Robust distal tip cell pathfinding in the face of temperature stress is ensured by two conserved microRNAs in *Caenorhabditis elegans*

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ABSTRACT

Biological robustness, the ability of an organism to maintain a steady state output as genetic or environmental inputs change, is critical for proper development. MicroRNAs have been implicated in biological robustness mechanisms through their post-transcriptional regulation of genes and gene networks. Previous research has illustrated examples of microRNAs promoting robustness as part of feedback loops and genetic switches, and by buffering noisy gene expression resulting from environmental and/or internal changes. Here we show that the evolutionarily conserved microRNAs mir-34 and mir-83 (homolog of mammalian mir-29) contribute to the robust migration pattern of the distal tip cells in Caenorhabditis elegans by specifically protecting against stress from temperature changes. Furthermore, our results indicate that mir-34 and mir-83 may modulate the integrin signaling involved in distal tip cell migration by potentially targeting the GTPase cdc-42 and the beta-integrin pat-3. Our findings suggest a role for mir-34 and mir-83 in integrin-controlled cell migrations that may be conserved through higher organisms. They also provide yet another example of microRNA-based developmental robustness in response to a specific environmental stress, rapid temperature fluctuations.

INTRODUCTION

The ability of a living system to maintain a steady state output in the face of environmental and physiological stresses is referred to as biological robustness (Kitano 2004). Organisms must be able to compensate for adverse changes in gene expression caused by environmental stresses and internal gene expression noise if development is to proceed unchanged. The nematode Caenorhabditis elegans (C. elegans) is a useful
model for studying such robustness. *C. elegans* development has been mapped to a cell-by-cell level, such that we know exactly when cell divisions occur and what the fate of each cell is (Sulston 1976; Sulston and Horvitz 1977; Kimble and Hirsh 1979; Sulston *et al.* 1983). There is also extensive research on the worm’s responses to stress, including stress-induced alternative larval developmental choices such as stage-specific diapause (Baugh and Sternberg 2006; Fukuyama *et al.* 2006; Ruaud and Bessereau 2006; Schindler *et al.* 2014) and proceeding to the dauer larvae stage, an alternative third larval stage that allows *C. elegans* to lengthen their lifespan and survive food deprivation or heat stress (Cassada and Russell 1975; Liu *et al.* 1995).

MicroRNAs (miRNAs) are single-stranded RNAs of approximately 22 nucleotides that negatively regulate the translation of their target messenger RNAs (mRNAs) by binding to their 3’ untranslated region (3’ UTR) as part of a protein-RNA complex called the miRNA-induced silencing complex (miRISC). Target recognition is determined by the miRNA’s seed sequence, nucleotides two through seven (reviewed in Bartel 2004; Ambros 2004). miRNAs with the same seed sequence can presumably regulate the same target mRNAs, and are grouped together within a miRNA family (reviewed in Bartel 2009). In addition to being regulated by multiple members of a miRNA family, an mRNA can be co-targeted by multiple distinct miRNAs families, if it contains the corresponding distinct seed-complementary sites. Such inter-family and intra-family co-targeting is considered one reason why most single miRNA gene deletion mutants in *C. elegans* do not display apparent phenotypes; although one negative regulator is deleted, the target mRNAs in question remain under the regulation of additional, functionally redundant miRNAs. (Miska *et al.* 2007; Alvarez-Saavedra and Horvitz
Findings reported by the Abbott lab exemplified this phenomenon. They screened for developmental phenotypes resulting from miRNA deletions in genetically sensitized backgrounds, in which the miRNA-specific argonaute protein \textit{alg-1} was no longer functional (Brenner et al. 2010). In the \textit{alg-1} mutant background, overall miRNA production is partially compromised, such that the additional deletion of an otherwise redundant single miRNA can result in target deregulation and visible phenotypes.

In their study, the Abbott Lab identified six miRNAs involved in gonad morphogenesis (Brenner et al. 2010), a normally robust developmental process that is dependent on the migration of two distal tip cells (DTCs) (reviewed in Wong and Schwarzbauer 2012). One of these miRNAs included \textit{mir-83}, a miRNA that is highly conserved in animals, including mammals (known as \textit{mir-29}, Figure S1) (Lagos-Quintana et al. 2001; Lau 2001; Mourelatos et al. 2002; Lagos-Quintana et al. 2002; Dostie et al. 2003; Lim et al. 2003; Lim 2003; Michael et al. 2003; Suh et al. 2004; Poy et al. 2004; Landgraf et al. 2007; Lui et al. 2007). Mammalian \textit{mir-29} has been previously implicated in regulating cellular proliferation, differentiation, apoptosis, and the extracellular matrix (reviewed in Boominathan 2010; Kriegel et al. 2012). In order to better understand how \textit{mir-83} functions we set out to determine its mRNA targets and miRNA co-regulators in \textit{C. elegans}. Using mirWIP (Hammell et al. 2008), we noticed an overlap between the predicted mRNA targets for \textit{mir-83} and \textit{mir-34}, another miRNA that is conserved between nematodes and mammals (Figure S1, Table S1) (Lau 2001; Lim et al. 2003; Lim 2003; Grad et al. 2003; Ambros et al. 2003; Houbaviy et al. 2003; Landgraf et al. 2007). \textit{mir-34} has been shown to have tumor suppressor activity in mammalian systems. There its transcription is activated by p53 and it functions to
reinforce p53 negative regulation (reviewed in He et al. 2007; Yamakuchi and Lowenstein 2009; Hermeking 2009; Rokavec et al. 2014).

To further understand the roles of mir-83 and mir-34, we tested for genetic redundancy by creating the double mutant and looking at developmental phenotypes. We also identified potential targets of mir-34 and mir-83 by tests of genetic suppression. mir-83(n4638); mir-34(gk437) double mutants display a defect in gonad morphogenesis. In addition, this defect reflects a loss of developmental robustness; the mir-83(n4638); mir-34(gk437) phenotype is significantly enhanced in response to temperature changes but not by other environmental stresses that we tested. Based on our results, we conclude that mir-34 and mir-83 function together to help create or maintain robust function of the genetic network controlling gonad morphogenesis, such that the development of this important organ is protected from the temperature changes C. elegans may encounter, either the rapid oscillations tested here or subtler changes experienced in the wild. Furthermore, we observed that mir-83(n4638); mir-34(gk437) mutants have a decreased lifespan and decreased fecundity, suggesting that the loss of these two miRNAs has repercussions for the biological fitness of the animals.

MATERIALS AND METHODS

C. elegans strains: The C. elegans Bristol N2 strain was used as wild-type in the study (Brenner 1974). Additional strains are listed in Table S2. Both the n4638 and gk437 alleles were backcrossed to N2 four times upon receipt. The VT2595 strain was used as the mir-83(n4638); mir-34(gk437) double mutant except when VT3289 is explicitly discussed in Figure 2C.
**C. elegans maintenance:** Strains were maintained using standard procedures on nematode growth media (NGM) plates seeded with *Escherichia coli* (*E. coli*) strain HB101 (Brenner 1974), unless explicitly stated as being raised on OP50. Strains were raised at 20º unless otherwise stated when temperature was oscillated.

**Sequence alignments, target prediction, and statistical significance:**
MicroRNA sequences were supplied by miRBase (Lagos-Quintana *et al.* 2001; Lau 2001; Mourelatos *et al.* 2002; Lagos-Quintana *et al.* 2002; Dostie *et al.* 2003; Lim *et al.* 2003; Lim 2003; Grad *et al.* 2003; Ambros *et al.* 2003; Sempere *et al.* 2003; Aravin *et al.* 2003; Houbaviy *et al.* 2003; Michael *et al.* 2003; Griffiths-Jones 2004; Suh *et al.* 2004; Poy *et al.* 2004; Griffiths-Jones *et al.* 2006; Landgraf *et al.* 2007; Lui *et al.* 2007; Griffiths-Jones *et al.* 2008; Kozomara and Griffiths-Jones 2011; 2014) and aligned by eye. Predicted targets were identified using mirWIP (Hammell *et al.* 2008). Throughout the manuscript, three significance stars (***) are used for a p-value of .005 or less, two (**) for a p-value greater than .005 and less than or equal to .01, and one (*) for a p-value greater than .01 and less than or equal to .05.

**Migration defective phenotype scoring:** Hypochlorite treatment (Stiernagle 2006) was used to isolate embryos. Where noted, synchronized populations were created by allowing embryos to hatch in M9 buffer for approximately 24 hours (Johnson *et al.* 1984). Embryos or starved L1s were then plated on HB101-seeded NGM plates and raised to adulthood in the temperature scheme noted. Day one adults were paralyzed in 100mM levamisole, mounted on 2% agarose pads, and scored using a Zeiss Axioskop differential interference contrast (DIC) microscope and a 63x objective. A 2-proportion z-test was used to determine significant differences between counts.
except where triplicates are presented. In such cases mean values were compared using an unpaired t-test performed by PRISM.

**Temperature oscillations:** Temperature oscillations were performed using modified MJ Research Programmable Thermocyclers. First, the Thermocyclers’ lids were removed. Next, a 0.25 inch thick aluminum plate was attached to the heat block using silicone based thermal conductive grease. Original lids were replaced by insulating covers constructed out of styrofoam. HB101-seeded NGM plates containing worms were placed inside the modified device, in contact with the aluminum plate. To assess the thermal dynamics of the system, a thermometer was embedded in an NGM plate and the temperature of the agar was monitored during programmed temperature cycles. We observed that the temperature of NGM plates did cycle in accordance with the Thermocycler program, although not as quickly as the heat block itself; we observed an approximately 15 to 30 second delay as the NGM cools or heats. Stated temperature cycles refer to the program run by the Thermocycler.

**Electron microscopy:** N2 and mir-83(n4638) IV; mir-34(gk437) X embryos were isolated using hypochlorite treatment, hatched in M9 buffer for approximately 24 hours, and plated as synchronized L1s on HB101-seeded NGM plates. Worms were raised with temperature oscillations: 20° for 16 hours, [15° for 15 minutes, 25° for 15 minutes, repeat three additional times], 20° until young adulthood. Worms were fixed and prepared for electron microscopy as previously described (Irazoqui et al. 2010) except they were cut below the pharynx rather than in half to preserve gonad morphology. Electron microscopy work was performed by the UMass Medical School Electron Microscopy Core Facility.
Cloning and transgenics: Constructs were created using Life Technologies Gateway Cloning. Except where otherwise stated, gene fragments were first amplified from N2 genomic DNA using the primers listed in Table S3. PCR was next used to add the proper att sequences such that fragments could be moved into the appropriate Gateway DONR vector, as described in the Gateway Protocol. For the mir-34 promoter, the 5 kilobase promoter was digested from a separate plasmid and ligated to a modified 476p5Emcs (a gift from Nathan Lawson’s Lab, modified to remove unnecessary SalI cut sites) to add the necessary att sites. For mutated 3’UTRs, DNA fragments of the desired sequence were created de novo by GeneWiz. The constructed DONR vectors, along with two commercially available vectors, were then used for transgene construction as described in the Gateway Protocol (Table S4). The destination vectors were developed for the Mos1-mediated single-copy insertion technique (Frøkjaer-Jensen et al. 2008), which was used to generate transgenic strains (Table S2). Multi-copy arrays were generated for cdc-42 and pat-3 GFP/mCherry reporters. mir-83(n4638) IV; mir-34(gk437) X males were first crossed to EG6701 to create a unc-119(ed3) III; mir-83(n4638) IV; mir-34(gk437) X strain (VT3087). VT3087 was subsequently injected with a mix of pBluescript SK+ (30 ng/µL), pIF9 (15 ng/µL), pCFJ150 (30 ng/µL), pCFJ210 (30 ng/µL), and either pSLB054 (3 ng/µL) and pSLB056 (3 ng/µL) or pSLB071 (3 ng/µL) and pSLB075 (3 ng/µL). Array maEx246 was generated from the pSLB054 and pSLB056 containing injection mix, while array maEx247 was made from the pSLB071 and pSLB075 mix. The array carrying strains, VT3118 (for maEx246) and VT3145 (for maEx247), were crossed to N2 males in order to cross out the
mir-83(n4638) and mir-34(gk437) deletions, generating strains VT3136 and VT3178 respectively.

**RNAi:** RNA-mediated interference (RNAi) was performed by raising animals on dsRNA-producing *E. coli* as described in (Kamath *et al.* 2001). Synchronized L1s were placed on cdc-42 RNAi food, pat-3 RNAi food, or empty vector control food, and raised to young adulthood with the temperature scheme stated. Animals were scored as previously described.

**Target reporter scoring:** Hypochlorite treatment was used to isolate embryos, which were hatch in M9 to generate synchronized L1s. Worms were plated on HB101-seeded NGM plates and raised with temperature oscillations: 20º for 16 hours, [15º for 15 minutes, 25º for 15 minutes, repeat three additional times], 20º until time of scoring. L1 animals were scored starting 18 hours post-plating, immediately following temperature oscillations. L3 animals were scored starting 43 hours post-plating, shortly after the molt from L2 to L3. Adults were scored starting 67 hours post-plating, after egg-laying had commenced.

Worms were suspended in 100 mM levamisole, mounted on 2% agarose pads, and scored using a Leica TCS SPE confocal microscope. For cdc-42 reporters DTCs were first identified in the DIC setting by eye using the 63x objective. The confocal microscopy setting was subsequently used for imaging and quantifying the mean value florescence of each channel for a DTC using the Leica LAS AF software. For pat-3 reporters, the 10x objective was used to observe whole animals. A 30 step z-series was taken per worm. The Leica AF software was used to quantify mean value fluorescence
for each channel using the z-stack maximum projection. Data sets were compared using an unpaired t-test performed by PRISM.

**Brood size counts:** N2 and *mir-83(n4638) IV; mir-34(gk437) X* embryos were isolated using hypochlorite treatment and left in M9 buffer for approximately 24 hours to hatch. Arrested L1s were then plated on HB101-seeded NGM plates and either raised at 20° continuously or with temperature oscillations from hour 16 to hour 18 post-plating (labeled “Cycled” - 20° for 16 hours, [15° for 15 minutes, 25° for 15 minutes, repeat three additional times], 20° until death). Worms were individually plated as L4s, and the number of live offspring were counted from the start of egg-laying until its end. Mean values were compared using an unpaired t-test performed by PRISM.

**Mating assays:** N2 and *mir-83(n4638) IV; mir-34(gk437) X* embryos were isolated using hypochlorite treatment and left in M9 buffer for approximately 24 hours to hatch. Arrested L1s were then plated on HB101-seeded NGM plates and either raised at 20° continuously or with temperature oscillations from hour 16 to hour 18 post-plating (labeled “Cycled” - 20° for 16 hours, [15° for 15 minutes, 25° for 15 minutes, repeat three additional times], 20° until death). Once they reached adulthood, hermaphrodites were transferred daily away from progeny until the ability to lay eggs was exhausted (day four of adulthood). Each hermaphrodite was then moved to an individual plate, to which four one-day-old adult N2 males were added. Animals were allowed to mate for three days, at which point the males were removed. The total number of offspring that hatched were counted for each hermaphrodite from the start of the assay until its death.

**Lifespan assays:** Survival assays were performed similarly to previously described (Kenyon *et al.* 1993). N2 and *mir-83(n4638) IV; mir-34(gk437) X* embryos
were isolated using hypochlorite treatment and left in M9 buffer for approximately 24 hours to hatch. Arrested L1s were then plated on HB101-seeded NGM plates and either raised at 20° continuously or with temperature oscillations from hour 16 to hour 18 post-plating (labeled “Cycled” - 20° for 16 hours, [15° for 15 minutes, 25° for 15 minutes, repeat three additional times], 20° until death). To track survival, one hundred worms per replicate and three replicated per condition were moved to a new HB101-seeded NGM plate as L4s. Worms were transferred to a new plate daily during the course of the assay to avoid overcrowding from the offspring. Worms were scored as alive or dead based on reaction to a gentle nose prod using a standard worm pick. Animals were tracked until their death. Any worms that died as a consequence of crawling up the side of the plate were removed from the assay. Replicates were used to calculate percent survival and the standard deviation for each day. Mean values for individual days were compared using a two sample t-test for means.

**Additional stresses:** Animals was raised on NGM plates containing 0.03% sodium arsenite to induce oxidative stress (Sahu *et al.* 2013). Incubation in 1% SDS for 30 minutes was used to isolate dauer animals from starved plates (Stiernagle 2006). Animals were raised on *P. auruginosa* PA14 plates as previously described (Powell and Ausubel 2008).

**RESULTS**

**Improper distal tip cell migration paths in mir-83(n4638); mir-34(gk437) mutants:** The shape of the *C. elegans* adult hermaphrodite gonad is determined by the migration of two distal tips cells (DTCs), somatic gonadal cells at the tip of each gonad arm that drag the proliferative portion of the gonad with them as they migrate during the
second through fourth larval stages of hermaphrodite development (Kimble and Hirsh 1979; Kimble and White 1981; Hedgecock et al. 1987). Both DTCs are born near the mid-body during the first larval stage, and begin to migrate along the ventral body wall muscles towards the head and tail during the second larval stage, referred to as phase 1 of migration (Hirsh et al. 1976; Kimble and Hirsh 1979; Kimble and White 1981).

During phase 2, DTCs first turn dorsally to migrate from the ventral to the dorsal body wall muscle, crossing the hypodermis in the process (Hedgecock et al. 1987). A second turn is required to continue migration along the dorsal body wall muscles and return to the mid-body (phase 3), creating two U-shaped gonad arms (Figure 1A) (Hirsh et al. 1976; Kimble and Hirsh 1979; Hedgecock et al. 1987). The final shape of the gonad arms can be used to deduce the path taken by each DTC. In a fraction of \textit{mir-83(n4638); mir-34(gk437)} mutants (26% at 20º, quantified in Figure 2), anterior and posterior gonad arms appear displaced at various positions, improperly crossing the dorsal-ventral axis (Figure 1B). These misplaced gonad arms suggest DTCs wandered from the proper path during phase 1 and/or phase 3 of migration, and often migrated too far and past the developing vulva. The penetrance of this overextension phenotype was highly variable and overextension was observed to occur either with or without wandering during phase 1 and/or phase 3. Unlike some classes of \textit{C. elegans} migration mutants, DTCs in \textit{mir-83(n4638); mir-34(gk437)} mutants execute both their first (dorsal) and second (anterior/posterior) turns at the proper positions in the animals and at the proper times in development. The migration defect of these mutants therefore reflects a defect in the precise pathfinding of the DTCs as they migrate longitudinally along the ventral or dorsal muscle. To score this phenotype we categorized gonad arms as either

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migration-defective or normal. Gonad arms were categorized as migration-defective if their abnormal shape indicated that the DTC had improperly crossed the dorsal-ventral axis during its migration, or that the DTC migrated completely past the vulva before stopping. Previously published findings indicated a low level of migration defects in the N2 strain (Peters et al. 2013). These included such phenotypes as DTCs stopping slightly short of the vulva and a slight ventral-ward “dip” of the DTCs when migration ceases. We observed such phenotypes as well, but due to their occurrence in N2 we classified such cases as within normal variation and did not score them as migration defects. It is also important to note that in the previously mentioned study (Peters et al. 2013), strains were raised at 23° rather than 20°.

DTC wandering results in displaced gonad arms, which is reflected in a reorganization of the internal organs. To visualize this reorganization in mir-83(n4638); mir-34(gk437) mutants, we performed electron microscopy on cross-sections of adult worms. We first collected approximately 20 mir-83(n4638); mir-34(gk437) worms whose anterior arm displayed a migration defect. These mutants and age-matched N2s were fixed and processed for electron microscopy. As previously described, in N2 worms the intestine spans the dorsal-ventral axis (Figure 1C) (Hall and Altun 2008). The oocyte-containing proximal gonad is located ventrally while the distal gonad is dorsal (Hall and Altun 2008). In order for a DTC to wander from the appropriate migration path, it must displace the intestine. In addition, the somatic gonad itself will be displaced as it trails behind the migrating DTC. This rearrangement of internal organs, with the intestine, distal gonad arm, and proximal gonad arm all displaced, was visible in both of the two mir-83(n4638); mir-34(gk437) mutants sectioned and visualized (Figure 1C).
The penetrance of the migration defect is significantly enhanced by oscillating temperature: We first sought to quantify the penetrance of the migration defect in \textit{mir-83(n4638); mir-34(gk437)} worms raised in standard laboratory conditions, namely on \textit{E. coli}-seeded NGM plates kept at 20\(^\circ\) (Brenner 1974). As expected, the DTCs migrate properly in N2 worms in the majority of worms (2 +/- 2% migration defective, performed in triplicate, Figure 2A). Both \textit{mir-34(gk437)} and \textit{mir-83(n4638)} single mutant populations display migration defects at a low frequency, 10.67 +/- 1.15\% and 8 +/- 0\% respectively. The phenotype’s penetrance is significantly enhanced to 26 +/- 7.21\% in the \textit{mir-83(n4638); mir-34(gk437)} double mutant (p \leq .05, unpaired t-test), suggesting that the two miRNAs contribute partially redundant functions in the context of DTC migration.

Due to the low penetrance of the phenotype in \textit{mir-83(n4638); mir-34(gk437)} double mutants (Figure 2A), we reasoned that the functions of these miRNAs in DTC migration could be relatively unimportant under standard laboratory conditions, but perhaps more critical under stressful conditions. Therefore, we tested for enhancement of the DTC migration phenotype in worms exposed to various stresses. We observed no change in the penetrance of the phenotype when mutants were exposed continuously throughout development to high temperature (25\(^\circ\)) or low temperature (15\(^\circ\)), or to a diet of pathogenic \textit{Pseudomonas aeruginosa} (\textit{P. aeruginosa}), oxidative stress (0.03% arsenic), or starvation during early larval stages to induce dauer formation (Table S5). However, an enhanced phenotype was observed when worms developed under a changing temperature regimen (Figure 2B), a phenomenon previously observed for \textit{mir-7 Drosophila} mutants in the context of \textit{Drosophila} eye development (Li \textit{et al.} 2009).
Semi-synchronized populations of developing larvae were exposed to a regimen of temperature changes every 15 minutes - first from 15° to 25°, and then back to 15°, and so on. The DTC migrations of N2 worms were unaffected by temperature oscillations (0 +/- 0%). All three mutant strains (mir-34(gk437) and mir-83(n4638) single mutants, and the mir-83(n4638); mir-34(gk437) double mutant) showed an enhanced penetrance of the DTC migration defective phenotype under oscillating temperature compared to constant temperature (20°); from 10.67 +/- 1.15% to 28 +/- 5.29% for mir-34(gk437), from 8 +/- 0% to 16.67 +/- 4.16% for mir-83(n4638), and from 26 +/- 7.21% to 62.67 +/- 4.16% for mir-83(n4638); mir-34(gk437). A similar pattern was observed for animals grown on OP50 rather than HB101 E. coli, suggesting that the migration defect and its enhancement by oscillating temperature are independent of food source (Figure S2).

We also examined a second mir-34 null allele, n4276, and obtained similar results as that for gk437 (Figure S3).

To confirm that the DTC migration defects of mir-34 and mir-83 mutants are a consequence of the loss of the mir-34 and mir-83 genes, we generated single-copy integrated rescuing transgenes in their respective single mutant (Table S2). Both the mir-34 transgene, mals387, and the mir-83 transgene, mals391, rescue the migration defects of their respective single mutant (Figure 2C). Although this finding supports the conclusion that the migration defects can be explicitly attributed to the mir-34 and mir-83 loss of function, a caveat remained that the migration defect was due to background mutations, outside the mir-34 or mir-83 loci, that had been present in the single mutants and were segregated away during construction of the transgene-containing strains. In this scenario, the hypothetical background mutations would have been maintained
during the construction of the \textit{mir-83(n4638)}; \textit{mir-34(gk437)} double mutant, strain VT2595. To address this caveat we crossed the \textit{mals387; mir-34(gk437)} and \textit{mals391; mir-83(n4638)} strains together to generate a new doubly-mutant \textit{mir-83(n4638)}; \textit{mir-34(gk437)} strain (VT3289) lacking both transgenes, and a sibling doubly-mutant strain carrying both transgenes (VT3294) (Figure 2C). We observed that VT3289 exhibited a 56% penetrance of migration defective, which is not statistically different from 62% migration defective exhibited by the original \textit{mir-83(n4638)}; \textit{mir-34(gk437)} double mutant, VT2595 (Figure 2C, 2-proportion z-test, p > .05). If the migration defect had been due to background mutations rather than the \textit{mir-34} and \textit{mir-83} deletions we would not expect the newly isolated double to have the defect. As expected, the double mutant carrying both rescue constructs, \textit{mals387; mals391; mir-83(n4638)}; \textit{mir-34(gk437)}, appears mostly normal (4% migration defective).

The above results suggest that DTC migrations may be inherently sensitive to unstable temperature, and that the activity of \textit{mir-34} and \textit{mir-83} helps protect the genetic network controlling this migration from environmental temperature changes. Although the penetrance of the phenotype was increased in \textit{mir-83(n4638)}; \textit{mir-34(gk437)} mutants worms experiencing oscillating temperatures (from 26% at constant 20° to 62.67% in oscillating temperatures), the migration defect is still not fully penetrant in \textit{mir-83(n4638)}; \textit{mir-34(gk437)} mutants, even with temperature oscillations. It was previously shown that for ALG-1 dominant-negative alleles \textit{ma192} and \textit{ma202}, respectively 71% and 73% of worms have DTC migration defects, strongly suggesting that other miRNAs are involved in the regulation of DTC migrations (Zinovyeva \textit{et al.} 2014). Therefore the additional miRNAs implicated in gonad migration by the Abbott Lab...
(Brenner et al. 2010) were examined to see if being raised in changing temperatures enhanced any of the single mutants as it did for mir-34(gk437) and mir-83(n4638) mutants. We found that only the mir-259(n4106) migration defect appears to be enhanced by oscillating temperatures (Figure 3A), although the enhancement is not significant (p = .086, 2-proportion z-test). Additionally, the migration defects exhibited by mir-259(n4106) mutants are similar to those of mir-34(gk437) and mir-83(n4638) single and double mutants; DTCs improperly cross the dorsal/ventral axis during phase 1 and phase 3 of migration and migrate past the vulva. However, the mir-259 deletion does not enhance the penetrance of these defects in a triple mutant mir-83(n4638); mir-259(n4106); mir-34(gk437) compared to the mir-83(n4638); mir-34(gk437) double mutant (Figure 3B, p ≥ .05, 2-proportion z-test). This suggests that, although mir-259 regulates targets involved in DTC migration in a manner that is sensitive to temperature changes and could be involved in the same or a similar process as mir-34 and mir-83, mir-259 appears to not regulate the same targets as mir-34 and mir-83. If the three miRNAs were acting in parallel on the same set of mRNA targets, we would expect an elevated penetrance of the phenotype upon removal of mir-259 from the double mutant as the target mRNAs in question would be further derepressed. One caveat regarding this conclusion could be that the lack of enhancement seen in the mir-83(n4638); mir-259(n4106); mir-34(gk437) triple compared to the mir-83(n4638); mir-34(gk437) double (Figure 3B) might reflect common targets that were fully derepressed in the mir-83(n4638); mir-34(gk437) double, such that the loss of an additional regulator had no effect. In this regard, we note that the migration defect penetrance in both the mir-259(n4106); mir-34(gk437) and the mir-83(n4638); mir-259(n4106) double mutants
are weaker than that of the \textit{mir-83(n4638); mir-34(gk437)} mutant (Figure 3C, \(p \leq .005\), 2-proportion z-test). This suggests that the relevant mRNA target sets for \textit{mir-34} and \textit{mir-83} may overlap more with each other than with \textit{mir-259}. For these reasons, we chose to focus on the functionally-interacting miRNAs \textit{mir-34} and \textit{mir-83} for the remainder of this study.

**The temperature-sensitivity of DTC migration is restricted to a two hour period during the L1 stage:** We next sought to determine if the entire DTC migration process was temperature-sensitive in \textit{mir-83(n4638); mir-34(gk437)} mutants, or if there was a more restricted developmental period during which DTCs are sensitive to changing temperature in these mutants. Synchronized populations of N2 and mutant worms were produced by hatching eggs in the absence of food. L1 larvae arrest in such conditions, and will not proceed developing until the reintroduction of food (Johnson \textit{et al.} 1984; Baugh 2013). Once food is reintroduced, it takes the larvae approximately twenty-four hours at 20º to reach the first molt into the L2 larval stage. Using the DTC-specific reporter, \textit{P\textit{lag-2::GFP}} (Siegfried and Kimble 2002), we found that the DTCs are born approximately 16 to 16.5 hours after the reintroduction of food (data not shown) in both N2 and \textit{mir-83(n4638); mir-34(gk437)} worms. This agrees with previous reports for the timing of DTC birth in N2s (Sulston and Horvitz 1977; Kimble and Hirsh 1979). By examining the effects of two-hour time windows during which the temperature oscillated between 15º and 25º every 15 minutes, we found that the temperature-sensitive period for DTCs overlapped with the approximate time of their births. When the two hour oscillating temperature regimen occurred before 14 hours of development (Figure 4A) or after 20 hours of development (Figure 4C), \textit{mir-83(n4638); mir-34(gk437)} mutants
displayed the migration phenotype at a penetrance similar to that seen in the non-enhanced 20° condition (see Figure 2A). However, when temperature oscillations occurred from hour 16 to hour 18 of development (Figure 4B) we observed an enhanced penetrance of the phenotype similar in magnitude to that of animals that had experienced temperature oscillations throughout larval development (see Figure 2B). This implies that the enhancement observed with temperature oscillations throughout development likely resulted from changing temperature in the interval between hour 16 to hour 18. Indeed, animals that experienced temperature oscillations throughout most of larval development but excluding the two hour period from hour 16 to hour 18 did not exhibit phenotypic enhancement (Figure S4).

Our results indicate that cycling between 15° and 25° captures the full enhancement of the migration phenotype under the conditions we have tested. Increasing the upper temperature to 37°, thereby heat shocking the worms in 15 minute increments, did not further enhance the penetrance of the phenotype (Figure S5). We also found that the phenotypic enhancement occurred whether the first temperature change was a decrease (Figure 4) or an increase (Figure S6) in temperature and was largely independent of the magnitude of the temperature change; ten degree or five degree changes in temperature resulted in similar phenotype enhancement (Figure S7). We observed that a single oscillation cycle during the hour 16 to hour 18 period was insufficient to enhance the phenotype (Figure S8), suggesting that multiple cycles are necessary to elicit the stress that compromises the integrity of gonad morphogenesis in the absence of mir-34 and mir-83.
**Tissue specificity of mir-34 and mir-83:** To determine the anatomical site of action of mir-34 and mir-83 in regulating the integrity of DTC migrations we generated constructs expressing each of these miRNAs driven by its natural promoter or by promoters from genes expressed in the hypodermis (*dpy-7*), DTCs (*lag-2*, also expressed in some vulval cells), gonadal sheath cells (*lim-7*), or muscle (*myo-3*, also expressed in muscle-like sheath cells 3 - 5) (Johnstone *et al.* 1992; Henderson *et al.* 1994; Hall *et al.* 1999; Fox *et al.* 2007; Ono *et al.* 2007; Dupuy *et al.* 2007). These constructs were built in Mos1-mediated single-copy insertion (MosSCI) destination vectors and were used to generate integrated transgenic lines (Frøkjær-Jensen *et al.* 2008) (Table S2).

As expected, both mir-34 or mir-83 expressed under their cognate natural promoters rescued the DTC migration defect of the respective single mutants (Tables 1 and 2, p ≤ .005, 2-proportion z-test). mir-34(*gk437*) and mir-83(*n4638*) phenotypes were also rescued to varying degrees by tissue specific heterologous promoters. mir-34 expressed in the DTCs (driven by the *lag-2* promoter; Plag-2::mir-34) partially rescued the mir-34(*gk437*) defect, from 28.3% to 11.7% (Table 1, p ≤ .05, 2-proportion z-test). The lack of full rescue could be due to a difference in expression levels and/or a requirement for mir-34 in more than one tissue. mir-83 expressed in either the DTCs (Plag-2::mir-83) or muscle (Pmyo-3::mir-83), significantly rescued the migration defect (from 30% in the mutant to 5% in each rescue line, Table 2, p ≤ .005, 2-proportion z-test). Potential explanations for this result, where mir-83 appears to function in either the DTCs or the muscle, are discussed in the Discussion section.
Mir-34 and mir-83 regulate two key proteins involved in DTC migrations: Our findings that DTC-specific expression of mir-34 or mir-83 can rescue the gonad migration defect of mir-34(gk437) or mir-83(n4638) mutants suggests that mir-34 and mir-83 may regulate a gene or genes whose activity impacts the fidelity of the DTC migration process, and that the migration defect reflects abnormal pathfinding by the DTCs during larval development. An alternative hypothesis, that the displaced gonad arms observed in mutant adults resulted from the shifting of internal organs after otherwise proper migrations, was tested by examining whether the animals' movement impacted their gonad morphology. We observed that the migration defective phenotype was not affected in genetically paralyzed worms compared to fully active worms, arguing against a contribution of movement-derived structural damage as a cause of the migration defect (Figure S9).

To identify potential targets of mir-34 and mir-83 in the regulation of DTC migration, we tested for suppression of the phenotype in mir-83(n4638); mir-34(gk437) mutants by RNAi knockdown of genes predicted by mirWIP (Hammell et al. 2008) to be targets of both mir-34 and mir-83 (Table S1), prioritizing genes known to be involved in cell migrations, larval development, or miRNA function. cdc-42 and pat-3, both expressed in the DTCs and body wall muscle and known to be involved in their migration (Lee et al. 2001; Cram et al. 2006; Lucanic and Cheng 2008), are predicted targets of mir-34 and mir-83 and were subsequently examined.

cdc-42 is a GTPase shown to be downstream of integrin signaling. Upon activation by integrin, cdc-42 acts to bring about the actin cytoskeleton rearrangements associated with cell migration (Van Aelst and D'Souza-Schorey 1997; Price et al. 1998; Ren et al. 2001).
1999). Previously it has been shown that when a constitutively active allele of *cdc-42* (unable to hydrolyze GTP to GDP) is expressed in the DTCs, those DTCs display pathfinding defects (Peters *et al.* 2013). If *cdc-42* is a direct target of *mir-34* and *mir-83*, we would expect its expression to be elevated in animals mutant for these two miRNAs. Therefore, we hypothesized that by lowering the level of *cdc-42* in *mir-83(n4638); mir-34(gk437)* mutants we could suppress the gonad migration defect. This is in fact the case. The *cdc-42* null allele *gk388* deletes a portion of *cdc42’s* 5’ UTR, the first exon, and a portion of the first intron (Kimata *et al.* 2012). *mir-83(n4638); mir-34(gk437)* mutants heterozygous for the *cdc-42(gk388)* allele were significantly rescued, from 53% of the population displaying the gonad migration defective phenotype to 23% (Figure 5A, *p* ≤ .005, 2-proportion z-test). Mutants containing one copy of *gk388* and wild-type copies of both *mir-34* and *mir-83* also are migration defective (wandering in phase 1 and/or phase 3) at a low penetrance (10% of the population). This is expected as *cdc-42* is a critical regulator of the integrin signaling network (Van Aelst and D’Souza-Schorey 1997; Price *et al.* 1998; Ren *et al.* 1999). We confirmed this suppression using RNAi-induced knockdown of *cdc-42* (Figure 5B); *cdc-42(RNAi)* in a *mir-83(n4638); mir-34(gk437)* mutant significantly suppressed the penetrance of the migration defect from 70% to 37% (*p* ≤ .005, 2-proportion z-test). N2 worms on *cdc-42* RNAi food exhibit wandering phenotypes similar to that observed in the balanced heterozygote. This result supports that idea that the reduction of *cdc-42* is responsible for the suppression seen in *mir-83(n4638); mir-34(gk437)* rather than being the result of the inclusion of the balancer chromosome *mln1.*
C. elegans has one β-integrin gene, pat-3 (Williams and Waterston 1994; Gettner et al. 1995). Integrin signaling is the major regulatory network involved in phase 1 and phase 3 of DTC migration (Baum and Garriga 1997; Lee et al. 2001; Cram et al. 2006). Using the pat-3 null allele st564 (Williams and Waterston 1994), we created a strain homozygous for deletions of both mir-34 and mir-83 and carrying only one functional copy of pat-3. In this case, partial loss of pat-3 reduced the migration defect from 52% to 25% (Figure 5C, p ≤ .005, 2-proportion z-test), supporting the conclusion that pat-3 overexpression contributes to the migration defect in mir-83(n4638); mir-34(gk437) mutants. Note that 18% of worms with one functional copy of pat-3 are migration defective (we observed wandering during phase 1 and phase 3 and overextension, but no defects in phase 2 turns), indicating that the fidelity of DTC migration may depend critically on pat-3 dosage. RNAi against pat-3 also significantly suppressed the migration defect in a mir-83(n4638); mir-34(gk437) mutant from 65% to 40% (Figure 5D, p ≤ .01, 2-proportion z-test), confirming that the reduction of pat-3 is responsible for the suppression.

The suppression in mir-83(n4638); mir-34(gk437) mutant phenotype by genetic reduction of either cdc-42 or pat-3 activity supports the supposition that cdc-42 and pat-3 function downstream of these miRNAs in the regulation of DTC migration. The fact that both cdc-42 and pat-3 are predicted to contain sites for both mir-34 and mir-83 in their 3’ UTR sequences strongly suggests direct targeting by these miRNAs. In an attempt to gather additional data in support of direct regulation, we constructed a set of nuclear localized mCherry and GFP reporters using the promoters and 3’ UTR sequences of each target (see supplemental methods). mCherry was paired with the
wild-type target 3’ UTR while GFP was fused to a 3’ UTR where the predicted mir-34 and mir-83 binding sites were mutated. Specifically, the 3’ UTR bases predicted to be part of the mRNA-miRNA seed base pairing were mutated to the complementary base, A to T, C to G, G to C, and U to A, such that mir-34 and mir-83 loaded miRISCs would no longer to be able to bind the mutated 3’ UTRs. For each wild type 3’ UTR reporter, the corresponding GFP construct with a mutated 3’ UTR served as an internal control, as it should not be subjected to regulation by either miRNA. The mCherry construct however, should be regulated by both miRNAs and therefore, should be expressed differently in their presence or absence. We generated multi-copy arrays (single-copy transgenes were unsuccessful, see Discussion) containing both constructs in mir-83(n4638); mir-34(gk437) mutants and crossed these arrays into N2 worms for comparison to the mutant background. GFP and mCherry fluorescence was subsequently quantified within DTCs or for the whole animal. We did not observe a difference in the ratio of mCherry to GFP fluorescence expressed in DTCs for the cdc-42 reporters, in any of the stages observed (Figure S10A, p ≥ .05, unpaired t-test).

As for the pat-3 reporters, the results were indicative but not definitive. Expression of the pat-3 reporters was not detectable in DTCs in larval stages. In adults, pat-3 reporter expression was very dim and bleached rapidly, necessitating scoring whole animals. In whole adult animals, there was a slight increase in the ratio of mCherry to GFP fluorescence for the pat-3 reporters when mir-34 and mir-83 were deleted (Figure S10B, p ≤ .05, unpaired t-test), suggesting that the two miRNAs do directly regulate pat-3. Although these reporter experiments could not confirm direct regulation of both cdc-42 and pat-3 by mir-34 or mir-83, there were numerous technical issues that limited
the sensitivity and fidelity of the reporter assays (see Discussion). It is possible that
\textit{cdc-42} and \textit{pat-3} could be acting in parallel pathways that indirectly oppose the
activities of \textit{mir-34} and \textit{mir-83}, such that the observed suppression is the result of
decreasing the activity of an opposing pathway. However, based on the computational
prediction of co-targeting by \textit{mir-34} and \textit{mir-83}, combined with the fact that both \textit{cdc-42}
and \textit{pat-3} are known to be required for proper pathfinding, we propose that these
predicted \textit{mir-34} and \textit{mir-83} common targets function downstream of the two miRNAs in
conferring robustness to DTC migration in the face of temperature changes.

\textit{mir-83(n4638); mir-34(gk437)} mutants display reduced cross progeny

\textbf{ FECUNDITY: } In addition to guiding the morphology of the adult gonad, DTCs are also
required to signal to the germline and regulate the production of germ cells (reviewed in
Hubbard and Greenstein 2000). We therefore tested whether the deletion of \textit{mir-34} and
\textit{mir-83} affected the function of the hermaphrodite germline by quantifying offspring. We
scored the total number of viable progeny, animals that hatched from laid eggs. We did
not observe a noticeable difference in the number of dead eggs laid by \textit{mir-83(n4638)};
\textit{mir-34(gk437)} mutants versus N2 worms, therefore we did not include them in our
quantification. When raised at 20\degree or with temperature oscillations occurring during the
previously described two hour temperature-sensitive period (referred to as “cycled”),
there was no statistical difference in the number of viable self progeny produced by
\textit{mir-83(n4638); mir-34(gk437)} mutants compared to wild type (Figure 6A, \(p \geq .05,\)
unpaired t-test).

The self-fertility of a \textit{C. elegans} hermaphrodite is limited by the number of sperm
that it produces. Once self sperm are exhausted, the hermaphrodite’s total reproductive
capacity is defined by the number of additional oocytes it can produce that are competent to produce viable cross progeny upon mating to males (Brenner 1974). To investigate cross progeny production, N2 and \textit{mir-83(n4638); mir-34(gk437)} mutants were first either raised at 20º or cycled. After day 4 of adulthood, animals had exhausted their own supply of sperm and could no longer produce fertilized embryos (Byerly \textit{et al.} 1976). At this point, young adult N2 males were mated to aged animals to assess their fertility (Figure 6B). Temperature oscillations did not affect the number of cross progeny produced by wild type hermaphrodites crossed to wild type males (247.25 +/- 77.52 versus 236.5 +/- 103.44 at 20º, \( p \geq .05 \), unpaired t-test). Additionally, there was no significant difference between the number of cross progeny produced by \textit{mir-83(n4638); mir-34(gk437)} worms raised in either constant temperature or with temperature oscillations when mated to wild type males (141.85 +/- 84.01 at 20º versus 118.65 +/- 51.99 cycled, \( p \geq .05 \), unpaired t-test). There were however, significant differences between strains in total cross progeny produced. Specifically, the number of cross progeny produced by \textit{mir-83(n4638); mir-34(gk437)} mutants when mated to wild type males was significantly less than the number of cross progeny produced by wild type hermaphrodites mated to wild type males regardless of the temperature regime during development (\( p \leq .005 \), unpaired t-test).

If the fecundity defect in \textit{mir-83(n4638); mir-34(gk437)} hermaphrodites was a direct consequence of the gonad migration defect, we would expect to see a greater number of cross progeny produced by \textit{mir-83(n4638); mir-34(gk437)} mutants when raised at 20º compared to temperature cycled as temperature cycling dramatically decrease the percentage of animals with normal gonad morphology. Moreover, we
would expect to see two populations of animals within the cycled conditioned, a lower number of viable progeny for the approximately 60% of the population expected to have a gonad migration defect and a higher number of viable progeny for the 40% of the population expected to lack any defect. However, the reduced fecundity of *mir-83(n4638); mir-34(gk437)* hermaphrodites was unaffected by temperature regimen and the population distribution of fecundity for *mir-83(n4638); mir-34(gk437)* worms was not consistent with a 60%/40% split. Thus it appears that the fecundity defect in *mir-83(n4638); mir-34(gk437)* mutants is likely independent of the gonad migration defect. It is possible that *mir-34* and *mir-83* may regulate targets other than *cdc-42* and *pat-3* within the DTCs that affect their ability to signal to the germline for the regulation of oocyte production. Alternatively, there may be subtle changes in the mating behavior of *mir-83(n4638); mir-34(gk437)* hermaphrodites that could be detected with closer study.

**mir-83(n4638); mir-34(gk437) mutants have a decreased lifespan:** A rearrangement of internal organs (as seen in Figure 1C) might be expected to have negative consequences for the overall fitness and viability of the affected worm. Moreover, it has been shown that an animal’s fecundity can correlate with their longevity (Hsin and Kenyon 1999; Berman and Kenyon 2006; Kenyon 2010). To explore this possibility, we measured the lifespan of N2 and *mir-83(n4638); mir-34(gk437)* mutants raised at either 20° continuously (Figure 7A) or with temperature oscillations during the time sensitive period (Figure 7B). Although *mir-83(n4638); mir-34(gk437)* worms raised at 20° appeared to have a slightly shortened lifespan compared to N2, the difference was only marginally statistically significant (Figure 7A, ***p < 0.005, **0.005 < p ≤ 0.01,* .
01 < p ≤ .05, unpaired t-test). However, for animals subjected to oscillating temperatures during the temperature-sensitive period (from 16-18 hours post-plating) the difference between N2 and mir-83(n4638); mir-34(gk437) mutant worms was more pronounced and statistically significant from day seven to day twenty-one (with the exception of days nine and seventeen, Figure 7B, *** p ≤ .005, ** .005 < p ≤ .01, * .01 < p ≤ .05, unpaired t-test). The difference in lifespans may be related to the DTC migration phenotype. A relation between the two would explain the slight, less significant difference in lifespan at 20°, when only ~20% of the mir-83(n4638); mir-34(gk437) population is migration defective, versus the significant difference in lifespan when animals undergo temperature changes, as now ~60% of the population is migration defective. This hypothesis has yet to be explored as we have not scored individual worms for both the migration phenotype and lifespan due to the fact that scoring for the migration phenotype involves paralyzing the worms and is potentially detrimental to their lifespan. It is possible that the more pronounced difference in lifespan between mir-83(n4638); mir-34(gk437) and N2 worms when raised with temperature oscillations does not reflect a direct relationship between lifespan and gonadal migration but instead could reflect a general decrease in the fitness of mir-83(n4638); mir-34 (gk437) worms when experiencing temperature changes.

DISCUSSION

There is an obvious benefit for building biological robustness into genetic networks; the fitness of an organism critically depends on the fidelity of developmental processes and reproductive capacity in the face of environmental changes. Previous research across numerous model organisms has described evolutionarily conserved
responses to stresses such as food deprivation, heat shock, and various forms of toxicity (reviewed in Lant and Storey 2010). miRNAs are thought to contribute to ensuring the fidelity of gene expression programs in the face of both external stresses and internal transcriptional noise (Li et al. 2009) (reviewed in Hornstein and Shomron 2006; Ebert and Sharp 2012; Posadas and Carthew 2014). Previous studies have shown how miRNAs can participate in regulatory loops to precisely regulate gene expression levels (reviewed in Tsang et al. 2007; Ebert and Sharp 2012) or establish genetic switches. miRNAs have also been shown to repress leaky transcription, thereby dampening noise within genetic networks (reviewed in Hornstein and Shomron 2006; Posadas and Carthew 2014).

The precise spatio-temporal program of DTC migration is controlled by a complex genetic network including genes pat-3 and cdc-42 (Cram et al. 2006), which encode, respectively, β-integrin and a GTPase downstream of integrin signaling. Genetic networks need to be robust so as to compensate for adverse changes in gene expression that can result from stress (reviewed in Lant and Storey 2010; Zhou et al. 2011). Cells can compensate for stress-induced positive fluctuations in mRNA levels through the post-transcriptional repressive action of miRNAs (Figure 8A). Likewise, negative fluctuations in mRNA levels can be compensated for by activating transcription and/or translation, including releasing inhibition imparted by miRNAs. Thus miRNAs can contribute to the robustness of a genetic network by fine tuning the expression levels of networked genes and buffering their expression from environmentally-driven adverse perturbations.
For DTCs, phase 1 and phase 3 of their migration is regulated by cell-intrinsic integrin signaling (Baum and Garriga 1997; Lee et al. 2001; Cram et al. 2006; Meighan and Schwarzbauer 2007). We propose that both mir-34 and mir-83 help protect the robustness of this genetic network by regulating cdc-42 and pat-3 (Figure 8B), particularly when the network is stressed by temperature changes. Our finding that DTC expression of mir-34 and mir-83 can rescue the migration defect of their respective mutants supports the hypothesis that the two miRNAs regulate cdc-42 and pat-3 within the DTCs. Although these rescue data strongly suggest that mir-34 and mir-83 function within the DTCs, we were not able to confirm expression of these miRNAs in DTCs. Transgenes with either the mir-34 or mir-83 promoter driving GFP did not produce detectable expression of GFP in the DTCs (although expression in body wall muscle cells was detected for mir-34 driven GFP). It is possible that the endogenous mir-34 and mir-83 genes are expressed in the DTCs, but these transgenic constructs may express GFP at levels below limits of detection.

Interestingly, we also observed rescue of the migration defect in mir-83(n4638) mutants by expression of mir-83 in muscle cells using the myo-3 promoter. This could reflect a low level of activity of the myo-3 promoter in the DTCs. Alternatively, it is possible that mir-83 may perform functions within both the DTCs and muscle, and that function in either cell type can rescue DTC migration. A third possibility is that mir-83 may function only in the DTCs, but that the mir-83 miRNA can be supplied either cell intrinsically or cell extrinsically from muscle expressed mir-83. Intriguingly, another known component of the DTC migration gene network, the metalloprotease mig-17, is secreted by body wall muscle cells and localizes to the gonadal basement membrane.
It is also possible that mir-83 synthesized in the muscle could be transported to the DTCs to function, explaining why mir-83 expression in either of these two tissues can result in similar levels of rescue.

For technical reasons, our fluorescent reporter transgene approach did not permit us to confirm 3’ UTR-dependent regulation of cdc-42 or pat-3 by mir-34 or mir-83. Both genes are widely expressed; cdc-42 is most highly expressed in the intestine and muscles while pat-3 expression is particularly high in muscles (data not shown and (Plenefisch et al. 2000). Since the two DTCs make up such a small fraction of the worm, protein expression changes within the DTCs are not easily analyzed by Western blotting as expression throughout the entire worm will mask any small cell-specific changes. We used fluorescent reporters due to the difficulty of seeing cell-specific changes. We first attempted to generate single copy insertions of the four reporters. For both the pat-3 GFP reporter and the cdc-42 mCherry reporter, we were not able to generate single copy integrated transgenes that expressed at detectable levels. Therefore we were forced to use multi-copy arrays, which most certainly reflect an overexpression of mRNA. We suspect that the absence of measurable response of these reporters to mir-34 and mir-83 activity in our experiments likely reflects an overexpression of the reporters, in excess to the endogenous miRNA levels. It is also possible that mir-34 and mir-83 may regulate their targets in a fashion too dynamic to visualize using fluorescent reporters.

Here we showed that mir-34 and mir-83 both contribute to keeping DTC migrations robust and protecting the fidelity of DTC migratory behavior from changes in temperature. In addition to organizing the morphogenic processes that shape the
mature gonad, DTCs are also responsible for regulating meiosis in the germline (Hubbard and Greenstein 2000). Therefore DTCs have a direct impact on *C. elegans* reproductive capacity, and there is a direct benefit for the worm to protect these cells from external stresses. Interestingly, although *mir-83(n4638); mir-34(gk437)* mutants produced the normal number of self-progeny in our experiments, they nevertheless exhibited reduced numbers of cross-progeny when mated to wild type males. Thus, the overall maximum reproductive capacity of *mir-83(n4638); mir-34(gk437)* hermaphrodites is compromised. Our results suggest that this maximum fecundity defect is not a direct consequence of the gonad migration defect, as the penetrance of the fecundity defect is higher than that of the migration defect and is not enhanced by temperature oscillations. Additionally the fecundity defect may reflect a role for *mir-34* and *mir-83* in regulating fertility that is entirely unrelated to their role in regulating DTC migrations. We do not yet know the pathway or pathways through which *mir-34* and *mir-83* affect fertility, or whether the fertility phenotypes represent functions within the germline, DTCs, or both.

We identified a two hour temperature-sensitive period, during which temperature oscillations can induce the enhanced DTC migration phenotype of *mir-83(n4638); mir-34(gk437)* hermaphrodites. This temperature-sensitive period overlaps with the birth of the DTCs during the L1 larval stage, and is well before the DTCs begin their migration (Sulston 1976; Sulston and Horvitz 1977; Kimble and Hirsh 1979; Sulston et al. 1983). We have not determined what aspect of DTC specification and/or differentiation may be inherently sensitive to temperature changes such that in the absence of *mir-34* and *mir-83*, DTCs display a stronger defect in pathfinding fidelity than when under constant temperature. It is intriguing that this defect is enhanced by
fluctuating temperature specifically during the L1 stage, suggesting that DTCs are particularly sensitive to the stress of unstable temperature prior to the execution of their migratory program. It is known that the rate of development is closely tied to the environmental temperature in *C. elegans*. Temperature-sensitivity during the L1 stage may reflect a requirement to buffer noise within the integrin genetic network early in development. A lack of proper initial buffering may sensitize DTCs such that their subsequent migration is no longer robust. Alternatively, early temperature fluctuations may lead to changes in gene expression that are subsequently buffered by *mir-34* and *mir-83* as the DTCs actively migrate during later larval stages.

In mammals, both *mir-34* and *mir-29* (the mammalian *mir-83* homolog) have been implicated in cancer, and *mir-29* has also been implicated in regulating fibrosis and cell-cell interactions (reviewed in Boominathan 2010; Kriegel et al. 2012). Mammalian *mir-34* homologs are transcriptionally activated by p53 and mediate post-transcriptional regulatory processes downstream of p53 (reviewed in He et al. 2007; Hermeking 2009; Boominathan 2010). *mir-34a* overexpression has been shown to reduce lung cancer tumor cell proliferation and tumor volume (Xue et al. 2014). Human cells contain four paralogs of *mir-29/mir-83*, namely *mir-29a*, *mir-29b-1*, *mir-29b-2*, and *mir-29c* (reviewed in Kriegel et al. 2012). Expression of the *mir-29* miRNAs are regulated by both c-Myc and NF-κB, and have been shown to regulate genes involved in apoptosis, cell proliferation, differentiation, and extracellular matrix components (reviewed in Kriegel et al. 2012). The *mir-29* miRNAs have been shown to be downregulated in a variety of cancers, including cervical, colon, liver, leukemia, lung, and melanoma (Calin et al. 2005; Yanaihara et al. 2006; Cummins et al. 2006; Pekarsky et al. 2006; Garzon et al. 2006).
2008; 2009; Xiong et al. 2010; Nguyen et al. 2011; Li et al. 2011). They have also been shown to be up regulated in certain breast cancers. Human CDC42 was shown to be a direct target of the \textit{mir-29} miRNAs in work from the Kim lab (Park et al. 2008). Additionally, they showed that the suppression of CDC42 by \textit{mir-29} lead to p53 up regulation and increased apoptosis. Taken together with the work in mammals showing that \textit{mir-34} reinforces p53 negative regulation, this suggests that the co-regulation of a genetic network by \textit{mir-34} and \textit{mir-83/mir-29} is evolutionarily ancient and conserved. Furthermore, TargetScan (Lewis et al. 2005; Grimson et al. 2007; Friedman et al. 2009; Garcia et al. 2011) predicts conserved targeting of human ITGB1 (integrin beta-1), ITGA11 (integrin alpha-11), and ITGA6 (integrin alpha-6) by the \textit{mir-29} family and conserved targeting for human ITGB8 (integrin beta-8) and ITGA10 (integrin alpha-10) by the \textit{mir-34} family.

Here we show a link between \textit{mir-34}, \textit{mir-83/mir-29}, and integrin-controlled cell migration. Integrin and extracellular matrix misregulation is a key factor in epithelial to mesenchymal transition (EMT) (reviewed in Seguin et al. 2015). The implication of \textit{mir-34} and \textit{mir-83/mir-29} in various cancers may reflect a conserved involvement in metastasis through altered EMT that has yet to be explored. The wandering DTC defect in \textit{C. elegans} \textit{mir-34} and \textit{mir-83/mir-29} mutants, which we have shown results from misregulation of \textit{pat-3} and \textit{cdc-42}, may reflect a homologous miRNA-integrin axis in tumor formation, proliferation, and metastasis in higher animals, including humans.

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AUTHOR CONTRIBUTIONS
S.L.B. performed the experiments and analyzed the data. S.L.B., M.H., and V.A designed experiments; S.L.B. and V.A. wrote the paper.

LITERATURE CITED


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Figure 1. *mir-83(n4638); mir-34(gk437)* mutants have gonad migration defects.
(A) The *C. elegans* gonad consists of two U-shaped gonad arms. The shape of each arm is created by the migration path of the DTC. The two DTCs are initially located ventrally near the mid-body. Each DTC migrates away from the mid-body before turning dorsally, migrating to the dorsal body wall muscle, and then migrating back towards the mid-body. The path of each DTC can be inferred by the location of the respective gonad arm. Body wall muscle in red, germline in gray, DTCs in black, uterus designated with "U". (B) *mir-83(n4638); mir-34(gk437)* mutants have a gonad migration defect. The improper location of the mature gonad arms implies that DTCs did not migrate along the normal path. In the arm pictured (bottom), the DTC (black arrow) migrated too far, passing the vulva (white asterisk), and is displaced ventrally, as compared to N2 (top). Penetrance quantified in Figure 2. (C) Improper DTC migration causes a rearrangement of internal organs, due to space constraints. In N2 worms, the intestine is positioned laterally with respect to the dorsal-ventral axis (indicated by a white, dashed line), and the proximal and distal segments of the gonad are positioned ventrally and dorsally, respectively, on the other side from the intestine. In the *mir-83(n4638); mir-34(gk437)* worm shown here, the displaced gonad caused a displacement of the intestine. Black arrowheads point to the adult lateral alae, which are positioned on the left and right sides of the animal.
Figure 2. The gonad migration defect is significantly enhanced in \textit{mir-83(n4638); mir-34(gk437)} double mutants. Gonad arm morphology in young adult hermaphrodites was used to score for defects in DTC migrations during larval development at (A) 20\(^\circ\) or (B) under an oscillating temperature regimen (15\(^\circ\) for 15 minutes, 25\(^\circ\) for 15 minutes, repeated from plating eggs until young adulthood). (C) Integrated transgenes expressing either \textit{mir-34}, \textit{maIs387}, or \textit{mir-83}, \textit{maIs391}, under their natural promoters rescue single mutants. Both VT3289 and VT3294 \textit{maIs387; maIs391; mir-83(n4638); mir-34(gk437)} were produced by crossing VT3106 \textit{maIs387; mir-34(gk437)} to VT3110 \textit{maIs391; mir-83(n4638)}, shown in (D). As expected the newly isolated \textit{mir-83(n4638); mir-34(gk437)} double mutant, VT3289, displays the migration defect while the double mutant carrying both rescue transgenes, VT3294, appears normal. Significance stars compare to N2. Penetration in VT2595 is not significantly different from penetration in VT3289. Animals were cycled as described in Part B. *** \textit{p-value} \leq .005, * .01 < \textit{p} \leq .05, not significant (n.s.) if \textit{p} > .05.
Figure 3. Other miRNAs implicated in gonad migration function in separate pathways. (A) Previously implicated miRNA mutants were tested for enhancement of gonad migration defects by temperature oscillations. Worms were either maintained at a steady 20°C throughout development, or were subjected to oscillating temperature (15°C for 15 minutes, 25°C for 15 minutes) throughout development ("cycled"). (B) The mir-259(n4106) mutation was crossed into the mir-83(n4638); mir-34(gk437) strain to assess potential genetic interactions. Worms were subjected to an oscillating temperature regimen (15°C for 15 minutes, 25°C for 15 minutes, repeated until young adulthood). The difference in mig phenotype penetrance between mir-83(n4638); mir-34(gk437) and mir-83(n4638); mir-259(n4106); mir-34(gk437) is not significant. *** p-value ≤ .005. (C) Phenotype penetrance for the mir-259(n4106) mutation in combination with either the mir-34(gk437) mutation or the mir-83(n4638) mutation was compared to that in the mir-83(n4638); mir-34(gk437) double mutant. Worms raised at 20°C or cycled during the temperature sensitive period discussed in Figure 4 (20°C for 16 hours, [15°C for 15 minutes, 25°C for 15 minutes, repeated three additional times], 20°C until young adulthood). *** p-value ≤ .005.
Figure 4. Temperature oscillations within a limited two hour window cause the migration defective phenotype enhancement in *mir-83(n4638); mir-34(gk437)* mutants.

The temperature was oscillated every fifteen minutes for a total of two hours (15° for 15 minutes, 25° for 15 minutes, repeated four times). (A) Temperature oscillations occurred prior to the birth of the DTCs (12 to 14 hours after plating starved L1s on HB101-seeded NGM plates). (B) Temperature oscillations occurred over an interval (16 to 18 hours after plating starved L1s on HB101) corresponding to the time of DTC birth. (C) Temperature oscillations occurred after the birth of the DTCs (20 to 22 hours after plating starved L1s on HB101). *** p-value ≤ .005, ** .005 < p ≤ .01, significance stars compare N2 to *mir-83(n4638); mir-34(gk437)* mutants.
Figure 5. The mir-83(n4638); mir-34(gk437) migration defect is suppressed in cdc-42 and pat-3 heterozygotes. The penetrance of the migration defective phenotype is significantly reduced in mir-83(n4638); mir-34(gk437) mutants when (A) carrying one copy of cdc-42, (B) raised on cdc-42 RNAi food as compared to empty vector control, (C) carrying one copy of pat-3, or (D) raised on pat-3 RNAi food as compared to empty vector control. Arrested L1s were plated on HB101-seeded NGM plates to restart development. Once plated, temperature was held at 20º for 16 hours, then cycled as follows: [15º for 15 minutes, 25º for 15 minutes, four times], then held at 20º until young adulthood. *** p-value ≤ .005, ** .005 < p ≤ .01, * .01 < p ≤ .05, p > .05 not significant (n.s.).
Figure 6. *mir-83(n4638); mir-34(gk437)* mutants produce normal sized self-broods and reduced cross-broods. (A) The total number of living offspring was counted to determine brood size for N2 and *mir-83(n4638); mir-34(gk437)* worms raised at 20º throughout development or subjected to temperature cycles (15º for 15 minutes, 25º for 15 minutes, repeated four times) from 16 to 18 hours of development after plating starved L1s on HB101. There is no statistically significant difference between any of the groups. (B) Individual four-day-old adult hermaphrodites that had exhausted their self progeny were crossed to four one-day-old N2 males and number of cross-brood progeny was counted. The average cross-brood size was reduced for *mir-83(n4638); mir-34(gk437)* hermaphrodites whether they were raised at 20º or subjected to temperature cycles (15º for 15 minutes, 25º for 15 minutes, repeated four times) from 16 to 18 hours of development. Averages were compared using an unpaired t-test, performed by PRISM. *** p-value ≤ .005. Each group was compared to the three others. The absence of significance stars denotes a lack of statistical significance.
Figure 7. mir-83(n4638); mir-34(gk437) mutants have a decreased lifespan compared to wild type. Wild type (N2) and mir-83(n4638); mir-34(gk437) embryos were harvested by hypochlorite treatment and synchronized L1 larvae were obtained by hatching overnight in M9. Larvae were plated on HB101-seeded NGM plates and raised (A) continuously at 20º or (B) subjected to temperature cycles (15º for 15 minutes, 25º for 15 minutes, repeated four times) from 16 to 18 hours of development after plating starved L1s on HB101. For each longevity assay shown in panels A and B, 100 animals per replicate, three replicates per condition, were plated as L4s (Day 0) and tracked until their death. Alive or dead was determined by prodding worms on their nose and looking for a reaction. Daily averages were compared using a two sample t-test for means. *** p-value ≤ .005, ** .005 < p ≤ .01, * .01 < p ≤ .05.
Figure 8. A model proposing robustness functions for *mir-34* and *mir-83* through dampening noisy *cdc-42* and *pat-3* expression in the face of temperature changes. 

(A) Environmental stresses, such as changing temperatures, may lead to fluctuations in the expression levels of various transcripts, which could challenge cellular proteomic homeostasis. Repression of protein production by miRNAs can provide a means of stabilizing protein output from fluctuating target transcripts. (B) In the case of *mir-34* and *mir-83*, the fidelity of DTC migration is proposed to be maintained in part by inhibiting noisy *cdc-42* and *pat-3* expression incited by unstable environmental temperature.
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Table 1. Tissue specific mir-34 rescue
Synchronized L1s were raised with oscillating temperatures - 20º for 16 hours, [15º for 15 minutes, 25º for 15 minutes, repeated three additional times], 20º until young adulthood. *** p-value ≤ .005, * .01 < p ≤ .05, significance stars compare rescue strains to mir-34(gk437) mutants.
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Table 2. Tissue specific mir-83 rescue
Synchronized L1s were raised with oscillating temperatures - 20º for 16 hours, [15º for 15 minutes, 25º for 15 minutes, repeated three additional times], 20º until young adulthood. *** p-value ≤ .005, significance stars compare rescue strains to mir-83(n4638) mutants.