individual *C. elegans* miRNA genes are apparently dispensable under laboratory growth conditions, there is accumulating evidence that specific miRNAs play integral roles in a variety of stress response pathways (Ambros and Ruvkun, 2018; Miska et al., 2007; Alvarez-Saavedra and Horvitz, 2010). Previous studies found that loss of miR-71, miR-246, miR-80 or the miR-229, -64, -66 cluster resulted in increased sensitivity to heat stress (Nehammer et al., 2015; de Lencastre et al., 2010). We observed that miR-71 is upregulated 1.4-fold in HS (Supplementary Table S2), which would be consistent with a survival role for this miRNA in elevated temperatures. Conversely, we detected an over 5-fold decrease in miR-246 levels in response to HS, which seems contrary to the profound sensitivity of miR-246 mutants to HS (de Lencastre et al., 2010). Another counterintuitive change in expression was observed for miR-239a/b. We, and others, detected substantial increases in the levels of these related miRNAs in response to HS (Nehammer et al., 2015). Additionally, we present evidence that these miRNAs are transcriptionally induced by HSF-1. Yet, the miR-239a/b(nDf62) strain, which lacks both miRNAs, was previously reported to have an enhanced survival phenotype when subjected

to HS (Nehammer et al., 2015; de Lencastre et al., 2010). The identification of direct targets of the differentially regulated miRNAs will be necessary to understand how their altered expression during HS relates to their functional roles.

Most of the miRNAs we found to be up- or downregulated by HS have not yet been ascribed biological functions. Of the miRNAs that increase in HS, previous reports have documented that miR-247 promotes survival upon exposure to graphene oxide and miR-235 regulates developmental arrest in response to starvation (Liu et al., 2019; Kasuga et al., 2013). Our observation that these miRNAs increase in HS suggests that they may have roles in multiple stress response pathways. Downregulation of miR-34 in response to our HS conditions was surprising given its reported roles in promoting dauer survival in response to food deprivation and ensuring normal development in animals subjected to rapid temperature fluctuations (Isik et al., 2016; Burke et al., 2015). However, miR-34 mutants have also been found to exhibit increased and decreased radiosensitivity in the soma and germline, respectively, suggesting that the function of this miRNA is highly context dependent (Kato et al., 2011).

By far, the most changed miRNA was miR-4936, which increased over 100-fold in HS. Although the dramatic fold change is linked to the virtually undetectable levels in control conditions, this miRNA did accumulate to appreciable levels, as validated by Northern blot. Curiously, this miRNA exhibited more heterogeneity in its mature forms than most other miRNAs. In fact, the predominant species detected in our HS studies differs from the sequence currently present in miRbase (Kozomara et al., 2019). While the miR-4936 sequence resides in a predicted hairpin that resembles other miRNA precursors, a corresponding passenger strand has not yet been identified that would be indicative of canonical Dicer processing. Regardless of these peculiarities, it is evident that HS induces massive upregulation of this RNA, making it a contender for a role in the HSR.

Unlike the comparable numbers of up- and downregulated miRNAs, members of the longer ncRNA classes predominately increased in HS. Considering that very few functions have been assigned to any *C. elegans* long ncRNAs, the ones that increase in HS are good candidates for potential roles in this stress condition. The lincRNA *tts-1* (transcribed telomeric sequence) has previously been shown to be important for the extended lifespan of animals with reduced insulin signaling (*daf-2* mutants) or mitochondrial activity (*clk-1* mutants) (Essers et al., 2015). As the levels of *tts-1* are increased in long lived *daf-2* mutants and in animals grown in the presence of bacterial pathogens, the over 10-fold increase in *tts-1* RNA induced by HS could reflect a general response of this lincRNA to stress (Essers et al., 2015; O'Rourke et al., 2006).

Some mammalian long ncRNAs, including pseudogene RNAs, have been assigned roles as competitive endogenous RNAs (ceRNAs) (Grüll and Massé, 2019). In this role, long ncRNAs have the potential to sequester and sometimes destabilize small RNAs, such as miRNAs, through base-pairing interactions. A caveat to broad regulation of miRNA availability by ceRNAs, is the typically much lower abundance of lincRNAs and pseudogene RNAs (Denzler et al., 2016). Thus, dramatic upregulation of these types of RNAs, as we observed for specific lincRNAs, unclassified ncRNAs and pseudogenes during HS, could boost their regulatory potential. Notably, the upregulated linc-7 contains five sites that support seed pairing to miR-239a-5p and miR-239b-5p. The linc-82 contains repeating elements that can pair with the other half of miR-239a, referred to as the -3p or passenger strand, as well as with miR-230-3p. Considering that these lincRNAs and their potential miRNA partners are all upregulated in HS, mutual RNA stabilization could be an outcome of these interactions.

There is precedent for repeat RNAs being expressed at higher levels in response to increased temperature (Jolly et al., 2004; Goenka et al., 2016; Hogan et al., 1995;

Klosin et al., 2017). In *C. elegans*, even a mild temperature elevation can trigger aberrant expression of repetitive loci. Growth at 25°C, instead of the standard condition of 20°C, resulted in increased levels of RNA from some transposons (Klosin et al., 2017). Given the importance of silencing transposons and maintaining genome integrity, it is not surprising that multiple small RNA and chromatin remodeling pathways act to prevent the mobilization of repetitive elements under normal and stress conditions (Klosin et al., 2017; McMurchy et al., 2017). Nonetheless, the upregulation of RNAs from multiple repeat families during HS indicates that these silencing pathways become compromised in harsh environments. Interestingly, the HS-induced change in repeat RNA expression can be inherited and last for multiple generations in the absence of the original heat stress (Klosin et al., 2017). It has been proposed that certain repeat RNAs or transposon mobilization could be advantageous during stress (Horváth et al., 2017). For example, SINE RNAs in human and mouse cells are induced by HS and act as transcriptional repressors by binding directly to RNA Pol II (Mariner et al., 2008; Yakovchuk et al., 2009). Additionally, changes in genomic arrangement caused by stress induced expression of transposons could foster beneficial changes in the progeny of stressed parents. Evidence for this idea was recently documented in Schizosaccharomyces pombe, where certain forms of stress induced the mobility of transposable elements and the new insertions were linked to enhanced adaptation to the assault (Esnault et al., 2019). As elevated temperatures have been associated with increased genetic mobility in *C. elegans*, it is possible that relaxed silencing of transposons during HS could sometimes provide an advantageous genetic change in the progeny of stressed animals (Matsuba et al., 2013).

3.5.3 HSF-1 regulates the expression of diverse ncRNAs

Activation of the transcription factor HSF-1 in response to HS, and a variety of other environmental perturbations, is essential for driving the expression of factors needed to survive the stress (Vihervaara et al., 2018). While numerous studies, including our own, have observed the upregulation of hundreds of PCGs in response to HS, recent work in mammalian cells and budding yeast has shown that HSF1/Hsf1 is directly responsible for the transcriptional induction of only a small subset of these genes, which mostly encode molecular chaperones (Vihervaara et al., 2018; Mahat et al., 2016; Solís et al., 2016). Although it is yet to be established if HSF-1 has a similar limited set of direct essential targets in intact multicellular organisms, it is evident that HS induces widespread changes in HSF-1 binding across the genome in *C. elegans* (Li et al., 2016). Here we show that some of these HSF-1-bound loci are associated with ncRNA genes. We identified 2 miRNA, 11 unclassified ncRNA, 5 pseudogene and 5 repeat family genes that were upregulated in HS and contained HSEs centered within HSF-1 ChIP-seq peaks. Furthermore, we confirmed that the upregulation of miR-239b, *Helitron1_CE* and pseudogene *dct-10* , induced by HS was sensitive to HSF-1 levels. A previous study found that six *C. elegans* miRNAs were dependent on HSF-1 for increased expression in HS (Brunquell et al., 2017). Thus, the network of miRNAs, and other ncRNAs, controlled by HSF-1, both directly and indirectly, expands the repertoire of genes regulated by this transcription factor during the HSR. Furthermore, the dramatic, and in some cases rapid, accumulation of specific ncRNAs in response to heat stress in *C. elegans* suggests that functions for some regulatory RNAs may only surface when needed for organismal survival in the natural world.

3.6 Data Availability

The RNA-seq datasets generated in this study are available from GEO under accession number GSE132876. Other data and reagents are available upon request.

3.7 Acknowledgements

We thank Dr. Cindy M. Voisine and members of the Pasquinelli Lab for helpful discussions and critical reading of the manuscript.

Chapter 3, in full, is a reprint of the material as it occurs in *Nucleic Acids Research*, W. P. Schreiner, D. C. Pagliuso, J. M. Garrigues, A. P. Aalto, J. S. Chen, and A. E. Pasquinelli, Oxford University Press, 2019. Schreiner W.P. was the primary author.

Chapter 4

Identifying Targets of ALG-1 and ALG-2 Associated MicroRNAs in Aging

4.1 Introduction

Aging is a part of life—albeit a not always enjoyable part. Aging and the health related declines associated with it, do not affect everyone equally. A very lucky few age gracefully with very few health problems. However, for many, aging is associated with a decline in health, and aging is the chief risk factor for many diseases such as cancer and heart disease (Niccoli and Partridge, 2012). By studying aging and the processes that regulate it, researchers can better elucidate the mechanisms that lead to aging and related diseases as well as identify therapies that could lessen the burden of aging.

C. elegans is an excellent model organism for the study of aging. Many of the genes that regulate human aging are conserved in *C. elegans* (Tissenbaum, 2015). The average lifespan of *C. elegans* is approximately 17 days and does not have too much deviation (Tissenbaum, 2015). Thus, researchers can examine the effects of various

genetic backgrounds on aging on a relatively quick timescale. *C. elegans* aging is regulated by many genes and environmental conditions with the insulin signaling pathway at the center (Inukai and Slack, 2013; Tissenbaum, 2015).

The miRNA pathway is also involved in *C. elegans* aging (Inukai and Slack, 2013). Mutations in *lin-4*, the first miRNA ever discovered, lead to a shortened lifespan (Inukai and Slack, 2013). This shortened lifespan is caused by an increase in one of the primary targets of lin-4, *lin-14* (Inukai and Slack, 2013). MicroRNA profiling in aged worms also identified additional miRNAs that are associated with aging such as *miR-71* and *miR-239a/b* (de Lencastre et al., 2010). Mutations in *miR-71* are associated with a reduced lifespan whereas mutations in *miR-239* are associated with an increased lifespan (de Lencastre et al., 2010). In addition to miRNAs, Argonaute proteins in *C. elegans* are also associated with aging. In *C. elegans*, ALG-1 and ALG-2 are the primary effectors of the miRISC complex (Grishok et al., 2001). ALG-1 is thought to be the primary AGO, as *alg-1* mutants have severe developmental phenotypes, while *alg-2* mutants appear phenotypically normal except for a reduced brood size (Bukhari et al., 2012; Vasquez-Rifo et al., 2012; Grishok et al., 2001). *C. elegans* lacking both ALG-1 and ALG-2 are not viable (Grishok et al., 2001; Vasquez-Rifo et al., 2012). Most of the experiments characterizing *alg-1* and *alg-2* have been in larval stage worms. Recently, our lab explored the role of these AGO proteins in aging (Aalto et al., 2018). Surprisingly, *alg-1* and *alg-2* have divergent phenotypes in aging: *alg-1* mutants are short-lived whereas *alg-2* mutants are long-lived (Aalto et al., 2018). ALG-1 and ALG-2 also exhibit differing protein expression patterns, with ALG-1 levels declining in age and ALG-2 levels remaining the same as worms age (Aalto et al., 2018). To better understand the divergent lifespan phenotypes exhibited by these Argonautes, I searched for genes misregulated in *alg-1* mutants that could be targeted by ALG-1 associated miRNAs. I found that genes misregualted in day 5 *alg-1* deficient worms, are enriched for putative

MiRNA Binding Sites (MBS) of ALG-1 associated miRNAs. These results may partially explain the shortened lifespan of *alg-1* loss of function mutants.

4.2 Experimental Procedures

I obtained 3'UTR sequences for *C. elegans* genes from version WS263 of the genome (Lee et al., 2018). For each gene I considered the longest isoform and did not consider genes with UTRs shorter than 20 nucleotides. To search for genes targeted by aging miRNAs, I searched for sites complementary to the seed sequence (nt 2-7) of the miRNA. Analysis was done using the python programming language and relied on the NumPy and pandas data science libraries. After searching for seed matches in all *C. elegans* genes for which we have UTR information, enrichment was determined for different classes of genes (such as genes up or down regulated) enriched in *alg-1* and *alg-2* loss of function mutants at day 5 of adulthood.

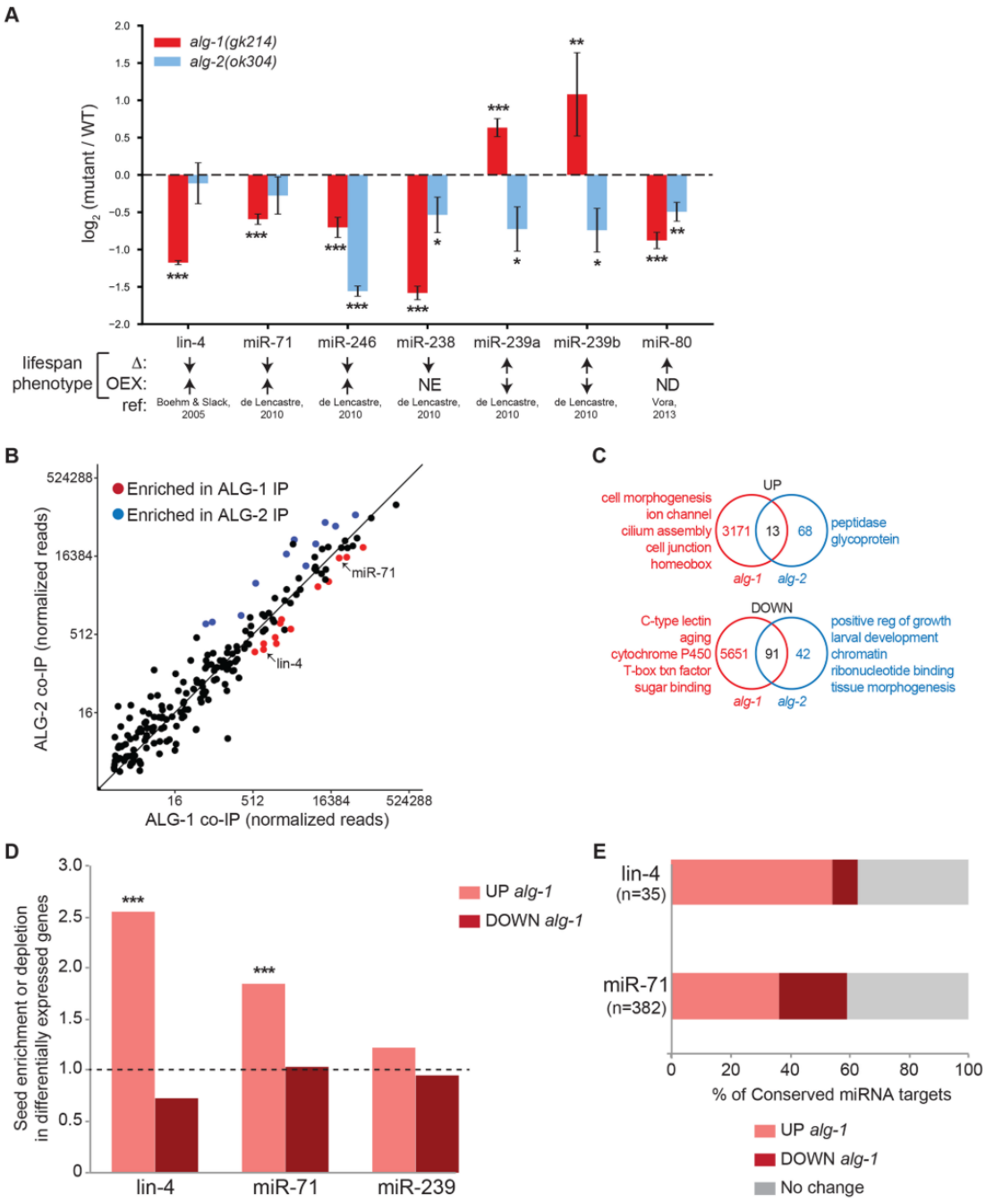
4.3 Results

In addition to the divergent aging phenotypes, expression patterns, and tissue localization, ALG-1 and ALG-2 also differ in the miRNAs that they associate with. Co-immunoprecipitation assays of ALG-1 and ALG-2 in day 5 animals showed that ALG-1 and ALG-2 bind 13 and 11 different miRNAs respectively (Figure 4.1B). Interestingly some of these miRNAs have known aging phenotypes. For instance, lin-4 and miR-71 were both enriched in the ALG-1 pull down and loss of these miRNAs is associated with reduced lifespan (Inukai and Slack, 2013). Furthermore, transcriptome profiling in day 5 *alg-1(gk214)* and *alg-2(ok304)* mutants revealed that different sets of protein coding genes

were misregulated in these mutants (Figure 4.1C). In *alg-1* mutants 3,184 genes were upregulated and 5,742 downregulated (Figure 4.1C). In *alg-2*, fewer genes were up and down regulated compared to *alg-1* mutants, and there was not a large overlap in the genes misregulated in day 5 *alg-1* and *alg-2* mutants (Figure 4.1C). Interestingly, at the L4 stage of adulthood, *alg-1* and *alg-2* mutants display a large overlap in the number of genes that are misregulated in both backgrounds (Brown et al., 2017). Using seed analysis, I looked for possible MBS in genes both up and down regulated in *alg-1* mutants. Interestingly genes upregulated in *alg-1* were enriched for putative lin-4, and miR-71 binding sites (Figure 4.1D). These miRNAs are also depleted in *alg-1* mutants and are associated with ALG-1 in day 5 (Figure 4.1A-B). These findings suggest that the shortened lifespan of *alg-1* mutants could be caused by the misregulation of lin-4 and miR-71 targets.

Strikingly, four genes upregulated in *alg-1* deficient worms are predicted to be targeted by both miR-71 and lin-4. One of these targets is *lin-14*, which when upregulated in aging antagonizes longevity (Inukai and Slack, 2013). In *alg-1* mutants, miR-239 is upregulated (Figure 4.1 A). However, I did not see an enrichment in miR-239 targets in genes downregulated in *alg-1*. A similar analysis for genes misregulated in *alg-2* mutants is not displayed as there are too few genes misregulated in this background to draw meaningful relationships.

Figure 4.1: Altered miRNA and mRNA expression in *alg-1* and *alg-2* mutants. (A) TaqMan analyses of the indicated miRNAs in *alg-1(gk214)* (red) and *alg-2 (ok304)* (blue) compared to WT animals at day 5 ofadulthood, averaged from five independent experiments. The error bars represent SEMs. *P<0.05, **P<0.01, ***P<0.001 (t-test). The published lifespan phenotypes observed with reduced expression (delta symbol), overexpression (OEX), and corresponding references (ref) are indicated. (increased lifespan), # (decreased lifespan), NE (no effect), ND (not determined). (B) Enrichment of specific miRNAs with ALG-1 (red) or ALG-2 (blue) detected by sequencing ofsmall RNAs that co-immunoprecipitated (co-IP) with each AGO protein at day 5 ofadulthood averaged from 2 independent experiments. See S2 Table for a complete list of miRNAs reproducibly enriched for association with ALG-1 or ALG-2. (C) Overlap of all genes significantly (P<0.05) up- or down-regulated at day 5 ofadulthood in *alg-1(gk214)* (red) and *alg-2(ok304)* (blue) compared to WT animals. Overlap of down- regulated genes in *alg-1(gk214)* and *alg-2(ok304)* is more than expected by chance (P<0.0001, hypergeometric test). The top ranked gene ontology terms identified by DAVID analysis are listed. See S3 Table and S4 Table for complete RNA-seq and DAVID results. (D) Enrichment and depletion of seed pairing (nucleotides 2-7) for the indicated miRNAs with genes differentially expressed in *alg-1(gk214)* versus WT animals. The fold difference shown is in comparison to the fraction of seed-pairing sites detected in genes with unchanged expression patterns in the *alg-1* mutants. ***P<0.0001 (Chi Squared with Yates Correction). (E) Percent of conserved lin-4 and miR-71 targets predicted by TargetScan (Lewis et al., 2005; Jan et al., 2011) that are differentially regulated in *alg-1(gk214)*.


A
alg-1(gk214)
alg-2(ok304)
log2 (mutant / WT)
lin-4
miR-71
miR-246
miR-238
miR-239a
miR-239b
miR-80
lifespan phenotype
Δ:
OEX:
NE
ND
ref:
Boehm & Slack, 2005
de Lencastre, 2010
Vora, 2013
B
Enriched in ALG-1 IP
Enriched in ALG-2 IP
ALG-2 co-IP (normalized reads)
ALG-1 co-IP (normalized reads)
miR-71
lin-4
C
UP
cell morphogenesis
ion channel
cilium assembly
cell junction
homeobox
3171
13
68
peptidase
glycoprotein
alg-1
alg-2
DOWN
C-type lectin
aging
cytochrome P450
T-box txn factor
sugar binding
5651
91
42
positive reg of growth
larval development
chromatin
ribonucleotide binding
tissue morphogenesis
D
Seed enrichment or depletion in differentially expressed genes
UP alg-1
DOWN alg-1
lin-4
miR-71
miR-239
E
lin-4 (n=35)
miR-71 (n=382)
% of Conserved miRNA targets
UP alg-1
DOWN alg-1
No change

4.4 Discussion

The upregulation of lin-4 and miR-71 targets in *alg-1* loss of function mutants suggests that the *alg-1* aging phenotype is caused by loss of the association of these miRNAs with ALG-1 in the *alg-1* deficient background. Future studies can expand upon this finding by further exploring the miR-71 and lin-4 targets that contribute to the decreased longevity phenotype of *alg-1* in aging. The upregulation of *lin-14* in *lin-4* mutants likely contributes to the shortened lifespan in *alg-1* mutants as increased *lin-14* levels caused by deletion of *lin-4* has already been shown to lead to a decreased lifespan (Inukai and Slack, 2013). Four genes, including *lin-14*, upregulated in *alg-1* mutants are predicted to be targeted by both *lin-4* and *miR-71*. Future experiments could ask if these genes contribute to the lifespan phenotype of *alg-1* mutants, by knocking down these genes in *alg-1* mutants individually or in various combinations. If knockdown of these targets rescues the decreased lifespan phenotype, this would provide strong evidence that loss of regulation of the target gene by ALG-1 is responsible for the decreased lifespan phenotype. Biochemical experiments, such as ALG-1 RNA Immunoprecipitations or CLIP-seq, could be used to confirm that ALG-1 directly regulates these targets. If global techniques such as CLIP are used, researchers will be able to identify all ALG-1 targets in aging.

Target prediction analysis can be useful for exploratory data analysis, but its scope can be limited owing to a lack of complete UTR annotations for *C. elegans* genes and the fact that it can miss putative non-canonical miRNA-target binding interactions. Future *in silico* experiments could repeat the seed searching analysis with different parameters to compensate for this shortcoming and to identify additional putative ALG-1 targets. For instance, researchers could search for seed matches but allow for one mismatched base pair. Although, the seed is considered the primary determinant in miRNA-target binding, 3'

sequences also can confer specificity to targets (Bartel, 2018). For example, biochemical evidence from human Ago2 shows that nt 13 through 16 of the miRNA are available for base pairing (Sheu-Gruttadauria et al., 2019). Future analysis, could take this into consideration in searching for potential miRNA targets which could then be experimentally validated.

4.5 Acknowledgements

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Chapter 5

Conclusions

The Heat Shock Response has been studied for over fifty years, and the transcriptional response to HS has been especially well studied. However, there is less known with respect to the role of non-coding RNAs in the HSR. Here I have presented evidence that there are massive changes in the transcriptome during HS. Fascinatingly, the repertoire of HSF-1 targets appears to be more diverse than previously thought as specific non-coding RNAs of different classes appear to be directly regulated by HSF-1, including a repeat RNA, a miRNA, and a pseudogene. The direct regulation of these genes by HSF-1 suggests that they function in the HSR. Careful studies should examine the effects of knockdown and overexpression of these genes on *C. elegans* viability during HS.

In addition to these direct HSF-1 targets, we found additional ncRNAs that change in HS. Despite a general block on transcription in HS, some of these genes are massively upregulated. Below, I speculate on the possible function of the different classes of ncRNAs that change in HS and describe methods that researchers can use to elucidate the function of these ncRNAs in HS.

5.1 MicroRNAs and the Heat Shock Response

MicroRNAs are part of complex gene regulatory networks, and in humans miRNAs are predicted to target at least 50% of the transcriptome (Friedman et al., 2009). It should perhaps come as no surprise that miRNAs appear to also be involved in the response to HS. In fact, certain unique features of miRNA mediated target regulation lend themselves exceedingly well to the sweeping changes in gene expression that occur in HS. On one hand, miRNAs are rapidly transcribed and very stable relative to other genes—properties that could aid in the rapid response to HS (Reichholf et al., 2019). On the other hand, miRNAs can also regulate their targets in a more nuanced role and reduce the levels of target genes by small but significant ways. In this role, miRNAs are often analogized as "sculptors" of the genome, chipping away at specific targets to achieve a desired balance of gene expression (Bartel, 2018). It is easy to speculate that this more nuanced role of miRNAs could be leveraged in HS to finely tune the levels of specific RNAs to the needs of *C. elegans* undergoing HS. For instance, transcription of HSPs in HS appears to be stochastic, in that isogenic animals subjected to the same HS treatment display vastly different levels of HSP induction (Rea et al., 2005). In this way, miRNAs could act as fine tuners to standardize the expression of genes whose levels are stochastically upregulated in HS.

MicroRNAs can regulate their targets through mRNA destabilization or translational inhibition (Ambros and Ruvkun, 2018). Although, mRNA destabilization is thought to be the main method of target repression, whether this is the case in HS is unknown. In *C. elegans* deprived of food there is evidence that miRNA mediated target regulation shifts towards translational inhibition (Holtz and Pasquinelli, 2009). Inhibition of translation, uncoupled with mRNA destabilization in HS would presumably allow for the continued translation of

miRNA targets upon return to homeostasis. Future studies of the HSR in *C. elegans* should make use of ribosome profiling as well as RNA-seq to uncover how miRNA mediated target repression is achieved in HS. Ribosome profiling experiments could also shed light on the contribution of translational vs. transcriptional upregulation of Protein Coding Genes (PCGs) in HS. For instance, in *Streptomyces coelicolor*, translational, not transcriptional upregulation of genes appears to be more important for the expression of HS induced genes (Bucca et al., 2018).

Although certain miRNAs are implicated in the response to thermal stress, the function of these miRNAs in HS remains undetermined. *In silico* analysis can shed light on the possible targets of miRNAs in HS. One idea is that miRNAs that increase in HS could be responsible for regulating the expression of genes that decrease in HS, whereas genes that increase in HS could be targeted by miRNAs that decrease in HS. Through miRNA target prediction analysis, we found putative miRNA target interactions that fit this pattern. For instance, HSP mRNAs are targeted by some of the miRNAs that decrease in HS. There is some evidence that excess levels of HSPs can be toxic which would explain why miRNAs might target HSPs in basal conditions (Krebs and Feder, 1997). In HS, where upregulation of HSPs is crucial, it would make sense to down regulate these miRNAs to maximize HSP expression. Measuring the levels of predicted miRNA targets in *C. elegans* deficient for miRNAs of interest could be used to test this hypothesis. Further experiments that disrupt the putative miRNA binding site on the target mRNA, and then ask if miRNA mediated regulation is lost would be an excellent way to provide strong evidence of direct miRNA-target regulation.

While prediction methods prove useful in hypothesis generation, to precisely determine the function of HS regulated miRNAs high throughput biochemical techniques such as individual-nucleotide resolution Cross-Linking and ImmunoPrecipitation (iCLIP)

will be necessary to determine the global targets of the miRISC complex during HS. A benefit of using iCLIP, is that a certain percentage of sequencing reads are chimeric in that the miRNA is ligated to the AGO targeted mRNA (Broughton et al., 2017). This allows researchers to not only determine the mRNA targets of AGO in HS, but also the targeting miRNA (Broughton et al., 2017). This information will give a comprehensive view of miRNA targeting in HS and identify novel miRNA-target interactions that could be critical for the HSR.

5.2 The Role of Repeat RNAs in Heat Shock

In Chapter 3, we show that HS affects the expression of repeat RNAs. As opposed to other classes of ncRNAs that we examined, a similar amount of repeat RNAs are up as well as down regulated. A simple explanation for the changes in repeat RNA seen in heat stress could be that pathways that regulate transposon levels are repressed leading to changes in transposon RNA levels. Or changes in repeat RNA in HS could be caused by aberrant transcription. In the case of Helitron RNA this seems less likely, as we present evidence that transcription of Helitrons is directly regulated by HSF-1. Another possibility is that Helitron and/or additional HS induced repeat RNAs could regulate certain biological processes in HS. Repeat RNAs in mammalian (sat III) and insect (omega) cell culture congregate in the nucleus of heat stressed cells and form Nuclear Stress Bodies (nSBs) along with HSF-1 (Goenka et al., 2016; Jolly and Lakhotia, 2006; Jolly et al., 2004; Hogan et al., 1995). One possible role for nSBs is the inhibition of transcription and splicing as transcription associated factors localize to nSBs (Biamonti and Vourc'h, 2010). Titrating specific splicing factors in HS could help remodel splicing or RNA processing to fit with the necessary gene expression programs for survival in HS (Biamonti and Vourc'h, 2010). I believe Helitrons

may play a similar role because they are directly regulated by HSF-1 and massively induced in heat shock like sat III repeats. In *C. elegans*, HSF-1 is constitutively expressed and localizes to nuclear foci in HS, but it is unknown if repetitive RNAs take place in this process in worms (Morton and Lamitina, 2013). Future studies could examine whether Helitron RNA colocalizes with HSF-1 foci in HS using Fluorescence In Situ Hybridization (FISH). RNAi against Helitron RNAs in HS could determine if repeat RNAs are necessary for the formation of HSF-1 foci. If Helitron RNA localizes to nSBs, determination of its protein interacting partners, if any, would be of paramount importance to determine the function of Helitron nSBs in HS.

In mammalian cell culture, repeat RNAs also repress transcription in HS through the inhibition of pol II (Mariner et al., 2008; Yakovchuk et al., 2009). The repeat RNAs transcribed by pol III repress pol II transcription by inhibiting the ability of pol II to bind DNA (Mariner et al., 2008; Yakovchuk et al., 2009). *In vitro* experiments could determine if Helitron RNA is capable of hindering the ability of pol II to interact with DNA.

If TEs are active in HS, they may be capable of insertion into the genome. Normally, this is viewed as a negative consequence of Transposable Elements and is part of the reason for their "selfish element" moniker (Orgel and Crick, 1980). Intriguingly, in times of environmental stress, like thermal shock, this bug might just be a feature (Figure 5.1.). This idea, which was first proposed by Barbara McClintock, posits that in the germline during stressful periods, the progeny of the stressed parents could accumulate transposon derived mutations on which natural selection could act (McClintock, 1984). In some cases, these mutations could enhance fitness in the new stressful environment. There is a precedent for evolution favoring genetic diversity in times of stress in *C. elegans*. *C. elegans* reproduce asexually the majority of the time, and 99.9% of *C. elegans* exist as hermaphrodites. The lack of genetic diversity in primarily self-crossing animals could disadvantage them in

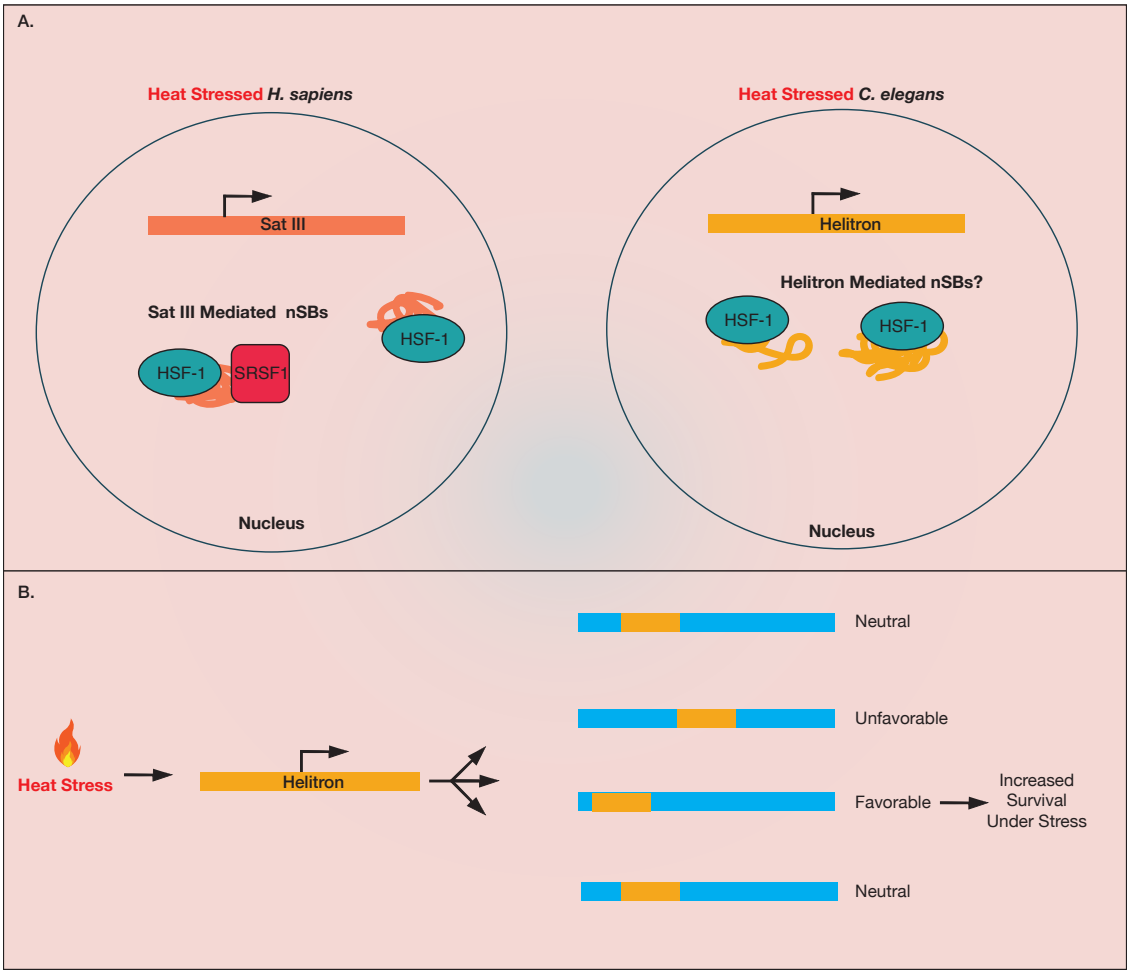

Figure 5.1: Possible Roles of Helitrons in Heat Shock. (A) In humans, some repeat RNAs form nuclear Stress Bodies (nSBs) in heat shock. It is not known if Helitrons serve a similar function. (B) Transposition of transposons is postulated to help promote genetic diversity in stressful environments. Helitrons could create mutations in the *C. elegans* germline. Mutations could be neutral, or deleterious. However some mutations could promote enhanced survival in stress.

adapting to new environments (Morran et al., 2009). *C. elegans* subjected to stress such as HS and starvation produce an increase in the percentage of males which presumably bolsters genetic diversity and potential adaptation to the new environment (Morran et al., 2009). In this way, the generation of mutations by repetitive elements in HS could be another way in which *C. elegans* generate genetic diversity in times of stress. Too high a mutation rate under basal conditions can affect fitness, and in prokaryotes strains prone to mutation only seem to fare well under stress conditions, where their ability to diversify the genome could be seen as favorable for enhanced survival (Matic, 2019). Repeat driven mutations in HS would, in a sense, be ideal because they could potentially increase the ability of organisms to adapt to heat stress without compromising fitness under unstressed conditions when repeat RNAs would be less highly expressed. Esnault and colleagues provide evidence for such a mechanism in *Schizosaccharomyces pombe* subjected to cobalt chloride stress where they found that stress adaptation was driven by TE insertion (Esnault et al., 2019). While there is not currently evidence that Helitrons drive mutation in HS to the benefit of the host, there is evidence that increased temperature in *C. elegans* can cause genetic mobility (Matsuba et al., 2013). Thus, it seems worth testing if Helitrons and other HS induced elements can be mobilized in HS to the benefit of the host as this could represent a new mechanism by which *C. elegans* adapt to HS.

5.3 Non-Coding RNAs and the Heat Shock Response

5.3.1 LincRNAs and the Heat Shock Response

We noticed that many lincRNAs changed in expression in HS. However, the role of lincRNAs in the *C. elegans* HSR is at present undetermined. In mammalian cell culture,

lincRNAs are found dispersed in most cellular compartments, and fulfill a variety of roles (Quinn and Chang, 2016). I hypothesize that functional *C. elegans* lincRNAs would also likely fulfill a variety of roles. An important step in studying HS induced lincRNAs would involve using the great genetic tool kit available to researchers to disrupt lincRNA expression and study both their tissue specific and sub-cellular localization. Care needs to be taken when generating lincRNA mutant lines, since ablation of a lincRNA locus could also disrupt critical DNA regulatory sequences. A clever method to create more anodyne mutants involves using CRISPR to place an early polyadenylation signal immediately downstream of the Transcription Start Site of the lincRNA which hinders the production of a full length lincRNA, while keeping possible regulatory features of the DNA sequence intact (Engreitz et al., 2016). If the study of lincRNAs in HS is extended to mammalian cell culture, CRISPRi can be used to disrupt lincRNA transcription and study the response of lincRNAs in HS at a genome wide level (Liu et al., 2017).

5.3.2 Pseudogenes and the Heat Shock Response

At first glance, pseudogenes may appear to be relics that have long since decayed into uselessness. However, the fact that they are expressed at all begs the question: What are they doing in HS? In the case of *dct-10*, we have somewhat of an answer; *dct-10* is up regulated following HS and is regulated by HSF-1. This pseudogene is also regulated by *daf-16*, the *C. elegans* homolog of the FOXO3 transcription factor which is also implicated in stress responsiveness (Pinkston-Gosse and Kenyon, 2007). The regulation of *dct-10* by two stress transcription factors alludes to a role in the response to HS. Knockdown and overexpression during HS is an obvious next step to determine its role in the HSR.

The regulation and possible roles of the dozens of other pseudogenes that rapidly increase in HS is less clear. The study of pseudogene biology is relatively new; however,

one trend that seems to hold with many of the biologically relevant pseudogenes is that they have a functional protein coding counterpart whose expression pattern is interlinked with the pseudogene (Pink et al., 2011). Thus, knockdown or overexpression of the HS pseudogene would tell researchers if the pseudogene is linked to the regulation of its protein coding partner—if it has such a counterpart. Interestingly, pseudogene mediated regulation of its protein coding counterpart can be achieved in a variety of ways. For example, pseudogenes that share cis elements with a cognate protein coding gene can titrate away regulators that interact with these cis elements such as miRNAs or RNA binding proteins (Pink et al., 2011). Pseudogenes can also affect the expression of their protein coding partner by folding into a double stranded RNA that can be recognized by Dicer (Pink et al., 2011). Dicer then recognizes the dsRNA and generates siRNAs from the pseudogene that can then regulate the cognate protein coding gene (Pink et al., 2011). Finally, transcription antisense to a pseudogene can also lead to inhibition of translation of its protein coding partner by base pairing to the mRNA (Pink et al., 2011). Thus, HS induced pseudogenes could potentially regulate the expression of protein coding genes in multiple ways. To ask if HS induced pseudogenes regulate the function of a protein coding partner, researchers should determine if HS induced pseudogenes have protein coding partners. Then experiments that examine the effect of pseudogene levels on the protein coding partner mRNA levels should be conducted. If the levels of a HS induced pseudogene affect the levels of its protein coding homolog, researchers can assay whether any of the above methods of pseudogene mediated regulation are at play.

5.3.3 Leveraging the abilities of RNA for HSR regulation?

RNA can form unique secondary structures that are temperature dependent. In bacteria there are numerous examples of organisms leveraging this ability to control the

expression of protein coding genes. For instance, secondary structures such as stem loops can obstruct the ribosome (Kortmann and Narberhaus, 2012). Changes in temperature can relax these structures allowing for ribosomal entry (Kortmann and Narberhaus, 2012). Most of the evidence for this phenomenon is documented in prokaryotes; however, there is some evidence that the 5' UTR of the *Drosophilia Hsp90* mRNA may also function as an RNA thermometer (Ahmed and Duncan, 2004). Whether non-coding RNAs in eukaryotes act as RNA thermometers is currently unknown. Many of the RNAs that we detected in our study are highly induced in HS and not abundant at control temperatures. One would expect of RNA thermometers to be abundant or at least expressed to a certain extent under normal growth temperatures to sense changes in temperature. SHAPE-seq, a technique that can probe the structure of the entire transcriptome, could be used to assay the changes in structure for all non-coding RNAs in control and HS conditions (Lucks et al., 2011). Many functional non-coding RNAs are conserved in structure and function but not at the nucleotide sequence level (Quinn and Chang, 2016). Thus, these experiments could help to identify RNAs that undergo interesting and perhaps functional changes in HS. As a substitute to transcriptome wide methods, the structure of RNAs that are experimentally validated to have physiological or molecular phenotypes could instead be probed to help elucidate how these non-coding RNAs might function.

Fascinatingly, there is evidence that RNA itself could assist in protein folding and function as a chaperone. All of the chaperones previously discovered until this point have been protein in origin. In fact, the term RNA chaperone, refers to proteins that help RNAs fold, not the other way around. But there is no specific reason to preclude nucleic acids from helping to combat the pernicious effects of HS induced protein misfolding. There is evidence that nucleic acids, including both DNA and RNA, can help misfolded proteins fold (Docter et al., 2016; Choi et al., 2008). RNA is negatively charged and in, the crowded cellular

environment, there are likely electrostatic interactions, whether accidental or intentional between proteins and RNA that could assist in protein folding (Franzmann and Alberti, 2019). Interestingly, nucleic acid binding proteins seem to be particularly sensitive to stress (Franzmann and Alberti, 2019). Strikingly, of all classes of proteins in Yeast RNA binding proteins are the most sensitive to heat (Wallace et al., 2015). An interesting but unproven idea postulated by Franzmann and Alberti is that RNA binding proteins themselves may have been "shaped by evolution" to unfold at higher temperatures perhaps serving as a type of HS thermometer (Franzmann and Alberti, 2019). Interestingly, RNA can aid in the disaggregation of specific RNA binding proteins. For instance, *in vitro* evidence shows that ssRNA can prevent aggregation of TDP-43, a protein prone to aggregation in neurological protein folding diseases (Huang et al., 2013). Whether RNA in HS is acting as a chaperone for specific misfolded proteins or misfolded proteins in general is unknown but represents another possible role of non-coding RNA in HS.

5.4 Bioinformatic Approaches to Characterize Stress Induced Non-Coding RNAs

One relatively quick way to learn more about HS induced ncRNAs and identify additional stress responsive non-coding RNAs would be to analyze transcriptome profiling experiments from *C. elegans* subjected to stresses other than HS. HSPs are induced by other stresses in addition to HS. It is possible that HS induced non-coding RNAs could be induced by other types of stress as well, and knowledge of the types of stress that elicit these non-coding RNAs could further help characterize them. For instance, an ncRNA that is induced by almost every type of stress could be a general stress response factor. Whereas an RNA that is only active in HS may have a more specific function. Fortuitously,

there exists published RNA-seq data sets for multiple types of stresses in *C. elegans*, and the bioinformatic methods outlined in Chapter 3 to characterize HS induced ncRNAs could be used to analyze these data sets as well.

In chapter 3 we note that the expression of specific protein coding genes in HS, appears to be caused by the expression of Downstream of Genes (DoGs) and present a bioinformatic pipeline to filter out these erroneous gene calls. This pipeline could be used and adapted for two important purposes. One, future studies using RNA-seq to study gene expression in heat stressed *C. elegans* could make use of this pipeline to help filter out erroneous gene calls. Two, this pipeline could be adapted to see if DoGs or a similar phenomenon that causes read through transcription, are caused by additional types of stress in *C. elegans*. In addition to our study, Melnick and colleagues also noticed the formation of DoGs in HS in *C. elegans* (Melnick et al., 2019). Initial studies on DoGs found that they were caused by osmotic and oxidative stress in addition to HS (Vilborg et al., 2015, 2017). Thus it seems likely that this phenomenon will occur in other types of stress in *C. elegans*, and it could be important for those undertaking transcriptome wide studies in stress conditions to know if it occurs, so they can analyze their data properly and prepare samples accordingly.

5.4.1 Identifying novel Stressed Induced Non-Coding RNAs

Our study comprehensively examined many classes of *C. elegans* non-coding RNAs in HS. To identify changes in ncRNA expression, we used annotations from various databases. However, if novel previously unidentified non-coding RNAs are elicited in HS our analysis would not have detected them. Thus, future studies could make use of our data set and other HS data sets to discover novel HS induced ncRNAs. The first study to characterize lincRNAs in *C. elegans* used mixed stage worms at normal growth temperatures and found

more than 150 lincRNAs, and a more recent study reanalyzed numerous RNA-seq data sets to uncover potentially thousands more lincRNAs (Nam and Bartel, 2012; Akay et al., 2019). However, neither of these studies looked at stress conditions to identify lincRNAs. Thus by applying similar identification pipelines in stress RNA-seq data sets, researchers could potentially identify novel stress induced lincRNAs. A similar strategy could be used for the identification of novel HS induced miRNAs.

5.5 Future Directions

Immediate follow up experiments to the work presented here should focus on the identification of physiologically significant ncRNAs, followed by mechanistic studies to determine how they act in HS. By expanding research of the Heat Shock Response to include ncRNAs, researchers can uncover novel insights as to how the HSR is regulated, fill knowledge gaps, and better understand how organisms respond to environmental stress. In humans misregulation of the HSR is associated with diseases such as cancer. Clinicians could study HSR regulation by ncRNAs to uncover new avenues to explore clinically.

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