

# microRNAs: small molecules with big roles – *C. elegans* to human cancer

Masaomi Kato and Frank J. Slack<sup>1</sup>

Department of Molecular, Cellular and Developmental Biology, Yale University, New Haven, CT 06520, U.S.A.

miRNAs (microRNAs) were first discovered as critical regulators of developmental timing events in *Caenorhabditis elegans*. Subsequent studies have shown that miRNAs and cellular factors necessary for miRNA biogenesis are conserved in many organisms, suggesting the importance of miRNAs during developmental processes. Indeed, mutations in the miRNA-processing pathway induce pleiotropic defects in development, which accompany perturbation of correct expression of target genes. However, control of gene expression in development is not the only function of miRNAs. Recent work has provided new insights into the role of miRNAs in various biological events, including aging and cancer. *C. elegans* continues to be helpful in facilitating a further understanding of miRNA function in human diseases.

## Introduction

When and where genes are expressed are very important subjects in the study of development. The discovery of a new mode of gene regulation during development has revolutionized this field. *Caenorhabditis elegans* *lin-4* (*linage-4*) and *let-7* (*lethal-7*), the first miRNAs discovered, were identified on the basis of their roles in developmental timing (Lee et al., 1993; Reinhart et al., 2000). From these humble beginnings, the study of miRNAs has blossomed into a greater understanding of the regulation of gene expression in all aspects of biology. miRNAs are ~21–23-nucleotide non-protein-coding RNA molecules that control gene expression at the post-transcriptional level (reviewed in Nilsen, 2007). miRNAs are initially produced from RNA polymerase II transcripts that form a stem-loop structure and undergo processing

by a protein complex containing the RNase III enzyme Drosha and the double-stranded RNA-binding protein Pasha in the nucleus. After initial cleavage, pre-miRNAs are exported into the cytoplasm by exportin-5, followed by further processing by RNase III endonuclease Dicer. Eventually, mature miRNAs of approx. 22 nt are incorporated into miRISC (miRNA-induced silencing complex). Here, they act to negatively regulate gene expression through mRNA degradation when they find perfect complementary sequences in target mRNAs, or through translational inhibition when they find imperfect complementary sequences in the 3' UTR (untranslated region) of target mRNAs.

Recent computational predictions and systematic cloning of miRNAs have revealed that hundreds of genes encoding miRNAs exist in the genome, and they are envisaged to control a significant number of genes (Carthew, 2006). Currently, 112 miRNA genes have been identified in *C. elegans*, which have been grouped into 63 families on the basis of the sequence similarity at the 5' end of the miRNA (Ruby et al., 2006). Many of these miRNAs are conserved in other organisms. Interestingly, in some cases, not only is the miRNA sequence, but also its temporal regulation, conserved across phylogeny: for example, the *let-7* RNA is detected at late larval stages in *C. elegans* and *Drosophila*, at 48 h after fertilization in *Danio rerio*

<sup>1</sup> To whom correspondence should be addressed (email frank.slack@yale.edu).

**Key words:** aging, *Caenorhabditis elegans*, cancer, development, microRNA.  
**Abbreviations used:** *alg*, argonaute-like gene; ASEL, left ASE; ASER, right ASE; *ceh*, *C. elegans* homeobox; *cog*, connection of gonad defective; *daf*, abnormal dauer formation; *die*, dorsal intercalation and elongation defect; EGF, epidermal growth factor; FOX, forkhead box; *gcy*, guanylate cyclase; *gfp*, green fluorescent protein; *hbl*, hunch-back-like; HSF, heat-shock factor; IGF, insulin-like growth factor; L1, L2 etc., stage, first, second etc. larval stage; *let*, lethal; *lin*, *linage*; *lsy*, laterally symmetric; miRNA, microRNA; PHA-4, pharynx development defect-4; RNAi, RNA interference; UTR, untranslated region; VPC, vulva precursor cell.

(zebrafish) and at embryonic day 10.5 in mouse, suggesting the possibility that miRNAs, including *let-7*, have common functions during development in different species (Pasquinelli et al., 2000; Schulman et al., 2005; Ruby et al., 2006).

As exemplified by *lin-4* and *let-7*, the primary function of miRNAs had been believed to be the control of gene expression in development. Indeed, mutations in *alg-1* (argonaute-like gene-1), a gene necessary for miRNA biogenesis, cause pleiotropic developmental abnormalities in *C. elegans* (Grishok et al., 2001). However, the control of gene expression in development makes up only a small part of the reach of miRNAs. Accumulating evidence demonstrates that miRNAs mediate diverse biological functions, including metabolism and aging, and are also human disease loci. This review focuses on the various roles of miRNAs, especially those in *C. elegans*, and how this knowledge has been used to understand biology in more complex organisms. We can expect many more contributions from *C. elegans* miRNA research that will bring us closer to understanding common biological events in animals, such as neuronal differentiation and apoptosis, and also human disease.

### Developmental timing: temporal patterning by miRNAs

Temporal patterning, as well as spatial patterning, of cell fates are key events in establishing the fundamental body plan. The *C. elegans* heterochronic pathway regulates the temporal aspect of development. Heterochronic genes are required for the normal transition from one developmental stage to another. Loss-of-function mutations in heterochronic genes result in precocious or retarded execution of developmental programmes; this has been observed most easily in hypodermal seam cells, which undergo cell division patterns that are synchronized with the four larval molts and terminally differentiate during the final molt. Genetic screening for mutants defective in developmental timing transitions led to the discovery of the first known miRNAs, *lin-4* and *let-7* (Lee et al., 1993; Reinhart et al., 2000).

The *lin-4* miRNA controls the proper progression from the first larval (L1) to the second larval (L2) stage by directly repressing the activity of its target *lin-14* (Lee et al., 1993; Wightman et al., 1993; Feinbaum and Ambros, 1999; Olsen and Ambros, 1999). *lin-4*

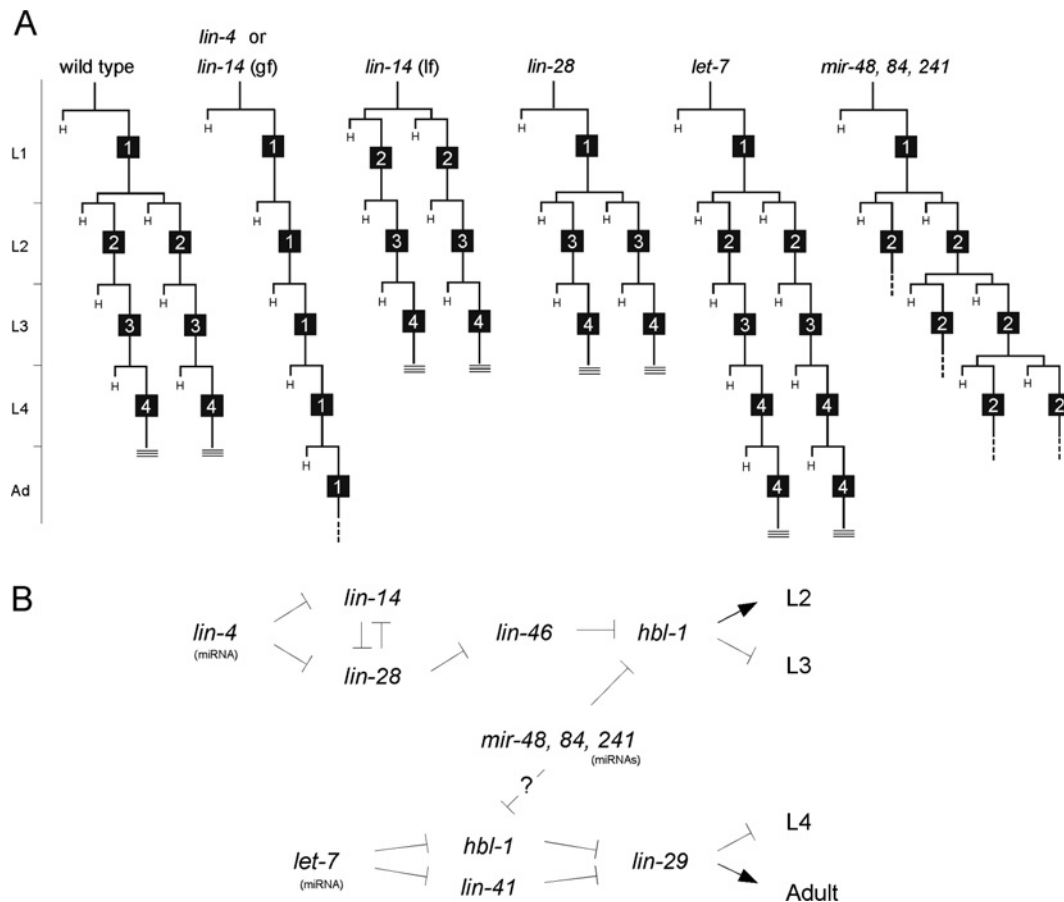
loss-of-function mutants repeat the L1-specific cell division in seam cells and fail to terminally differentiate (Figure 1A). Similarly, *lin-14* gain-of-function mutants, which lack *lin-4*-binding sites in the *lin-14* 3' UTR, also have the identical retarded phenotype. Conversely, *lin-14* loss-of-function mutations result in a precocious phenotype, where the seam cell division skips the L1 stage, indicating that *lin-14* activity is required to specify L1-specific cell division patterns (Figure 1A).

The second known miRNA, *let-7*, functions later in larval development; it is most abundantly expressed at the fourth larval (L4) stage to initiate the larval-to-adult transition (Reinhart et al., 2000). The *let-7* miRNA down-regulates *bbl-1* (hunch-back-like-1) (Abrahante et al., 2003; Lin et al., 2003) and *lin-41* (Slack et al., 2000; Vella et al., 2004), resulting in the expression of the transcription factor LIN-29 (Figure 1B). LIN-29 accumulation in the hypodermis directs the L4-to-adult transition, which is marked by lateral seam cell fusions, the secretion of cuticular structures called alae and the cessation of molting (Rougvié and Ambros, 1995). *let-7* loss-of-function mutants show a retarded phenotype, where the seam cells fail to terminally differentiate and, instead, repeat the L4 stage programmes once more (Figure 1A). Similar to *let-7*, *lin-29* mutants display a retarded phenotype.

Six more miRNAs have been identified in *C. elegans* on the basis of the sequence similarity to the 5' end of the *let-7* miRNA. These miRNAs are called miR-48, miR-84, miR-241, miR-793, miR-794 and miR-795 (Lim et al., 2003; Ruby et al., 2006). Although little is known about the latter three miRNAs, the remaining set of miRNAs shows a temporal expression pattern that is partially overlapping with that of *let-7* (Esquela-Kerscher et al., 2005). In agreement with this observation, a mutant allele of *mir-48*, which contains a point mutation in its possible promoter region, causes a weak precocious hypodermal phenotype due to the early expression of *mir-48* (Li et al., 2005), suggesting that these *let-7* family members also play a role in developmental timing. Another study demonstrated that these miRNAs function redundantly in the heterochronic pathway (Abbott et al., 2005). *mir-48*, *mir-84* and *mir-241* are abundantly expressed at the L3 stage, and the triple mutants develop extra seam cells, because of the inappropriate repetition of L2-specific

**Figure 1 | Regulation of developmental timing by miRNAs**

(A) The pattern of seam cell divisions in wild-type and heterochronic mutants. Horizontal bars indicate the time when cell division occurs in each larval stage. Larval stages (L) are shown on the left-hand side and in squares on the vertical axis. H signifies cells that have fused to the hypodermis. The three horizontal bars at the bottom of cell lineages represent the cessation of larval-specific cell division, followed by alae formation. (B) A genetic pathway of heterochronic genes, including miRNAs. The *lin-4* miRNA also targets *lin-28*, which functions in early larval development together with *lin-14*. *lin-46* acts downstream of *lin-28*. The function of *mir-48*, *mir-84* and *mir-241* for the control of *hbl-1* activity has not been confirmed in later larval development.



seam cell divisions during the L3 stage (Figure 1A). These results indicate that three *let-7* family members act in the L2-to-L3 transition, raising the possibility that the heterochronic gene *lin-28* is a possible target of *mir-48*, *mir-84* and *mir-241*, because the *lin-28* and *let-7* family miRNAs have opposite activities. Expression of *lin-28* is down-regulated during the L2-to-L3 stage in wild-type, and L2-specific seam cell divisions are skipped in *lin-28* mutants. (Moss et al., 1997) (Figure 1A). However, temporal down-regulation of LIN-28 is not affected in a *mir-48*, *mir-*

*84* and *mir-241* triple-mutant background, suggesting that *lin-28* is not a direct target of these miRNAs. Other candidate targets of these *let-7* family miRNAs are *hbl-1* and *lin-41*, because the expression of both genes is down-regulated through *let-7* complementary sites in their 3' UTRs, promoting the larval-to-adult transition. A *hbl-1* mutation suppresses the reiteration of L2-specific cell fates in a *mir-48*, *mir-84* and *mir-241* triple-mutant background. However, a *lin-41* mutation has little effect. These observations indicate that *mir-48*, *mir-84* and

*mir-241* control the L2-to-L3 transition by repressing *bbl-1*, but not *lin-41* or *lin-28* (Figure 1B).

Temporal control of programmed cell death is necessary to ensure that specific cells die at the correct time in normal development. Developmental timing defects can also manifest themselves in the timing of non-apoptotic cell death (Abraham et al., 2007). The male-specific linker cell, one of the cells destined to die during development in *C. elegans*, is produced during the L2 stage in the central region of worm. As the cell migrates, it leads the extension of the male gonad behind it and eventually dies within 2 h after the L4-to-adult transition in wild-type animals (Sulston et al., 1980). However, the death of the linker cell is blocked in *lin-29* and *let-7* mutants, suggesting that *let-7* is also involved in non-apoptotic cell death events through its role in the developmental timing pathway (Abraham et al., 2007).

### Left-right asymmetry in sensory neurons: spatial patterning by miRNAs

Although *lin-4*, *let-7* and their family members regulate temporal patterning in cell-fate determination during development, two novel miRNAs, *lly-6* and miR-273, were shown to regulate spatial patterning in a pair of taste receptor sensory neurons in *C. elegans*. Bilaterally symmetrical structures are a common feature in the nervous system; however, left-right asymmetrical expression of genes often correlates with the functional lateralization of some neurons (Bargmann and Horvitz, 1991; Hobert et al., 2002). One such example is a pair of head neurons, the ASEs, where the guanylate-cyclase-receptor genes are asymmetrically expressed (Yu et al., 1997). In adult *C. elegans*, *gcy-6* (guanylate cyclase-6) and *gcy-7* are only expressed in the left ASE (ASEL), whereas *gcy-5* is specifically expressed in the right ASE (ASER). A genetic screen for mutants that disrupted ASEL-specific *gcy-7* expression revealed the novel miRNA, *lly-6* (laterally symmetric-6) (Johnston and Hobert, 2003). *lly-6* mutants have a two-ASER phenotype. ASEL fails to show the normal expression of *gcy-7*, but, instead, displays ectopic expression of ASER-specific *gcy-5* (Figure 2B), although ASE neuronal specification itself is unaffected.

Expression of the *lly-6* miRNA is normally observed in ASEL, but not in ASER, in adult worms. When *lly-6* is ectopically expressed in both ASE cells

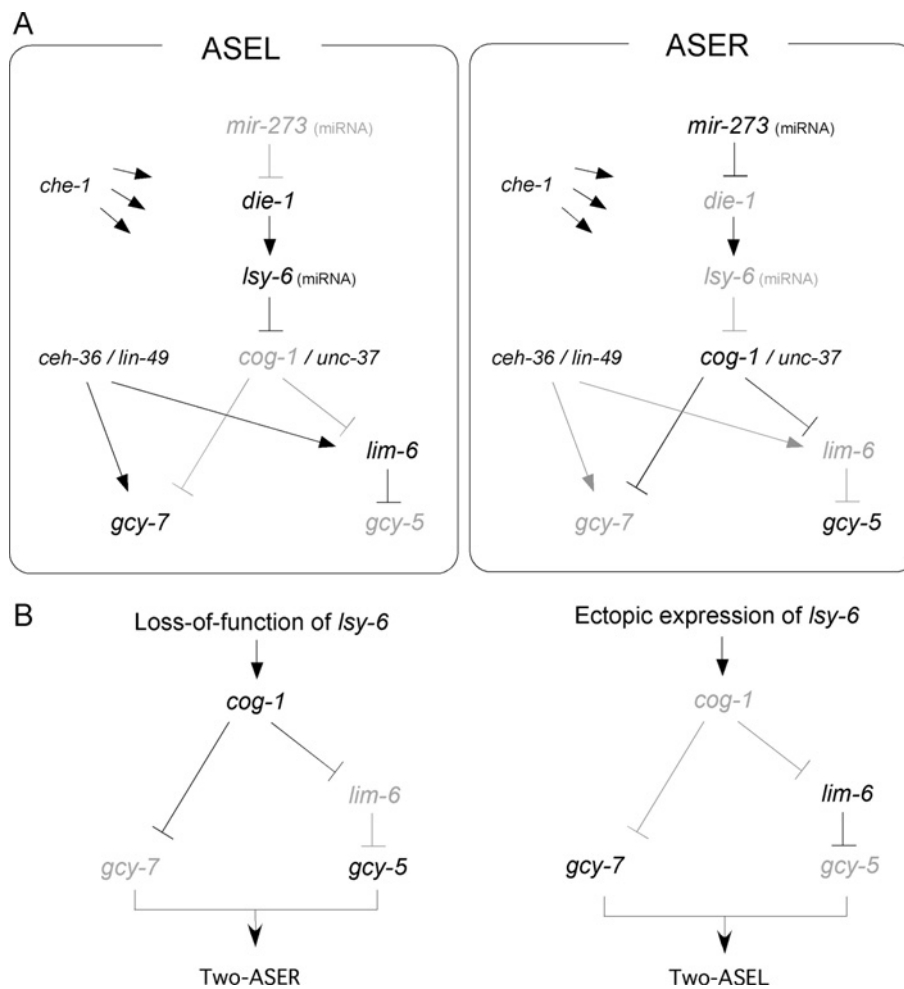
in a *lly-6* loss-of-function mutant background, *gcy-7* expression is restored and ectopic expression of *gcy-5* is repressed in the ASER. Moreover, ASER-specific *gcy-5* expression disappears and *gcy-7* expression is ectopically activated in ASER, causing a two-ASEL phenotype (Johnston and Hobert, 2003) (Figure 2B).

The left-right asymmetrical features in the ASE neuron are established by the proper transcriptional activation and/or inactivation of a regulatory cascade. Previous studies have shown that the Nkx6-type homeobox gene *cog-1* (connection of gonad defective-1) acts to repress the ASEL cell fate in ASER, partially through the repression of the *lim-6* homeobox gene, which suppresses the ASER cell fate (Chang et al., 2003) (Figure 2A). On the contrary, the OTX-type homeobox gene *ceb-36* (*C. elegans* homeobox-36) acts to promote the left cell fate in ASEL (Chang et al., 2003). The expression of these factors was examined in *lly-6* mutants in order to determine the extent of the *lly-6* miRNA regulatory network. Consistent with the change of cell fate, ASEL to ASER in *lly-6* mutants, ASEL-specific expression of *lim-6* is lost following the ectopic activation of *cog-1*. In addition, *lly-6* represses the expression of *cog-1* in the ASEL, and this regulation is dependent on the presence of *lly-6* complementary sites in the 3' UTR of *cog-1*. These results demonstrated that the *lly-6* miRNA functions in determining left-right asymmetry in the sensory neuron through the negative regulation of the downstream target *cog-1* (Johnston and Hobert, 2003) (Figure 2A).

Another miRNA acting in asymmetric neuronal differentiation was identified through genetic analysis with *die-1* (dorsal intercalation and elongation defect-1) (Chang et al., 2004). *die-1* mutants also have a two-ASER phenotype as observed in *lly-6* mutants; both ASE cells have ASER-specific expression of *gcy-5* and lose ASEL-specific expression of *gcy-7*. *die-1* is expressed more strongly in the ASEL (Figures 2A and 2B). Loss of *die-1* function causes a lack of ASEL-specific *lim-6* and *lly-6* expression and de-repression of *cog-1* expression in the ASEL. On the contrary, ectopic *die-1* expression in the ASER causes ectopic expression of *lly-6*, followed by the repression of *cog-1*. These observations can be interpreted as the presence of a regulatory pathway in which *die-1* up-regulates the *lly-6* miRNA in order to repress the expression of *cog-1* in the ASEL. In support of this, the expression of *die-1* in the ASER is not able to repress *cog-1*

**Figure 2 | Asymmetrical neuronal differentiation by miRNAs**

(A) Reciprocal gene expression profiles in left and right ASE neurons are accomplished by spatially controlled miRNA expression. *cog-1*, a homeodomain transcription factor, acts with transcriptional co-repressor *unc-37* to repress the ASER cell fate in the ASEL. *ceh-36*, a homeodomain transcription factor, and *lin-49*, a co-activator, act together to promote the left-cell fate in the ASEL. *che-1* (chemotaxis defect-1) is thought to positively regulate transcription in both the ASEL and the ASER, although it remains unknown where *che-1* acts in the regulatory cascade. (B) Changes of gene expression pattern are shown in simplified genetic cascade when *lisy-6* is mutated or ectopically expressed in both ASE cells. Black and grey indicate genes which are up-regulated and down-regulated respectively in each cascade. *die-1* mutants and the ectopic expression of *mir-273* in both ASE cells also result in two-ASER phenotype through the up-regulation of *gcy-5* following the repression of *lisy-6* and de-repression of *cog-1*.



expression in a *lisy-6* mutant background (Chang et al., 2004).

Furthermore, a reporter assay where a *gfp* (green fluorescent protein) reporter gene is fused to the *die-1* 3' UTR exhibits significant down-regulation of GFP expression in the ASER, raising the possibility that the *die-1* 3' UTR contains elements that are subject to miRNA-regulated left/right-biased expression. Fur-

ther sequence comparison with the related nematode *C. briggsae* and computational prediction led to the identification of a possible miRNA, miR-273, which regulates the biased expression of *die-1*. *mir-273* activity is biased towards the ASER (Figure 2A). The ectopic expression of *mir-273* in both ASE cells from a bilateral promoter shows, not only down-regulation of *die-1* expression in the ASEL, but also the

two-ASER phenotype of *die-1* mutants. This suggests that ASER-biased expression of *mir-273* suppresses *die-1* activity, followed by the down-regulation of the *lgy-6* miRNA and expression of its target *cog-1*, resulting in the ASER cell-fate specification. Thus reciprocal expression of the miRNA, *lgy-6*, is controlled through another spatially regulated miRNA, miR-273, ensuring the laterality of the ASE sensory neurons (Figure 2A).

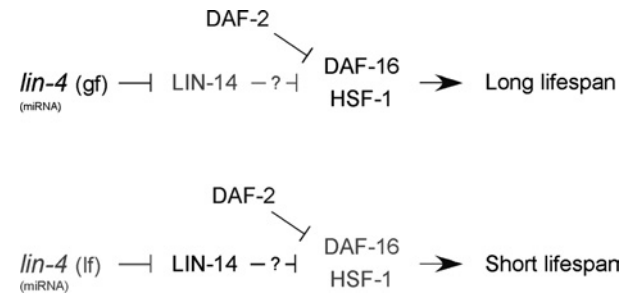
### Lifespan: metabolic modulation by miRNAs

Temporal and spatial cell-fate determinations are fundamental aspects of development that are accomplished by the proper execution of programmed genetic cascades. At the completion of the larval developmental stages, another temporal event, aging, is initiated. A major cause of aging is thought to result from the passive accumulation of damage to cells over time. Although the precise mechanisms regulating lifespan are still not well understood, one model suggests that temporal activation of a timer gene may initiate the aging programme early in the adult stage (McCarroll et al., 2004). This idea is reminiscent of temporal up-regulation of heterochronic genes in developmental timing, and, indeed, components in the miRNA-containing heterochronic pathway were shown to be involved in lifespan regulation in *C. elegans*.

Mutations in the *lin-4* miRNA and its target *lin-14* not only perturb developmental timing during larval development, but also affect the normal aging process (Boehm and Slack, 2005). Reducing the activity of *lin-4* shortens lifespan, whereas overexpression of *lin-4* extends lifespan (Figure 3). Moreover, disruption of *lin-14* activity, a target of *lin-4*, produces the opposite effect on lifespan. *lin-14* loss-of-function mutants have a longer lifespan, whereas gain-of-function mutants, which lack the *lin-4*-binding site in the *lin-14* 3' UTR, have a shorter lifespan. Therefore, *lin-4* normally acts to promote a long lifespan and *lin-14* normally acts to promote a short lifespan. Additionally, loss of *lin-14* activity suppresses the short-lived phenotype of *lin-4* mutants. These results suggest that a major role of *lin-4* in lifespan regulation is to repress the expression of its target *lin-14* (Figure 3). The lifespan changes that result from the disruption of *lin-4*/*lin-14* activity are not solely due to their roles in the regulation of developmental timing

### Figure 3 | Lifespan control by miRNA

*lin-4* miRNA and *lin-14* act in parallel to or upstream of DAF-2, and modulate lifespan by directly or indirectly repressing DAF-16 activity.



in larval stages, because, essentially, the same lifespan defects are found in worms whose *lin-14* activity is changed only after larval development (Boehm and Slack, 2005).

The molecular machinery first identified in the regulation of *C. elegans* lifespan is the insulin/IGF-1 (insulin-like growth factor-1) signalling pathway (Kenyon et al., 1993), and insulin signalling pathways are conserved in other organisms (Kenyon, 2005). The IGF-1 pathway controls lifespan through mechanisms that are dependent on the downstream FOXO (forkhead box O) transcription factor DAF-16 (abnormal dauer formation-16) and HSF-1 (heat-shock factor-1) in *C. elegans* (Kimura et al., 1997; Lin et al., 1997; Ogg et al., 1997; Hsu et al., 2003). Inhibiting *daf-16* or *hsf-1* activity shortens lifespan, whereas elevating their activities extends lifespan. DAF-16 is antagonized by signalling from DAF-2, the insulin/IGF-1 signalling receptor. Reduction in *daf-2* activity causes a more than 2-fold increase in lifespan, and its longevity is abolished by reduced activity of *daf-16*.

When the short-lived *daf-16* and *hsf-1* mutants are put into a *lin-14* background [RNAi (RNA interference)-based knockdown], *lin-14* loss-of-function mutants no longer display an extended lifespan, indicating that *daf-16* and *hsf-1* are required for the longevity caused by the *lin-14* mutation. Furthermore, the short-lived *lin-4* mutant phenotype is significantly suppressed by the *daf-2* RNAi-mediated longevity and displays a lifespan similar to the wild-type, suggesting that *daf-2* is necessary for the short lifespan of *lin-4* mutants. Long-lived

*daf-2* mutants, when combined with long-lived *lin-14* mutants, show the similar long lifespan of *daf-2* mutants. Taken together, these data support the idea that the *lin-4* miRNA and its target *lin-14* regulate lifespan through the insulin/IGF-1 signalling pathway, probably by repressing *daf-16* activity directly or indirectly (Boehm and Slack, 2005) (Figure 3).

*lin-4* is the first example of a miRNA associated with aging. This raises the possibility that other miRNAs may also function as key regulators of aging. Recently, a genome-wide transcriptional profile of miRNAs was performed using adult *C. elegans*. This profile found that approximately one third of the miRNAs in *C. elegans* exhibit changes in expression levels during adulthood. *lin-4* and *let-7* are included among these miRNAs (Ibanez-Ventoso et al., 2006).

Several additional uncharacterized miRNAs were shown to exhibit significant changes in relative abundance during adult life. For example, miR-1, a homologue of a *Drosophila* miRNA necessary for muscle development (Sokol and Ambros, 2005), exhibits a decline in expression during adulthood, suggesting that age-related muscle decline might be influenced by miRNA-dependent pathways. To help our further understanding of the role of miRNAs in aging, aging-associated genes were examined as possible targets of age-regulated miRNAs (Hamilton et al., 2005; Hansen et al., 2005). The insulin signalling pathway is a primary modulator of lifespan in *Drosophila*, mouse and *C. elegans* (Kenyon, 2005), and *C. elegans* has more than 30 insulin-related genes. Fourteen of them are predicted to be potential targets of age-related miRNAs. Moreover, *daf-16*, the major effector in insulin signalling pathway, is identified as a possible target for age-regulated miRNAs (Ibanez-Ventoso et al., 2006).

In addition to the insulin signalling pathway, caloric restriction also modulates lifespan in *C. elegans* (Lakowski and Hekimi, 1998). Recently, it was shown that a FOXA transcription factor, PHA-4 (pharynx development defect-4), is necessary for caloric-restriction-mediated longevity in *C. elegans* (Panowski et al., 2007). FOXA family members in mammals are known to act later in life to regulate glucagon production and glucose homeostasis, particularly in response to fasting (Kaestner et al., 1999; Shen et al., 2001). Interestingly, *pha-4* is one of the validated targets of the *let-7* miRNA (Grosshans

et al., 2005). Also, DAF-12, a nuclear hormone receptor acting downstream of DAF-16 to modulate lifespan and dauer formation, is also regulated by *let-7* (Grosshans et al., 2005). These data imply that *let-7* miRNA might help control lifespan, supporting its down-regulation in aging *C. elegans* (Ibanez-Ventoso et al., 2006).

### From vulval development to human cancer: cell proliferation control by miRNAs

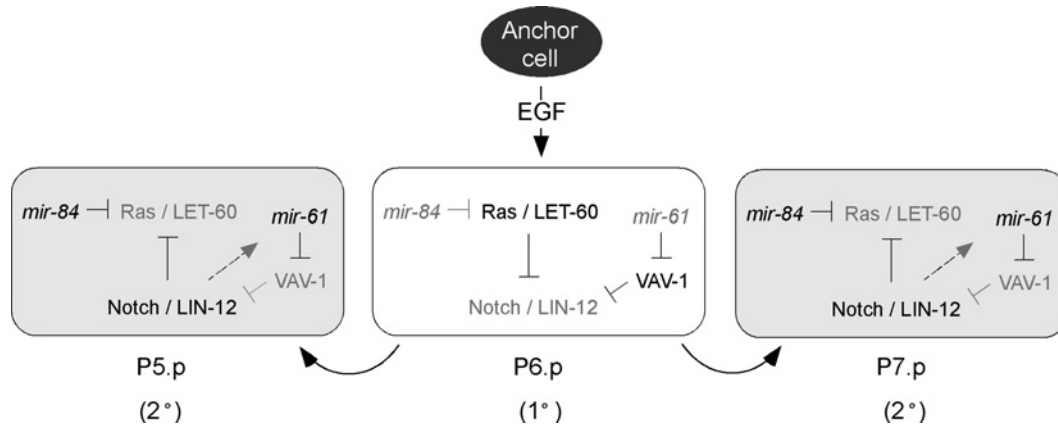
In *C. elegans*, the temporal up-regulation of *let-7* activity in the seam cell results in appropriate terminal differentiation. In contrast, reduced activity of *let-7* causes the failure of seam cells to exit the cell cycle and they continue to divide (Figure 1A). Inappropriate cell proliferation and differentiation are reminiscent of human cancer, and this opens up the possibility of miRNAs functioning as tumour suppressor genes or oncogenes.

The development of the vulva in the *C. elegans* hermaphrodite provides a tractable model system in which to understand cell proliferation and differentiation. Ras and Notch signalling pathways play key roles in vulval development through the proper control of cell division and cell fate (Sundaram, 2005). Several miRNAs, including *let-7* family members, act in the signalling cascade to regulate vulval morphogenesis (Esquela-Kerscher et al., 2005; Johnson et al., 2005; Yoo and Greenwald, 2005). Among these miRNAs, miR-84 and miR-61 target *let-60* and *vav-1* respectively, both of which are known orthologues of human oncogenes.

In the initial step of vulval development, six VPCs (vulva precursor cells), P3.p to P8.p, are specified in the 11 Pn.p cells, which are located in the ventral epidermis. These cells each adopt a vulval cell fate, primary (1°), secondary (2°) and tertiary (3°), which form a precise spatial pattern, 3°–3°–2°–1°–2°–3°. EGF (epidermal growth factor) signalling from the gonadal anchor cell, followed by the activation of the Ras–MAPK (mitogen-activated protein kinase) signalling cascade, specifies the 1° cell fate in P6.p cells, whereas the lateral signal from the 1° cell activates Notch/LIN-12 in P5.p and P7.p cells, promoting the 2° fate and inhibiting the 1° fate (Figure 4). The crosstalk between cells via the Ras and Notch signalling pathways ensures the precise patterning

**Figure 4 | An overview of Ras and Notch signalling network in vulval development**

EGF-Ras/LET-60 activation specifies P6.p to adopt 1° VPC fate that results in the production of a lateral signal shown by the curved arrows, which activates Notch/LIN-12 in P5.p and P7.p cells. Ras/LET-60 has an antagonistic interaction with Notch/LIN-12 in these VPCs. LIN-12 signal may positively control *mir-61* expression in a feedback loop in P5.p and P7.p cells.



during vulval morphogenesis (for further details regarding vulval development, see Sternberg, 2005).

The observation that miRNAs are expressed in the developing vulva (Esquela-Kerscher et al., 2005; Johnson et al., 2005) and of lateral signalling defects in the *alg-1* mutants led to the implication that miRNAs function in vulval cell differentiation. Bioinformatics and transcriptional reporter assays revealed that the *mir-61* miRNA is specifically expressed in P5.p and P7.p cells and is a transcriptional target of LIN-12 signalling (Figure 4). The ectopic expression of *mir-61* in P6.p cells, the presumptive 1° fate, induces the 2° cell fate instead of 1° cell fate, suggesting that *mir-61* promotes the adoption of the 2° fate (Yoo and Greenwald, 2005).

*vav-1*, a homologue of the Vav oncogene family in vertebrates (Tybulewicz, 2005), contains sequences in its 3' UTR that are complementary to miR-61. On the basis of reporter assays, *vav-1* was determined to be an authentic target for miR-61. The reporter construct, comprising the upstream sequence of *vav-1* fused to *gfp* and the *unc-54* 3' UTR, is found to be expressed in the VPCs, but when the *unc-54* 3' UTR is replaced with the *vav-1* 3' UTR, reporter expression is lost in P5.p and P7.p cells. This loss is dependent on the presence of miR-61 complementary sites in the *vav-1* 3' UTR. These observations suggest that *mir-61* promotes the 2° fate through the suppression of *vav-1* activity (Figure 4). Although a link between miRNAs and Vav has not been demonstrated in mammalian

cells, this work suggests that miRNAs may influence cancer through the regulation of Notch signalling (Yoo and Greenwald, 2005). Additional examples showing the relationship between miRNAs and human cancer came from work in our laboratory using *C. elegans*.

*let-60*, the Ras homologue in *C. elegans*, is required for normal vulval induction. From a computational prediction of *let-7* targets and a genetic screen for suppressors of the *let-7*-induced bursting vulva phenotype, *let-60* was found to be a target of miR-84, a *let-7* family miRNA (Johnson et al., 2005). *mir-84* expression starts during the early to mid-L3 stage in the anchor cell and in all vulval precursor cells (P3.p to P8.p) except for P6.p cells. In contrast with the weak expression of *mir-84* in P6.p cells, *let-60* is strongly expressed in P6.p cells (Figure 4). Gain-of-function mutations of *let-60* activate multiple VPCs to adopt 1° cell fates, leading to a multivulval phenotype. Over-expression of *mir-84* partially suppresses this multivulval phenotype, in a *let-60* 3'-UTR-dependent manner. Interestingly, another *let-7* family miRNA, *mir-48*, is also expressed in non-P6.p VPCs (Esquela-Kerscher et al., 2005), suggesting that miR-84 controls vulval cell-fate determination through the suppression of *let-60*, probably together with miR-48 or other miRNAs.

*let-60* is a *C. elegans* orthologue of the three human *ras* genes, *HRAS*, *KRAS* and *NRAS*, which are often commonly mutated in human cancer cells. Notably,

all of the human *ras* genes have complementary sites for human *let-7* family miRNAs in their 3' UTRs, suggesting that *ras* is regulated by *let-7* in a similar manner to *let-60* in *C. elegans* (Johnson et al., 2005). Consistent with this prediction, when human *let-7* is exogenously provided to, or when its function is blocked in, cultured human cells, the level of Ras protein is reduced or enhanced respectively. Furthermore, miRNA microarray analysis using tissues from cancer patients shows that *let-7* expression is reduced in a number of tumours, as compared with the normal adjacent tissues. Conversely, the Ras protein is reciprocally accumulated in these tumour tissues. Interestingly, all of the lung tumour tissues show lower levels of *let-7* expression. These observations suggest that the human *let-7* miRNA acts as a tumour suppressor by repressing *ras* oncogene activity, especially in the lungs. Indeed, studies have mapped *let-7* family members to human chromosomal sites that are related to various types of cancers, including lung cancer (Calin et al., 2004).

The importance of the p53 pathway in preventing tumour formation has been implied from the presence of mutations in the mammalian p53 pathway in almost all cancers (Hollstein et al., 1991). Multiple physiological stresses, including radiation, can cause the accumulation of p53 protein and activate a p53-mediated downstream cascade to induce growth arrest, promote apoptosis or facilitate DNA repair (Ko and Prives, 1996; Hanahan and Weinberg, 2000). One of the miRNA families in mouse, miR-34, acts in a stress-response pathway in a p53-dependent manner; DNA damage can induce the activation of miR-34 in a wild-type background, but not in a p53-null background (He et al., 2007). Further analysis indicated that the activation of miR-34 represses cell-cycle progression and suggests the possibility that p53 may suppress cell-cycle-related genes via induction of miR-34 activity. Consistent with this idea, another study using neuroblastoma tumours demonstrated that miR-34a, a member of the miR-34 family, directly targets E2F3, a potent transcriptional inducer of cell-cycle progression (Welch et al., 2007). This and two other recent works also demonstrated that the induction of miR-34a significantly increases caspase activity (Welch et al., 2007) and promotes apoptotic cell death (Chang et al., 2007; Raver-Shapira et al., 2007). It is known that members of the E2F family of transcription factors can trigger apoptosis

when they are inappropriately expressed (Jaquinta et al., 2005). These findings suggest that miR-34 plays an important role in the p53-mediated oncogenic stress response network. Although the role of *C. elegans mir-34* still remains unknown, it might function in a similar manner in *C. elegans*.

## Conclusions

New roles for miRNA in *C. elegans* and mammals are currently being discovered. For example, recent studies provide further evidence that miRNAs have additional roles in a neurodegenerative disorder, Parkinson's disease, and in an immune response to microbial infection in mammals (Kim et al., 2007; Taganov et al., 2007; Thai et al., 2007). These findings imply more diverse and more universal miRNA functions than we have previously imagined. Furthermore, a novel class of small RNAs, piRNA (Piwi-interacting RNA) and its interacting protein, Piwi proteins, have been found to be involved in transposon suppression during gametogenesis in several organisms, including mammals (Aravin et al., 2007; O'Donnell and Boeke, 2007). This might have implications in sterility and/or congenital abnormality in mammals. Since many important pathways commonly found in mammals, such as insulin signalling, Ras/Notch signalling and p53 pathways, as well as many miRNAs, are conserved in *C. elegans*, we believe *C. elegans* will help us further understand how miRNAs function in human disease and may illuminate new aspects of the small regulatory RNA world.

## Acknowledgements

We thank Lena Chin and Katherine Carter for comments on this manuscript. M.K. was supported by the postdoctoral fellowship from the Uehara Memorial Life Science Foundation and E.S. by grants from the NIH (National Institutes of Health), NSF (National Science Foundation) and Ellison Medical Foundation.

## References

- \*Articles of special interest  
Abbott, A.L., Alvarez-Saavedra, E., Miska, E.A., Lau, N.C., Bartel, D.P., Horvitz, H.R. and Ambros, V. (2005) The *let-7* microRNA family members *mir-48*, *mir-84*, and *mir-241* function together to regulate developmental timing in *Caenorhabditis elegans*. *Dev. Cell* **9**, 403–414  
Abraham, M.C., Lu, Y. and Shaham, S. (2007) A morphologically conserved nonapoptotic program promotes linker cell death in *Caenorhabditis elegans*. *Dev. Cell* **12**, 73–86

- Abrahante, J.E., Daul, A.L., Li, M., Volk, M.L., Tennesen, J.M., Miller, E.A. and Rougvie, A.E. (2003) The *Caenorhabditis elegans* hunchback-like gene *lin-57/hbl-1* controls developmental time and is regulated by microRNAs. *Dev. Cell* **4**, 625–637
- Aravin, A.A., Sachidanandam, R., Girard, A., Fejes-Toth, K. and Hannon, G.J. (2007) Developmentally regulated piRNA clusters implicate MILI in transposon control. *Science* **314**, 744–747
- Bargmann, C.I. and Horvitz, H.R. (1991) Chemosensory neurons with overlapping functions direct chemotaxis to multiple chemicals in *C. elegans*. *Neuron* **7**, 729–742
- \*Boehm, M. and Slack, F. (2005) A developmental timing microRNA and its target regulate life span in *C. elegans*. *Science* **310**, 1954–1957
- Calin, G.A., Sevignani, C., Dumitru, C.D., Hyslop, T., Noch, E., Yendamuri, S., Shimizu, M., Rattan, S., Bullrich, F., Negrini, M. and Croce, C.M. (2004) Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 2999–3004
- Carthew, R.W. (2006) Gene regulation by microRNAs. *Curr. Opin. Genet. Dev.* **16**, 203–208
- Chang, S., Johnston, Jr, R.J. and Hobert, O. (2003) A transcriptional regulatory cascade that controls left/right asymmetry in chemosensory neurons of *C. elegans*. *Genes Dev.* **17**, 2123–2137
- \*Chang, S., Johnston, Jr, R.J., Frokjaer-Jensen, C., Lockery, S. and Hobert, O. (2004) MicroRNAs act sequentially and asymmetrically to control chemosensory laterality in the nematode. *Nature* **430**, 785–789
- Chang, T.C., Wentzel, E.A., Kent, O.A., Ramachandran, K., Mullendore, M., Lee, K.H., Feldmann, G., Yamakuchi, M., Ferlito, M., Lowenstein, C.J., Arking, D.E., Beer, M.A., Maitra, A. and Mendell, J.T. (2007) Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis. *Mol. Cell* **26**, 745–752
- Esquela-Kerscher, A., Johnson, S.M., Bai, L., Saito, K., Partridge, J., Reinert, K.L. and Slack, F.J. (2005) Post-embryonic expression of *C. elegans* microRNAs belonging to the *lin-4* and *let-7* families in the hypodermis and the reproductive system. *Dev. Dyn.* **234**, 868–877
- Feinbaum, R. and Ambros, V. (1999) The timing of *lin-4* RNA accumulation controls the timing of postembryonic developmental events in *Caenorhabditis elegans*. *Dev. Biol.* **210**, 87–95
- Grishok, A., Pasquinelli, A.E., Conte, D., Li, N., Parrish, S., Ha, I., Baillie, D.L., Fire, A., Ruvkun, G. and Mello, C.C. (2001) Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control *C. elegans* developmental timing. *Cell* **106**, 23–34
- Grosshans, H., Johnson, T., Reinert, K.L., Gerstein, M. and Slack, F.J. (2005) The temporal patterning microRNA *let-7* regulates several transcription factors at the larval to adult transition in *C. elegans*. *Dev. Cell* **8**, 321–330
- Hamilton, B., Dong, Y., Shindo, M., Liu, W., Odell, I., Ruvkun, G. and Lee, S.S. (2005) A systematic RNAi screen for longevity genes in *C. elegans*. *Genes Dev.* **19**, 1544–1555
- Hanahan, D. and Weinberg, R.A. (2000) The hallmarks of cancer. *Cell* **100**, 57–70
- Hansen, M., Hsu, A.L., Dillin, A. and Kenyon, C. (2005) New genes tied to endocrine, metabolic, and dietary regulation of lifespan from a *Caenorhabditis elegans* genomic RNAi screen. *PLoS Genet.* **1**, 119–128
- \*He, L., He, X., Lim, L.P., de Stanchina, E., Xuan, Z., Liang, Y., Xue, W., Zender, L., Magnus, J., Ridzon, D., Jackson, A.L., Linsley, P.S., Chen, C., Lowe, S.W., Cleary, M.A. and Hannon, G.J. (2007) A microRNA component of the p53 tumour suppressor network. *Nature* **447**, 1130–1134
- Hobert, O., Johnston, Jr, R.J. and Chang, S. (2002) Left–right asymmetry in the nervous system: the *Caenorhabditis elegans* model. *Nat. Rev. Neurosci.* **3**, 629–640
- Hollstein, M., Sidransky, D., Vogelstein, B. and Harris, C.C. (1991) p53 mutations in human cancers. *Science* **253**, 49–53
- Hsu, A.L., Murphy, C.T. and Kenyon, C. (2003) Regulation of aging and age-related disease by DAF-16 and heat-shock factor. *Science* **300**, 1142–1145
- Iaquinta, P.J., Aslanian, A. and Lees, J.A. (2005) Regulation of the Arf/p53 tumor surveillance network by E2F. *Cold Spring Harb. Symp. Quant. Biol.* **70**, 309–316
- \*Ibanez-Ventoso, C., Yang, M., Guo, S., Robins, H., Padgett, R.W. and Driscoll, M. (2006) Modulated microRNA expression during adult lifespan in *Caenorhabditis elegans*. *Aging Cell* **5**, 235–246
- \*Johnson, S.M., Grosshans, H., Shingara, J., Byrom, M., Jarvis, R., Cheng, A., Labourier, E., Reinert, K.L., Brown, D. and Slack, F.J. (2005) RAS is regulated by the *let-7* microRNA family. *Cell* **120**, 635–647
- \*Johnston, R.J. and Hobert, O. (2003) A microRNA controlling left/right neuronal asymmetry in *Caenorhabditis elegans*. *Nature* **426**, 845–849
- Kaestner, K.H., Katz, J., Liu, Y., Drucker, D.J. and Schutz, G. (1999) Inactivation of the winged helix transcription factor HNF3 $\alpha$  affects glucose homeostasis and islet glucagon gene expression *in vivo*. *Genes Dev.* **13**, 495–504
- Kenyon, C. (2005) The plasticity of aging: insights from long-lived mutants. *Cell* **120**, 449–460
- Kenyon, C., Chang, J., Gensch, E., Rudner, A. and Tabtiang, R. (1993) A *C. elegans* mutant that lives twice as long as wild type. *Nature* **366**, 461–464
- Kim, J., Inoue, K., Ishii, J., Vanti, W.B., Voronov, S.V., Murchison, E., Hannon, G. and Abeliovich, A. (2007) A microRNA feedback circuit in midbrain dopamine neurons. *Science* **317**, 1220–1224
- Kimura, K.D., Tissenbaum, H.A., Liu, Y. and Ruvkun, G. (1997) *daf-2*, an insulin receptor-like gene that regulates longevity and diapause in *Caenorhabditis elegans*. *Science* **277**, 942–946
- Ko, L.J. and Prives, C. (1996) p53: puzzle and paradigm. *Genes Dev.* **10**, 1054–1072
- Lakowski, B. and Hekimi, S. (1998) The genetics of caloric restriction in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 13091–13096
- \*Lee, R.C., Feinbaum, R.L. and Ambros, V. (1993) The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. *Cell* **75**, 843–854
- Li, M., Jones-Rhoades, M.W., Lau, N.C., Bartel, D.P. and Rougvie, A.E. (2005) Regulatory mutations of *mir-48*, a *C. elegans let-7* family microRNA, cause developmental timing defects. *Dev. Cell* **9**, 415–422
- Lim, L.P., Lau, N.C., Weinstein, E.G., Abdelhakim, A., Yekta, S., Rhoades, M.W., Burge, C.B. and Bartel, D.P. (2003) The microRNAs of *Caenorhabditis elegans*. *Genes Dev.* **17**, 991–1008
- Lin, K., Dorman, J.B., Rodan, A. and Kenyon, C. (1997) *daf-16*: An HNF-3/forkhead family member that can function to double the life-span of *Caenorhabditis elegans*. *Science* **278**, 1319–1322
- Lin, S.Y., Johnson, S.M., Abraham, M., Vella, M.C., Pasquinelli, A., Gamberi, C., Gottlieb, E. and Slack, F.J. (2003) The *C. elegans* hunchback homolog, *hbl-1*, controls temporal patterning and is a probable microRNA target. *Dev. Cell* **4**, 639–650
- McCarroll, S.A., Murphy, C.T., Zou, S., Pletcher, S.D., Chin, C.S., Jan, Y.N., Kenyon, C., Bargmann, C.I. and Li, H. (2004) Comparing genomic expression patterns across species identifies shared transcriptional profile in aging. *Nat. Genet.* **36**, 197–204
- Moss, E.G., Lee, R.C. and Ambros, V. (1997) The cold shock domain protein LIN-28 controls developmental timing in *C. elegans* and is regulated by the *lin-4* RNA. *Cell* **88**, 637–646
- Nilsen, T.W. (2007) Mechanisms of microRNA-mediated gene regulation in animal cells. *Trends Genet.* **23**, 243–249
- O'Donnell, K.A. and Boeke, J.D. (2007) Mighty Piwis defend the germline against genome intruders. *Cell* **129**, 37–44

- Ogg, S., Paradis, S., Gottlieb, S., Patterson, G.I., Lee, L., Tissenbaum, H.A. and Ruvkun, G. (1997) The forkhead transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in *C. elegans*. *Nature* **389**, 994–999
- Olsen, P.H. and Ambros, V. (1999) The *lin-4* regulatory RNA controls developmental timing in *Caenorhabditis elegans* by blocking LIN-14 protein synthesis after the initiation of translation. *Dev. Biol.* **216**, 671–680
- Panowski, S.H., Wolff, S., Aguilaniu, H., Durieux, J. and Dillin, A. (2007) PHA-4/Foxa mediates diet-restriction-induced longevity of *C. elegans*. *Nature* **447**, 550–555
- \*Pasquinelli, A.E., Reinhart, B.J., Slack, F., Martindale, M.Q., Kuroda, M.I., Maller, B., Hayward, D.C., Ball, E.E., Degnan, B., Müller, P., Spring, J., Srinivasan, A., Fishman, M., Finnerty, J., Corbo, J., Levine, M., Leahy, P., Davidson, E. and Ruvkun, G.** (2000) Conservation of the sequence and temporal expression of *let-7* heterochronic regulatory RNA. *Nature* **408**, 86–89
- Raver-Shapira, N., Marciano, E., Meiri, E., Spector, Y., Rosenfeld, N., Moskovits, N., Bentwich, Z. and Oren, M. (2007) Transcriptional activation of miR-34a contributes to p53-mediated apoptosis. *Mol. Cell* **26**, 731–743
- \*Reinhart, B.J., Slack, F.J., Basson, M., Pasquinelli, A.E., Bettinger, J.C., Rougvie, A.E., Horvitz, H.R. and Ruvkun, G.** (2000) The 21-nucleotide *let-7* RNA regulates developmental timing in *Caenorhabditis elegans*. *Nature* **403**, 901–906
- Rougvie, A.E. and Ambros, V. (1995) The heterochronic gene *lin-29* encodes a zinc finger protein that controls a terminal differentiation event in *Caenorhabditis elegans*. *Development* **121**, 2491–2500
- Ruby, J.G., Jan, C., Player, C., Axtell, M.J., Lee, W., Nusbaum, C., Ge, H. and Bartel, D.P. (2006) Large-scale sequencing reveals 21U-RNAs and additional microRNAs and endogenous siRNAs in *C. elegans*. *Cell* **127**, 1193–1207
- Schulman, B.R., Esquela-Kerscher, A. and Slack, F.J. (2005) Reciprocal expression of *lin-41* and the microRNAs *let-7* and *mir-125* during mouse embryogenesis. *Dev. Dyn.* **234**, 1046–1054
- Shen, W., Searce, L.M., Brestelli, J.E., Sund, N.J. and Kaestner, K.H. (2001) Foxa3 (hepatocyte nuclear factor 3 $\gamma$ ) is required for the regulation of hepatic GLUT2 expression and the maintenance of glucose homeostasis during a prolonged fast. *J. Biol. Chem.* **276**, 42812–42817
- Slack, F.J., Basson, M., Liu, Z., Ambros, V., Horvitz, H.R. and Ruvkun, G. (2000) The *lin-41* RBCC gene acts in the *C. elegans* heterochronic pathway between the *let-7* regulatory RNA and the LIN-29 transcription factor. *Mol. Cell* **5**, 659–669
- Sokol, N.S. and Ambros, V. (2005) Mesodermally expressed *Drosophila microRNA-1* is regulated by *Twist* and is required in muscles during larval growth. *Genes Dev.* **19**, 2343–2354
- Sternberg, P.W. (2005) Vulval development, WormBook 1.6.1 (<http://www.wormbook.org/>)
- Sulston, J.E., Albertson, D.G. and Thomson, J.N. (1980) *The Caenorhabditis elegans* male: postembryonic development of nongonadal structures. *Dev. Biol.* **78**, 542–576
- Sundaram, M.V. (2005) The love–hate relationship between Ras and Notch. *Genes Dev.* **19**, 1825–1839
- Taganov, K.D., Boldin, M.P. and Baltimore, D. (2007) MicroRNAs and immunity: tiny players in a big field. *Immunity* **26**, 133–137
- Thai, T.H., Calado, D.P., Casola, S., Ansel, K.M., Xiao, C., Xue, Y., Murphy, A., Frendewey, D., Valenzuela, D., Kutok, J.L., Schmidt-Suppran, M., Rajewsky, N., Yancopoulos, G., Rao, A. and Rajewsky, K. (2007) Regulation of the germinal center response by microRNA-155. *Science* **316**, 604–608
- Tybulewicz, V.L. (2005) Vav-family proteins in T-cell signalling. *Curr. Opin. Immunol.* **17**, 267–274
- Vella, M.C., Choi, E.Y., Lin, S.Y., Reinert, K. and Slack, F.J. (2004) The *C. elegans* microRNA *let-7* binds to imperfect *let-7* complementary sites from the *lin-41* 3' UTR. *Genes Dev.* **18**, 132–137
- Welch, C., Chen, Y. and Stallings, R.L. (2007) MicroRNA-34a functions as a potential tumor suppressor by inducing apoptosis in neuroblastoma cells. *Oncogene* **26**, 5017–5022
- Wightman, B., Ha, I. and Ruvkun, G. (1993) Post-transcriptional regulation of the heterochronic gene *lin-14* by *lin-4* mediates temporal pattern formation in *C. elegans*. *Cell* **75**, 855–862
- \*Yoo, A.S. and Greenwald, I.** (2005) LIN-12/Notch activation leads to microRNA-mediated down-regulation of Vav in *C. elegans*. *Science* **310**, 1330–1333
- Yu, S., Avery, L., Baude, E. and Garbers, D.L. (1997) Guanylyl cyclase expression in specific sensory neurons: a new family of chemosensory receptors. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 3384–3387

Received 10 July 2007/12 September 2007; accepted 28 September 2007

Published on the Internet 21 January 2008, doi:10.1042/BC20070078