The evolution of our thinking about microRNAs

Victor Ambros

Should one example make the general case?

Our appreciation of the significance of microRNAs to biology at large continues to be an evolving process. The story began more than 15 years ago with the characterization of two genetic loci involved in the control of developmental timing in *Caenorhabditis elegans*: *lin-4* and *lin-14* (**Fig. 1**). Genetic analysis[1] had shown that *lin-4* functions as a repressor of *lin-14*, which encodes a novel protein[2]. So, when Rosalind Lee and Rhonda Feinbaum set out to clone the *lin-4* locus[3], our expectation was that *lin-4* should encode a conventional regulatory protein. Rosalind and Rhonda found that the locus was unusually small and did not seem to contain a conventional coding sequence. They exhaustively disrupted every predicted micro open-reading frame in a 700 base-pair (bp) *lin-4* rescuing construct to prove that the *lin-4* gene product was not a protein, but a small 22 nucleotide non-coding RNA that was apparently processed from a short hairpin precursor[3] (**Fig. 2a**, top). Meanwhile, the Ruvkun lab had identified, in the 3′ untranslated region (UTR) of the *lin-14* gene, conserved sequences that mediate a post-transcriptional developmental regulation of LIN-14 protein abundance[4,5]. By comparing conserved sequences of *lin-4* and *lin-14*, Gary and I spotted the partial sequence complementarity between the *lin-4* RNA and the *lin-14* 3′ UTR[3,5,6] (**Fig. 2b**, top).

While these findings, in retrospect, were sufficient to define the essential characteristics of microRNA regulation of gene expression, there did not seem to be good reasons to think that there should necessarily be other small antisense RNAs like *lin-4*. Although it would have been great if *lin-4* were an 'emissary' of other similar small noncoding regulatory RNAs yet to be discovered, as suggested by Takayama and Wickens[7], as time went on I did not believe it would be so. We had no evidence for *lin-4* sequences in organisms other than closely related nematodes[6], and as *lin-14*, the target of *lin-4*, encoded a novel protein that was not particularly conserved outside of nematodes[2], it seemed likely that the *lin-4*–*lin-14* partnership could be just a nematode-specific curiosity.

Why was I so pessimistic about the prospect of *lin-4* being the harbinger of a diverse class of regulatory molecules of broad importance? One reason was that we found *lin-4* in the process of exploring genetic pathways underlying developmental phenomena in the worm. There was no theoretical need to explain existing phenomena in terms of new mechanisms or new classes of molecules. Transcription factor–mediated regulation of cell fate was a successful model to account for developmental biology, and post-transcriptional mechanisms for gene expression regulation, including those involving 3′ UTR sequences, were satisfactorily conceived in terms of the activities of RNA-binding regulatory proteins.

Another reason not to think that *lin-4* could be the first example of a whole class of regulatory RNAs was the simple fact of its uniqueness. Between 1992 and 2000, despite extensive efforts by Rosalind to screen more distantly related nematodes for *lin-4*, it remained the only example of a small hairpin regulatory RNA. Despite decades of model system genetics and gene cloning, no other example of a small RNA gene product like *lin-4* had been identified. So, maybe *lin-4* really was a peculiarity of *Caenorhabditis* developmental timing mechanisms.

Emerging clues to the generality of the *lin-4* model

Between 1993 and 2000, a number of findings provided hints for a generality of the *lin-4*–*lin-14* style of gene-regulatory interaction. First, we found that *lin-4* regulates not only *lin-14* but also *lin-28*, another gene of the heterochronic pathway, through complementary elements in its 3′ UTR[8]. This finding showed that distinct genes could acquire functional *lin-4* sites and, hence, hinted at a potential evolutionary flexibility for this type of antisense interaction.

Another boost to the status of *lin-4* was the discovery of the central role of double-stranded RNA in RNA interference (RNAi)[9], together with the identification by Hamilton and Baulcombe[10] of small RNAs—antisense RNAs of about the same length as *lin-4*—associated with RNAi in plants. These findings established a context for imagining a relationship of *lin-4* to wider, evolutionarily conserved antisense RNA–mediated gene silencing. The *lin-4* precursor hairpin was essentially a double-stranded RNA. So, one could imagine that the 22-nucleotide *lin-4* RNA could be generated from its precursor and might even function using some of the same molecular machinery involved in RNAi[11]. Worms were indeed shown to be very good at RNAi, so, shouldn't there at least be other RNAs like this in *C. elegans*?

In 2000, the Ruvkun lab identified a second small RNA in *C. elegans,* the product of the *let-7* gene[12], another gene controlling developmental timing in the worm. Like the *lin-4* RNA, the 21-nucleotide *let-7* RNA seemed to be generated from a double-stranded hairpin precursor (**Fig. 2a**, bottom), and it controlled the production of yet another developmental timing regulatory molecule, *lin-41*. Furthermore, the *let-7* RNA also appeared to work through imprecise antisense base-pairing with 3′ UTR sequences of its regulatory target[13] (**Fig. 2b**, bottom). The finding that worms had two distinct examples

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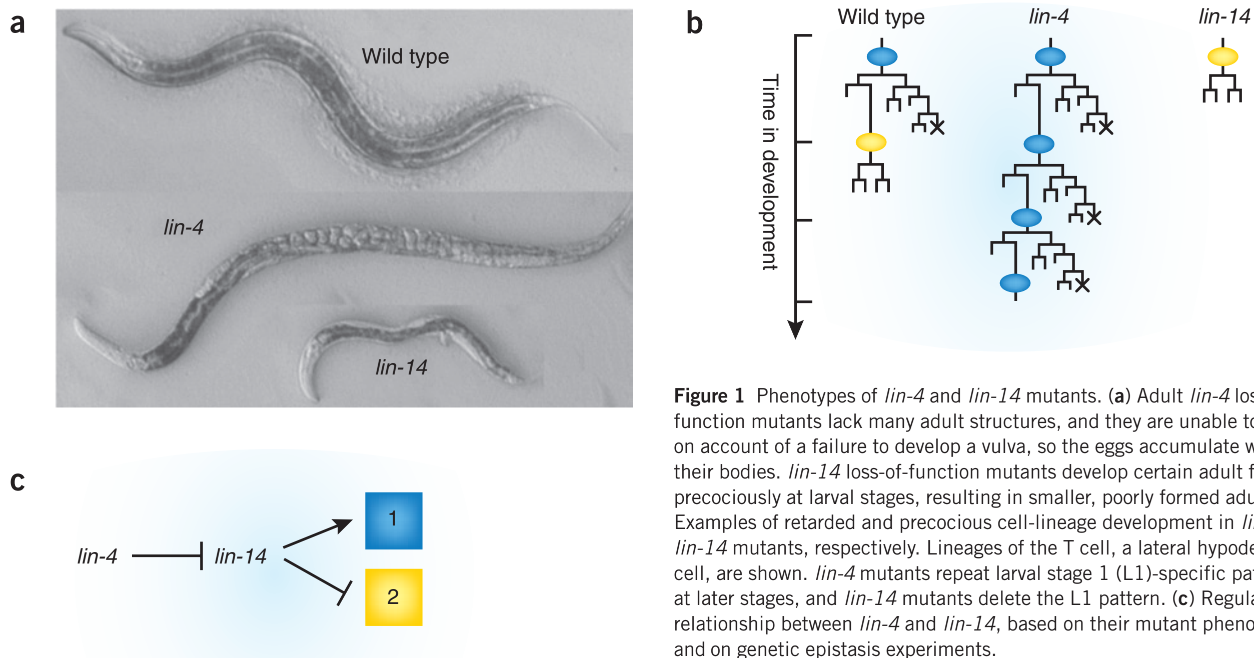

Figure 1 Phenotypes of *lin-4* and *lin-14* mutants. (**a**) Adult *lin-4* loss-of-function mutants lack many adult structures, and they are unable to lay eggs on account of a failure to develop a vulva, so the eggs accumulate within their bodies. *lin-14* loss-of-function mutants develop certain adult features precociously at larval stages, resulting in smaller, poorly formed adults. (**b**) Examples of retarded and precocious cell-lineage development in *lin-4* and *lin-14* mutants, respectively. Lineages of the T cell, a lateral hypodermal cell, are shown. *lin-4* mutants repeat larval stage 1 (L1)-specific patterns at later stages, and *lin-14* mutants delete the L1 pattern. (**c**) Regulatory relationship between *lin-4* and *lin-14*, based on their mutant phenotypes and on genetic epistasis experiments.

of these small RNAs that targeted genes by partial base pairing clearly showed versatility to this mode of gene regulation. Such versatility, considered in light of the idea that *lin-4* and *let-7* RNAs could represent an adaptation of evolutionarily ancient RNAi mechanisms, should have persuaded us that ancient animal and plants ancestors ought to have used this same hairpin-mediated scheme to produce small antisense regulatory RNAs.

Lingering skepticism

If the state of knowledge in early 2000 arguably linked the *lin-4* and *let-7* RNAs to the broader RNAi phenomena, suggesting that there could be other genes encoding RNAs like *lin-4* and *let-7*, why didn't we try harder to find them? One issue was technical: forward genetics had led to the identification of *lin-4* and *let-7*, but this had been a slow process, and new molecular approaches seemed to be required to crack the problem. We had tried strategies involving size selection and end-labeling, but we were exceedingly wary of what we imagined was a vast detritus of partially degraded RNAs in cells. This concern eventually turned out to be unfounded: cDNA libraries of small RNAs from animal cells contain for the most part microRNAs and other classes of small silencing RNAs[14].

A second issue was theoretical: should we expect RNAs like *lin-4* and *let-7* to be evolutionarily conserved in other animals? I had a nagging suspicion that *lin-4* and *let-7* could be members of a nematode-specific developmental pathway. Moreover, my thinking about the question of conservation was dominated by an erroneous assumption based on the sloppy base-pairing that characterized all of the *lin-4* and *let-7* complementary sites in *lin-14*, *lin-28* and *lin-41*. The middle of the microRNA didn't seem to matter much (**Fig. 2b**), and, indeed, *lin-4* and *let-7* seemed to have evolved to avoid precise base-pairing in order to preserve the stability of their targets. Hamilton and Baulcombe's discovery of small interfering RNAs (siRNAs) provided a satisfying explanation for this imprecise base-pairing. Whereas siRNAs match their target mRNAs precisely, and consequently elicit target destruction, *lin-4* and *let-7* match all their targets imprecisely and, hence, can engage in reversible, regulated inhibition of the synthesis of proteins from stable mRNAs[15]. The assumption was that even if other animals had small RNAs evolutionarily related to *lin-4* or *let-7*, their overall sequences would have drifted too much to be detected. Indeed, our attempts to detect *lin-4* by hybridization to nematode families other than *Caenorhabditis* had come up dry. For all these reasons, it seemed futile to look for orthologs of *lin-4* or *let-7* in other animals by database search.

A watershed discovery: deep conservation of *let-7*

Through a startlingly straightforward approach, using northern blot hybridization and genomic database searches, Gary Ruvkun and co-workers showed that the *let-7* RNA is perfectly conserved across a wide range of animal phyla[16] (**Fig. 3**). The conservation of *let-7* meant that an ancient common ancestor of animals from sea urchins to humans had a *let-7* RNA of precisely the same sequence as our *let-7* of today. After reading their paper in the autumn of 2000, I had to set aside 10 minutes to stare out the window and reorganize my view of the universe. Now we knew that *lin-4* and *let-7* RNAs were members of an evolutionarily ancient class of regulatory molecules, and so it was finally obvious that there must be other RNAs like them yet to be discovered in animals.

Rosalind Lee and I immediately set out to identify new microRNAs by sequencing cDNA libraries prepared from size-fractionated (~22-nucleotide) *C. elegans* RNA and also to search computationally for hairpin-forming sequences conserved between *C. elegans* and *C. briggsae* genomic sequences. In retrospect, we were terribly naive about potential competition in this effort to find new microRNAs; we thought nobody else was looking. Our complacency was derived from our having become accustomed over the years to little general interest in *lin-4* and *let-7*. Even though we were sure that others must have appreciated the implications of Ruvkun's finding that *let-7* was evolutionarily conserved, and we knew that others, notably Tom Tuschl[17] knew how to make cDNA libraries of tiny RNAs, we essentially assumed that we were the only ones motivated to do the search. I took to the extreme this head-in-the-sand attitude; in July 2001 I heard a rumor at a *C. elegans* meeting that David Bartel was trying to clone small

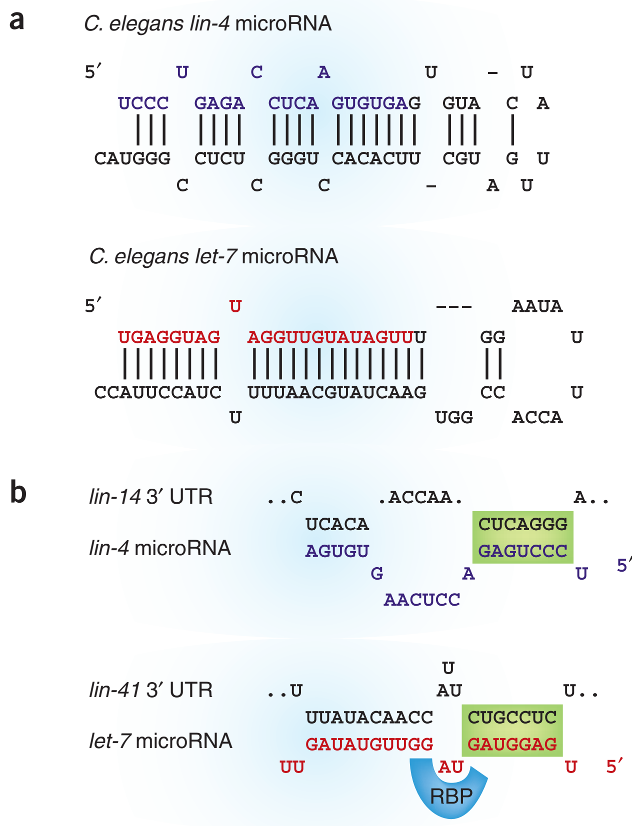

Figure 2 *C. elegans lin-4* and *let-7* microRNAs and their predicted base-pairing with sites in the 3′ UTRs of *lin-14* and *lin-41* mRNAs, respectively. (**a**) *lin-4* precursor hairpin (top), with sequence of the mature 22-nucleotide microRNA in blue and *let-7* precursor hairpin (bottom), with sequence of the mature 22-nucleotide microRNA in red. (**b**) Predicted base-pairing of microRNAs (5′ end to the right) to 3′ UTR target sites; 5′ seed sequence pairing is highlighted in green. Bulged bases in the microRNA target duplex may be recognized (bottom) by hypothetical RNA binding proteins (RBP).

RNAs from the worm, but I thought to myself, "Bartel? … He's not a worm person," and put the thought out of my mind.

As it turned out, we escaped from being spectacularly scooped only by a whisker. By August 2001, Rosalind and I were enjoying a period of blissfully ignorant success, with almost one new microRNA falling daily out of the few hundred clones we had manually sequenced and compared to the raw *C. briggsae* genome sequence that was released in late July. We had identified a dozen or so new *C. elegans* microRNAs that were phylogenetically conserved, some of them (such as *mir-1*) all the way to humans. Then on the afternoon of Monday, August 6, I received an e-mail from the editors of *Science* containing the abstract of a manuscript from Tom Tuschl's lab, asking me to serve as a referee. To our horror, the abstract of the paper[18] was bursting with reports of new microRNAs from flies and humans! I had to inform *Science* that I was unable to review the Tuschl paper on account of a conflict of interests because we had a manuscript in preparation with similar findings. We quickly decided to try an end run: Tuesday morning we sent a presubmission inquiry to *Cell* describing our identification of numerous new, phylogenetically conserved small RNAs like *lin-4* and *let-7*. The editor responded that afternoon with the opinion that our findings would probably not fly at *Cell*, as referees would want to see functional characterization of these new small RNAs. We went back to *Science* hat in hand, and, on Tuesday afternoon, the *Science* editors graciously agreed that, if we could submit our manuscript by Friday, it would be reviewed along with the Tuschl paper. And "by the way," an editor added, "there is also a third manuscript—from David Bartel." Oh, *that* David Bartel!

There was a serious problem with our promise to get the manuscript to *Science* by that coming Friday: it was already Tuesday night, and there was no manuscript, not even a preliminary draft. We had been planning to start writing perhaps in the autumn, after accruing an imagined 'whole story' about new worm microRNAs. So, for 60-odd hours straight, Rosalind and I did last-minute experiments, drew up figures, and typed while standing up at the bench to avoid falling asleep. We managed to send the manuscript off to *Science* Friday afternoon. It was accepted, along with the two other papers[18,19], although all three referees noted that our initial manuscript was poorly written to the extreme. Little did they know that it was the product of two-and-a-half days of fatigue-fogged frenzy.

Hundreds of millions of years of conservation

Our search for additional microRNAs was precipitated by Pasquinelli and Ruvkun's finding that the *let-7* RNA sequence is perfectly conserved across a vast evolutionary distance. At that time, there was no expectation that there would be sufficient evolutionary conservation over a 22-nucleotide sequence to detect homologous RNAs by hybridization or by BLAST search. Even today, we still have a rather inadequate understanding of the basis for complete conservation of a microRNA sequence.

One factor is certainly a multiplicity of targets. Nowadays we know that an individual microRNA can typically regulate dozens or even scores of functional targets[20–22]. This helps to account for evolutionary fixation of microRNA sequences: once a newly evolved microRNA acquires important targets in sufficient numbers to preclude coevolution of the microRNA and target sequences, that microRNA sequence becomes fixed. However, this argument does not account by itself for the perfect conservation of all 22 nucleotides of a microRNA, given the principle that base-pairing to the 5′ 'seed' part of the microRNA (**Fig. 2**) is a dominant factor in microRNA target recognition[23]. Multiplicity of seed interactions would be sufficient to account for the evolutionary fixation of families of microRNAs with similar seeds[24], but what about nucleotides outside the seed region?

Besides canonical 5′ seed interactions, the so-called 3′ compensatory interactions, where extensive 3′ base-pairing can compensate for relatively weak seed interactions[25,26] (**Fig. 2b**, bottom), provide a model for constraints on 3′ sequences. For bases in the middle region of the microRNA (for example, nucleotides 9–11), it is doubtful that base-paring alone would be the only source of selective pressure. This is because interactions that involve perfect base-pairing in the middle of animal microRNAs are rare. Another source of selection on microRNA sequence could be interactions with protein cofactors that bind to the microRNA, during its biogenesis and/or after it base-pairs to form the microRNA·target duplex (**Fig. 2b**,

bottom). The loops and bulges of the typical animal microRNA·target duplex could display bases for sequence-specific contacts with protein factors that serve to modulate the potency or the nature of the effect on the mRNA target. One distinction, therefore, between a conserved microRNA such as *let-7* and its more species-specific family members could be the conservation of base-specific contacts between the microRNA and RNA-binding protein cofactors.

Conclusions and prospects for the future

I will always by astonished by microRNAs, for which new and intriguing findings seem to emerge daily. The diverse physiological and developmental roles for microRNAs that have been uncovered are too extensive to discuss here. Nor is it possible to satisfactorily sum up all the current open questions about microRNAs in this small space. However, a number of key questions arise in relation to the deep conservation of microRNA sequences. In this regard, our challenge is to identify all the molecular interactions in which microRNAs engage, both RNA–RNA interactions with targets and interactions between microRNAs and proteins. In particular, the identification of microRNA regulatory cofactors by biochemical and genetic approaches will likely lead to advances in understanding the regulation of microRNA biogenesis, activity and specificity.

We are still unable to predict and classify with precision the functional targets of microRNAs. The 5′ seed model has been successful in identifying potential targets of microRNA families, but we need a better understanding of the roles of nucleotides outside the seed for parsing the roles of particular family members. Although we know that a microRNA can exert effects on mRNA stability and/or protein synthesis, we do not yet understand what features of the interaction and what associated factors determine the outcome. What governs whether a particular mRNA is permanently or reversibly silenced, and to what extent? In theory, regulatory proteins with diverse activities could be assembled around the core microRNA/Argonaute complex, and the constitution and activity of such complexes could be specified by sequence-specific binding proteins that recognize parts of the microRNA (**Fig. 2b**, bottom).

It will be important to calibrate the stoichiometry of microRNAs and their targets within cells and to carry out accurate genome-scale mRNA and proteomic profiling so that we can model interactions on the genome and network scale; ideally, our goal should be to acquire an understanding of microRNA·target interactions sufficient to successfully predict the quantitative impact on the proteome of

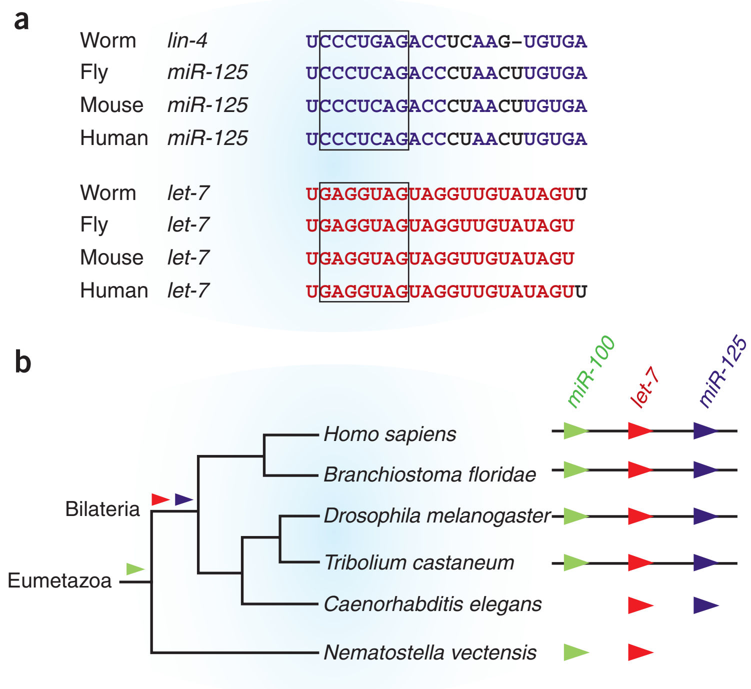

Figure 3 Phylogenetic conservation of *let-7* and *lin-4* (*miR-125*), in sequence and genomic organization[16,28,29,31]. (**a**) Sequence alignment of *lin-4/miR-125* and *let-7* microRNAs among diverse animals. Each of the indicated genomes contains additional paralogs of *miR-125* and *let-7*, some of which differ from the indicated sequences outside of the 5′ seed region (boxed). (**b**) *let-7* and *miR-125* were present, clustered with *miR-100*, in a common ancestor of bilaterians. *miR-100* is older, as it is found in a common ancestor of cnidarians and bilaterians. The relative order and tandem configuration of *miR-100*, *let-7* and *miR-125* have been widely maintained in the genomes of bilaterians, although the spacing between them varies from less than 1 kilobase to tens of kilobases[29]. In *Drosophila melanogaster*, the three microRNAs are transcribed together as a polycistronic primary transcript[29]. In the nematode lineage, *miR-100* appears to have been lost, and *lin-4/miR-125* and *let-7* have become unlinked. The human genome contains three *miR-100* and *miR-125* paralogs, and each is located near a *let-7*–encoding sequence. Nine other human *let-7* paralogs are unlinked from *miR-100* and *miR-125*.

defined changes in the abundances of specific microRNAs.

We don't understand why certain microRNAs occur in clusters in the genome, often transcriptionally coexpressed, and often from polycistronic primary transcripts. For example, in a curious reflection of the partnership that the *lin-4* and *let-7* RNAs helped forge between my lab and the Ruvkun lab, it turns out that *lin-4* and *let-7* have themselves engaged in an ancient genetic partnership. In most animals, except *C. elegans*, *lin-4* and *let-7* are clustered closely together in the genome, apparently co-regulated transcriptionally[27–29], along with an even more ancient microRNA, *miR-100* (ref. 24) (**Fig. 3**). Why is this configuration of *lin-4* and *let-7* so important as to be preserved so widely? Is it to coordinate regulation of their respective targets? How so, and why? Curiously, even in *C. elegans*, where *lin-4* and *let-7* are unlinked and expressed at distinct times in development, these two microRNAs have numerous shared targets.

Finally, we wish to understand the roles that microRNAs have played in the evolution of animals. The pattern of their emergence in animal phylogeny suggests that microRNAs may have been an important component of the genetic toolkit for the emergence of body-plan diversity[24]. Interestingly, evolutionary comparison of the repertoire of predicted targets of conserved microRNAs has revealed a remarkable flexibility in the particular genes targeted by the same microRNA from, say, worms, flies and mammals[30]. Apparently, while the microRNA becomes constrained in sequence owing to a constant multiplicity of important interactions, the sets of genes with *cis*-acting regulatory sites for the microRNA can shift profoundly during evolution, permitting flexibility in the evolutionary roles for a microRNA in shaping different aspects of development in different contexts.

It has been interesting to watch microRNAs rise from being nearly dead in 2001 to several years of gold rush activity, with hundreds of publications about them. This expansive phase of the field was characterized by an inclination to seek simple mechanisms and functional themes. The field has more recently progressed

to a more mature stage, where biochemical and genetic studies have begun to reveal a marvelous complexity of microRNA regulatory mechanisms and biological functions, involving multiple targets, protein cofactors and interactions with signaling pathways and other microRNAs. While microRNAs may not offer the simple answers that we may have hoped for five years ago, and decades of long hard work may lie ahead to uncover the secrets of microRNA, we know the answers will be that much more satisfying for the difficulty of the search.

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