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Noncoding RNA Regulation of Dormant States in Evolutionarily Diverse Animals

JULIE A. REYNOLDS

Department of Evolution, Ecology, and Organismal Biology, The Ohio State University, 400 Aronoff Lab, 318 West 12th Avenue, Columbus, Ohio 43201

Abstract. Dormancy is evolutionarily widespread and can take many forms, including diapause, dauer formation, estivation, and hibernation. Each type of dormancy is characterized by distinct features; but accumulating evidence suggests that each is regulated by some common processes, often referred to as a common "toolkit" of regulatory mechanisms, that likely include noncoding RNAs that regulate gene expression. Noncoding RNAs, especially microRNAs, are well-known regulators of biological processes associated with numerous dormancy-related processes, including cell cycle progression, cell growth and proliferation, developmental timing, metabolism, and environmental stress tolerance. This review provides a summary of our current understanding of noncoding RNAs and their involvement in regulating dormancy.

Introduction

Becoming dormant provides a means to survive changing environments and inhospitable conditions. Dormancy is widespread across all animal phyla, ranging from sponges to mammals; and it can take a number of forms that are broadly categorized as diapause, dauer, hibernation, or estivation, depending on the environmental cues (*e.g.*, changes in photoperiod, crowding, temperature stress, desiccation, or starvation) that initiate the dormancy and the circumstances under which dormancy is terminated (*e.g.*, endogenous mechanisms, improved food quality, or improved thermal environment). The specific characteristics that define dormancy for any given species are genetically determined but generally include some degree of metabolic depression, increased tolerance to environmental stress, and developmental arrest.

Dormancy is an interesting puzzle that has fascinated scientists for generations, and many years of investigating this topic have yielded answers about the mechanisms that mediate the changes in physiology, biochemistry, and gene expression that accompany the entry into and out of dormancy for evolutionarily diverse species. However, in spite of the progress that has been made understanding how dormant states are regulated (for review, see Denlinger, 2002; Carey *et al.*, 2003; Podrabsky and Hand, 2015; Hand *et al.*, 2016; Baumgartner and Tarrant, 2017; Tougeron, 2019), the underpinning molecular mechanisms that regulate these changes remain a conspicuous knowledge gap.

Recent advances in our understanding of regulatory RNAs (*i.e.*, noncoding RNAs that can regulate gene expression) provide new insight into molecular mechanisms that might mediate the entry into, maintenance of, and exit from dormant states. Noncoding RNAs, especially microRNAs (miRNAs), are increasingly recognized as important regulators of biological processes relevant to dormancy, such as cell cycle regulation and cell proliferation (Sun *et al.*, 2008), developmental timing (Bethke *et al.*, 2009; Hammel *et al.*, 2009; Resnick *et al.*, 2010), metabolism (*e.g.*, Xu *et al.*, 2003; Jones *et al.*, 2016; Hadj-Moussa *et al.*, 2018), and stress tolerance (*e.g.*, Morin *et al.*, 2017a; Riggs and Podbrabsky, 2017; Graham and Barreto, 2018; Riggs *et al.*, 2018). During the past 10 years, numerous studies have explored noncoding RNAs in the context of diapause, dauer formation, hibernation, and estivation. My goal for this review is to provide an overview of

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Email: reynolds.473@osu.edu.

Abbreviations: Ago, Argonaute; Akt, protein kinase b; BCAA, branched-chain amino acid; lncRNA, long noncoding RNA; miRNA, microRNA; mTOR, mammalian target of rapamycin; PI3-K, phosphoinositide 3-kinase; piRNA, piwi-interacting RNA; PTEN, phosphatase and tensin homolog; RISC, RNA-induced silencing complex; RNA-Seq, RNA sequencing; RT-qPCR, reverse transcriptase polymerase chain reaction; siRNA, small-interfering RNA; Smad, Small body size/mothers against decapentaplegic; sncRNA, small noncoding RNA; TE, transposable element; TGF, transformation growth factor; TOR, target of rapamycin; UTR, untranslated region.

our current understanding of contributions that noncoding RNAs make to the regulation of a variety of dormant stages by (1) providing a brief overview of the functions of noncoding RNAs, (2) summarizing what is currently known about noncoding RNAs in the context of dormant states, and (3) exploring next steps that are necessary for understanding small RNA regulation of dormancy.

Overview of Noncoding RNAs

Noncoding regulatory RNAs are broadly classified as small noncoding RNAs (sncRNAs; <200 nucleotides) or long noncoding RNAs (lncRNAs; >200 nucleotides). sncRNAs have been divided into multiple classes based on their size, structure, origin, location in the cell, and mode of action. Currently, the best-studied classes of regulatory RNAs are miRNAs, piwi-interacting RNAs (piRNAs), and small-interfering RNAs (siRNAs). miRNAs (18–25 nucleotides) originate from single-stranded, primary sequences that are transcribed by RNA polymerase II and folded into a characteristic hairpin structure. Primary-miRNAs are processed by Drosha to become precursor RNAs (pre-miRNAs) that are translocated into the cytoplasm, where they are processed by Dicer1 with the aid of Loquacious and R2D2. The resulting mature miRNAs bind to an Argonaute (Ago) protein within the RNA-induced silencing complex (RISC). In mammals, miRNAs can bind to a RISC containing either Ago1 or Ago2 (Czech and Hannon, 2011). In insects, miRNAs bind to a RISC containing Ago1, while siRNAs bind to a RISC containing Ago2 (Iwasaki *et al.*, 2009; Rubio *et al.*, 2018). Once bound to the RISC, miRNAs guide the complex to target transcripts that have a region in the 3′ untranslated region (UTR) that is complementary to the "seed sequence" in the 5′ end of the miRNA. The target transcripts are silenced through mRNA degradation or inhibition of translation (Fig. 1A; Chen *et al.*, 2018; O'Brien *et al.*, 2018; Gebert and MacRae, 2019).

Slightly larger piRNAs (23–30 nucleotides) negatively regulate expression and movement of transposable elements (TEs). They originate from single-stranded RNA precursors, including TEs (Thompson and Lin, 2009; Juliano *et al.*, 2011; Iwasaki *et al.*, 2015). Generation and processing of piRNAs are not well understood but require Spindle-E, an RNA-helicase (Kennerdell *et al.*, 2002; Olivier *et al.*, 2010; Handler *et al.*, 2013). The piRNA-RISC includes the mature piRNA and one of three Argonaute proteins—Piwi, Aubergine, or Ago3—that determines the piRNA's function. The majority of piRNAs studied have been located in the germ line or somatic cells in gonads; but a recent study has discovered an important role for piRNAs in somatic cells in the fat body of adults of *Drosophila melanogaster*, where they mediate metabolic homeostasis and lifespan (Jones *et al.*, 2016).

siRNAs (20–25 nucleotides) originate from double-stranded regions of lncRNAs. Endogenous siRNAs originate from TEs, while exogenous siRNAs originate from viruses or other outside sources. Precursor siRNAs are processed by Dicer2, and the resulting mature sequence binds to a RISC containing Ago2. Once associated with the RISC, siRNAs, which are fully complementary to their targets, promote transcript degradation. Although siRNAs are best known for being used to experimentally manipulate specific gene sequences in a laboratory setting, their "natural" function is blocking the activity of viruses, TEs, or other foreign sequences (Piatek and Werner, 2014).

Transcriptional and Proteomic Evidence for Small Noncoding RNA Regulation of Dormant States

Some evidence that sncRNAs are involved in animal dormancy comes from studies that evaluated protein levels or transcript abundance of core components of piRNA, miRNA, and siRNA pathways. In hibernating thirteen-lined ground squirrels (*Ictidomys tridecemlineatus*), Dicer protein increased nearly 3-fold in heart tissue and was reduced by ~60% in kidneys, relative to tissues from euthermic animals (Morin *et al.*, 2008).

In insects, there is transcriptional evidence that sncRNAs are involved in diapause of *Sarcophaga bullata*, *Dianemobius nigrofasciatus*, *Megachile rotundata*, and *Chymomyza costata* (Reynolds *et al.*, 2013; Poupardin *et al.*, 2015; Yocum *et al.*, 2015). During pupal diapause in the flesh fly *S. bullata*, *Ago1* is upregulated more than twofold compared to pupae not in diapause (Fig. 1B). The subsequent downregulation of this gene by more than 50% after diapause termination (Fig. 1C) suggests that miRNAs contribute to diapause maintenance in this species (Reynolds *et al.*, 2013). There is also a greater than twofold increase in transcript abundance of genes encoding two components of the piRNA pathway—PIWI and Spindle-E—in first instar larvae that are programed to enter diapause. These changes suggest that piRNAs contribute to initiating diapause and/or regulating the switch from a continuous, direct developmental trajectory to a discontinuous trajectory that includes diapause. *Piwi* and *spindle-E* mRNAs are also more abundant in embryos of the band-legged ground cricket, *D. nigrofasciatus*, that are programmed to enter diapause than in embryos programmed for continuous development (Shimizu *et al.*, 2018). These two studies suggest that piRNAs may have a role in diapause regulation that is evolutionarily widespread and may be part of a common "toolkit" of processes that regulate insect diapause in evolutionarily diverse species. Differential regulation of components of the siRNA biogenesis pathway—*argonaute2*, *drosha*, and *pasha*—in post-diapausing bees (*M. rotundata*) suggest a role for siRNAs in regulating diapause termination and post-diapause development in this species (Yocum *et al.*, 2015). Finally, in pre-diapause larvae of the drosophilid *Chymomyza costata*, *ago2* mRNA is less abundant than in larvae not programmed for diapause, indicating a possible role for siRNAs in programming diapause in this species as well (Poupardin *et al.*, 2015). At

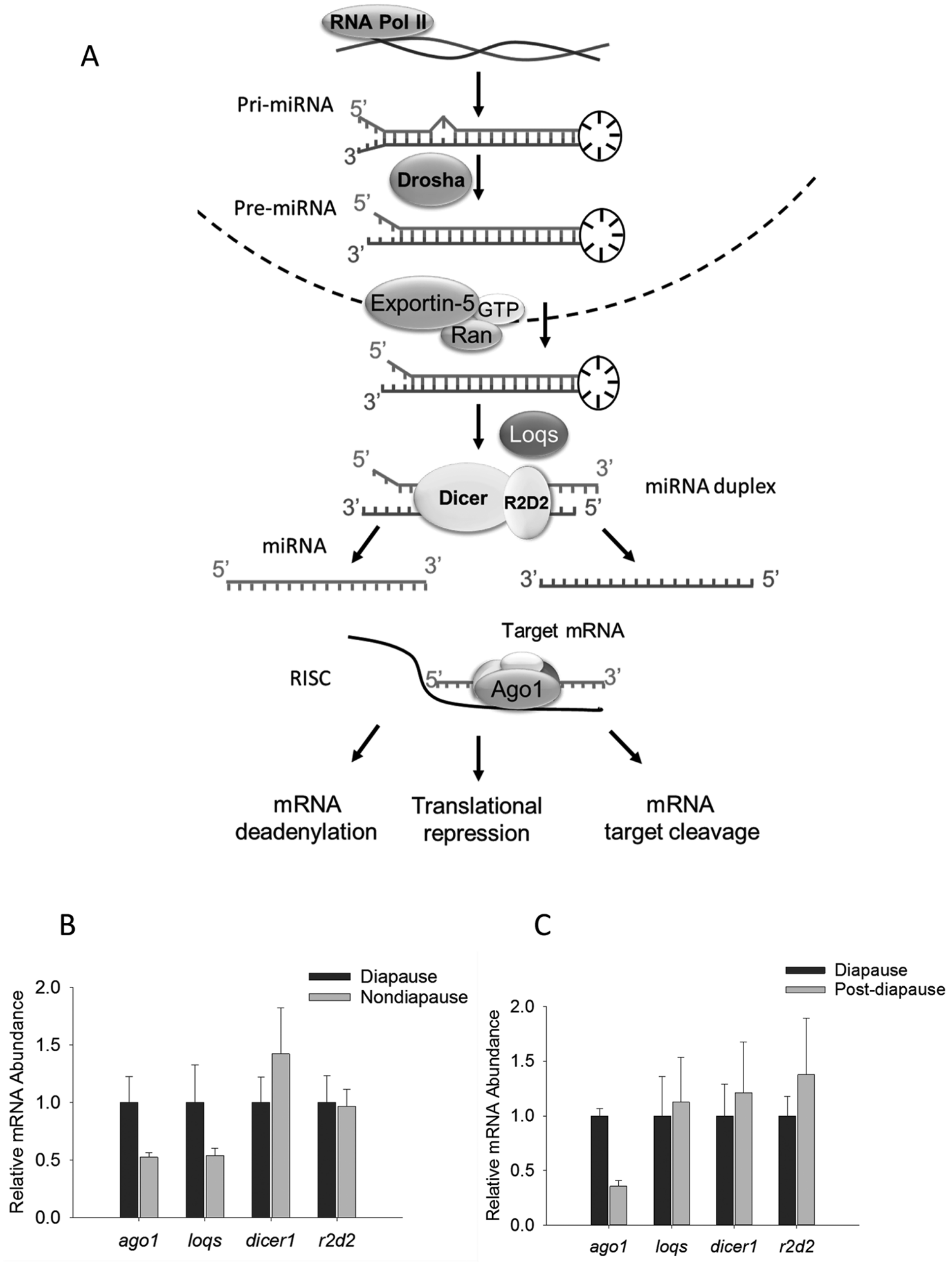

Figure 1. (A) Generalized microRNA biosynthesis pathway and mRNA expression of genes encoding core components from (B) diapause and nondiapausing pupae and (C) diapause and post-diapause pharate adults of *Sarcophaga bullata*. Ago1, Argonaute1; GTP, guanosine triphosphate; Loqs, loquacious; pol, polymerase; pre-miRNA, precursor miRNA; pri-miRNA, primary miRNA; Ran, RAs-related nuclear protein; RISC, RNA-induced silencing complex. Adapted from Reynolds (2017), with permission from Elsevier Books (http://www.elsevier.com).

present, the hypothesis that these sncRNA pathways regulate diapause is largely untested for insects. However, recent studies evaluating sncRNA profiles, rather than the mRNA expression of genes encoding core components, are beginning to shed light on the matter. The remainder of this review will focus on sncRNAs that are differentially regulated in dormant animals compared to their active counterparts and the possible roles that specific sncRNAs play in regulating diapause and other dormant states.

Regulation of Diapause by Small Noncoding RNAs

Animals that enter diapause (*e.g.*, rotifers, copepods, shrimp, insects, fish, and others) become dormant well before conditions become inhospitable. The diapause program, an alternative developmental pathway or trajectory, is initiated in response to token stimuli (*e.g.*, changes in photoperiod, cooling temperatures, or declining food quality) that predict the advent of a season of inimical environmental conditions. Diapausing individuals remain dormant, even though conditions might be permissive for growth and development, until diapause is terminated through a variety of mechanisms that are not yet well understood. The endogenous regulation of both diapause entry and termination distinguishes this type of dormancy from quiescence, which is a direct response to changes in the environment.

The diapause phenotype is genetically determined and manifested differently for each species. However, common characteristics of diapause for all species include arrested development, depressed metabolic rate, and increased tolerance of environmental stress. The physiological and biochemical changes (*e.g.*, metabolic rate, lipid composition, *etc.*) that occur before, during, and after diapause have been characterized for evolutionarily diverse species of insects (*e.g.*, Reynolds and Hand, 2009; Hahn and Denlinger, 2011; Reynolds *et al.*, 2012; Batz and Armbruster, 2018; Lehmann *et al.*, 2018), brine shrimp (Reynolds and Hand, 2004; Patil *et al.*, 2013; Podrabsky and Hand, 2015; MacRae, 2016), daphnia (Mariash *et al.*, 2017), copepods (Baumgartner and Tarrant, 2017), and annual killifish (Podrabsky and Hand, 2015). Additional studies have cataloged changes in gene expression that occur in association with diapause for a variety of species (*e.g.*, Denlinger, 2002; Tarrant *et al.*, 2008, 2016; Aruda *et al.*, 2011; Poupardin *et al.*, 2015; Tu *et al.*, 2015; Yocum *et al.*, 2015; Koštál *et al.*, 2017; Ragland and Keep, 2017; Roncalli *et al.*, 2018). Together, these studies have identified a number of common elements, including transcription factors, signaling pathways (*e.g.*, MAPK, Hippo, Wnt), and components of circadian clocks (Hand *et al.*, 2016; Ragland and Keep, 2017), that regulate diapause in evolutionarily diverse taxonomic groups and are often referred to as a toolkit of mechanisms.

The accumulating evidence from numerous studies published during the past five years suggests that sncRNAs should be considered part of the common molecular toolkit of processes that regulate diapause. Several studies have identified miRNAs that are differentially regulated in diapausing insects (*e.g.*, mosquitoes, flesh flies, and moths), brine shrimp, and annual killifish relative to their nondiapausing counterparts. To date, these studies have primarily focused on identifying and quantifying miRNAs in diapausing individuals and their nondiapausing counterparts, using both high-throughput sequencing and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR).

As a whole, studies of sncRNA regulation of insect diapause have examined changes in miRNA profiles of diapausing embryos, larvae, pupae, and adults in relatively few species (*e.g.*, Diptera and Lepidoptera). They also evaluated all three phases of diapause "development," or progression, as defined by Koštál (2006). Briefly, diapause induction, or initiation, begins when individuals perceive token stimuli that indicate the advent of an inhospitable season and initiate the switch from a direct developmental trajectory without dormancy to a trajectory that includes diapause. Diapause maintenance describes a period of sustained developmental arrest and metabolic depression; this phase continues, even though the environment may be permissive for continued development, until diapause termination occurs. Post-diapause refers to the period when development is re-initiated after diapause is terminated, either by natural phenomena (*e.g.*, exposure to cold or increased number of daylight hours) or by application of a diapause-terminating agent in a laboratory setting (*e.g.*, organic solvent). Each phase of diapause is characterized by coordinated up- or downregulation of certain genes (Denlinger, 2002; Koštál *et al.*, 2017); and, thus, it is likely that each phase is also characterized by unique changes in sncRNA abundance.

The Asian tiger mosquito, *Aedes albopictus*, enters diapause as a pharate first instar larvae after embryonic development is completed but before larvae emerge from the egg (Wang, 1966; Mori *et al.*, 1981). *Aedes albopictus* is an emerging model for studying diapause, and numerous studies have evaluated changes in mRNA expression that occur before, during, and after diapause (Poelchau *et al.*, 2011, 2013a, b; Reynolds *et al.*, 2012). It is likely that at least some of these changes in transcript abundance are mediated by miRNAs or other sncRNAs. Diapause in this species is maternally regulated, and females that are reared under a short-day photoperiod produce offspring that are programmed to enter diapause.

A recent study by Batz *et al.* (2017) used RNA-sequencing (RNA-Seq) to identify miRNAs that were differentially regulated in mature oocytes from mothers reared in diapause-inducing or diapause-averting environments (*i.e.*, that were associated with diapause induction). They also evaluated miRNA profiles of diapausing and nondiapausing pharate first instar larvae to identify miRNAs that may contribute to diapause maintenance in this species. Surprisingly, there were no differentially regulated miRNAs in mature oocytes, which suggests that maternal provisioning of miRNAs does not contribute to diapause induction in this species. However, there were seven miRNAs that were differentially regulated in pharate first instar larvae that had entered diapause. Three miRNAs (miR-283-5p, bantam, and miR-286b-3p) were more abundant in diapausing larvae than in ontogenetically matched nondiapausing larvae. Four miRNAs (miR-2942-3p, miR-282-5p, miR-14-3p, and miR-1-3p) were underexpressed.

The functional significance of miRNAs depends on the genes they target. Putative targets of differentially regulated miRNAs were identified using the *A. albopictus* genome, many of which have known roles regulating metabolism, cell cycle regulation, and apoptosis. Notably, the downregulation of miR-14-3p in diapausing pharate first instar larvae is consistent with the changes in lipid metabolism that characterize diapause in *A. albopictus* (Reynolds *et al.*, 2012; Batz and

Armbruster, 2018). In *Drosophila melanogaster*, miR-14 targets *sugarbabe*, which encodes a zinc finger protein that regulates insulin and fat accumulation. *Drosophila melanogaster* mutants lacking miR-14 have increased levels of lipids (Varghese *et al.*, 2010), and it is possible that miR-14 regulates lipid metabolism use in *A. albopictus* as well. Additional studies are needed to validate the putative targets of miR-14 and other differentially regulated miRNAs in *A. albopictus* and to confirm their role in regulating metabolism or cell cycle regulation during diapause.

Differentially regulated miRNAs have also been investigated in diapausing embryos of the silkworm *Bombyx mori*, an established model for studying embryonic diapause (Fan *et al.*, 2017). This study, which also used RNA-Seq, compared miRNA profiles of diapausing embryos to nondiapausing embryos that were generated by treating embryos programmed for diapause with hydrochloric acid to prevent diapause entry. In this case, HCl turned off, or terminated, diapause and resulted in differential regulation of 61 miRNAs. Following HCl treatment, 23 miRNAs were upregulated, and 38 were downregulated. Notably, miR-2761, which negatively regulates expression of *sorbitol dehydrogenase* (*sdh*), was fivefold more abundant in diapausing embryos. SDH is a key enzyme for converting sorbitol to glycogen to be used as an energy source and is found only in developing, nondiapausing embryos. Thus, miR-2761 inhibition of *sdh* expression is key for maintaining diapause in *B. mori* (Niimi *et al.*, 1992; Rubio *et al.*, 2011; Fan *et al.*, 2017). Some other biological processes regulated by the gene targets of these differentially regulated miRNAs include branched-chain amino acid (BCAA) metabolism, DNA replication, Notch signaling, target of rapamycin (TOR) signaling, and protein processing in endoplasmic reticulum (Fan *et al.*, 2017).

The miRNA regulation of pupal diapause has been investigated in the flesh fly *Sarcophaga bullata* (Reynolds *et al.*, 2017) and the corn earworm, *Helicoverpa zea* (Reynolds *et al.*, 2019). In *S. bullata*, RNA-Seq and qRT-PCR were used to identify and quantify small RNAs (15–40 nucleotides) during the pupal stage of development and to discover miRNAs that contribute to diapause maintenance. Surprisingly, only two miRNAs, miR-289-5p and miR-1-3p, were more abundant in diapausing pupae, while eight miRNAs (miR-9c-5p, miR-13b-3p, miR-31a-5p, miR-92b-3p, miR-275-3p, miR-276a-3p, miR-277-3p, and miR-305-5p) were less abundant compared to their nondiapausing counterparts. These miRNAs may regulate a number of KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways that contribute to the diapause phenotype, including Hippo signaling, Notch signaling, and fatty acid metabolism. Some specific genes that are putative targets of these differentially regulated miRNAs include *Insulin Receptor* (*InR*), *Heat shock protein 70* (*Hsp70*), and *Acetyl Coenzyme A synthase* (*AcCoAs*); these genes involved in metabolism are known to be up- or downregulated from other studies of diapausing *S. bullata* or *Sarcophaga crassipalpis*, a closely related species (Ragland *et al.*, 2010; Reynolds *et al.*, 2017). A notable example is *phosphoenolpyruvate carboxykinase* (*pepck*), a key metabolic enzyme and a putative target of miR-9c. *Pepck* is significantly upregulated in diapausing pupae, an observation consistent with downregulation of miR-9c (Ragland *et al.*, 2010; Reynolds *et al.* 2017; Spacht *et al.*, 2018). Additional validation experiments are needed to further explore the relationship between *pepck* and miR-9c.

Evaluating changes in abundance of candidate miRNAs (let-7, miR-289-5p, miR-13b-3p, miR-31a-5p, and miR-305-5p) after diapause termination provided a few clues about their role in post-diapause development in pupae and pharate adults of *S. bullata* (Reynolds *et al.* 2017). There was no change in abundance of several miRNAs evaluated even 48 hours after diapause was terminated with hexane. However, miR-289-5p, a miRNA that potentially targets several genes in both the Wnt and TOR pathways (Fig. 2), was significantly downregulated. Additional studies are needed to further explore possible interactions between miR-289-5p and putative target genes in these pathways during diapause termination, but changes in the activity of Wnt and TOR pathways are consistent with what is known about diapause termination in the apple maggot fly, *Rhagoletis pomonnella*, and the drosophilid *Chymomyza costata* (Ragland *et al.*, 2011; Koštál *et al.*, 2017). Transcriptome studies of these flies indicate that diapause termination includes starting metabolism and developmental processes through a number of signaling pathways, including Wnt and TOR pathways. Whether downregulation of miR-289 has a role in “restarting” these pathways in post-diapause insects remains to be tested.

Changes in miRNA abundance have also been examined in the context of diapause termination pupae of *H. zea* (Reynolds *et al.*, 2019). For this species, downregulation of miR-277 may be important for restarting metabolism after diapause. In this species, diapause can be terminated by injecting the steroid hormone ecdysone or the neuropeptide diapause hormone (DH). Following diapause termination with either agent resulted in a significant twofold downregulation of miR-277 (Reynolds *et al.*, 2019). Studies of miR-277 in *D. melanogaster* and *Aedes aegypti* mosquitoes implicate miR-277 in regulation of metabolism. In *D. melanogaster*, miR-277-3p regulates BCAA metabolism (*i.e.*, valine, leucine, isoleucine catabolism) and can influence lifespan (Esslinger *et al.*, 2013). BCAAs have not been studied in the context of diapause, but they are known to have roles in diapause-relevant processes, including mammalian target of rapamycin (mTOR) signaling, energy metabolism, and protein synthesis and turnover (Monirujjaman and Ferdouse, 2014). Therefore, it is possible that BCAAs mediate restructuring that occurs before, and after, diapause.

miR-277 also regulates lipid metabolism *via* the insulin-signaling pathway in *A. aegypti* (Ling *et al.*, 2017). Insulin signaling has been well studied in the context of diapause in insects (Sim and Denlinger, 2013; Zhang *et al.*, 2017),

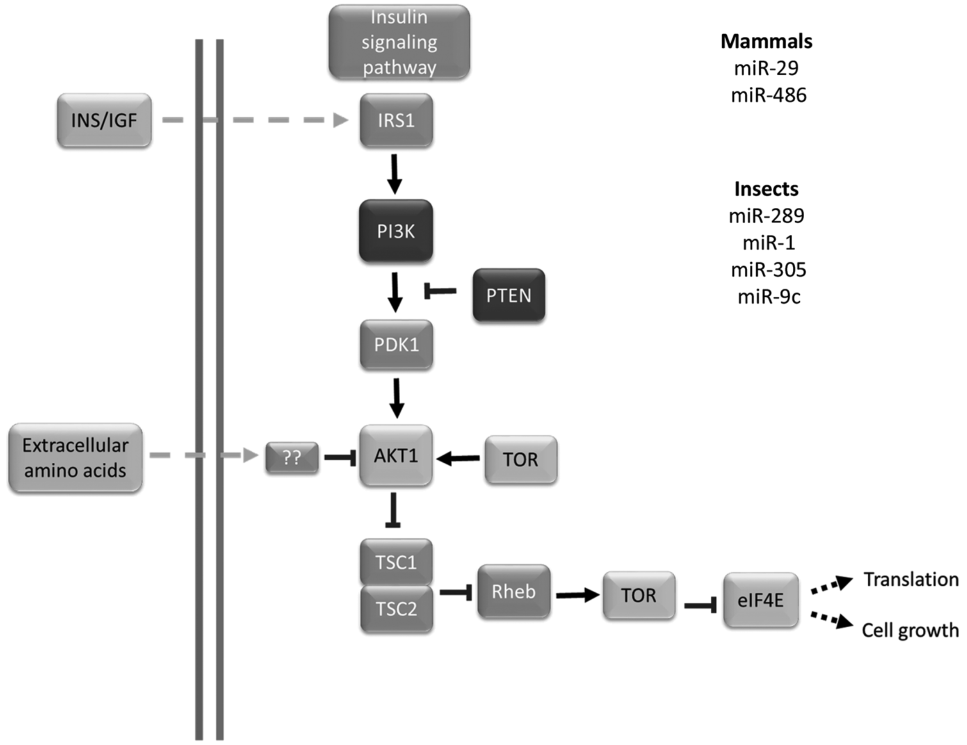

Figure 2. Generalized target of rapamycin (TOR) or mammalian target of rapamycin (mTOR) pathway. In mammals, miR-29 and miR-486 regulate the pathway by inhibiting phosphoinositide 3-kinase (PI3-K) and phosphatase and tensin homolog (PTEN), respectively; and miR-200 is regulated by mTOR. In insects, *AKT1*, *TOR*, *eIF4E*, and other genes are regulated by miR-289, miR-1, miR-305, and miR-9c.

and there is evidence of a role for incidence in the regulation of diapause in nematodes, copepods, and fish (Kimura *et al.*, 1997; Christie *et al.*, 2016; Woll and Podrabsky, 2017). In *H. zea*, insulin signaling is mediated in part by changes in reactive oxygen species (ROS) acting through protein kinase b (Akt) and the phosphoinositide 3-kinase (PI3-K) pathway (de Keizer *et al.*, 2011; Zhang *et al.*, 2017), and it seems likely that miR-277 adds another level of regulation to this pathway during and after diapause.

In addition to regulating insulin signaling, miR-277 may regulate metabolism by targeting genes in the mitochondria. A number of the ~700 genes that are putative targets of miR-277 in *D. melanogaster* (Agarwal *et al.*, 2018) encode metabolism-related proteins. Notably, acetyl-CoA C-acyltransferase (Yip2) and enoyl-CoA hydratase short chain 1 (Echs1) are involved in β-oxidation of fatty acids. In pupae of *H. zea*, both genes are upregulated two- to threefold after diapause termination with ecdysone (JR, unpubl. data). Together, these preliminary results suggest that miR-277 may have a role restarting metabolism after diapause termination in this species and possibly in others.

The miRNA regulation of adult diapause has been investigated in the mosquito *Culex pipiens*, using qRT-PCR to quantify the abundance of candidate miRNAs (see Table 1) in diapausing and nondiapausing females over the course of 22 days, beginning on the day of adult emergence (Meuti *et al.*, 2018). In *C. pipiens* the diapause program is initiated during the pupal stage in response to short-day photoperiods (<12 hours of daylight per 24 hours). However, diapause entry does not occur until about five days after females emerge, so this study includes changes of miRNA that potentially regulate both diapause induction and diapause maintenance

Table 1

Differentially regulated microRNAs (miRNAs) in pre-diapause and diapause Culex pipiens *mosquitoes relative to their nondiapausing counterparts*

	Days after adult emergence			
miRNA	0	5	12	22
miR-8-3p				↓
miR-13-3p		↓		
miR-14-3p	↓			
miR-124-3p				↓
miR-275-3p	↓	↓	↑	↓
miR-277-3p	↓		↑	
miR-289-5p				
miR-305-5p	↓			
miR-309-5p				
miR-375-3p				↓

Downward arrows indicate downregulation in diapausing females. A blank cell indicates no significant difference. Adapted from Meuti *et al.* (2018).

phases. At some point during the course of the study, the majority of miRNAs evaluated were differentially regulated in diapausing females compared to their nondiapausing counterparts. Notable differences were found for miR-14-3p, miR-277-3p, and miR-305-5p, which are involved in lipid metabolism and/or insulin signaling in other insects (Varghese *et al.*, 2010; Foronda *et al.*, 2014; Ling *et al.*, 2017). There were also significant changes in abundance of miR-8-3p, miR-275-3p, and miR-375-3p, which are necessary for ovarian development in other mosquito species (Bryant *et al.*, 2010; Hussain *et al.*, 2013; Lucas *et al.*, 2015). Lipid hypertrophy and arrested ovarian development are hallmark characteristics of diapause in *C. pipiens* (Spielman and Wong, 1973; Mitchell *et al.*, 1989); thus, it is likely that these miRNAs contribute to these changes.

In addition to identifying specific miRNAs that are differentially regulated in diapausing *C. pipiens* mosquitoes, there were more general findings that should be kept in mind for future studies of miRNA regulation of diapause in insects. First, miRNA abundance is dynamic and changes significantly as both diapausing and nondiapausing female mosquitoes age, and these changes should be kept in mind when selecting which developmental time points to analyze. Second, differentially regulated miRNAs were generally less abundant in diapausing females than in nondiapausing females. This result suggests that in nondiapausing females miRNAs are turning off some genes in order to allow development to continue uninhibited by diapause. This idea should be considered as an alternative to the hypothesis that miRNAs turn off developmental processes when insects become dormant. Finally, the most striking differences between diapausing and nondiapausing females occurred prior to diapause entry. This finding was in contrast to the results of Batz *et al.* (2017), who found that in *A. albopictus* differential regulation of miRNAs occurred only after diapause was established. Thus, it is important to keep in mind that, even though evolutionarily conserved miRNAs are part of the common toolkit of mechanisms regulating diapause, the stage when miRNAs act may be different for different species.

Diapause in Non-Insect Arthropods and Other Invertebrates

Diapause is widespread among non-insect Arthropods and has been investigated at the physiological, biochemical, and gene expression levels for brine shrimp (*e.g.*, *Artemia* spp.), daphnia, and copepods (Patil *et al.*, 2013; Podrabsky and Hand, 2015; MacRae, 2016; Baumgartner and Tarrant, 2017; Mariash *et al.*, 2017). However, relatively little is currently known about miRNAs in these species; and, except for *Artemia*, there are currently no published studies of miRNAs in the context of diapause or other dormant states.

Species of *Artemia* are anostracan crustaceans that inhabit highly saline bodies of water. In response to changes in photoperiod, salinity, and other factors that signal the advent of winter, females can produce encysted embryos that enter diapause in the gastrula stage (Berthelemy-Okazaki and Hedgecock, 1987). In one species, *Artemia parthenogenetica*, at least 107 miRNAs are differentially regulated in diapausing embryos compared to their nondiapausing counterparts. Two miRNAs—miR-100 and miR-34—have been implicated as important cell cycle regulators during diapause (Zhao *et al.*, 2015). miR-100, which is underexpressed in diapausing embryos, targets *polo-like kinase 1* (*plk1*) and thereby regulates downstream signaling pathways, including the mitogen-activated protein kinase-extracellular signal-regulated kinase-ribosomal S6 2 (MEK-ERK-RSK2) pathway (van Vugt and Medema, 2005; Ziv *et al.*, 2017). Downregulation of miR-100 during diapause inhibits cell division and promotes developmental arrest. miR-34, which is overexpressed in diapausing cysts relative to developing embryos, also inhibits development during diapause by targeting *cyclin K* and *RNA polymerase II*. Together, the results of this study show that the arresting of cellular processes during diapause is due to the coordinated up- and downregulation of miRNAs and their target genes. It will be interesting to see whether miR-100 and miR-34 have similar functions in other animals during diapause.

Diapause in the Annual Killifish *Austrofundulus limnaeus*

Austrofundulus limnaeus is an emerging model for studying diapause and environmental stress response in a vertebrate (Podrabsky and Arezo, 2017). This killifish, which is native to ephemeral ponds on the coast of Venezuela, can survive harsh environments that are characterized by large fluctuations in temperature, oxygen availability, and other environmental factors (reviewed by Podrabsky *et al.*, 2017). *Austrofundulus limnaeus* is unique among vertebrates in that embryonic development can occur along one of two alternative pathways. One pathway is discontinuous and includes one to three periods of diapause. The second pathway is continuous, and embryos develop directly to the pre-emergence stage without entering diapause. Which pathway is followed depends on both maternally provisioned factors and the incubation temperature of the embryos (Podrabsky *et al.*, 2010).

Maternal regulation of the developmental pathway depends on maternal age and appears to be mediated by differences in the population of RNAs provisioned by the mother during oogenesis (Romney and Podrabsky, 2017). RNA-Seq revealed that 1–2 cell stage embryos programmed for the diapause pathway, or trajectory, have a different RNA profile than ontogenetically matched embryos programed for the "escape" trajectory that lacks diapause. Differences between the two types of embryos include alternatively spliced mRNAs and diverse populations of sncRNAs (Romney and Podrabsky, 2017). There are a relatively small number of previously annotated mature miRNAs in these early 1–2 cell stage embryos compared to later stages of *A. limnaeus*. This may be either because there are primarily novel *A. limneaus*-specific miRNAs that are maternally provisioned or because maternal miRNAs are not part of the toolkit of mechanisms involved in the maternal determination of diapause (Romney and Podrabsky, 2017).

Although there was a conspicuous lack of miRNAs in 1–2 cell stage embryos, there was a diverse population of other small sncRNAs present. The most abundant annotated sequences were antisense RNAs, including one ST7 antisense RNA 1 conserved region 1 (RF02179), a lncRNA that, in humans, is involved in cellular response to DNA damage and acts as a tumor suppressor (Liu *et al.*, 2015). In *A. limnaeus*, this sequence aligns with several regions of the genome, including a zinc finger protein and a rRNA. Additional studies are needed to determine the function of this RNA in *A. limnaeus* and other fishes.

Four sncRNAs were differentially regulated in diapause-destined embryos compared to escape trajectory embryos, and their primary function appeared to be regulating genes that were important for cell proliferation. The only sncRNA that was relatively more abundant in diapause trajectory embryos likely regulated an uncharacterized protein that contained a SANT (switching-defective protein 3 [Swi3], adaptor 2 [Ada2], nuclear receptor co-repressor [N-Cor], and transcription factor [TF]IIIB) domain. Proteins with this motif are chromatin-remodeling enzymes that are abundant in proliferating cells during early development (Boyer *et al.*, 2002; Mo *et al.*, 2005), and they may be important for long-term regulation of multiple gene networks. The three sncRNAs that are relatively more abundant in escape trajectory embryos may also regulate cell cycle progression. It will be interesting to discover how these regulatory sequences influence developmental arrest in diapausing killifish and whether they have a similar function regulating dormant states in other animals.

The rearing environment experienced by embryos can also influence whether development is on the diapause (*i.e.*, discontinuous) or escape (*i.e.*, continuous) trajectory. RNA-Seq of small RNA libraries of embryos from seven morphological stages from the diapause and escape trajectories revealed significant differences in abundance of specific miRNAs, including several members of two miRNA families: miR-10 and miR-430. Members of a miRNA family share a common seed sequence but can vary along the remainder of the sequence. The miR-10 family, which targets *Hox* genes that regulate anterior-posterior patterning (Giusti *et al.*, 2016), was collectively the most abundant family of sequences across all time points for both diapause and escape embryos. Sequences of the miR-10 family appeared earlier in escape embryos than in diapause embryos, suggesting a link between their presence and developmental timing along each trajectory.

Members of the miR-430 family were also highly abundant during embryonic development, with higher overall abundance in escape embryos compared to diapause embryos. miR-430 is known to mediate the removal of maternal transcripts during the maternal-to-zygote transition during embryonic development in fish, and it may have a role in the embryos' "decision" to choose a diapause or escape trajectory (Bazzini *et al.*, 2012; Romney and Podrabsky, 2018). Predicted targets of miR-430 members are largely classified with protein-interacting functions; and they have protein-binding, signaling, and regulatory roles. Targets of specific interest include *Argonaute*, which is part of the small-RNA silencing pathway, and several genes encoding nuclear receptors (*e.g.*, *retinoic-acid-receptor* [*rar*] and *related orphan receptor* [*ror*]). It will be interesting to discover the roles for miR-430 members in regulating diapause in *A. limneaus* as our understanding of these miRNAs unfolds.

Nematode Dauer Formation

Nematodes, including *Panagrellus redivivus* and *Caenorhabditis elegans*, have diapause-like dormant states that are regulated by miRNAs (Karp *et al.*, 2011; Srinivasan *et al.*, 2013; Ambros and Ruvkun, 2018). miRNAs were first discovered in *C. elegans*; and, thus, their role in regulating dormancy has been well studied (see Ambros and Ruvkun, 2018). *Caenorhabditis elegans* has the potential to become dormant at two different times during the larval stage of the life cycle in response to inhospitable environmental conditions. Newly hatched L1 larvae may enter a dormant state, known as L1 diapause, if they hatch in an environment lacking food; they resume development when food becomes available. Older larvae may enter the dauer stage, an alternative L3, in response to starvation, overcrowding, or other conditions that are not permissive for growth and maturation (Cassada and Russell, 1975; Golden and Riddle, 1982).

Two miRNAs, miR-235 and miR-71, have been identified as regulators of L1 diapause. miR-235, which is required for entering L1 diapause, integrates environmental signals (*i.e.*, availability of food) with a downstream physiological response. The abundance of miR-235 is regulated through insulin or insulin-like growth factor signaling and is elevated in dormant worms. It declines when food becomes available and dormancy is terminated. miR-235 inhibits development by targeting *nhr-91*, a gene encoding a nuclear hormone receptor that is found in multiple tissues (Ambros and Ruvkun, 2018). miR-71 targets heterochronic pathway genes, including *lin-42* and *hbl-1*, that regulate developmental timing. miR-71 is necessary for proper development following L1 diapause (Zhang *et al.*, 2011), and it provides a possible link between the developmental program and the environmental conditions.

Dauer formation is regulated by several miRNAs, including miR-125, miR-214, miR-48, miR-34, and the miR-58 family (reviewed by Ambros and Ruvkun, 2018). miR-34, which also regulates diapause in *Artemia parthenogenetica*, is notable because it regulates cell cycle progression, lipid metabolism, and other dormancy-relevant processes (Rottiers and Näär, 2012; Chakraborty *et al.*, 2013; Xu *et al.*, 2015; Isik *et al.*, 2016). miR-34 is also tied to insulin signaling and metabolic homeostasis, which are dormancy-relevant processes in evolutionarily diverse animals (Chakraborty *et al.*, 2013; Sim and Denlinger, 2013; Hand *et al.*, 2016, Ragland and Keep, 2017). In *C. elegans*, miR-34, which is upregulated in the dauer stage, is required for survival. Its abundance regulates, and is regulated by, DAF-16/FOXO, a transcription factor regulated by insulin. Together, members of this feedback loop provide a robust response to the environment through formation of an alternate phenotype (Isik *et al.*, 2016).

Mammalian Hibernation

Hibernation occurs during the winter months in response to cold temperatures and shorter day lengths, and it is characterized by variable periods of torpor interspersed with periods of arousal. Physiological changes that define hibernation include metabolic depression, reduced body temperature, decreased heartbeat and respiration rate, and a reliance on fat stores for energy. As with other dormancies, hibernation is associated with coordinated changes in gene expression (Hampton *et al.*, 2011; Vermillion *et al.*, 2015); and several recent studies have begun to uncover changes in abundance of miRNAs and other noncoding RNAs underpinning the up- and downregulation of specific genes.

The regulation of mammalian hibernation by miRNAs has been explored in a number of species, including bats (Kornfeld *et al.*, 2012; Biggar and Storey, 2014; Yuan *et al.*, 2015), ground squirrels (Morin *et al.*, 2008, 2017b; Liu *et al.*, 2010; Lang-Ouellette and Morin, 2014; Luu *et al.*, 2016; Wu *et al.*, 2016), and a South American marsupial (Hadj-Moussa *et al.*, 2016). Using a variety of methods (*e.g.*, RNA-Seq, microarrays, and qRT-PCR) these studies have identified a total of 41 miRNAs that are differentially regulated in hibernating individuals compared to their non-hibernating, euthermic counterparts. In each study, miRNA abundance was measured in isolated tissues, including heart, kidney, liver, and white and brown adipose tissues. Together, these studies, summarized in Table 2, provide a catalog of miRNAs that are differentially regulated in tissues of hibernating mammals and that serve as a starting point for understanding how noncoding RNAs may regulate dormant states in mammals. There have been several recent reviews of miRNAs in hibernating mammals (Arfat *et al.*, 2018). The discussion here will focus on two pathways that are known to regulate dormant states in other animals: the PI3-K/Akt/mTOR pathway and the transformation growth factor β (TGF-β) signaling cascade.

The PI3-K/Akt/mTOR pathway (Fig. 2) has been implicated in the regulation of hibernation states in thirteen-lined ground squirrels and other mammals (Wu and Storey, 2012; Hadj-Moussa *et al.*, 2016; Lee *et al.*, 2018). The mTOR pathway is part of the insulin receptor network that includes insulin receptor and insulin-like growth factors PI3-K and Akt. PI3-K/Akt/mTOR signaling regulates cell growth and proliferation, protein synthesis, and transcription. It is primarily regulated through reversible phosphorylation but may also be regulated post-transcriptionally by miRNAs. miR-29, which is upregulated in skeletal muscles of hibernating bats, and miR-486, which is upregulated in liver of the arctic ground squirrel (Table 2), inhibit PI3-K activation, thus limiting mTOR activity and subsequent activation of transcription factors and eukaryotic initiation factor 4B (Park *et al.*, 2009; Small, 2010).

Akt activity also regulates the miR-200 family and miR-186 clusters in hibernating thirteen-lined ground squirrels. These miRNAs, which are underexpressed in brains of hibernating ground squirrels, likely mediate epithelial-mesenchymal transitions (EMT/MET) and improve tolerance of ischemia (*i.e.*, oxygen limitation resulting from limited blood flow), thereby protecting brain tissue during torpor (Lee *et al.*, 2018)

The PI3-K/Akt/TOR pathway also regulates diapause in insects and dauer formation in *Caenorhabditis elegans* (Song *et al.*, 2018; Kaplan *et al.*, 2019). Diapause induction in pupae of the cotton bollworm, *Helicoverpa armigera*, is mediated by phosphatase and tensin homolog (PTEN) inhibition of Akt (Song *et al.*, 2018), and diapause termination is associated with upregulation of genes related to the PI3-K/Akt/TOR pathway in *Chymomyza costata* (Košťál *et al.*, 2017). Genes in these pathways are computationally predicted targets of miR-289, miR-1, miR-305, and miR-9c and others that are differentially regulated in diapausing *Helicoverpa zea* and *Sarcophaga bullata* (Reynolds *et al.*, 2017, 2019). However, our understanding of miRNA regulation of this pathway is not well understood for any insect, and additional studies are needed to understand how these miRNAs are connected to the PI3-K/Akt/TOR pathway.

TGF-β signaling (Fig. 3) negatively regulates cell proliferation through the transcription factor, Small body size/mothers against decapentaplegic (Smad). Smad promotes expression of cyclin-dependent kinase inhibitors and other genes that induce cell cycle arrest, and it promotes apoptosis through upregulation of pro-apoptosis genes (Massagué *et al.*, 2005). Smad also positively regulates the production and maturation of miRNAs (Blahna and Hata, 2012), including several that are overexpressed in the liver of torpid thirteen-lined ground squirrels relative to their non-hibernating counterparts (Wu and Storey, 2018). miR-21, one miRNA that is upregulated in hibernating ground squirrels, has ~500 predicted targets that have a variety of functions, including DNA replication, cell proliferation, and ERK/MAPK (extracellular signal-regulated kinase/mitogen-activated protein kinase) signaling. Several of these putative target genes are differentially expressed in torpid arctic ground squirrels (*Urocitellus parryii*), which suggests that miR-21 (Liu *et al.*, 2010), acting downstream of TGF-β, is an important regulator of dormancy in multiple species of ground squirrels.

TGF-β regulates dormancy in other animals as well. This cytokine, which is regulated by members of the miR-58 family, regulates dauer formation in *C. elegans* (de Lucas *et al.*, 2015). In *Austrofundulus limnaeus*, the annual killifish, the miR-430 family members, which are downregulated in diapausing embryos compared to escape embryos, target genes in the TGF-β pathway (Romney and Podrabsky, 2018); but the pathway itself has not been closely studied in diapausing

Table 2

Summary of microRNAs (miRNAs) that are differentially regulated in hibernating mammals

	Ictidomys tridecemlineatus								*Dromiciops gliroides*	
miRNA	Heart[a]	Skeletal muscle[a,b]	Kidney[a]	White adipose tissue[c]	Brown adipose tissue[c]	Liver[b]	*Spermophilus parryii* liver[d]	*Myotis lucifugus* skeletal muscle[e]	Liver[f]	Skeletal muscle[b,f]
miR-24	↑									
miR-1a		↑	↑					↑		
miR-21		↑	↑		↓			↓		
miR-142-5p		↑								
miR-144		↑								
miR-122		↓								
miR-106b		↓				↓				↓
miR-24		↓								
miR-519d				↑						
miR-200a				↑						
miR-143				↑						
miR-222				↓						
miR-150				↓						
miR-107				↓	↓					
miR-31				↓	↓					
let-7a				↓						
let-7b				↓						
miR-138					↑					
miR-221					↓					
miR-125b					↓					
miR-103					↓					
miR-486							↑			
miR-451							↑			
miR-378							↓			
miR-320							↓			
miR-206								↑		
miR-181b								↑		
miR-128								↑		
miR-29b								↑		
miR-20								↑		
miR-483-5p									↑	
miR-190-5p									↑	
miR-181a-3p									↑	
miR-139-5p									↑	↑
miR-99b-5p									↑	↑
miR-1a1-5p									↑	↑
miR-1b-5p									↑	↑
miR-185-5p									↓	
miR-33a-5p									↓	
miR-22-5p									↓	
miR-16-1-3p									↓	

Downward arrows indicate downregulation in hibernating individuals. A blank cell indicates that the miRNA was not included in the referenced studies. Adapted from Arfat *et al.* (2018).

[a] Morin *et al.*, 2008.

[b] Maistrovski *et al.*, 2012.

[c] Wu *et al.*, 2014.

[d] Liu *et al.*, 2010.

[e] Kornfeld *et al.*, 2012.

[f] Hadj-Moussa *et al.*, 2016.

fish. In insects, TGF-β regulates diapause in pupae of *H. armigera* and in *Culex pipiens* mosquitoes (Hickner *et al.*, 2015; H. Y. Li *et al.*, 2017, 2018). It is currently unknown which, if any, miRNAs regulate TGF-β signaling in these species, but computational predictions of target genes with DIANA mirPath indicate miR-375, miR-289, miR-124, and miR-277 target genes in this pathway (Vlachos *et al.*, 2015). These miRNAs are differentially regulated in diapausing *C. pipiens* (Meuti *et al.*, 2018) and diapausing *H. zea*, a close relative of *H. armigera* (Reynolds *et al.*, 2019). Future studies

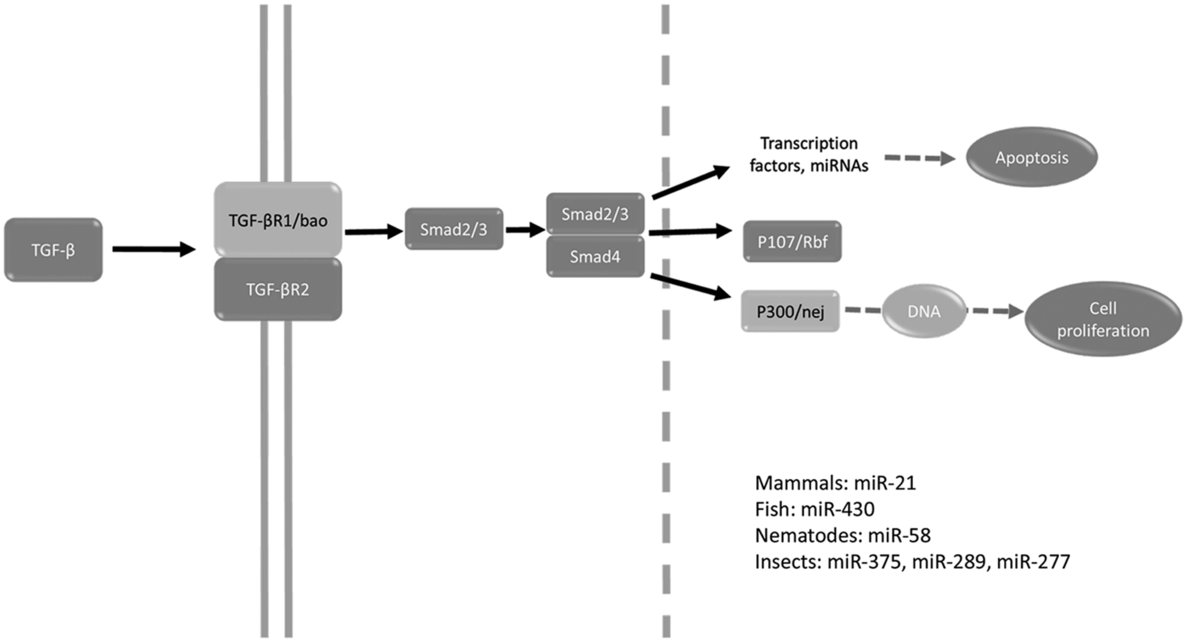

Figure 3. Generalized tranformation growth factor (TGF)-β pathway. In hibernating mammals, miR-21 is regulated by TGF-β and regulates downstream events. miR-58, miR-430, miR-375, miR-289, and miR-277 regulate components of this pathway and downstream processes during diapause in insects, *Caenorhabditis elegans*, and *Austrofundulus limneaus*.

that integrate transcriptome data with information about miRNA abundance will provide valuable information about the regulation of this important pathway.

Small RNA Regulation of Estivation

Estivation occurs during the summer months in response to high temperatures and/or arid conditions. It provides a means for animals to avoid hyperthermia and, other stresses that commonly occur in high temperatures (*e.g.*, dehydration and/or desiccation). Estivation is known to occur, and has been studied, in evolutionarily diverse species including snails, toads, and terrestrial mammals (Storey, 2002). The sea cucumber *Apostichopus japonicus* is an emerging model for studying estivation in a marine invertebrate, as well as an emerging model for studying miRNA function in echinoderms. *Apostichopus japonicus* estivates in response to elevated water temperatures (20–25 °C) and can remain dormant for several months (Yuan *et al.*, 2007). Estivation in this species is characterized by depressed oxygen consumption, degeneration of the alimentary canal, and changes in the immune system (Yang *et al.*, 2005, 2006; Bao *et al.*, 2010).

Recent studies have begun to identify the molecular mechanisms, including miRNAs, underpinning physiological and biochemical changes associated with estivation in *A. japonicus* (Chen *et al.*, 2013, 2018; Chen and Storey, 2014). During estivation, there are known changes in miRNA profiles in both the respiratory tree, which is important for both gas exchange and ammonia excretion (Robertson, 1972; Sisak and Sander, 1985), and the alimentary canal. A notable change in miRNA abundance includes overexpression of miR-200-3p in the alimentary canal of estivating individuals. A predicted target of miR-200-3p is a gene encoding enoyl-CoA hydratase and 3-hydroxyacyl CoA dehydrogenase (EHHADH), enzymes that are a necessary component of the peroxisomal fatty acid β-oxidation pathway. Integrating results from luciferase reporter assays, transcriptomics, and proteomics studies provides experimental evidence that miR-200-3p targets *ehhadh* and modulates fatty acid metabolism during estivation (Chen *et al.*, 2018).

Other notable changes in miRNA abundance in the alimentary canal include overexpression of miR-22 and underexpression of miR-10a during estivation. miR-22 may contribute to metabolic suppression by regulating PTEN and the PI3-K/Akt pathway (Bar and Dikstein, 2010). miR-10a may be involved in apoptosis, protein synthesis, and dormancy-relevant processes (Chen *et al.*, 2013). In the respiratory tree of estivating sea cucumbers, there is an overlap in the miRNAs that are differentially regulated during estivation and those that are differentially regulated in response to hypoxia (Chen and Storey, 2014; Huo *et al.*, 2017). miR-71 is overexpressed in estivating animals and is also upregulated in the respiratory tree of *A. japonicus* during exposure to oxygen-limited environments (Huo *et al.*, 2017). Studies on miR-71 in *C. elegans* indicate that this miRNA interacts with genes in the insulin and PI3-K pathways to mediate responses to environmental stresses, including heat shock and oxidative stress (Zhang *et al.*, 2011; Boulias and Horvitz 2012). It is not clear whether miR-71 has the same function in nematodes and sea cucumbers. However, comparative studies that evaluate conserved miRNAs in diverse species may provide clues about miRNA functions. In addition, recent studies with *A. japonicus* have identified miRNA : mRNA pairs that are involved in heat tolerance, hypoxia response, and immune response in *A. japonicus* that may provide clues about the function of key miRNAs during estivation (Huo *et al.*, 2017; Li and Xu, 2018; Zhou *et al.*, 2018). Together, these studies not only will provide valuable information concerning the functional significance of miR-71 and other miRNAs in estivating *A. japonicus* but also could provide information that can be extrapolated to evolutionarily diverse animals in response to stressful environments.

Roles for Other Classes of Noncoding RNAs

The studies reviewed above focus primarily on just one class of noncoding RNAs—miRNAs—and their role regulating dormant states. However, there is accumulating evidence that other noncoding RNAs—siRNAs, piRNAs, mitosRNAs, and lncRNAs—also contribute to dormancy as described in the studies referenced below.

In *C. elegans*, siRNAs are required for dauer formation. Worms lacking MUT-16, a protein necessary for siRNA biogenesis, or Argonaute CSR-1 in sensory neurons are unable to enter the dauer stage (Phillips *et al.*, 2012; Bharadwaj and Hall, 2017). Knockdown of MUT-16 or CSR-1 impairs the ability to detect the pheromone that is produced in response to overcrowding and is responsible for dauer formation under these conditions. It will be interesting to see whether there are also siRNAs that regulate the formation of dauers in response to starvation or high temperature.

piRNAs have been called the guardians of the genome because they limit the movement of TEs. In addition, recent studies on *Drosophila melanogaster* indicate that piRNAs have important roles in regulating metabolic homeostasis and lifespan (Jones *et al.*, 2016). Thus, it seems likely that piRNAs also have a role regulating insect diapause and possibly dormancy in other animals. Some evidence of this is known from changes in the mRNA expression of genes encoding components of the piRNA pathway of diapausing *Sarcophaga bullata* and pre-diapause embryos of *Dianemobius nigrofasciatus* (Reynolds *et al.*, 2013; Shimizu *et al.*, 2018). In addition, in some insects, piRNAs make up a large proportion of the population of sncRNAs. In mature oocytes of *Aedes albopictus*, piRNAs make up about 60% of sncRNAs, and a similar proportion is found in embryos of *Bombyx mori* and in pupae of *S. bullata* (Batz *et al.*, 2017; Fan *et al.*, 2017; Reynolds *et al.*, 2017). It is currently unknown whether the number and types of piRNAs are distinct in diapausing (or diapause-destined) individuals. However, tools for identifying and quantifying piRNAs in non-model organisms are continually improving, and it is likely that our questions about the role of piRNAs in development and dormancy will be answered in the not too distant future.

The mitosRNAs are a recently discovered class of sncRNAs that are known to occur in insects, humans, fish, and chickens (Ro *et al.*, 2013; Zhou *et al.*, 2014; Ma *et al.*, 2016; Bottje *et al.*, 2017; Riggs *et al.*, 2018). The mitosRNAs originate from the sense strand of mitochondrial genomes and are hypothesized to regulate mitochondrial gene expression and mRNA stability (Ro *et al.*, 2013). In the annual killifish, *Austrofundulus limnaeus*, mitosRNAs are upregulated in response to anoxia (Riggs *et al.*, 2018). However, their function remains elusive, and it is not known whether they have a role in dormant states.

Finally, lncRNAs are 200–100,000-nucleotide RNAs that may have a significant role in regulating dormant states, including insect diapause and mammalian hibernation. lncRNAs are evolutionarily widespread, but little is known about lncRNAs as a class of regulators, because they are difficult to identify and because their function is complex and difficult to predict from the sequence. In general, lncRNAs regulate gene expression, but mechanisms vary and include recruitment of histone-modifying proteins, RNA stabilization, and mediating cytoplasm-to-nucleus trafficking (Ulitsky, 2016; De *et al.*, 2019).

There is accumulating evidence from insects and fish that lncRNAs regulate diapause. lncRNAs are among the maternally provisioned RNAs in early *A. limneaus* embryos that may direct embryos to either the diapause or escape trajectory (Romney and Podrabsky, 2018). In addition, two RNA-Seq studies on *Tetrapedia diversipes*, a neotropical solitary bee that enters diapause during the fifth larval instar, identified ~900 putative lncRNA sequences in pre-diapause larvae and in diapausing larvae (Araujo *et al.*, 2018; Santos *et al.*, 2018). However, the proportions of each lncRNA sequence were not evaluated in either study, so additional work is necessary to discover the identity and the regulatory function of lcnRNAs in this species and their role in regulating diapause.

In skeletal muscle of hibernating bats (*Myotis lucifugus*), levels of the lncRNA antisense hypoxia-inducible transcription factor (aHIF) are reduced compared to muscles of active bats (Maistrovski *et al.*, 2012). aHIF is complementary to the $3'$ UTR of *hif*-α, and its binding to *hif*-α mRNA promotes degradation (Rossignol *et al.*, 2002). HIF-α protein amounts and activity are elevated during hibernation, and the reduction of aHIF is likely linked to these changes (Morin and Storey, 2005).

In thirteen-lined ground squirrels (*Ictidomys tridecemlineatus*), the lncRNA *taurine-upregulated gene 1* (*TUG1*) is upregulated in skeletal muscle relative to euthermic individuals (Frigault *et al.*, 2016). TUG1 promotes cell proliferation and inhibits apoptosis in cancer cells (Niu *et al.*, 2017; T. Li *et al.*, 2018) and may prevent cold-induced tissue damage in mice (Su *et al.*, 2016). TUG1 also functionally regulates mitochondria biogenesis (Long *et al.*, 2016) and may have additional roles in the response to environmental stress. TUG1 is thought to regulate expression of *heat shock factor 2* (*hsf2*), a transcription factor that promotes expression of heat shock proteins, by acting as a decoy for miR-144 and by reducing its ability to regulate *hsf2* through competitive inhibition (Cai *et al.*, 2015). Additional studies are needed to determine the roles that TUG1 and other lncRNAs play in mammalian hibernation.

Summary and Necessary Future Directions

Taken together, the studies reviewed above illustrate the foundation that is being built for understanding the role for noncoding RNAs in regulating dormancy in evolutionarily diverse animals, including invertebrates (*i.e.*, worms, crustaceans, insects, and sea cucumbers) and vertebrates (fish and mammals). They provide evidence that the profiles of noncoding RNAs—including but probably not limited to miRNAs, piRNAs, mitosRNAs, and lncRNAs—are unique for dormant individuals compared to their non-dormant counterparts. Many of the differentially regulated miRNAs and other noncoding RNAs regulate a variety of processes related to dormancy, including metabolic suppression, developmental arrest, and environmental stress tolerance. Notably, noncoding RNAs regulate a number of pathways that are important for dormancy; and they have been studied in detail, including TGF-β, mTOR, Wnt, and insulin signaling pathways.

There is still much work to be done before we have a full understanding of noncoding RNAs in the context of dormancy. Considerable progress has been made in recent years toward understanding noncoding RNA biogenesis pathways, turnover (*i.e.*, stability and decay), and function in mammals and model invertebrates (*e.g.*, *Drosophila* and *C. elegans*); but sncRNA biology is still in its infancy for most non-model species, including many that are described in this review. However, the field is advancing rapidly, and our understanding of the biological relevance of sncRNAs, and the tools for studying them, is improving. Specifically, small RNA-Seq libraries and other tools for studying them are quickly becoming available to facilitate our understanding of their role in dormant states. Priorities for studying sncRNA regulation of dormancy should include cataloging changes in the abundance of sncRNAs in dormant and active individuals in additional taxa and striving to discover the functional significance of differentially regulated sncRNAs. Improved annotations of miRNAs, piRNAs, mitosRNAs, and lncRNAs would significantly aid our ability to profile sncRNAs. Improved methods for manipulating the amount of sncRNAs, especially miRNAs, by using inhibitors or other synthetic molecules, would allow progress in discovering the function of differentially regulated miRNAs and other sncRNAs. Even the tools we have for studying miRNAs, which are comparatively well studied compared to other classes of sncRNAs, are often insufficient for studying their function in often challenging invertebrate systems.

A particular challenge for understanding the role of miRNAs in dormant animals is discovering the genes they target. Not only can each miRNA potentially target hundreds of mRNAs, but also each mRNA can be regulated by multiple miRNAs. Processes—such as a dual-luciferase assay, commonly used for experimentally validating interactions between a particular miRNA and its putative targets—are tedious, and selecting genes to validate in this way is challenging. Computational tools used to predict miRNA targets generate a large number of false positives (Seitz, 2017), and it is not clear whether miRNA targets are conserved between even relatively closely related species (Agrawal *et al.*, 2018). Together, these challenges make identifying miRNA:mRNA pairs seem like looking for a needle in a haystack. However, the growing number of well-annotated animal genomes and improved programs for predicting miRNA targets will significantly advance our ability to identify *bona fide* targets. These advances will significantly advance our understanding not only of noncoding RNAs but also of the gene networks that regulate dormant states in animals.

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