Review

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The *let-7* family of microRNAs

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The first two known microRNAs (miRNAs), *lin-4* and *let-7*, were originally discovered in the nematode *Caenorhabditis elegans* and control the timing of stem-cell division and differentiation. *let-7* was subsequently found as the first known human miRNA. *let-7* and its family members are highly conserved across species in sequence and function, and misregulation of *let-7* leads to a less differentiated cellular state and the development of cell-based diseases such as cancer. Although much research has been devoted to *let-7* target prediction and to understanding its biological role, research into what regulates *let-7* has only just begun. Here, we review *let-7*-family conservation and the recent advances in understanding how *let-7*-expression is regulated at the transcriptional and post-transcriptional levels across species. A greater understanding of what controls *let-7* expression might enable the development of treatments to fight or prevent many cancers.

Introduction

MicroRNAs (miRNAs) are a rapidly growing class of ~22 nucleotide (nt), non-coding RNAs that post-transcriptionally regulate downstream mRNAs (mRNAs) through 3′ untranslated regions (UTRs) of the target mRNAs (for reviews, see Refs [1,2]). Since the discovery in 2000 that miRNAs are a conserved class of genes [3,4], >6000 miRNA entries across 72 virus, plant and animal species have been recorded in miRBase release 11.0 (http://microrna.sanger.ac.uk/sequences/). Early estimates grouped miRNAs into 48 families, based on similarities in nucleotides 2–8 at their 5′ end, referred to as the 'seed sequence' [1,5] (Figures 1a,2). At least a third of miRNA families are highly conserved across species [5], and 60% of miRNA loci are conserved from mouse to human [6]. miRNAs are involved in diverse biological functions, including development, life span, cell proliferation, differentiation, signaling pathways, apoptosis and metabolism [7]. Concomitant with their many biological roles is their involvement in many diseases, such as viral infections, genetic disorders and many types of cancer [2,7–9].

miRNA genes can be expressed as single genes (monocistronic) or as clusters of miRNAs from within one locus (polycistronic) [10], and the closely linked miRNAs can be from different families. miRNAs have been found in intergenic regions and within introns and exons of other genes. Furthermore, they can be transcribed from their own promoters, promoters of nearby genes or from promoters of 'host' genes [10]. Most miRNAs are transcribed by RNA polymerase-II, then 5′-methyl G-capped and polyadenylated, like most protein-coding genes [11] (for a review, see Ref. [10]).

The primary miRNA (pri-miRNA) is the initial transcript and is the longest of the three miRNA forms [10]. Within the pri-miRNA is a hairpin structure that contains the mature miRNA. This hairpin is the precursor miRNA (pre-miRNA) that is processed in the nucleus from the pri-miRNA by the microprocessor complex (Box 1), which includes the RNase-III enzyme Drosha and its RNA-binding partner, Pasha, also known as DGCR8 (DiGeorge syndrome critical region 8) [12–14]. Drosha is the catalytic center of the microprocessor that cleaves the double-stranded-RNA (dsRNA) stem of the hairpin, whereas DGCR8 ensures that the complex is correctly positioned for cleavage [14,15]. DGCR8 directly binds the dsRNA–single-stranded-RNA (ssRNA) junction at the base of the hairpin stem, but Drosha is thought to only transiently contact the pri-miRNA [16]. Although it is not necessary for processing, a terminal loop more than ten nts results in the most efficient processing [16].

With the initial pri-miRNA processing being completed in the nucleus, pre-miRNAs are exported from the nucleus into the cytoplasm by exportin-5 [17], except in *Caenorhabditis elegans*, which does not have a recognizable exportin-5 and, currently, does not have an identified transport receptor [18]. In the cytoplasm, the pre-miRNA is bound by a second dsRNA-cleaving, RNase-III enzyme Dicer which processes the pre-miRNA into a 22-nt dsRNA [19]. Through a currently unknown mechanism, the single-stranded mature miRNA is incorporated from the dsRNA into the miRNA RNA-induced silencing complex (miRISC). The miRNA-loaded miRISC then regulates its target mRNAs by affecting mRNA translation and/or stability.

An overview of the *let-7* family

The *lethal-7* (*let-7*) gene was initially discovered as an essential developmental gene in *C. elegans* and, later, as one of the first miRNAs [3,20] (Figure 1). Soon afterwards, a simple BLAST (basic local alignment search tool) search revealed exact matches to the mature *let-7* miRNA sequence in the emerging *Drosophila melanogaster* and human genomes [4,21]. It is now known that mature *let-7* is highly conserved across animal species [4,21–23].

The *let-7* family of miRNAs is often present in multiple copies in a genome (Table 1). To distinguish between multiple isoforms, a letter is placed after *let-7* to indicate a *let-7* with a slightly different sequence, and a number at the end denotes that the same sequence is present in multiple genomic locations. For example, humans have ten mature *let-7*-family sequences (Figure 3) that are produced from 13 precursor sequences (Figure 4). Three separate precursors produce the mature *let-7a* sequence (*let-7a-1, let-7a-2* and *let-7a-3*) and precursors from two different genomic locations produce the *let-7f* (*let-7f-1* and

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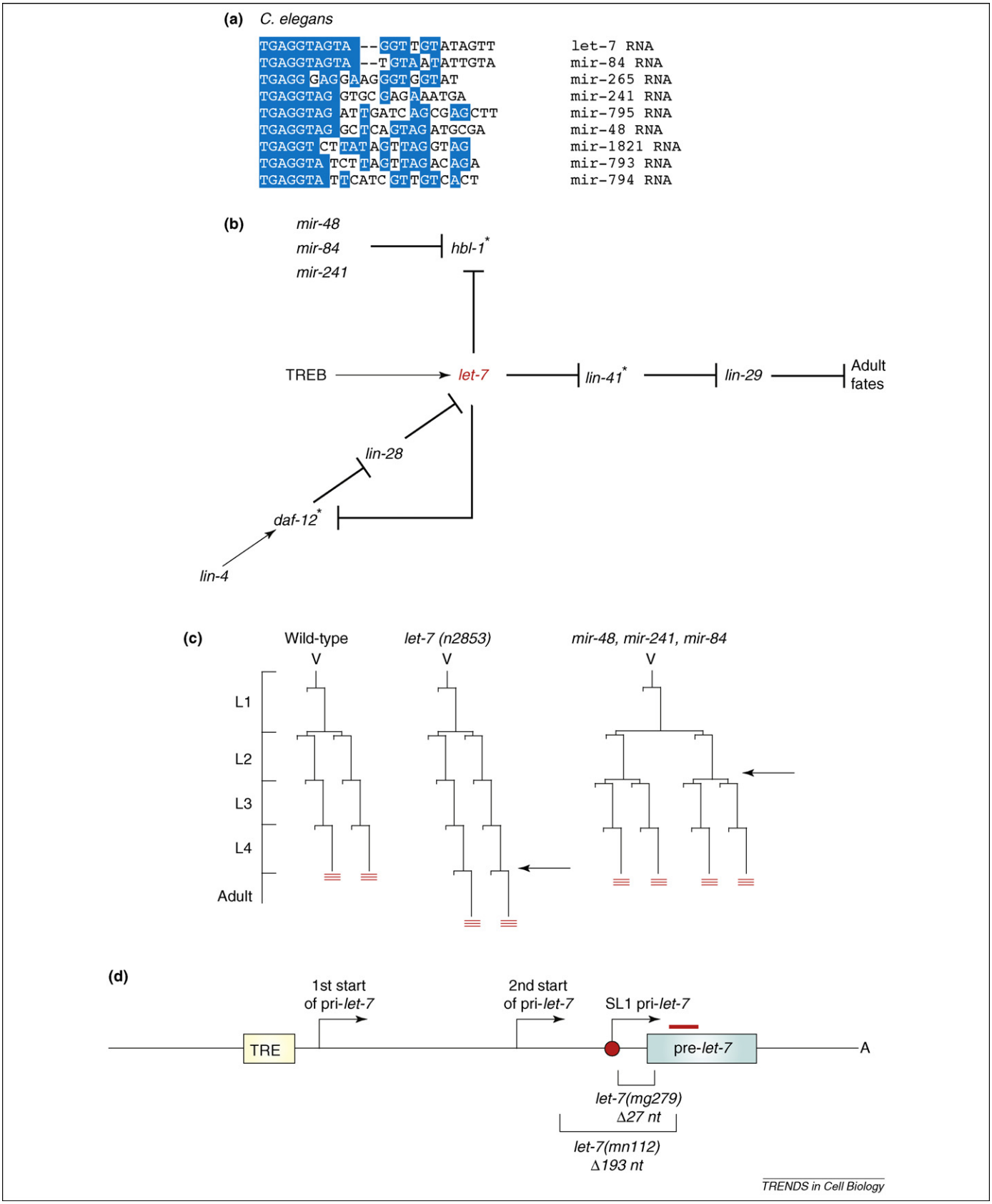

Figure 1. The *C. elegans let-7* family. **(a)** DNA sequences of mature *let-7*-family members. Sequence conservation is indicated by blue shading. The seed sequence is nucleotides 2–8 (unbroken blue bar). **(b)** Genes of the heterochronic pathway control developmental timing in *C. elegans* [3,36]. Several regulators of *let-7* expression, such as *lin-4* and *daf-12* [46,49], and *let-7* targets, such as *lin-41*, *daf-12* and *hbl-1*, indicated by (*) are shown [3,38–41]. TREB is an unknown protein that is a possible activator of *let-7* transcription [49]. **(c)** Seam cells in wild-type animals divide at each larval stage in a set, stem-cell-like manner. *let-7(n2853)* is a temperature-sensitive mutant and *mir-48, mir-241, mir-84* is a triple knockout. Both mutants show retarded seam-cell phenotypes. *let-7(n2853)* has an extra seam-cell division at the L4-to-adult transition (arrow) before terminally differentiating (red bars) [3]. The triple mutant reiterates an L2-like seam cell division in L3 (arrow) [36]. **(d)** There are three *C. elegans* pri-*let-7* miRNAs. The two longer pri-*let-7* miRNAs are *trans*-spliced to a splice leader 1 (SL1) sequence to form the third. The temporal regulatory element (TRE) is a *cis*-acting element that

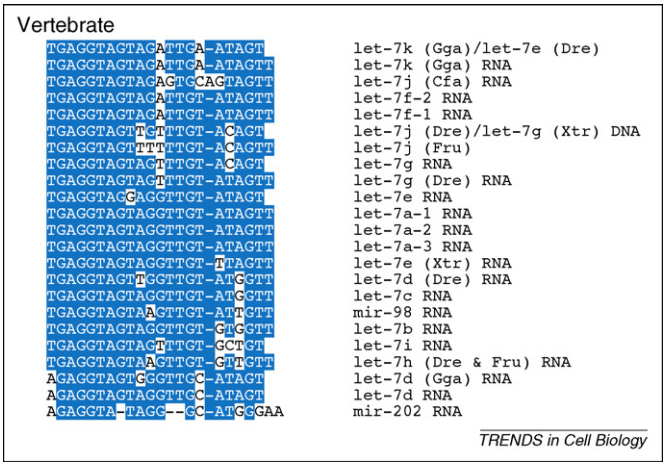

Figure 2. The vertebrate *let-7* family. DNA sequences of mature *let-7*-family members. Sequence conservation is indicated by blue shading. The seed sequence is nucleotides 2–8 (unbroken blue bar). Unless otherwise noted, the vertebrate sequences given are common to all vertebrates, including human, that express these miRNAs. Where shown, *let-7* molecules with identical sequences are distinguished based on having derived from unique pre-*let-7* molecules. Sequences unique to a species are followed by the three letter species symbol: Cfa, *Canis familiaris* (dog); Dre, *Danio rerio* (zebrafish); Gga, *Gallus gallus* (chicken); Fru, *Fugu rubripes* (pufferfish); Xtr, *Xenopus tropicalis* (frog).

let-7f-2) sequence. However, the size of the family can differ between organisms. *Drosophila* (fly) has only one *let-7*, whereas the zebrafish *Danio rerio* has 11 mature sequences in 19 loci throughout its genome (http://micro-rna.sanger.ac.uk/sequences/) [6] (Table 1).

Biological functions for *let-7* in animals include the regulation of stem-cell differentiation in *C. elegans* [3], neuromusculature development and adult behaviors in flies [24,25], limb development in chicken and mouse [26–28], and cell proliferation and differentiation [9,24,29–32]. Moreover, many *let-7*-family members function as tumor suppressors in a variety of cancers [8,9].

The *C. elegans* let-7 family

In *C. elegans*, *let-7* is the founding member of the *let-7* family, which consists of genes encoding nine miRNAs: *let-7*, *mir-48*, *mir-84*, *mir-241*, *mir-265, mir-793*, *mir-794*, *mir-795* and *mir-1821* [5,33,34] (Figure 1a). *let-7*, *mir-84*, *mir-48* and *mir-241* are all members of the *C. elegans* heterochronic pathway (Figure 1b), a set of genes that function sequentially to control developmental timing of cell fates at different larval transitions (for a review, see Ref. [35]). *mir-48*, *mir-84* and *mir-241* control the larval 2 (L2)-to-larval 3 (L3) transition, and *let-7* itself controls the larval 4 (L4)-to-adult transition [3,36] (Figure 1c). At each larval stage of *C. elegans* development, hypodermal skin cells, known as seam cells, divide in a stem-cell-like manner with one daughter cell differentiating and the other daughter cell self-renewing and continuing with the proliferative program (Figure 1c). At the L4-to-adult transition, the self-renewing daughter cell ceases proliferation and terminally differentiates. Once the seam cells have differentiated, they secrete ridges, called alae, that run along the sides of the animal. In precocious heterochronic mutants, programmed seam-cell divisions are skipped and the cells terminally differentiate and secrete alae prematurely. In retarded heterochronic mutants, cell divisions are reiterated, leading to an increased seam-cell number, a delay in terminal differentiation and incomplete alae [35] (Figure 1c).

Seam cells in loss-of-function *let-7* mutants fail to exit the cell cycle at the L4-to-adult transition and display extra seam-cell divisions (Figure 1c) and delayed and incomplete

Box 1. The microprocessor

The core proteins of the ~500–650-kDa microprocessor are Drosha and Pasha, which is also known as DiGeorge syndrome critical region 8 (DGCR8) [82]. Drosha is an RNase-III enzyme that cleaves dsRNA, leaving a characteristic two nucleotide 3′ overhang. The binding partner of Drosha, DGCR8, is a dsRNA-binding protein that binds to the RNase-III domains and central region of Drosha (Figure I). The necessity of these proteins in pri-miRNA processing was demonstrated using RNAi knockdown experiments in several animal systems, in which the reduction of either protein caused an increase in pri-miRNAs levels and a decrease in pre- and mature-miRNA levels [83]. In general, pri-miRNA-specific processing *in vitro* requires both these two proteins, but no other accessory factors. However, if DGCR8 is not present, then Drosha has a non-specific RNase activity [83].

Drosha is the catalytic center of the microprocessor and has two RNase-III domains that make up a catalytic site with each domain making one of the two cuts necessary to excise the pre-miRNA. Drosha excises the pre-miRNA from the pri-miRNA by cutting at the base of the dsRNA stem–loop of the pre-miRNA [82]. The two nucleotide 3′ overhang left by the cutting is necessary for exportin-5 recognition and transfer of the pre-miRNA out of the nucleus and into the cytoplasm [83]. DGCR8 functions like a molecular ruler, measuring from the ssRNA–dsRNA junction at the base of the stem to ensure that Drosha cuts at the correct distance from the loop to create the ~70-nt pre-miRNA hairpin [82].

Recently, it has been shown that for a subset of miRNAs involved in vascular smooth muscle cell differentiation, which includes the oncogenic miRNA miR-21, pri-miRNA processing can be enhanced when some SMAD proteins are ligand-bound [84]. The increase in pri-miRNA processing seems to be from an association of the ligand-bound SMAD proteins to helicase p68, a microprocessor protein that is needed to process a subset of miRNAs. Exactly how this association increases microprocessor activity for this set of miRNAs is still being investigated.

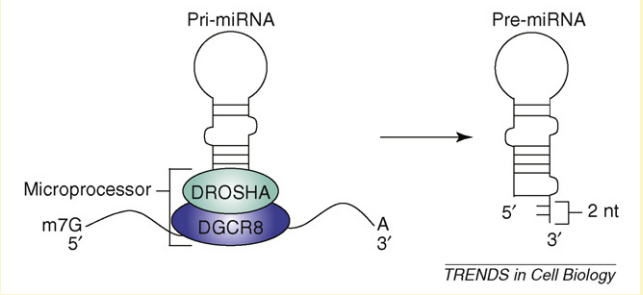

Figure I. The microprocessor The core proteins of the microprocessor are Drosha and DGCR8 (for reviews, see Refs [82,83]). DGCR8 binds at the base of the miRNA-stem loop at the ssRNA–dsRNA junction in the pri-miRNA. Drosha makes two cuts to cleave the dsRNA, leaving a 2 nt 3′ overhang that is characteristic of RNase-III enzymes, and liberating the ~70 nt pre-miRNA. Abbreviations: A, polyA tail; m7G, 7-methyl guanosine cap.

positively regulates the temporal expression of *let-7* in the seam cells [49]. *let-7(mg285)* and *let-7(mn112)* mutants both affect the SL1 splice site (red dot) that is important for proper pri-*let-7* formation and recognition by the microprocessor [11]. These mutants were obtained from genetic screens for developmentally defective *C. elegans* and show developmental timing defects [20]. Pri-*let-7* start sites are indicated by arrows. The sizes of the deletions are noted below their name. 'A' is the polyadenylation site. The red bar represents the location of the mature 22-nt *let-7*. Figure not to scale.

Table 1. The sequence and genomic distribution of many *let-7* family members is conserved[a]

	C. elegans (nematode)[b]	*D. melanogaster* (fly)	*D. rerio* (zebrafish)	*G. gallus* (chicken)	*H. sapiens* (human)	*M. musculus* (mouse)	*X. tropicalis* (frog)
let-7a	1	1	6	4[c]	3	2	1
let-7b	–	–	1	1	1	1	1
let-7c	–	–	2	1	1	2	1
let-7d[d]	–	–	2	1	1	1	–
let-7e[e]	–	–	1[f]	–	1	1	2
let-7f	–	–	1	1	2	2	1
let-7 g[g]	–	–	2	1	1	1	1[h]
let-7h	–	–	1	–	–	–	–
let-7i	–	–	1	1	1	1	1
let-7j	–	–	1[h]	1[c]	–	–	–
let-7k	–	–	–	1[f]	–	–	–
mir-98	–	–	–	–	1	1	1
mir-202[i]	–	–	1	1	1	2	1
Cluster 1	–	1	4	1	3	3	2
Cluster 2	–	–	–[j]	1	1	1	–
Cluster 3	–	–	1	1	1	–[k]	–
Cluster 4	–	–	–	–	1	1	1

[a]The *let-7* family members and their genomic organization is highly conserved [4,21,53–55,66,67]. However, the total number of *let-7* genes varies between organisms, with more complex animals having the greatest numbers. The numbers in the table represent the number of copies a given *let-7* family member has annotated in mirBase release 11.0 (http://microrna.sanger.ac.uk/sequences/). A dash (–) indicates that there are currently no annotations for this member in mirBase release 11.0. Cluster 1 is the *miR-100* and *99*, *let-7*, and *miR-125*. Cluster 2 is *let-7a*, *let-7d*, and *let-7f*. Cluster 3 is *let-7a-3* and *let-7b*. Cluster 4 is *let-7f* and *miR-98*.
[b]mir-48, mir-84, mir-241, mir-793, mir-794 and mir-795 not shown.
[c]Chicken *let-7j* has the same sequence as the canonical *let-7a*, but is not included in this number.
[d]Even though all species are labeled *let-7e*, human and mouse have the same sequence, but zebrafish and frog each have different sequences.
[e]Even though all species are labeled *let-7d*, human and mouse have the same sequence, but zebrafish and chicken each have different sequences.
[f]Zebrafish *let-7e* and chicken *let-7k* have the same sequence.
[g]Even though all species are labeled *let-7g*, human, mouse and chicken have the same sequence, but zebrafish and frog each have different sequences.
[h]Frog *let-7g* and zebrafish *let-7j* have the same sequence.
[i]Even though all species are labeled *miR-202*, they each have a different sequence.
[j]*let-7a* and *let-7*f are clustered.
[k]*let-7b* is clustered with *let-7c-2*.

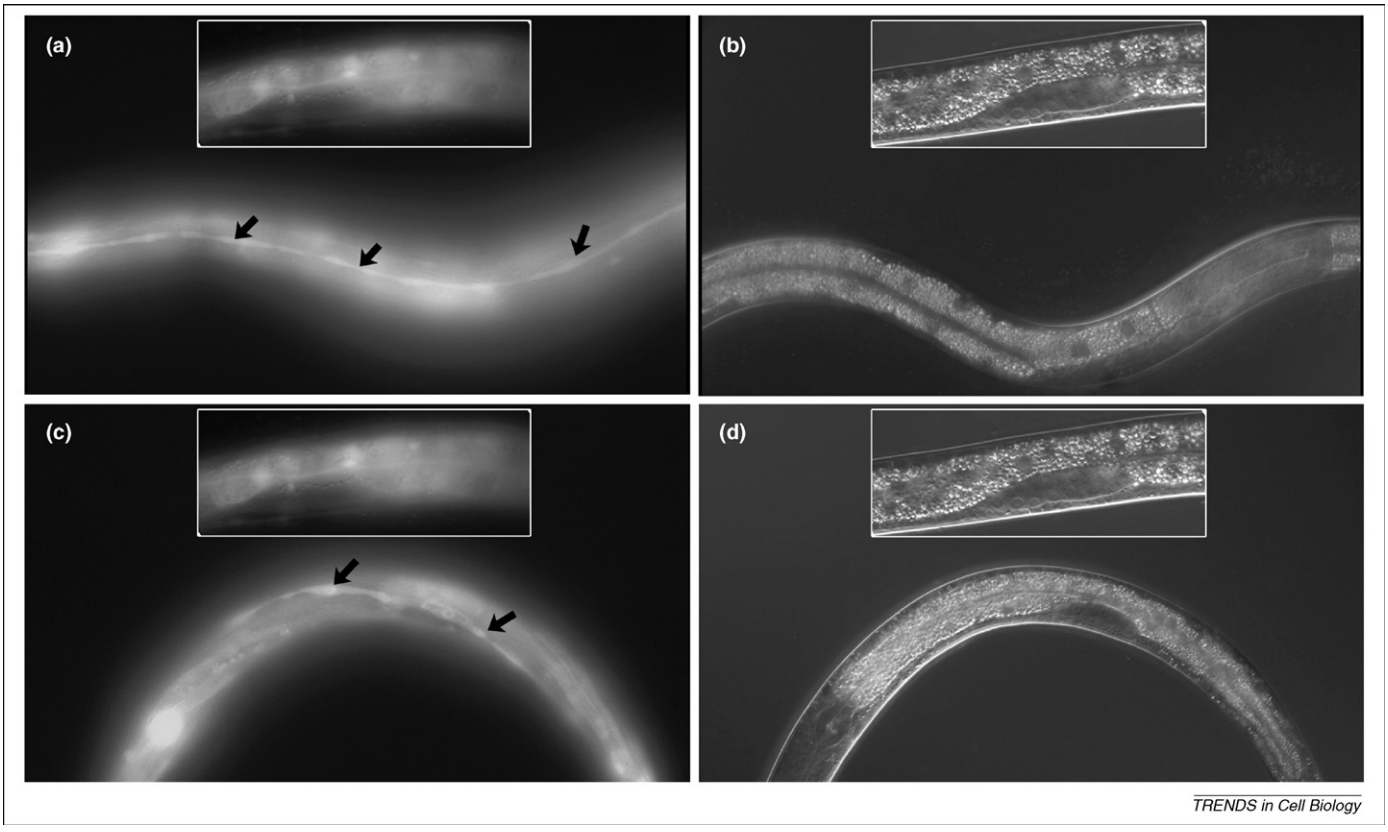

Figure 3. Temporal expression of *let-7* in *C. elegans*. Hypodermal seam cells are used for studying developmental timing in *C. elegans*. *let-7* loss-of-function mutants exhibit a retarded phenotype where the seam cells reiterate a larval 4 (L4)-stage cell division at the L4-to-adult transition when they normally terminally differentiate [3]. A GFP transcriptional fusion construct driven by the *let-7* promoter is not expressed in the hypodermal seam cells during the early larval 3 (L3) stage [inset in (a) and (c)]. **(a)** In a wild-type genetic background, GFP marker expression in seam cells is seen in L4. **(d)** When crossed into a *lin-28* loss of function mutant, there is precocious GFP-marker expression in the seam cells in the early L3 [49]. **(a,c)** GFP images. **(b,d)** Corresponding Nomarski images of (a,c), respectively. Inset images are the GFP transcriptional fusion in a wild-type genetic background. Arrows indicate the line of seam cells.

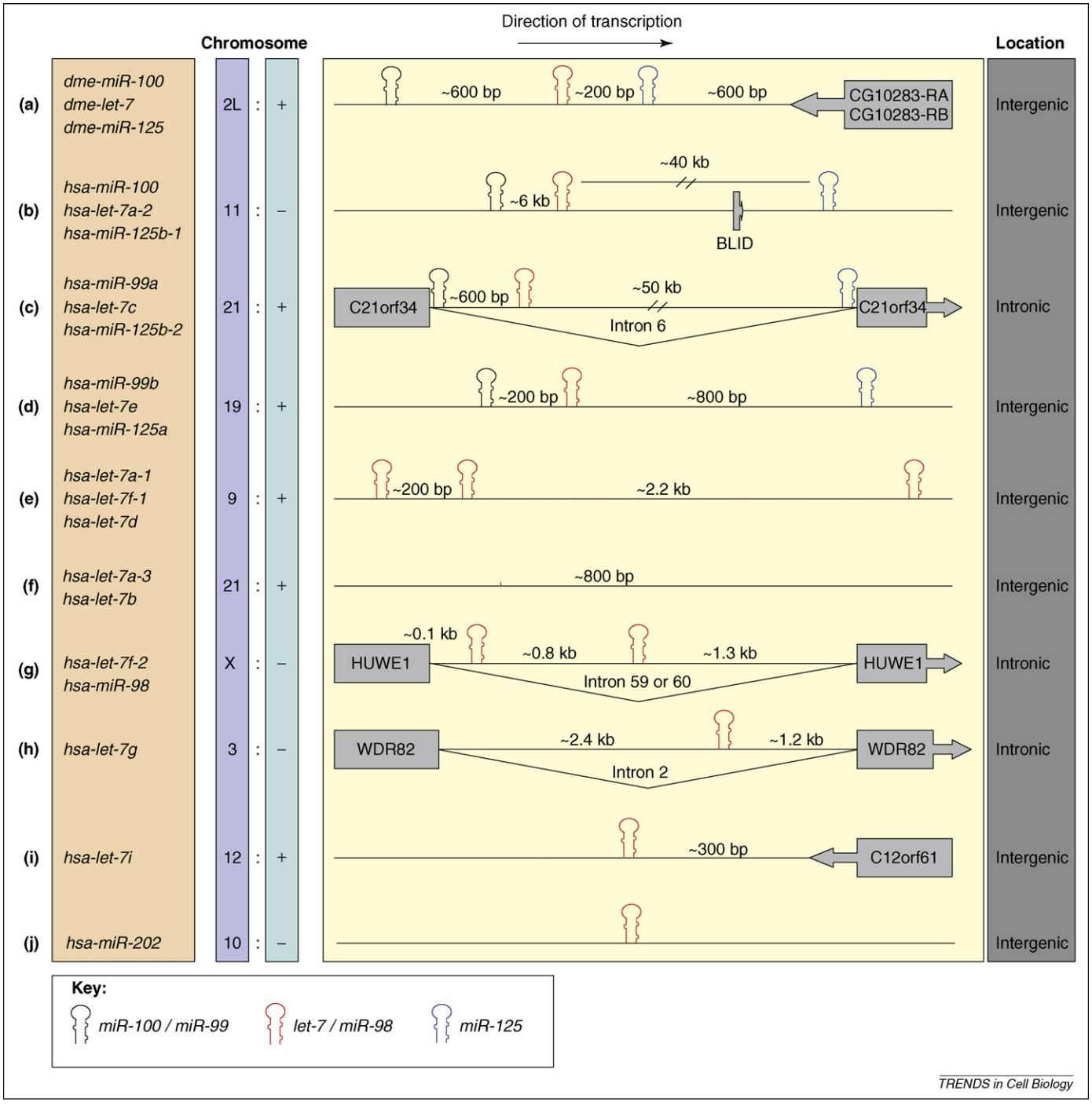

Figure 4. Genomic organization of the *let-7* family in *Drosophila* and humans. *let-7*-family member sequences and clustering patterns are conserved across many species. **(a)** There is only one *let-7* in *Drosophila*. It is in an intergenic cluster with *miR-100* and *miR-125*. (a–d) The conserved *miR-100* or *miR-99*, *let-7* or *miR-98* and *miR-125* cluster is repeated three times in the human genome, but only once in flies. However, unlike in flies, in humans, *miR-100* or *miR-99* and *let-7* are much closer to each other than to *miR-125*, which is often many kilobases (kb) away. **(b)** There is a small gene in the antisense direction between *let-7* and *miR-125b-1*. **(c)** All three miRNAs are in the sense orientation in intron 6 of *C21orf34*. **(d–f)** These clusters are all intergenic and not close to other genes; therefore, the flanking genes are not shown. **(g)** The cluster is in the sense orientation within an intron of *HUWE1*. **(h)** *let-7g* is a key gene in many cancers and its genomic location within an early intron of a WD40 gene, such as *WDR82*, is conserved across many organisms. **(i,j)** *let-7i* and *miR-202* are intergenic and not within any clusters. Red, black and blue hairpins represent *miR-100* or *miR-99* family members, *let-7* family members and *miR-125* family members, respectively. The approximate distances between miRNAs or close protein-coding genes are given. Chromosome strands are indicated by + or –. Figure not to scale. Figure information sourced from http://microrna.sanger.ac.uk/sequences/.

formation of adult alae [3]. A majority of the loss-of-function animals die by bursting through their vulvas and the null mutant is, therefore, lethal (Let). It is currently unknown exactly why *let-7* animals burst through the vulva but, normally, vulval cells connect to seam cells for structural integrity of the vulva, and these connections might be perturbed in *let-7* mutants.

Single mutants for the closely related *let-7*-family member genes *mir-48*, *mir-84* and *mir-241* exhibit low-penetrance developmental defects, although these single-

mutant animals do display a strong radio-resistant phenotype in vulval cells, probably owing to their repression of RAS (rat sarcoma) (see later) [37]. The *mir-48*, *mir-84* and *mir-241* triple-knockout mutant exhibits a retarded seam-cell phenotype in which the seam cells repeat an L2-type division in L3 (Figure 1c). The triple mutant also has incomplete adult alae and dies by vulval bursting at the L4-to-adult transition [36]. The triple mutant has a higher penetrance of the retarded phenotype compared with single or double knockouts of these miRNAs, indicating cooperation or functional redundancy among the three miRNAs.

In *C. elegans*, many of the *let-7*-family members target additional members of the heterochronic pathway [3,36,38–41] (Figure 1b). Some targets of *let-7* in *C. elegans* are also conserved targets in other organisms [27,42–44]. One important example is the mRNA encoding the *C. elegans* Ras GTPase-family member *let-60*, which was also shown to be a target for *let-7* in humans. This provided the first evidence of a miRNA functioning in a tumor-suppressor pathway to curb the oncogenic potential of the human *KRAS* (Kirsten-RAS) oncogene [45].

Expression patterns of the *let-7* family in *C. elegans*

Expression and regulation of *let-7*-family miRNAs are best understood in *C. elegans*. *let-7* is necessary for regulating the proper timing of *C. elegans* seam development at the last larval stage and, as such, expression of *let-7* is restrained until that time in development. *let-7* can first be detected by northern blotting at the L3 stage of development and reaches maximal expression during the L4 stage, consistent with its role in promoting cell-cycle exit and terminal differentiation of seam cells at the end of the L4 stage [46].

In *C. elegans*, there are three primary *let-7* transcripts (Figure 1d). The two longest primary transcripts are *trans*-spliced to a splice leader 1 (SL1) sequence to create the third, and smallest, primary transcript [11]. *Trans*-splicing is less common than *cis*-splicing and involves the splicing of one of two leader RNA sequences onto the 5′ end of some mRNAs [47,48]. *Trans*-splicing has also been documented in other phyla, including Euglenozoa, Platyhelminthes, and rare instances in chordates [47]. It is this SL1 transcript that is the substrate for Drosha (also known as DRSH-1) processing. Two *let-7* mutations, *let-7(mn112)* null [20] and *let-7(mg279)* loss-of-function [3], obtained from genetic screens for developmentally defective *C. elegans*, affect the *trans*-splicing site and primary transcript structure (Figure 1d). The *let-7(mn112)* null deletes 193 nt, including the splice acceptor sequence, and is unable to make the SL1 transcript. The splice acceptor sequence is unaffected by the 27-nt deletion of *let-7(mg279)*, but the context of the sequence is altered [11]. This mutant is able to make the SL1-transcript but at levels lower than for wild type. To date, *C. elegans let-7* is the only miRNA shown to be *trans*-spliced.

Green fluorescent protein (GFP)-reporter constructs driven by the *let-7* promoter show *let-7* expression in many tissues, including the seam cells, vulva precursor cells (VPCs) and vulva cells, distal tip cells and neurons throughout the animal [46,49]. Importantly, there is a change in GFP expression in the seam cells and VPCs over time [46,49] (Figure 2). In these tissues, expression of the GFP marker is not seen until L3, which corresponds well to expression data collected by northern blots and reverse transcriptase (RT)–PCR analyses [11,46,49,50]. The seam cells and VPCs are commonly used tissues for studying the timing of development by the heterochronic pathway, and *let-7* loss-of-function mutants have developmental defects in these tissues [3]. Therefore, the temporal expression of GFP in the seam cells and VPCs correlates with the role of *let-7* in the development of these tissues.

Promoter deletion analyses using the GFP-reporter construct described earlier revealed a temporal regulatory element (TRE) that is conserved in several nematode species and is responsible for the timing of GFP expression specifically in the seam cells [49]. The TRE is a 116-nt sequence located ~1200 nts upstream of the mature *let-7* sequence. Removal of TRE from the *let-7* GFP transcriptional fusion construct causes a seam-cell-specific loss of GFP expression. Additionally, electromobility shift assay (EMSA) data, using TRE as the probe, indicate that the TRE is bound by an unknown *C. elegans* protein designated the temporal regulatory element binding protein (TREB). Together, the GFP and EMSA data indicate that the TRE sequence is a necessary enhancer element that could be bound by a seam-cell-specific transcription factor essential for activating *let-7* expression in the seam cells [49].

As mentioned, the *let-7*-family members *mir-48*, *mir-84* and *mir-241* are also part of the heterochronic pathway in *C. elegans* [36] (Figure 1b), with *mir-48*, *mir-84* and *mir-241* functioning early in the heterochronic pathway to control the L2-to-L3 transition in the hypodermis [36] (Figure 1c). Northern blots show that mature *mir-48* and *mir-241* are first expressed at early L2, with expression increasing as the animal develops [46,51]. *mir-84* is expressed even earlier, with low levels at L1, and expression gradually increases over the course of development [46].

Transcriptional GFP fusions with promoters from *mir-48*, *mir-84* and *mir-241* reveal overlapping, yet distinct, expression patterns. *mir-48* and *mir-84* are expressed in the vulva and VPCs, starting at stages L3 or L4, but differ depending on the individual VPC in which they are expressed [46]. *mir-241* is also expressed in the vulva at L4, but the exact VPC identity of the VPCs has not been determined [51]. *mir-48* and *mir-84* are both expressed in the seam cells but at different stages: L2 and L4, respectively. *mir-84* is expressed in many parts of the somatic gonad at L3, including distal tip cells, uterine cells, spermatheca and the anchor cell [45,46]. Finally, *mir-241* is temporally expressed in the body-wall muscles at L2 and the excretory canal at L3 [51].

mir-48 is the only family member, beyond *let-7* itself, for which anything is known about *cis*-elements in the promoter. *C. elegans* strains *lin-58(ve33)* and *lin-58(ve12)* each contain a point mutant in a guanine and cytosine (GC)-rich inverted repeat upstream of the mature miR-48 sequence [51]. This repeat is a sequence that is 5′ to most *C. elegans* miRNAs, but it is not known if a mutation in this region affects the expression of any other *C. elegans* miRNA [51,52]. The stronger allele, *ve33*, causes early transcrip-

tion of the *mir-48* gene and an increase in miR-48 levels without any defect in miRNA processing. When this mutation is placed in a transcriptional fusion reporter, it causes precocious and increased expression of GFP in the seam cells and vulva. These data indicate that the *lin-58* alleles are mutations in a binding site for a repressor of *mir-48* expression [51]. Interestingly, *mir-48* and *mir-241* lie in the same direction 1.7 kb apart (Figure 4c), but are apparently regulated differently at the transcriptional level because levels of *mir-241* are unchanged in *lin-58(ve33)* [46,51].

Although the *let-7*-family members *let-7*, *mir-48*, *mir-84* and *mir-241* begin their expression at different times, they all seem to be regulated by other members of the heterochronic pathway. Loss of the heterochronic genes *lin-4* or *daf-12*, the latter encoding a nuclear hormone receptor, leads to a decrease in expression of the mature form of all of these *let-7*-family members on northern blots [46,49]. To date, no direct gene regulators of these miRNAs, transcriptional or post-transcriptional, have been identified in *C. elegans*.

Currently, nothing is known about the expression and regulation of the other *C. elegans let-7*-family members: *mir-793*, *mir-794*, *mir-795*, *mir-1821* and *mir-265*. Like the *C. elegans let-7*-family members mentioned, all of these family members are intergenic. Most of them occur as individual genes in the genome, but *mir-794* and *mir-795* are clustered together and *mir-1821* is clustered with an unrelated miRNA, *mir-792* (http://microrna.sanger.ac.uk/sequences/) (Figure 4).

let-7 in more complex organisms

Although many characteristics of *let-7* are conserved across species, there are some noticeable differences between the *let-7* family genes in *C. elegans* and orthologous genes in more complex organisms. First, the size of the *let-7* families in invertebrates is smaller: only nine related family members in nematode (Figure 1a) and one *let-7* in fly [33,53], compared with 13 closely related *let-7* family genes in humans (Figure 3). Second, patterns of *let-7* gene clustering and genomic positioning in the genomes of flies and higher organisms are highly conserved [53–55] (Table 1), but this organization is not conserved in *C. elegans.*

The appearance of mature *let-7* later in development is preserved in many organisms [4,56–58], including zebrafish and mouse. Given this common feature of *let-7* in animals, one might expect the same to be true in humans, but this has not been tested. Although this expression characteristic is clear, the mechanisms used for this temporal regulation are not always conserved. Research has shown that the temporal expression of mature *let-7* could be regulated at both the transcriptional and post-transcriptional levels [11,49,57,59]. In many lower eukaryotes, such as *C. elegans* and *Drosophila* (see later), temporal regulation occurs at the level of pri-*let-7* transcription [49,55,56]. Pri- and pre-*let-7* expression is not seen until later developmental stages, and this expression is concurrent with the appearance of mature *let-7* [11,55]. Whereas the amount of mature *let-7* processed from the pre-miRNA seems not to be post-transcriptionally controlled in *C. elegans* [11], in mammals, expression of mature *let-7* seems to be, in part, controlled post-transcriptionally (see later) [59,60]. The pri-*let-7a-1*– pri-*let-7d*– pri-*let-7f-1* cluster, the *let-7f-2* –*mir-98* cluster, *let-7g* and *let-7i* are all expressed throughout development. However, the mature forms of these miRNAs are not seen until later in embryonic development, indicating that regulation of temporal expression of mature *let-7* in these organisms has a post-transcriptional component (see later) [59,60].

let-7 in Drosophila

The *Drosophila* genome contains only a single *let-7* gene, and the mature sequence is 100% identical to *let-7* from *C. elegans,* as mentioned earlier [53]. *Drosophila let-7* is first detected by northern blots at the end of the 3rd larval instar [55,56], and a role for *let-7* in the transition from the 3rd instar to the pupal stage has just now become apparent. Recently, *let-7* deletion mutants were described that demonstrate a role for *let-7* in regulating the timing of formation of neuromuscular junctions in the abdominal muscles [24,25] and cell-cycle exit in the wing [25]. The neuromusculature in *let-7* mutants retains juvenile features into adulthood and results in defects in the adult behaviors of flight, motility and fertility. Furthermore, *let-7* exerts this function through the down-regulation of the *abrupt* gene to ensure that the abdominal neuromusculature is remodeled correctly at the larval-to-adult transition [24,25].

Like *C. elegans, Drosophila* is also an ecdysozoan. The defining feature of ecdysozoans is that their development involves a series of molts in which they shed their cuticles in a process known as ecdysis [61]. In *Drosophila*, a pulse of the ecdysteroid hormone ecdysone is released before each molt [62]. Expression of pri-, pre- and mature *let-7* coincide with the ecdysone pulse at the late-third-larval instar and prepupae stages, indicating that temporal expression is transcriptionally controlled [54–56]. The pri-miRNA is transiently expressed, whereas the mature form is present through pupal development and into adulthood [55]. The long-term presence of mature *let-7* could be caused by increased stability of the miRNA resulting from continual involvement with miRISC. Whether ecdysone is the cause of *let-7* expression is the subject of debate [54,55].

Like *Drosophila*, all forms of *let-7* in *C. elegans* are detected by northern blots and RT–PCR analyses in a late larval developmental stage, L3 [3,11], and the pri-miRNA is transiently transcribed, whereas the mature *let-7* continues to be expressed [11]. However, *C. elegans* does not have ecdysone, and the identity of the ecdysteroid hormone that presumably controls the timing of each molt is unknown [63]. Nevertheless, there are *C. elegans* genes in which expression does fluctuate with the molting cycle [35,63].

let-7 *in vertebrates*

Although more complex metazoans do not go through cuticular molts, they do have specific developmental events that need to occur at the correct time and distinct timed events that need to be regulated, such as organogenesis [35]. Whereas *let-7*-family miRNAs have a distinct temporal pattern of expression in vertebrate development

[7,26,30,56], a direct role for *let-7* in vertebrate development has not been shown to date. The delay in identifying this role is probably caused by the difficulties associated with knocking-out multiple *let-7*-family members (see later) in the same animal and the possible redundancy associated with such large families.

One of the major functions of *let-7*, and perhaps miRNAs in general, is to promote differentiation of cells [9,24,29–32]. In *C. elegans*, this role is demonstrated by the necessity of *let-7* in halting the stem-cell-like divisions of the seam cells and their consequent adoption of a fully differentiated state [3]. In higher organisms, the levels of *let-7* rise during embryogenesis [27], and pri-*let-7a* and pri-*let-7e* and mature *let-7a*, *let-7c* and *let-7e* are upregulated during mouse brain development [30]. In another study, *let-7* was found to be expressed in breast-stem-cell progenitors as they differentiate [64]. In addition, reduced expression of multiple *let-7* members have been found to be associated with human cancers [9] (see later) and with cancer stem cells [29], leading to the strong suggestion that, as in *C. elegans*, *let-7* genes function to promote terminal differentiation in development and function as tumor suppressors.

In vertebrates, the *let-7* family has many more members than in *C. elegans* and *D. melanogaster* [53] (Table 1), including multiple forms that seem to be modified post-transcriptionally (not shown) [65]. Interestingly, not only are the sequences and timing of expression of *let-7* and many of its family members conserved across species [4,53], but the genomic organization and clustering of miRNAs for many of the *let-7* members is also conserved [54,55,66,67] (Table 1). The selective pressure to conserve these clusters further underscores the importance of these genes and the need to maintain their correct expression patterns both spatially and temporally.

A cluster of *miR-100* or *miR-99*, *let-7* and *miR-125* family members commonly occurs in almost all *let-7*-expressing species, including *Drosophila* but not *C. elegans* [54] (Table 1 and Figure 5a–d), Although the order of the miRNAs on the chromosomes is conserved among animal phyla, the spacing between the miRNAs is not well conserved, with *mir-125* sometimes many kilobases away (Figure 5a–d). *Drosophila* is the only organism, so far, in which it has been shown that all three miRNAs are transcribed as one pri-miRNA transcript before processing by the microprocessor complex [24,54]. It is unknown whether the microprocessor recognizes each hairpin in the polycistronic transcript or if other processing must occur before Drosha can recognize the individual pri-miRNAs.

Another conserved cluster is that of *let-7a*, *let-7d* and *let-7f* (Table 1 and Figure 5e). This in cluster could be involved in B-cell lymphomas because it seems to be directly regulated by the oncogenic transcription factor MYC (v-myc myelocytomatosis viral oncogene homolog) [68]. MYC is thought to negatively regulate this cluster and a few other miRNAs by binding directly to their promoters (Figure 6a). High expression of MYC leads to a large downregulation of these miRNAs, which in turn might

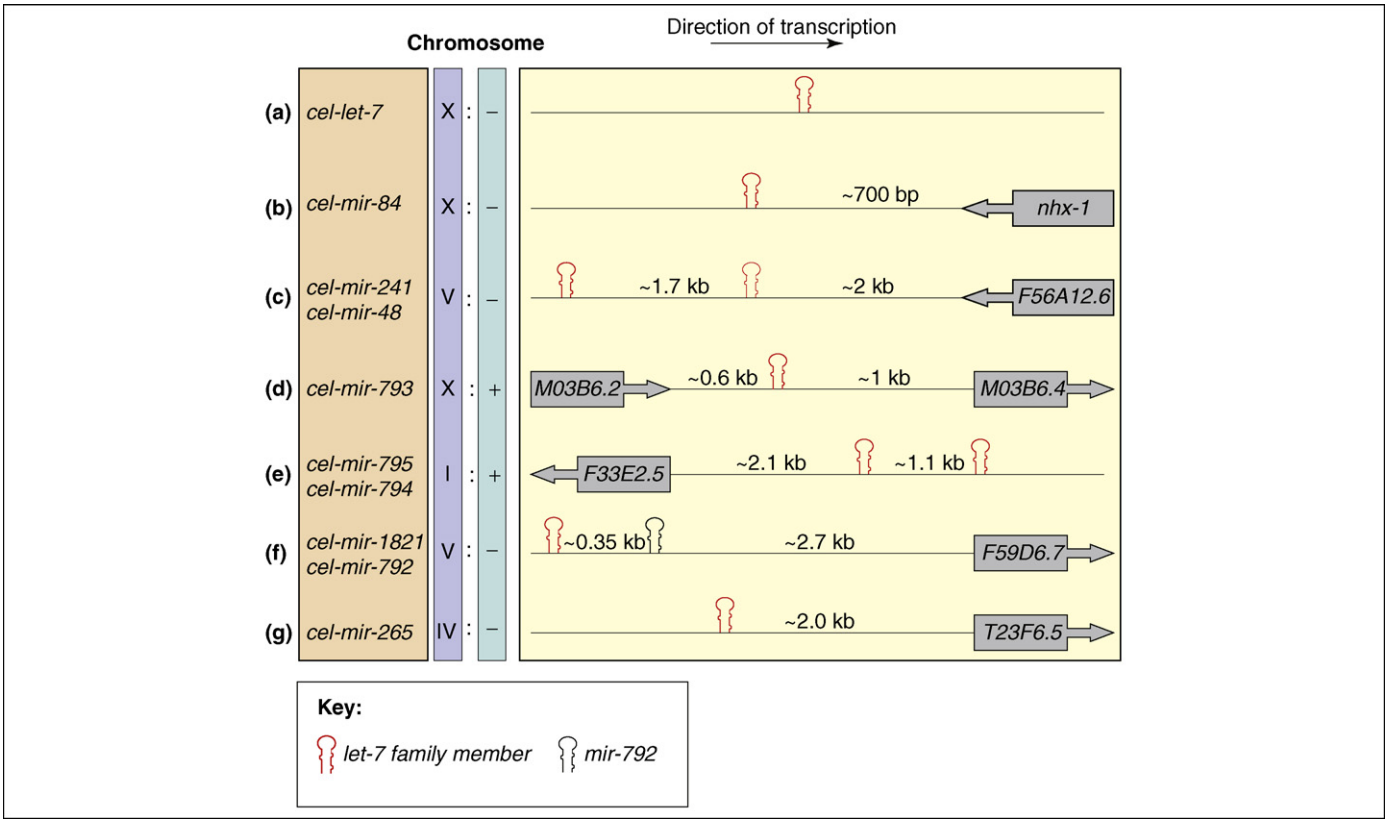

Figure 5. Genomic organization of the *let-7* family in *C. elegans*. All of the C. elegans *let-7* family members are intergenic. **(a,b,d,g)** let-7, mir-84, mir-793 and mir-265 all occur as individual genes in the genome. (There are three linked pairs of *let-7*-family miRNAs.) **(c)** Cluster 1 containing mir-241 and mir-48. Despite their close proximity, these two miRNAs seem to be transcriptionally regulated separately [51]. **(e)** Cluster 2 containing mir-795 and mir-794. **(f)** Cluster 3 containing mir-1821 and an unrelated miRNA, mir-792. Red and black hairpins represent *let-7*-family members and mir-792, respectively. The approximate distances between miRNAs or close protein-coding genes (gray boxes) are given. Chromosome strands are indicated by + or –. Figure not to scale. Figure information sourced from http://microrna.sanger.ac.uk/sequences/.

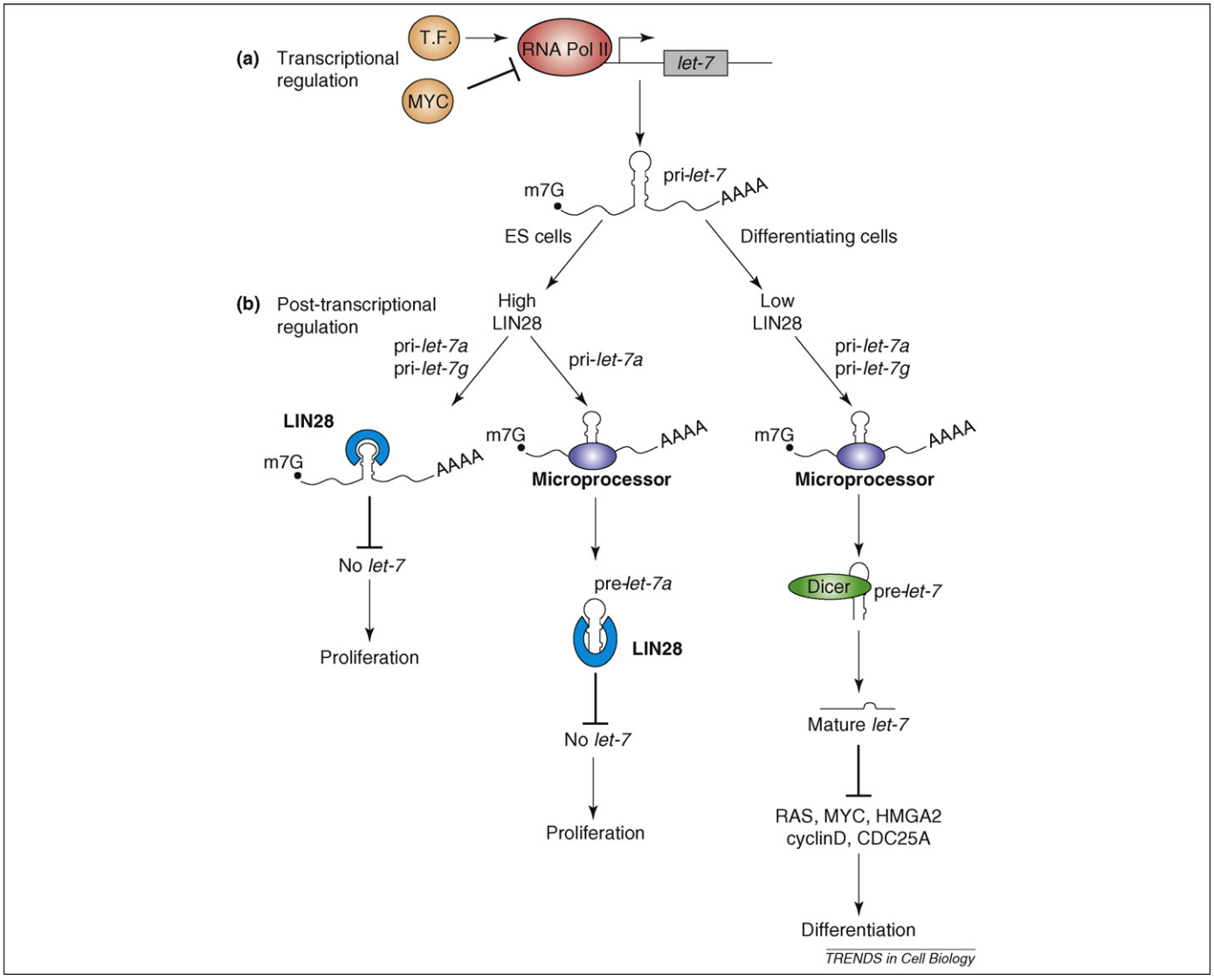

Figure 6. Regulation of certain members of the *let-7* family in mammals. Members of the *let-7* family are regulated at both the transcriptional and post-transcriptional levels. **(a)** In general, *let-7* genes are transcribed by RNA polymerase-II (Pol II) as the capped and polyadenylated pri-*let-7* RNA [10]. Transcription of several pri-*let-7* genes, including the *let-7a*, *let-7d* and *let-7f* cluster and *let-7g*, is repressed by direct binding of MYC [68]. To date, no transcription factors that directly enhance *let-7* transcription have been identified. In *C. elegans*, a hypothetical protein called temporal regulatory element (TRE)-binding protein (TREB) is believed to regulate transcription of *let-7* positively through the TRE *cis*-element [11]. **(b)** *let-7* is post-transcriptionally regulated by LIN28. When LIN28 is present, as in embryonic stem (ES) cells or undifferentiated cells, it binds to the loop portion of the *let-7a* and *let-7g* stem–loops [60,77]. LIN28 binding inhibits the microprocessor from processing pri-*let-7a* and pri-*let-7g*, but the exact mechanism behind this is unknown [59,60,77]. Some pri-*let-7a* processing into pre-*let-7a* is not inhibited but, in this case, LIN28 binds to the pre-*let-7a* stem and prevents Dicer processing, so no mature *let-7a* is made [78]. As cells differentiate, the levels of LIN28 decrease, enabling the microprocessor to bind and process pri-*let-7* into the pre-*let-7* hairpin [59]. At this time, because LIN28 and *let-7* are in a feedback loop [78], it is unclear whether the decrease of LIN28 or the increase in *let-7* is the initiating factor for differentiation. Once the mature miRNA has been processed from the pre-miRNA, *let-7* is incorporated into miRISC and post-transcriptionally downregulates its targets, such as the cell-cycle effectors RAS, MYC, HMGA2 (high mobility group AT-hook 2), cyclin D and CDC25A [8,9], enabling the cells to terminally differentiate.

promote tumorigenesis [68]. Further evidence has shown that *let-7a* represses expression of MYC in a Burkitt lymphoma cell line, indicating that MYC and *let-7a* work in a negative-feedback loop [69].

When both are present, *let-7b* and *let-7a-3* are also clustered together across several species (Table 1). Although they are separated by 1 kb (Figure 5f), they seem to be regulated separately through epigenetic methylation. The methylation state of CpG islands (areas of the genome that have a high content of cytosine–guanine dinucleotides) is an epigenetic mechanism used to control gene expression, with hypermethylation inhibiting expression and hypomethylation enabling more expression [70]. *let-7a-3* is within a CpG island that is usually heavily methylated [71,72], although *let-7b* does not seem to be within a CpG island. However, one study found that, in two out of eight lung adenocarcinomas and in the two lung-cancer-cell lines tested, *let-7a-3* was hypomethylated. In the hypomethylated state, pri-*let-7a-3* expression was upregulated threefold, and the increased expression of *let-7a-3* had an oncogenic effect, explaining the reason for its tight epigenetic regulation [71]. An oncogenic role for *let-7a-3* is unusual because most other *let-7* miRNAs seem to function as tumor suppressors [9,71]. For example, *let-7b* seems to work in a tumor-suppressor role, because a low level of *let-7b* is a poor prognostic indicator of survival in serous

ovarian cancer, the most commonly occurring subtype [73]. Perhaps the decreased expression of *let-7b* contributes to a less differentiated cellular state that results in a more aggressive cancer.

Contradictory to these results, another study examining *let-7a-1, let-7a-2* and *let-7a-3* in ovarian cancer found no significant change in the overall expression of mature *let-7a* when comparing tumor samples of patients with <42% methylation to >90% methylation [72]. An important difference between the lung cancer and ovarian cancer studies was that the levels of pre-*let-7a-3* were specifically measured in the lung cancer study [71], whereas the ovarian cancer study measured total mature *let-7a* levels and did not distinguish between the pre-*let-7a-1*, pre-*let-7a-2* and pre-*let-7a-3* isoforms [72]. The hypomethylation of *let-7a* in these ovarian tumors still led to an increase in other cancer prognostic-gene products and a decrease in patient survival, thus corroborating the lung cancer data [72].

Finally, an intriguing monocistronic family member is *let-7g* (Figure 5h). This miRNA is involved in the development of lung cancer and B-cell lymphoma [74] and is directly repressed by MYC [68] (Figure 6a). In animals that have *let-7g*, this miRNA is always in an intron of a gene containing WD-40 repeats, usually the second intron. The WD-40-containing gene is often identified as *WDR82*, as in human, or its possible homologs in other organisms [57,60]. WD-40-repeat-containing genes are involved in a wide variety of functions, including signaling, regulation of transcription and the cell cycle, and apoptosis [75]. The WDR82 protein is involved in recruiting the Setd1A histone H3–Lys4 methyltransferase complex to initiation sites for transcription by RNA polymerase-II in human cells, which leads to formation of histone modifications that mark the transcription initiation site of actively transcribed genes [76]. It is tempting to speculate that there is a biological reason for the conservation of the genomic location of *let-7g* within this one class of genes. Perhaps *let-7g* regulates *WDR82* mRNA expression or, maybe, both genes are involved in the same biological process, such as promoting cellular differentiation.

Post-transcriptional regulation of *let-7* miRNAs in mammalian cells

Temporal regulation of *let-7* in mammals seems to be, in part, controlled at a post-transcriptional level because the levels of the pri-*let-7a-1*–pri-*let-7d*–pri-*let-7f-1* cluster, pri-*let-7f-2–mir-98* cluster, pri-*let-7g* and pri-*let-7i* hold constant throughout development (as mentioned earlier) [57,59]. Also, during differentiation of mouse embryonic stem (ES) cells and embryocarcinoma (EC) cells, the levels of pri-*let-7a* and pri-*let-7e* increased in the differentiated cells in comparison with those of the undifferentiated cells. However, corresponding increases in the levels of mature *let-7* were not seen, indicating the presence of post-transcriptional regulation. Similarly, a slight increase in the pri-*let-7* levels that was not matched by an increase in mature levels was also detected during brain development [30].

Recent studies have found that a homolog of one of the *C. elegans* heterochronic genes, *LIN28*, is directly involved in the post-transcriptional regulation of the *let-7* family of miRNAs in mammalian cells [59,60,77] (Figure 6b). Researchers found that, in mouse ES cells and mouse postnatal day (P)19 embryonic carcinoma cells, pri-*let-7g* is plentiful, but there is no mature *let-7g* [59,60]. However, mature *let-7g* was easily detected after these cells had differentiated [59]. Similarly, HeLa cells, which have some level of differentiation, also have high mature *let-7g* levels [60]. In undifferentiated P19 cellular extracts, LIN28 was identified as a protein factor that bound to either pre-*let-7g* [59] or a conserved loop portion of the stem–loop [60,77]. LIN28 binding the loop region of the pri-*let-7* stem–loop sequesters the pri-*let-7* and prevents its further processing by the microprocessor [60,77] (Figure 6b). The result of this LIN28-mediated regulation is a steady level of pri-*let-7* and an undetectable level of mature *let-7* [59,60]. The exact mechanism of how LIN28 binding prevents microprocessor processing is unknown.

The function of *LIN28* in mammals has only recently been elucidated, whereby it was found to be one of the four genes necessary for the reprogramming of human somatic cells back to an undifferentiated state [31]. Research now indicates that LIN28 exerts this function by regulating *let-7*, which is known to be important for promoting cellular differentiation. As ES cells differentiate, LIN28 levels decrease at the same time that the levels of mature *let-7* increase [59]. High LIN28 levels prevent the processing of either pri-*let-7* and/or pre-*let-7,* possibly depending on which *let-7* isoform (*let-7a* or *let-7g*) is measured [59,60,78]. For *let-7a* and *let-7g*, LIN28 binds to the loop region of what will become the pre-miRNA hairpin and inhibits Drosha processing of the pri-*let-7*s in the nucleus [60]. As levels of LIN28 decrease, the processing is no longer inhibited, thus, enabling the microprocessor to convert these pri-*let-7*s into their pre-miRNA forms [59,60] (Figure 6b). However, in the case of *let-7a*, not all of the pri-miRNA processing is inhibited and some pre-*let-7a* is made [60,78]. LIN28 also regulates this pre-*let-7a* by preventing the pre-*let-7* from being turned into mature *let-7a* too early by binding to the pre-*let-7a* stem and preventing Dicer processing in the cytoplasm [78]. This series of post-transcriptional regulations is possible because LIN28 is known to shuttle between the nucleus and cytoplasm [79]. Post-transcriptional regulation of miRNAs by LIN28 is currently specific to the *let-7* family and *mir-128* [59,60,78].

Therefore, it is possible that one of the reasons for the mis-regulation of *let-7* in cancer is increased LIN28 activity [59]. In cancer, increased levels of LIN28 could limit the amount of mature *let-7* made [80]. Reduced *let-7* levels prevent it from functioning as an effective tumor suppressor, enabling unchecked cellular growth, and promoting a less differentiated state.

Three possibilities as to why the production of mature *let-7g* in ES and EC cells is controlled at the post-transcriptional level (as opposed to applying transcriptional controls) have been proposed. First, it could enable for the rapid production of mature *let-7g* and other *let-7*-family members at the exact time they are needed. Second, if even a small amount of *let-7* could promote differentiation, then post-transcriptional regulation could provide an extra step to ensure tight regulation of *let-7* expression to maintain an

undifferentiated state. Third, it enables an intronic miRNA, such as *let-7g*, to have an expression pattern that is distinct from that of its host gene [59].

Concluding remarks

let-7 remains one of the key miRNA regulators in development and cancer. The *let-7*-family miRNAs are highly conserved across animal species in sequence, at what developmental stage they are expressed and in their organization as clusters or within specific coding genes in the genome. *Drosophila* and *C. elegans let-7*-family members are expressed in later larval stages when cells, such as *C. elegans* seam cells, end their stem-cell-like programming and terminally differentiate. In more complex organisms, *let-7* expression dramatically increases as cells become more differentiated. Given its conserved sequence, expression and roles in fundamental biological aspects across phylogeny, it is no surprise that *let-7* is carefully regulated in space and time.

Interestingly, in addition to regulation of *let-7* expression at the transcriptional level, as in *C. elegans* and *Drosophila,* regulation can also occur at the post-transcriptional level, as seen in mammals. Recently, the stem-cell factor LIN28 has been shown to be a major contributor to the post-transcriptional regulation of mammalian *let-7* at both the pri-*let-7* and pre-*let-7* processing stages. Interestingly, *C. elegans* LIN28 protein is a known negative regulator of *let-7* with a direct or indirect role in transcriptional regulation of *let-7* (Figure 1b), because loss of LIN28 leads to an increase in mature *let-7* levels and early expression of a GFP transcriptional fusion driven by the *let-7* promoter [49] (Figure 2). However, it is not clear whether it functions to regulate processing of *let-7*, given that pre-*let-7* and mature *let-7* levels are co-regulated. It is possible that LIN28 regulates *let-7* subtly or regulates processing of other *let-7* family members in *C. elegans*.

The emergence of LIN28 as a post-transcriptional regulator of the *let-7* family raises many more questions to be investigated. What factors and conditions dictate if LIN28 will regulate at the microprocessor and/or Dicer processing steps? Does LIN28 regulate all *let-7* family members in the same manner, or is it isoform specific, such as might be the case with *let-7a* and *let-7g*? What features of pri- and pre-*let-7*s does LIN28 specifically recognize that are not present in other miRNAs? Does LIN28 regulate more than the *let-7* family and *mir-128* and, if so, is there a common characteristic among their functions, such as promoting differentiation, or cell types? Finally, are there other proteins that function like LIN28 to regulate other miRNAs?

Another recent development is that miRNA transcription and microprocessor processing seem to be coupled in mammalian cells, and transfected pri-miRNA constructs are more efficiently processed to mature miRNAs if they are retained at the transcription site due to lack of cleavage and polyadenylation signals, or are flanked by exons [81]. It has yet to be investigated if the four out of 13 human *let-7*s that are intronic are more efficiently processed from pri-*let-7* to mature *let-7* than the other nine human *let-7*s. It would be interesting to learn if LIN28 regulation decouples transcription and processing and the mechanism by which LIN28 might do it.

Clearly, the function of *let-7* as a tumor suppressor in many types of cancers makes the understanding of how *let-7* expression is regulated extremely important. We postulate that this understanding could lead to the development of novel cancer treatments. For example, small molecules or miRNAs and siRNAs that target LIN28 or MYC would be expected to promote or reactivate *let-7* expression in tumors with potentially beneficial effects on tumor growth.

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