miRNA collection

Unlocking the Secrets of microRNAs

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As illustrated below, miRCURY™ LNA microRNA Arrays produce very specific signals. You can create an effective, valid, and uniquely specific microRNA profile with minimal cross-hybridization from just 30 ng total RNA.

Figure 1

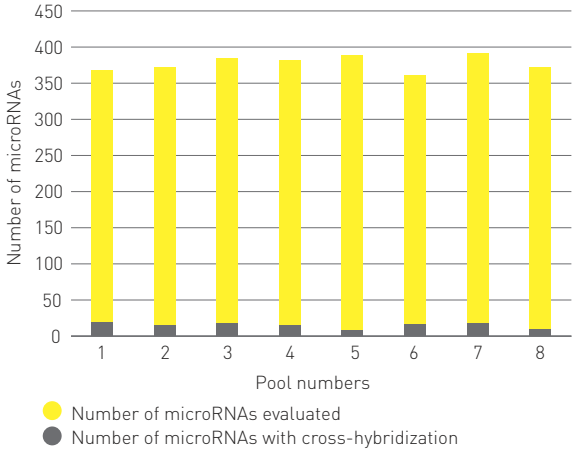

Figure 1: The unique specificity of the miRCURY™ LNA microRNA Arrays A total of 431 microRNAs were divided into eight pools with 40-71 distantly related microRNAs in each pool and analyzed using miRCURY™ LNA microRNA Arrays. The resulting data demonstrate the high specificity of the miRCURY™ LNA microRNA Arrays and a maximum cross-hybridization of just 4.9% across all eight pools, with an average of 3.9%.

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Contents

INTRODUCTIONS

ARTICLES

TECHNICAL NOTES

On the cover: Graphical representation of the transcription, processing, and targeting of miRNA within the cell. [Illustration: Cameron Slayden, Image from cover of *Science* (20 December 2002)]

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Small Wonders

In October 2001, three seminal papers, reproduced in their entirety in this booklet, appeared in the same issue of *Science*, together solidifying the evidence for an intriguing form of RNA. Named, by mutual agreement, microRNAs (miRNAs), these single-stranded small RNA molecules were isolated simultaneously by the three laboratories under the supervision of Thomas Tuschl at the Max Planck Institute in Göttingen, Germany, David Bartel at the Whitehead Institute in Cambridge, Massachusetts, and Victor Ambros from Dartmouth Medical School in Hanover, New Hampshire.

Using a number of common model organisms including *Drosophila melanogaster* and *Caenorhabditis elegans,* as well as human HeLa cells, the three groups isolated a variety of miRNA molecules, a portion of which were similar to previously described stRNAs (short temporal RNAs). The first stRNA (now regarded as part of the miRNA family), was named *lin-4* and was discovered in 1993 in the lab of Victor Ambros. It is complementary to the 3′ untranslated region of the mRNA transcribed from the *lin-14* gene in *C. elegans.* It wasn't until 2000 that a second stRNA from *C. elegans, let-7*, was found in Gary Ruvkun's lab at Massachusetts General Hospital in Boston, further priming the field for subsequent discoveries. The availability of the full genome sequence for *C. elegans,* completed in 1998, played a critical role and made identification and comparison of miRNA sequences possible.

Previously dismissed as degradation products from RNA isolation, small RNAs including miRNAs have been found to be more ubiquitous and abundant than anyone expected. To date, over 4,500 miRNAs have been deposited in the central Sanger Institutes' miRBase, and the number is climbing steadily. The number of miRNAs found in humans has reached 500 and, although some believe we are reaching the point of diminishing returns as far as the discovery of new miRNAs is concerned, this number is still increasing.

Publication of the 2001 papers established a new way of thinking about not only RNA function, but also about the regulation of all cellular functions, from differentiation to apoptosis. In the short space of six years research has advanced markedly. A recent explosion of papers describing and characterizing miRNAs has occurred, and research demonstrating their potential for the treatment of disease is becoming more compelling with each publication.

Novel forms of knockout and knockdown animals are now being developed in which regions of DNA coding for miRNAs are being deleted or their expression reduced, respectively. The resulting aberrant phenotypes are unexpectedly broad and variable. Many knockouts appear to have developmental defects and problems with the immune system, as described in papers reproduced here. The knowledge gained from these experiments—and the implication of miRNAs in several human disease pathologies—points to promising opportunities for therapeutic intervention through the targeting of miRNAs. An optimistic future can be imagined where previously refractory diseases, including certain cancers and neurodegenerative disorders, are rendered tractable through the application of anti-miRNA treatments.

Sean Sanders
Commercial Editor, *Science*

Unlocking the Secrets of microRNAs

MicroRNAs constitute a class of short endogenous noncoding RNA molecules that aid post-transcriptional regulation of gene expression by base-pairing with the 3' untranslated region of target mRNAs. This base-pairing results in the degradation or repressed translation of the target mRNAs, inhibiting gene expression. Substantial research over recent years has established that microRNAs play a central regulatory role during development, differentiation, and metabolism, providing valuable diagnostic and prognostic indicators as well as new drug targets for human diseases.

MicroRNA research presents several major challenges. The short nature (~22 nt) of microRNA sequences makes it difficult for traditional DNA-based analysis tools to achieve the required target sensitivity. In addition, closely related microRNA family members differ by as little as 1 nt in sequence, emphasizing the need for high selectivity and single base-pair mismatch discrimination.

With our miRCURY™ LNA product portfolio, Exiqon is the pioneer of microRNA tools, supplying the research and diagnostics community with leading-edge products and services, based on our proprietary Locked Nucleic Acid (LNA™) technology. The key feature of LNA containing probes is that the introduction of LNA monomers into oligonucleotides increases the target affinity significantly while at the same time increasing the level of selectivity. Thus, miRCURY™ LNA research tools offer unmatched sensitivity and selectivity for microRNA targets.

Within this Collection, we are pleased to present some of the groundbreaking scientific papers and reviews, published in *Science*, that have made a significant contribution to understanding the role of microRNA in regulating gene expression. Three papers published in 2001 from the research groups of Tüschl *et al.*, Bartel *et al.*, and Ambros *et al.* were the first to show the key role of 21- to 24-nucleotide noncoding microRNAs in *Drosophila, C. elegans* and human HeLa cells, respectively. The papers significantly advance our understanding of highly conserved microRNAs and suggest their key role in sequence-specific posttranscriptional regulatory mechanisms, in particular during embryogenesis and development.

Following these initial discoveries, in a groundbreaking paper from 2005, the research group of Plasterk showed that the temporal and spatial expression of microRNAs is highly tissue-specific during zebrafish development, suggesting a key role for microRNA in differentiation or maintenance of tissue identity. A paper by Rajewsky in 2007 further suggested that individual microRNAs can exert critical control over mammalian differentiation processes, e.g., by regulating cytokine production in the mammalian immune system. In addition, Bradley *et al.* (2007) highlighted the importance of microRNAs in nondevelopmental regulation, suggesting that they play a key role in the homeostasis and function of the immune system, while Hébert and De Strooper (2007) summarize recent research that suggests a role for microRNAs in the survival of postmitotic cells targeted in certain neurodegenerative disorders.

As the importance of microRNA research grows, new tools and methodologies will be needed to refine our understanding of gene regulation. By working with *Science* to bring you this Collection, we hope to illustrate that microRNA analysis is now an important part of understanding any living system and will be required to complement ongoing research initiatives.

Lars Kongsbak, President & CEO of Exiqon

In an interview with Exiqon, Dr. Thomas Tüschl, of the Rockefeller University, describes the current and future advances in microRNA technology, and the challenges faced in this field by the research community today.

Q: What do you consider to be the most important advance in the microRNA field?

The generation of a complete catalog of all microRNA genes and the definition of their cell–type specific expression pattern has been the most important advance in the microRNA field. These efforts are still ongoing, but are nearing completion.

Q: What is the biggest challenge facing the microRNA community at present?

This would fall in two categories. First of all, experimental definition of micro-RNA targeted genes, i.e., determining which genes are regulated by which microRNAs, is of key importance. An additional challenge is the determination of the biological function of micro-RNAs, both for cell–type specific as well as the more ubiquitously expressed microRNAs. Secondly, I would say the quantification of microRNA copy numbers per cell is a major challenge, which will then allow assessment of the expected degree of suppression exerted by these microRNAs.

Q: How do you see the potential for microRNAs developing in the future as biomarkers for disease diagnosis and prognosis?

MicroRNAs, because of their high copy numbers per cell and their defined expression patterns, represent good biomarkers for specific cell types and probably for diseased tissues originating from such cell types. There is a potential to facilitate diagnosis of some types of metastatic tumors, but it is still an open field.

Q: Can you speculate how the field of microRNA may develop to investigate other classes of noncoding RNA in the future?

The technologies developed for microRNA identification and characterization are readily adaptable for characterizing other types of small RNAs, so I think we are in a good position to investigate the role of other noncoding RNAs.

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Glimpses of a Tiny RNA World

Gary Ruvkun

Over the years, a steady stream of structural and regulatory RNAs have been identified. Three papers published in this booklet on pages 6, 11, and 15 from the Tuschl, Bartel, and Ambros labs continue the tradition, but now prospecting for tiny RNAs of ~22 nucleotides (nt) (*1–3*). The chain of reasoning that simultaneously attracted these groups to 22 nt is convoluted but interesting.

The first 22-nt RNAs, *lin-4* and *let-7*, were identified by genetic analysis of *Caenorhabditis elegans* developmental timing (*4, 5*). The expression of the *lin-4* RNA during the first larval stage and the *let-7* RNA during the fourth larval stage triggers the down-regulation of target mRNAs via 3′-untranslated region (UTR) elements that are complementary to each regulatory RNA to specify the temporal progression of cell fates (*6, 7*). The *let-7* RNA, as well as its temporal regulation, are conserved across much of animal phylogeny (*8*). These 22-nt RNAs are called small temporal RNAs or stRNAs

Tiny RNAs also emerged from the biochemical analysis of RNA interference (RNAi) by experimentally induced double-stranded RNA (dsRNA): 21- to 25-nt small interfering RNAs (siRNAs) are processed from dsRNA and act as templates for their own amplification and the degradation of target mRNAs during RNAi (9, *10*). *lin-4* and *let-7* are predicted to be processed from partially double-stranded precursors as well (*4, 5, 8*). The common size of ~22 nt for stRNAs and siRNAs suggested that they are generated and perhaps act by a common mechanism. In fact, the same Dicer ribonuclease (RNase) that is required to process dsRNA to siRNAs also processes the stRNAs from their precursors (*11, 12*). The developmental defects caused by Dicer mutations in plants and animals may be due to defects in processing of other endogenous tiny regulatory RNAs (*11–13*).

The three teams use a range of biochemical techniques to clone 21- to 25-nt RNAs (*1–3*) from three different organisms, and thus reveal the richness of the tiny RNA world. They detect almost 100 new tiny RNAs—microRNAs or miRNAs. The Tuschl group identified 14 new miRNAs from the *Drosophila* embryo and 19 miRNAs from HeLa cells (*1*). The expression of all the new miRNAs was verified. The Bartel group identified 55 new miRNAs from mixed-stage *C. elegans* and verified the expression of 20 out of 22 miRNAs tested (*2*). Lee and Ambros cloned and verified the expression of 15 *C. elegans* miRNAs, 10 of which were also identified by the Bartel group *(3*). While the entry point of these studies was biochemical, complete genome sequences were key in the analyses. All three groups used the genome sequences of a variety of organisms to determine that these miRNAs are not breakdown products of mRNAs or structural RNAs, to infer precursors, to determine the genetic locations of the new genes, and to determine whether the miRNAs are conserved in evolution.

All of these miRNAs are predicted to be processed from multiply bulged and partially duplex precursors, like the stRNA precursors. Therefore, they are likely to be processed by Dicer, as demonstrated for two of the new miRNAs (*3*). More of the miRNAs are processed from the 3′ region of the precursor stem loop than from the 5′ region, from which stRNAs are processed. One precursor produces miRNAs from both stems (*2*). Thus, as in siRNA processing from dsRNA, Dicer probably processes both strands of these precursors, but in many cases only one strand may be stable. Some of the miRNAs are expressed only as longer precursors at some developmental stages (*2, 3*), suggesting possible regulation of processing rather than transcription.

Members of the RDE-1/Argonaute superfamily of proteins may also function in the maturation of miRNAs. The *C. elegans* Argonaute orthologs are required for the maturation and function of *let-7* and *lin-4 (11*), and *C. elegans* RDE-1 and *Arabidopsis* Ago1 are necessary for RNAi (*14, 15*). These proteins may form a complex with Dicer, as has been shown for *Drosophila* Argonaute2 (*16*). Genome sequences suggest that there are 24 *C. elegans* RDE-1/Argonaute genes, 7 in *Arabidopsis,* 4 in *Drosophila*, and 4 in humans. The distinct RDE-1/Argonautes may be specialized for processing subsets of miRNA genes. The developmental defects caused by mutations in *Drosophila* or *Arabidopsis* Argonaute genes may be due to defective processing of particular miRNAs (*17, 18*).

Like *let-7*, a number of the miRNA genes are conserved in evolution. About 12% of the miRNAs are conserved between nematodes, flies, and mammals, but more than 90% of the *C. elegans* miRNAs are conserved in the *9*0% complete *Caenorhabditis briggsae* sequence (*2*). To detect these conserved segments in genome sequence comparisons, only one or two mismatches could be tolerated. But one of the new miRNAs, *mir-84,* is 5 nt diverged from *let-7*, temporally regulated like *let-7*, and conserved in flies and humans (*2*). Such a paralog could only be detected in the rarified sequence space of the miRNA sequence collection.

One of the more subtle results comes from what the papers did not find: There is almost no evidence of siRNAs diagnostic of RNA interference in normally growing animals (*2*). Thus, Dicer and its cofactors are normally used for miRNA production, and are only recruited for RNAi upon viral or other dsRNA induction.

Some of the miRNAs, like *lin-4* and *let-7*, are temporally regulated. A number of the *Drosophila* and *C. elegans* miRNAs are only expressed in germ line or early embryos, hotbeds of translational control. In addition, the analysis of miRNA expression in cell lines and tissues suggests cell type–specific expression (*1, 3*). The regulated expression patterns of these miRNAs suggests functions in developmental control. However, some of the miRNAs are uniformly expressed, which could indicate more general roles in gene regulation, although in situ expression analysis has not been done to reveal possible cell-specific functions. One argument against an miRNA role in housekeeping gene regulation is that inhibition of *C. elegans* or *Arabidopsis* Dicer function, expected to generally decrease miRNA levels, causes developmental defects rather than cell-lethal defects (*11, 13*).

Some of the miRNA genes are arranged in tandem clusters (*1–3*), as had been observed for the human *let-7* orthologs (*8*). The genes in the tandem clusters that have been studied are co-expressed, for example, in the germ line and early embryo of *C. elegans* and *Drosophila* (*1, 2*). In fact, a set of seven highly related *C. elegans* miRNA genes that are expressed only in germ line and embryos are so tightly clustered within 1 kb that they are predicted to form a precursor from which all seven mature miRNAs might be processed (*2*).

From the biochemical activities of *let-7*, *lin-4*, and the siRNAs, the new miRNAs are expected to regulate the translation or stability of other mRNAs (see the figure). The targets of the *let-7* and *lin-4* miRNAs emerged from genetic analysis of suppressors of the *let-7* or *lin-4* heterochronic mutant phenotypes (*6, 7*). These target mRNAs bear regions of complementarity to the *let-7* and *lin-4* RNAs, but with bulges and loops that make their informatic detection in total genome sequences difficult. The significance of the complementary sites has been proven by mutation that renders the site unresponsive to the regulatory RNA (*6, 7*). A variety of mRNAs bearing regulatory 3′ UTRs have been identified in the germ line and early embryo of *Drosophila* and *C. elegans*. A search of these 3′ UTRs for regions complementary to the embryonically expressed miRNAs could reveal potential targets and genetic pathways. Consistent with such a role, inhibition of the *C. elegans* Dicer gene *dcr-1* causes sterility and embryonic lethality (*11*).

miRNAs could act in other pathways, such

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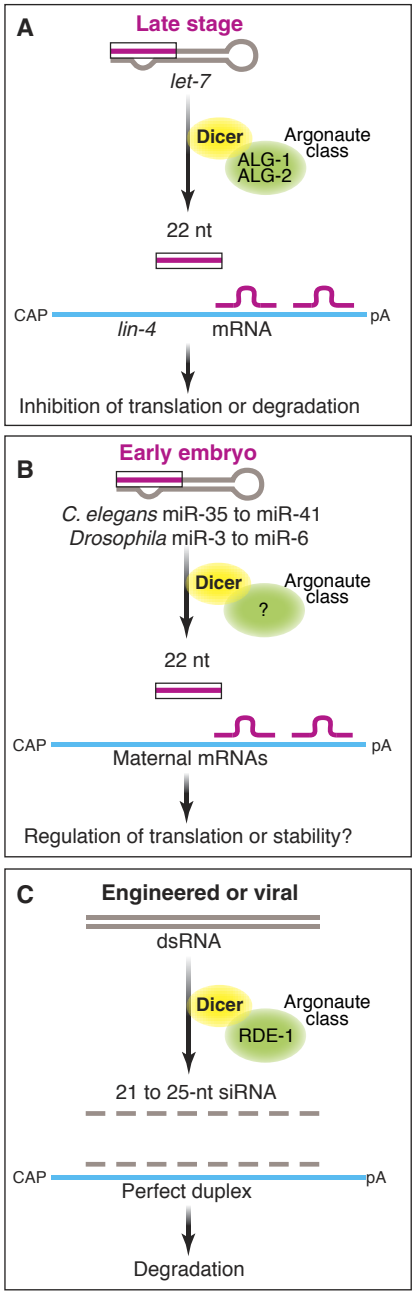
as in the translational control of mRNAs tethered in dendritic regions of neurons, which has been proposed to mediate synaptic plasticity (*19*). The 3′-UTR sequences that mediate dendritic translational control of calcium-calmodulin–dependent kinase II have been determined (*20*), and it would be interesting to see if there are miRNAs that are complementary to these cisacting sequences. Finally, because siRNAs in plants mysteriously regulate target gene transcription as well as mRNA stability (*9*), it is possible that the new miRNAs regulate target gene transcription as well.



Slicing and dicing miRNAs. (**A**) The two founder miRNAs from *C. elegans*, *lin-4* and *let-7*, are processed from partially duplexed precursors by the Dicer/Argonaute complex, and then down-regulate the expression of protein-coding mRNAs by base-pairing to partially complementary elements on those mRNAs. The new miRNAs are expected to function similarly (**B**). For example, the *Drosophila* and *C. elegans* miRNAs that are expressed during embryonic stages may regulate the translation of maternal mRNAs that pattern early development. (**C**) The small interfering RNAs (siRNAs) that are intermediates in RNAi are also 21 to 25-nt long, processed by a Dicer/RDE-1 complex, and target mRNAs. The molecular components of RNAi may have been recruited for viral and transposon surveillance from an original role in miRNA regulation, or vice versa.

The most definitive test of the function of the new miRNAs is to isolate mutations in these genes, and there may be genetic loci that have not been molecularly analyzed at the genetic location of these miRNAs in *C. elegans* or *Drosophila*. Gene knockout, or perhaps RNAi, increased gene dosage, or misexpression strategies, also could be used to establish the function of these miRNAs.

Because genes that encode gene-regulatory proteins constitute a large fraction of *C. elegans* and *Drosophila* genetically identified loci, why have more of the miRNAs not been revealed by genetic analysis? One view is that they constitute a small genetic target, although multiple *lin-4* and *let-7* mutant alleles emerged from relatively limited genetic screens. Alternatively, given that less than 5% of the many *C. elegans* KH or RRM domain RNA-binding protein genes have been genetically identified, the miRNA genes may be emerging from genetics at about the expected rate. And if these miRNA genes regulate the expression of fewer target mRNAs than other RNA-regulatory proteins, or if multiple miRNAs regulate adjacent sites on common target mRNAs, mutations in them may cause more subtle phenotypes. Or perhaps researchers have simply been focusing on the canon of protein-coding genes.

Why use a tiny RNA to regulate the expression of target genes? First, the expression of a miRNA of 70 nt occurs much more rapidly than that of a typical protein-coding primary transcript of 1 to 1000 kb and is not further delayed by translation. A transcriptional cascade of miRNA genes—for example, during the short cell cycles of *Drosophila* or *C. elegans* early embryogenesis—could trigger developmental transitions by translational control of preexisting maternal mRNAs through translational control. The expression of miRNAs after a synaptic transmission may regulate the translation of mRNAs tethered in a dendrite before the decay of a phosphorylation event that may mark a recently fired synapse. Second, the 22-nt siRNAs are remarkably potent at mRNA inactivation. There is good evidence that they can act systemically in plants and nematodes, with probable amplification (*9*). Given that the miRNAs use the same processing and presentation machinery as the siRNAs, the miRNAs also may be amplified from their precursors, and may spread throughout an organism from a single source of expression.

How complete are these miRNA surveys? They are just the first steps and not proposed to be saturating. But many of the miRNAs identified by the Ambros and Bartel group coincide, and *let-7* constituted about 30% of the 100 miRNAs isolated from HeLa cells (*1*). While these biochemical procedures are skewed toward abundant miRNAs, the multiple isolations of particular miRNAs circumscribe the abundant miRNA world. On the other hand, many miRNAs were isolated just once, suggesting more miRNAs expressed at lower levels. Many of the miRNAs were isolated from mixed-stage RNA preparations (*2, 3*). Biochemical collection of miRNAs from selected cell types or finely staged preparations may reveal rare miRNAs that act in particular cells or at particular times. But the comprehensive detection of miRNAs expressed in few cell types or under particular conditions may demand informatic approaches based on the now-extensive training set of miRNAs revealed in these papers.

In fact, the number of genes in the tiny RNA world may turn out to be very large, numbering in the hundreds or even thousands in each genome. Tiny RNA genes may be the biological equivalent of dark matter—all around us but almost escaping detection, until first revealed by *C. elegans* genetics and then more comprehensively charted by these papers. The next step is to figure out whether these regulatory RNAs use principles of amplification and systemic spread that have selected for their conservation as well as their ramification into so many apparently new sequences.

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Identification of Novel Genes Coding for Small Expressed RNAs

Mariana Lagos-Quintana, Reinhard Rauhut, Winfried Lendeckel, Thomas Tuschl*

In *Caenorhabditis elegans*, *lin-4* and *let-7* encode 22- and 21-nucleotide (nt) RNAs, respectively, which function as key regulators of developmental timing. Because the appearance of these short RNAs is regulated during development, they are also referred to as small temporal RNAs (stRNAs). We show that many 21- and 22-nt expressed RNAs, termed microRNAs, exist in invertebrates and vertebrates and that some of these novel RNAs, similar to *let-7* stRNA, are highly conserved. This suggests that sequence-specific, posttranscriptional regulatory mechanisms mediated by small RNAs are more general than previously appreciated.

Two distinct pathways exist in animals and plants in which 21- to 23-nt RNAs function as posttranscriptional regulators of gene expression. Small interfering RNAs (siRNAs) act as mediators of sequence-specific mRNA degradation in RNA interference (RNAi) (*1*–*5*), whereas stRNAs regulate developmental timing by mediating sequence-specific repression of mRNA translation (*6*–*11*). siRNAs and stRNAs are excised from double-stranded RNA (dsRNA) precursors by Dicer (*12*–*14*), a multidomain ribonuclease III protein, thus producing RNA species of similar sizes. However, siRNAs are believed to be double-stranded (*2*, *5*, *12*), whereas stRNAs are single-stranded (*8*).

We previously developed a directional cloning procedure to isolate siRNAs after processing of long dsRNAs in *Drosophila melanogaster* embryo lysate (*2*). Briefly, 5′ and 3′ adapter molecules were ligated to the ends of a size-fractionated RNA population, followed by reverse transcription polymerase chain reaction (PCR) amplification, concatamerization, cloning, and sequencing. This method, originally intended to isolate siRNAs, led to the simultaneous identification of 16 novel 20- to 23-nt short RNAs, which are encoded in the *D. melanogaster* genome and are expressed in 0- to 2-hour embryos (Table 1). The method was adapted to clone RNAs in a similar size range from HeLa cell total RNA (*15*), which led to the identification of 21 novel human microRNAs (Table 2), thus providing further evidence for the existence of a large class of small RNAs with potential regulatory roles. Because of their small size, and in agreement with the authors of two related papers in this issue (*16, 17*), we refer to these novel RNAs as micro-RNAs (miRNAs). The miRNAs we studied are abbreviated as miR-1 to miR-33, and the genes encoding miRNAs are named *mir-1* to *mir-33*. Highly homologous miRNAs are referred to by the same gene number, but followed by a lowercase letter; multiple genomic copies of a *mir* gene are annotated by adding a dash and a number.

The expression and size of the cloned, endogenous short RNAs were also examined by Northern blotting (Fig. 1 and Tables 1 and

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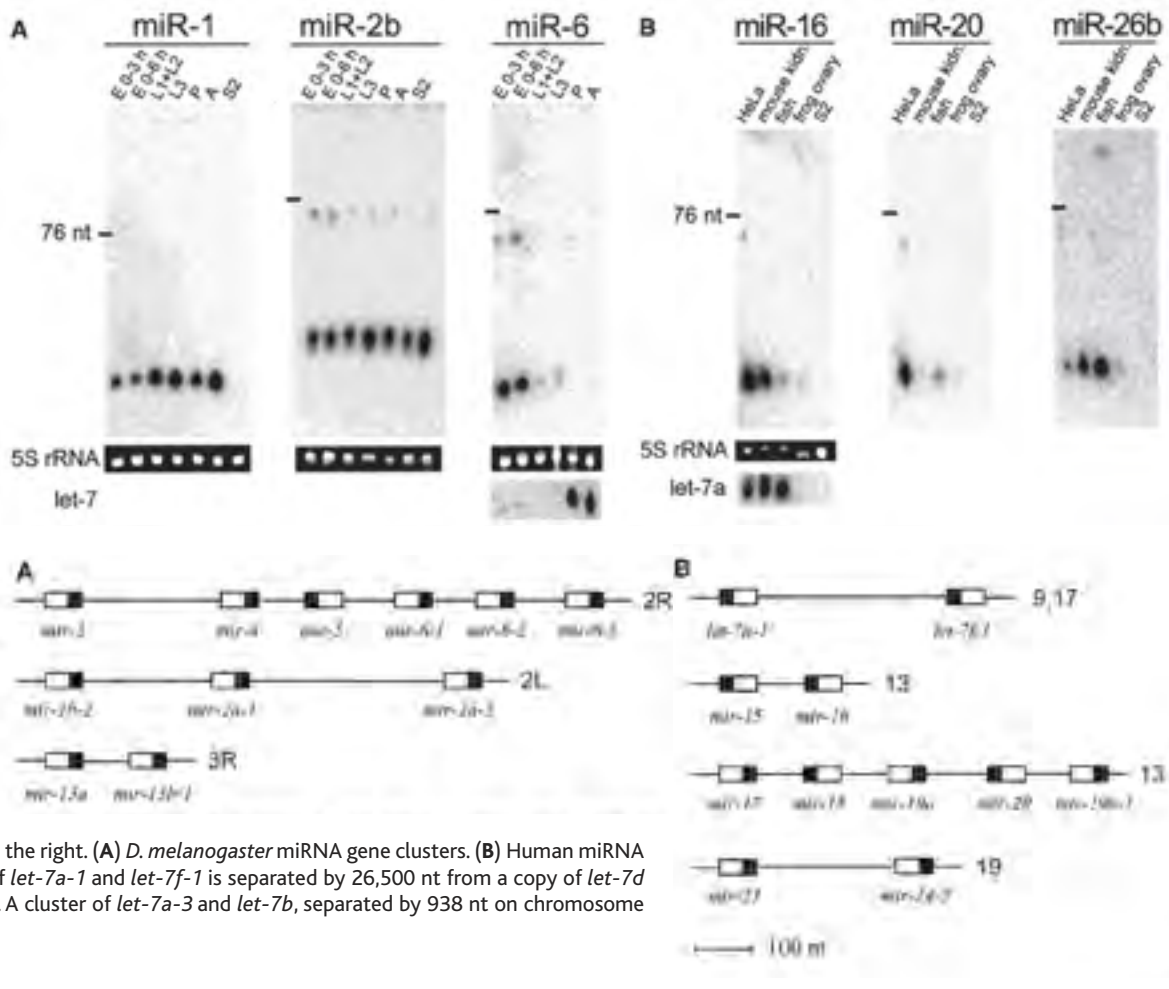

Fig. 1. Expression of miRNAs. Representative examples of Northern blot analysis are depicted (*21*). The position of 76-nt val-tRNA is indicated on the blots; 5*S* rRNA serves as a loading control. (**A**) Northern blots of total RNA isolated from staged populations of *D. melanogaster*, probed for the indicated miRNA. E, embryo; L, larval stage; P, pupa; A, adult; S2, Schneider-2 cells. (**B**) Northern blots of total RNA isolated from HeLa cells, mouse kidneys, adult zebrafish, frog ovaries, and S2 cells, probed for the indicated miRNA.

Fig. 2. Genomic organization of miRNA gene clusters. The precursor structure is indicated as a box, and the location of the miRNA within the precursor is shown in black; the chromosomal location is also indicated to the right. (**A**) *D. melanogaster* miRNA gene clusters. (**B**) Human miRNA gene clusters. The cluster of *let-7a-1* and *let-7f-1* is separated by 26,500 nt from a copy of *let-7d* on chromosomes 9 and 17. A cluster of *let-7a-3* and *let-7b*, separated by 938 nt on chromosome 22, is not illustrated.

2). For analysis of *D. melanogaster* RNAs, total RNA was prepared from different developmental stages, as well as from cultured Schneider-2 (S2) cells, which were originally derived from 20- to 24-hour *D. melanogaster* embryos (*18*) (Fig. 1 and Table 1). miR-3 to miR-7 are expressed only during embryogenesis and not at later developmental stages. The temporal expression of miR-1, miR-2, and miR-8 to miR-13 was less restricted. These miRNAs were observed at all developmental stages, and significant variations in the expression levels were sometimes observed. Interestingly, miR-1, miR-3 to miR-6, and miR-8 to miR-11 were completely absent from cultured S2 cells, whereas miR-2, miR-7, miR-12, and miR-13 were present in S2 cells, therefore indicating cell type–specific miRNA expression. miR-1, miR-8, and miR-12 expression patterns are similar to those of *lin-4* stRNA in *C. elegans*, as their expression is strongly upregulated in larvae and sustained to adulthood (*19*). miR-9 and miR-11 are present at all stages but are strongly reduced in the adult, which may reflect a maternal contribution from germ cells or expression in one sex only.

The *mir-3* to *mir-6* genes are clustered (Fig.2A), and *mir-6* is present as triple repeat with slight variations in the *mir-6* precursor sequence but not in the miRNA sequence itself. The expression profiles of miR-3 to miR-6 are highly similar (Table 1), which suggests that a single embryo-specific precursor transcript may give rise to the different miRNAs or that the same enhancer regulates miRNA-specific promoters. Several other fly miRNAs are also found in gene clusters (Fig. 2A).

The expression of HeLa cell miR-15 to miR-33 was examined by Northern blotting using HeLa cell total RNA, in addition to total RNA prepared from mouse kidney, adult zebrafish, *Xenopus laevis* ovary, and *D. melanogaster* S2 cells (Fig. 1B and Table 2). miR-15 and miR-16 are encoded in a gene cluster (Fig. 2B) and are detected in mouse kidney, adult zebrafish, and very weakly in frog ovary, which may result from miRNA expression in somatic ovary tissue rather than in oocytes. *mir-17* to *mir-20*

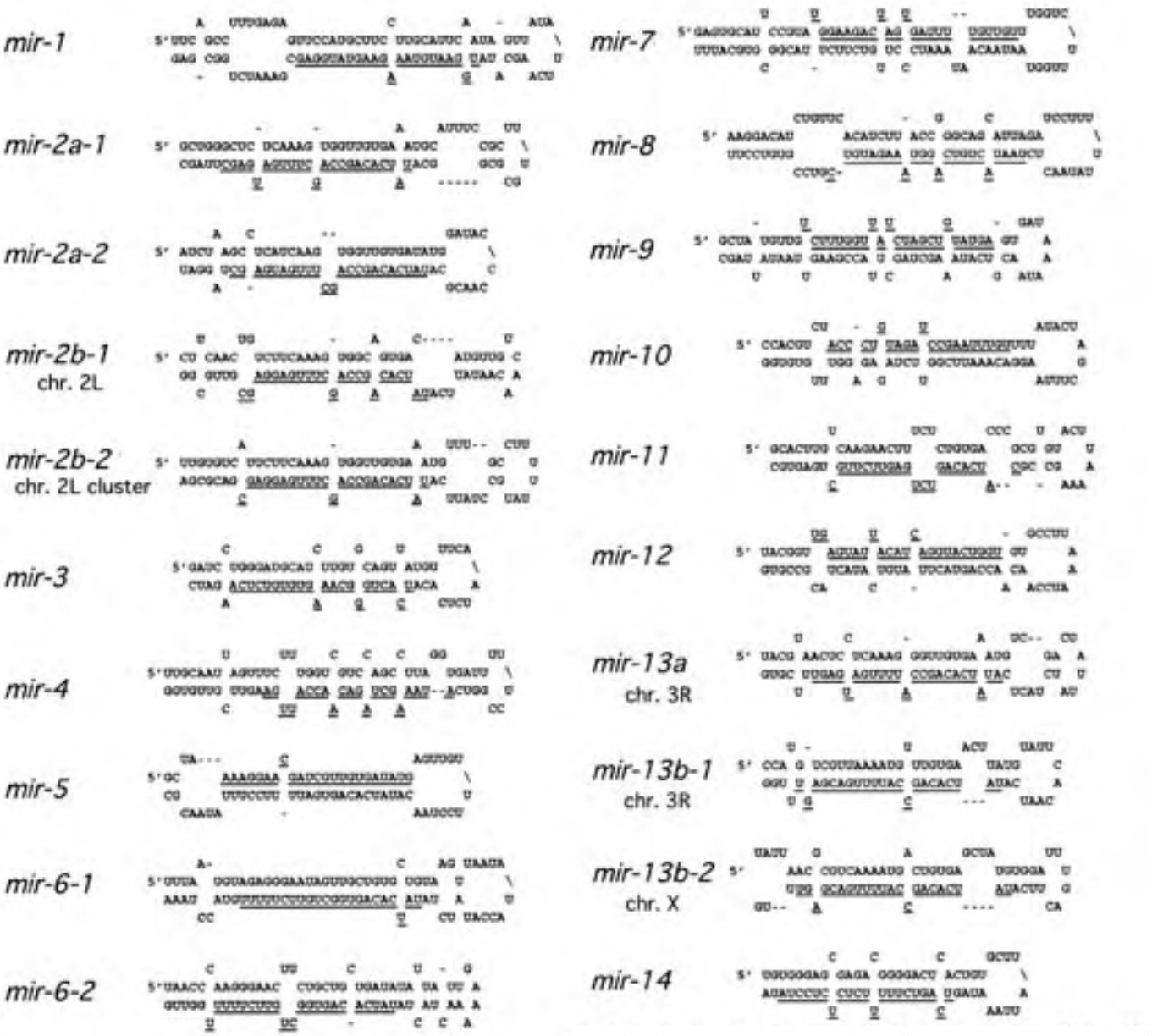

Fig. 3. Predicted precursor structures of *D. melanogaster* miRNAs. RNA secondary structure prediction was performed using mfold version 3.1 (*32*) and manually refined to accommodate G/U wobble base pairs in the helical segments. The miRNA sequence is underlined. The actual size of the stem-loop structure is not known experimentally and may be slightly shorter or longer than represented. Multicopy miRNAs and their corresponding precursor structures are also shown.

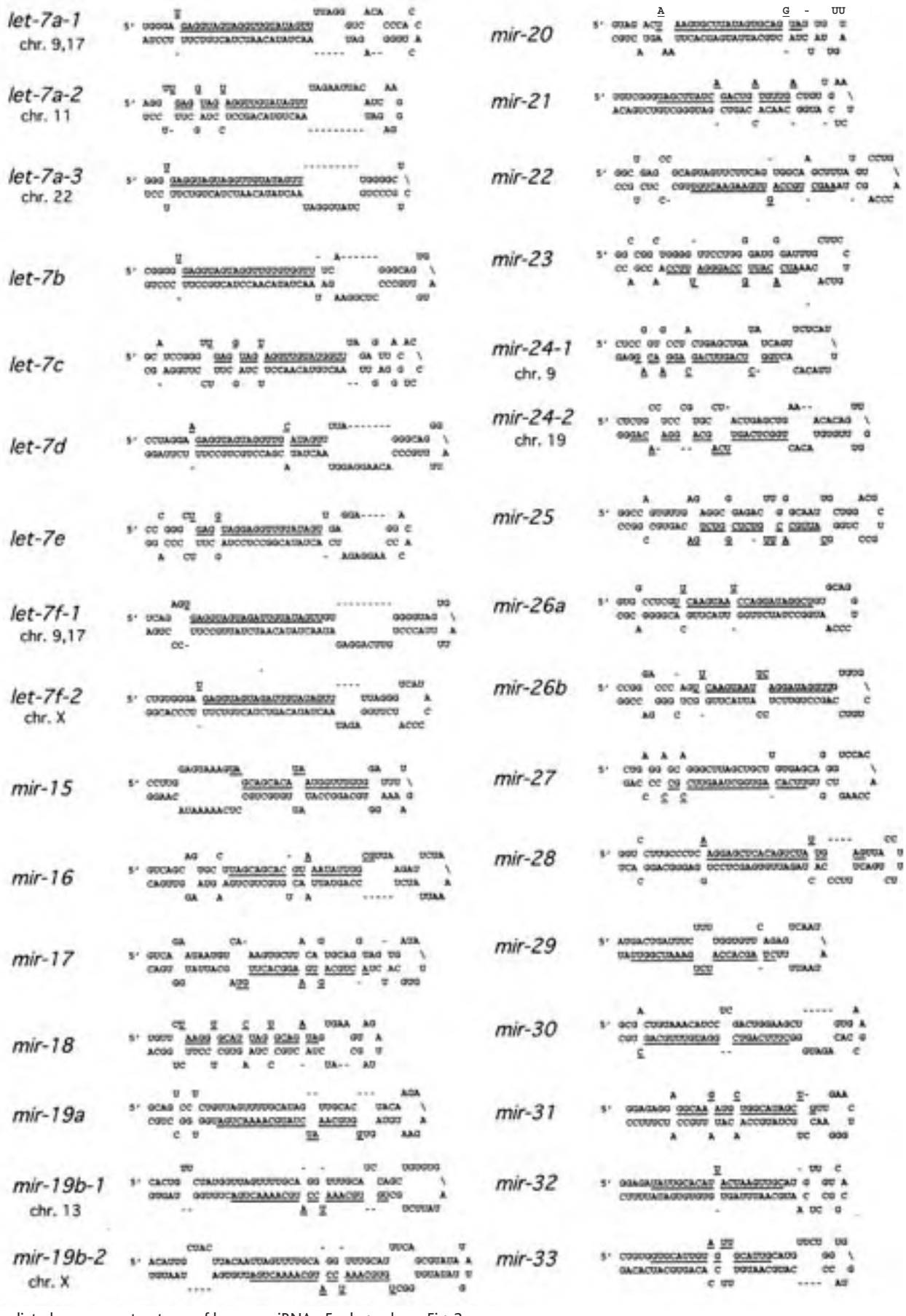

Fig. 4. Predicted precursor structures of human miRNAs. For legend, see Fig. 3.

Table 1. *D. melanogaster* miRNAs. The sequences given represent the most abundant, and typically longest, miRNA sequence identified by cloning; miRNAs frequently vary in length by one or two nucleotides at their 3′ termini. From 222 short RNAs sequenced, 69 (31%) corresponded to miRNAs, 103 (46%) to already characterized functional RNAs (rRNA, 7SL RNA, and tRNA), 30 (14%) to transposon RNA fragments, and 20 (10%) sequences had no database entry. The frequency for cloning a particular miRNA as a percentage relative to all identified miRNAs is indicated. Results of Northern blotting of total RNA isolated from staged populations of *D. melanogaster* are summarized. E, embryo; L, larval stage; P, pupa; A, adult; S2, Schneider-2 cells. The strength of the signal within each blot is represented from strongest (+++) to undetected (–). *let-7* stRNA was probed as the control. GenBank accession numbers and homologs of miRNAs identified by database searching in other species are provided in (*21*).

miRNA	Sequence (5′ to 3′)	Freq. (%)	E 0 to 3 hours	E 0 to 6 hours	L1 + L2	L3	P	A	S2
miR-1	UGGAAUGUAAAGAAGUAUGGAG	32	+	+	+++	+++	++	+++	–
miR-2a*	UAUCACAGCCAGCUUUGAUGAGC	3							
miR-2b*	UAUCACAGCCAGCUUUGAGGAGC	3	++	++	++	+++	++	+	+++
miR-3	UCACUGGGCAAAGUGUGUCUCA	9	+++	+++	–	–	–	–	–
miR-4	AUAAAGCUAGACAACCAUUGA	6	+++	+++	–	–	–	–	–
miR-5	AAAGGAACGAUCGUUGUGAUAUG	1	+++	+++	+/–	+/–	–	–	–
miR-6	UAUCACAGUGGCUGUUCUUUUU	13	+++	+++	+/–	+/–	–	–	–
miR-7	UGGAAGACUAGUGAUUUUGUUGU	4	+++	++	+/–	+/–	+/–	+/–	+/–
miR-8	UAAUACUGUCAGGUAAAGAUGUC	3	+/–	+/–	+++	+++	+	+++	–
miR-9	UCUUUGGUUAUCUAGCUGUAUGA	7	+++	++	+++	+++	+++	+/–	–
miR-10	ACCCUGUAGAUCCGAAUUUGU	1	+	+	++	+++	+/–	+	–
miR-11	CAUCACAGUCUGAGUUCUUGC	7	+++	+++	+++	+++	+++	+	–
miR-12	UGAGUAUUACAUCAGGUACUGGU	7	+	+	++	++	+	+++	+/–
miR-13a*	UAUCACAGCCAUUUUGACGAGU	1	+++	+++	+++	+++	+	+++	+++
miR-13b*	UAUCACAGCCAUUUUGAUGAGU	0							
miR-14	UCAGUCUUUUUCUCUCUCCUA	1	–	–	–	–	–	–	–
let-7	UGAGGUAGUAGGUUGUAUAGUU	0	–	–	–	–	+++	+++	–

*Similar miRNA sequences are difficult to distinguished by Northern blotting because of potential cross-hybridization of probes.

are also clustered (Fig. 2B) and are expressed in HeLa cells and adult zebrafish, but undetectable in mouse kidney and frog ovary (Fig. 1 and Table 2), and therefore represent a likely case of tissue-specific miRNA expression.

The majority of vertebrate and invertebrate miRNAs identified in this study are not related by sequence, but a few exceptions do exist and are similar to results previously reported for let-7 RNA (*8*). Sequence analysis of the *D. melanogaster* miRNAs revealed four such instances of sequence conservation between invertebrates and vertebrates. miR-1 homologs are encoded in the genomes of *C. elegans*, *C. briggsae*, and humans and are found in cDNAs from zebrafish, mice, cows, and humans. The expression of *mir-1* was detected by Northern blotting in total RNA from adult zebrafish and *C. elegans*, but not in total RNA from HeLa cells or mouse kidney (Table 2) (*20*). Interestingly, although *mir-1* and *let-7* are both expressed in adult flies (Fig. 1A) (*8*) and are both undetected in S2 cells, only *let-7* is detectable in HeLa cells. This represents another case of tissue-specific expression of an miRNA and indicates that miRNAs may play a regulatory role not only in developmental timing but also in tissue specification. miR-7 homologs were found by database searches of the mouse and human genomes and of expressed sequence tags (ESTs). Two mammalian miR-7 variants are predicted by sequence analysis in mice and humans and were detected by Northern blotting in HeLa cells and adult zebrafish, but not in mouse kidney (Table 2). Similarly, we identified mouse and human miR-9 and miR-10 homologs by database searches but only detected *mir-10* expression in mouse kidney.

The identification of evolutionarily related miRNAs, which have already acquired multiple sequence mutations, was not possible by standard bioinformatic searches. Direct comparison of the *D. melanogaster* miRNAs with the human miRNAs identified an 11-nt segment shared between *D. melanogaster* miR-6 and HeLa miR-27, but no further relationships were detected. It is possible that most miRNAs only act on a single target and therefore allow for rapid evolution by covariation. Highly conserved miRNAs may act on more than one target sequence and therefore have a reduced probability for evolutionary drift by covariation (*8*). An alternative interpretation is that the sets of miRNAs from *D. melanogaster* and humans are fairly incomplete and that many more mi-RNAs remain to be discovered, which will provide the missing evolutionary links.

lin-4 and *let-7* stRNAs were predicted to be excised from longer transcripts that contain stem-loop structures about 30 base pairs in length (*6, 8*). Database searches for newly identified miRNAs revealed that all miRNAs are flanked by sequences that have the potential to form stable stem-loop structures (Figs. 3 and 4). In many cases, we were able to detect the predicted precursors (about 70 nt) by Northern blotting (Fig. 1). Some miRNA precursor sequences were also identified in mammalian cDNA (EST) databases (*21*), indicating that primary transcripts longer than 70-nt stem-loop precursors also exist. We never cloned a 22-nt RNA complementary to any of the newly identified miRNAs, and it is as yet unknown how the cellular processing machinery distinguishes between an miRNA and its complementary strand. Comparative analysis of the precursor stem-loop structures indicates that the loops adjacent to the base-paired miRNA segment can be located on either side of the miRNA sequence (Figs. 3 and 4), suggesting that neither the 5′ nor the 3′ location of the stem-closing loop is the determinant of miRNA excision. It is also unlikely that the structure, length, or stability of the precursor stem is the critical determinant because the base-paired structures are frequently imperfect and interspersed by G/U wobbles and less stable, non–Watson-Crick base pairs such as G/A, U/U, C/U, and A/A. Therefore, a sequence-specific recognition process is a likely determinant for miRNA excision, perhaps mediated by members of the Argonaute (RDE-1/AGO1/PIWI) protein family. Two members of this family, ALG-1 and ALG-2, have recently been shown to be critical for stRNA processing in *C. elegans* (*13*). Members of the Argonaute protein family are also involved in RNAi and posttranscriptional gene silencing. In *D. melanogaster*, these include Argonaute2, a component of the siRNA-endonuclease complex (RISC) (*22*), and its relative Aubergine, which is important for silencing of repeat genes (*23*). In other species, these include RDE-1 in *C. elegans* (*24*); Argonaute1 in *Arabidopsis thaliana* (*25*); and QDE-2 in *Neurospora crassa* (*26*). In addition to the RNase III Dicer (*12, 13*), the Argonaute family represents another evolutionary line between RNAi and miRNA maturation.

Despite advanced genome projects, computer-assisted detection of genes encoding

Table 2. Human miRNAs. From 220 short RNAs sequenced, 100 (45%) corresponded to miRNAs, 53 (24%) to already characterized functional RNAs (rRNA, snRNA, and tRNA), and 67 (30%) of the sequences had no database entry. Results of Northern blotting of total RNA isolated from different vertebrate species and S2 cells are indicated. For legend, see Table 1.

miRNA	Sequence (5′ to 3′)	Freq. (%)	HeLa cells	Mouse kidney	Adult fish	Frog ovary	S2
let-7a*	UGAGGUAGUAGGUUGUAUAGUU	10	+++	+++	+++	–	–
let-7b*	UGAGGUAGUAGGUUGUGUGGUU	13					
let-7c*	UGAGGUAGUAGGUUGUAUGGUU	3					
let-7d*	AGAGGUAGUAGGUUGCAUAGU	2	+++	+++	+++	–	–
let-7e*	UGAGGUAGGAGGUUGUAUAGU	2	+++	+++	+++	–	–
let-7f*	UGAGGUAGUAGAUUGUAUAGUU	1					
miR-15	UAGCAGCACAUAAUGGUUUGUG	3	+++	++	+	+/–	–
miR-16	UAGCAGCACGUAAAUAUUGGCG	10	+++	+	+/–	+/–	–
miR-17	ACUGCAGUGAAGGCACUUGU	1	+++	–	–	–	–
miR-18	UAAGGUGCAUCUAGUGCAGAUA	2	+++	–	–	–	–
miR-19a*	UGUGCAAAUCUAUGCAAAACUGA	1	+++	–	+/–	–	–
miR-19b*	UGUGCAAAUCCAUGCAAAACUGA	3					
miR-20	UAAAGUGCUUAUAGUGCAGGUA	4	+++	–	+	–	–
miR-21	UAGCUUAUCAGACUGAUGUUGA	10	+++	+	++	–	–
miR-22	AAGCUGCCAGUUGAAGAACUGU	10	+++	+++	+	+/–	–
miR-23	AUCACAUUGCCAGGGAUUUCC	2	+++	+++	+++	+	–
miR-24	UGGCUCAGUUCAGCAGGAACAG	4	++	+++	++	–	–
miR-25	CAUUGCACUUGUCUCGGUCUGA	3	+++	+	++	–	–
miR-26a*	UUCAAGUAAUCCAGGAUAGGCU	2	+	++	+++	–	–
miR-26b*	UUCAAGUAAUUCAGGAUAGGUU	1					–
miR-27	UUCACAGUGGCUAAGUUCCGCU	2	+++	+++	++	–	–
miR-28	AAGGAGCUCACAGUCUAUUGAG	2	+++	+++	–	–	–
miR-29	CUAGCACCAUCUGAAAUCGGUU	2	+	+++	+/–	–	–
miR-30	CUUUCAGUCGGAUGUUUGCAGC	2	+++	+++	+++	–	–
miR-31	GGCAAGAUGCUGGCAUAGCUG	2	+++	–	–	–	–
miR-32	UAUUGCACAUUACUAAGUUGC	1	–	–	–	–	–
miR-33	GUGCAUUGUAGUUGCAUUG	1	–	–	–	–	–
miR-1	UGGAAUGUAAAGAAGUAUGGAG	0	–	–	+	–	–
miR-7	UGGAAGACUAGUGAUUUUGUUGU	0	+	–	+/–	–	+/–
miR-9	UCUUUGGUUAUCUAGCUGUAUGA	0	–	–	–	–	–
miR-10	ACCCUGUAGAUCCGAAUUUGU	0	–	+	–	–	–

*Similar miRNA sequences are difficult to distinguish by Northern blotting because of potential cross-hybridization of probes.

functional RNAs remains problematic (*27*). Cloning of expressed, short functional RNAs, similar to EST approaches (RNomics), is a powerful alternative and probably the most efficient method for identification of such novel gene products (*28–31*). The number of functional RNAs has been widely underestimated and is expected to grow rapidly because of the development of new functional RNA cloning methodologies.

The challenge for the future is to define the function and the potential targets of these novel miRNAs by using bioinformatics as well as genetics and to establish a complete catalog of time- and tissue-specific distribution of the already identified and yet to be uncovered miRNAs. *lin-4* and *let-7* stRNAs negatively regulate the expression of proteins encoded by mRNAs in which 3′ untranslated regions contain sites of complementarity to the stRNA (*9–11*). Because these interaction domains are only 6 to 10 base pairs long and often contain small bulges and G/U wobbles (*9–11*), the prediction of miRNA target mRNAs represents a challenging bioinformatic and/or genetic task. A profound understanding of the expression, processing, and action of miRNAs may enable the development of more general methods to direct the regulation of specific gene targets and may also lead to new ways of reprogramming tissues.

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An Abundant Class of Tiny RNAs with Probable Regulatory Roles in *Caenorhabditis elegans*

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Two small temporal RNAs (stRNAs), ***lin-4*** and ***let-7***, control developmental timing in ***Caenorhabditis elegans***. We find that these two regulatory RNAs are members of a large class of 21- to 24-nucleotide noncoding RNAs, called microRNAs (miRNAs). We report on 55 previously unknown miRNAs in ***C. elegans***. The miRNAs have diverse expression patterns during development: a ***let-7*** paralog is temporally coexpressed with ***let-7***; miRNAs encoded in a single genomic cluster are coexpressed during embryogenesis; and still other miRNAs are expressed constitutively throughout development. Potential orthologs of several of these miRNA genes were identified in ***Drosophila*** and human genomes. The abundance of these tiny RNAs, their expression patterns, and their evolutionary conservation imply that, as a class, miRNAs have broad regulatory functions in animals.

Two types of short RNAs, both about 21 to 25 nucleotides (21–25 nt) in length, serve as guide RNAs to direct post-transcriptional regulatory machinery to specific mRNA targets. Small temporal RNAs (stRNAs) control developmental timing in *Caenorhabditis elegans* (*1–3*). They pair to sites within the 3′ untranslated region (3′ UTR) of target mRNAs, causing translational repression of these mRNAs and triggering the transition to the next developmental stage (*1–5*). Small interfering RNAs (siRNAs), which direct mRNA cleavage during RNA interference (RNAi) and related processes, are the other type of short regulatory RNAs (*6–12*). Both stRNAs and siRNAs are generated by processes requiring Dicer, a multidomain protein with tandem ribonuclease III (RNase III) domains (*13-15*). Dicer cleaves within the double-stranded portion of precursor molecules to yield the 21–25 nt guide RNAs.

lin-4 and *let-7* have been the only two stRNAs identified, and so the extent to which this type of small noncoding RNA normally regulates eukaryotic gene expression is only beginning to be understood *(1–5)*. RNAi-related processes protect against viruses or mobile genetic elements, yet these processes are known to normally regulate only one other mRNA, that of *Drosophila Stellate* (*16–20*). To investigate whether RNAs resembling stRNAs or siRNAs might play a more general role in gene regulation, we isolated and cloned endogenous *C. elegans* RNAs that have the expected features of Dicer products. Tuschl and colleagues showed that such a strategy is feasible when they fortuitously cloned endogenous *Drosophila* RNAs while cloning siRNAs processed from exogenous dsRNA in an embryo lysate (*12*). Furthermore, other efforts focusing on longer RNAs have recently uncovered many previously unknown noncoding RNAs (*21*, *22*).

Dicer products, such as stRNAs and siRNAs, can be distinguished from most other oligonucleotides that might be present in *C. elegans* by three criteria: a length of about 22 nt, a 5′-terminal monophosphate, and a 3′-terminal hydroxyl group (*12*, *13*, *15*). Accordingly, a procedure was developed for isolating and cloning *C. elegans* RNAs with these features (*23*). Of the clones sequenced, 330 matched *C. elegans* genomic sequence, including 10 representing *lin-4* RNA and 1 representing *let-7* RNA. Another 182 corresponded to the *Escherichia coli* genomic sequence. *E. coli* RNA clones were expected because the worms were cultured with *E. coli* as the primary food source.

Three hundred of the 330 *C. elegans* clones have the potential to pair with nearby genomic sequences to form fold-back structures resembling those thought to be needed for Dicer processing of *lin-4* and *let-7* stRNAs (Fig. 1) (*24*). These 300 clones with predicted fold-backs represent 54 unique sequences: *lin-4*, *let-7*, and 52 other RNAs (Table 1). Thus, *lin-4* and *let-7* RNAs appear to be members of a larger class of noncoding RNAs that are about 20–24 nt in length and are processed from fold-back structures. We and the two other groups reporting in this issue of the journal refer to this class of tiny RNAs as microRNAs, abbreviated miRNAs, with individual miRNAs and their genes designated miR-# and *mir-#*, respectively (*25*, *26*).

We propose that most of the miRNAs are expressed from independent transcription units, previously unidentified because they do not contain an open reading frame (ORF) or other features required by current gene-recognition algorithms. No miRNAs matched a transcript validated by an annotated *C. elegans* expressed sequence tag (EST), and most were at least 1 kb from the nearest annotated sequences (Table 1). Even the miRNA genes near predicted coding regions or within predicted introns are probably expressed separately from the annotated genes. If most miRNAs were expressed from the same primary transcript as the predicted protein, their orientation would be predominantly the same as the predicted mRNA, but no such bias in orientation was observed (Table 1). Likewise, other types of RNA genes located within *C. elegans* intronic regions are usually expressed from independent transcription units (*27*).

Whereas both *lin-4* and *let-7* RNAs reside on the 5′ arm of their fold-back structures (*1*, *3*), only about a quarter of the other miRNAs lie on the 5′ arm of their proposed fold-back structures, as exemplified by miR-84 (Table 1 and Fig. 1A). All the others are on the 3′ arm, as exemplified by miR-1 (Table 1 and Fig. 1B). This implies that the stable product of Dicer processing can reside on either arm of the precursor and that features of the miRNA or its precursor—other than the loop connecting the two arms—must determine which side of the fold-back contains the stable product.

When compared with the RNA fragments cloned from *E. coli*, the miRNAs had unique length and sequence features (Fig. 2). The *E. coli* fragments had a broad length distribution, ranging from 15–29 nt, which reflects the size-selection limits imposed during the cloning procedure (*23*). In contrast, the miRNAs had a much tighter length distribution, centering on 21–24 nt, coincident with the known specificity of Dicer processing (Fig. 2A). The miRNA sequence composition preferences were most striking at the 5′ end, where there was a strong preference for U and against G at the first position and then a deficiency of U at positions 2 through 4 (Fig. 2B). miRNAs were also generally deficient in C, except at position 4. These composition preferences were not present in the clones representing *E. coli* RNA fragments.

The expression of 20 cloned miRNAs was examined, and all but two (miR-41 and miR-68) were readily detected on Northern blots (Fig. 3). For these 18 miRNAs with detectable expression, the dominant form was the mature 20–24 nt fragment(s), though for most, a longer species was also detected at the mobility expected for the fold-back precursor RNA. Fold-back precursors for *lin-4* and *let-7* have also been observed, particularly at the stage in development when the stRNA is first expressed (*1*, *14*, *15*).

Because the miRNAs resemble stRNAs, their temporal expression was examined. RNA from wild-type embryos, the four larval stages (L1 through L4), and young adults was probed. RNA from *glp-4 (bn2)* young adults, which are severely depleted in germ cells (*28*), was also

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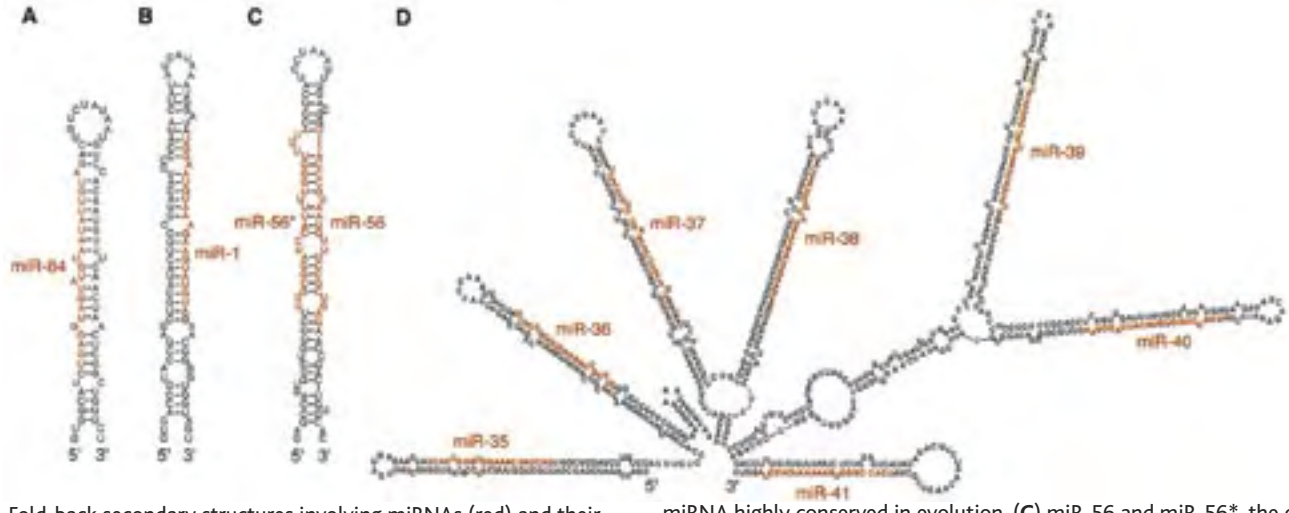

Fig. 1. Fold-back secondary structures involving miRNAs (red) and their flanking sequences (black), as predicted computationally using RNAfold (*35*). (**A**) miR-84, an miRNA with similarity to *let-7* RNA. (**B**) miR-1, an miRNA highly conserved in evolution. (**C**) miR-56 and miR-56*, the only two miRNAs cloned from both sides of the same fold-back. (**D**) The *mir-35–mir-41* cluster.

probed because miRNAs might have critical functions in the germ line, as suggested by the finding that worms deficient in Dicer have germ line defects and are sterile (*14*, *29)*. Many miRNAs have intriguing expression patterns during development (Fig. 3). For example, the expression of miR-84, an miRNA with 77% sequence identity to *let-7* RNA, was found to be indistinguishable from that of *let-7* (Fig. 3). Thus, it is tempting to speculate that miR-84 is an stRNA that works in concert with *let-7* RNA to control the larval-to-adult transition, an idea supported by the identification of plausible binding sites for miR-84 in the 3′ UTRs of appropriate heterochronic genes (*30*).

Nearly all of the miRNAs appear to have orthologs in other species, as would be expected if they had evolutionarily conserved regulatory roles. About 85% of the newly found miRNAs had recognizable homologs in the available *C. briggsae* genomic sequence, which at the time of our analysis included about 90% of the *C. briggsae* genome (Table 1). Over 40% of the miRNAs appeared to be identical in *C. briggsae*, as seen with the *lin-4* and *let-7* RNAs (*1*, *3*). Those miRNAs not absolutely conserved between *C. briggsae* and *C. elegans* might still have important functions, but they may have more readily co-varied with their target sites because, for instance, they might have fewer target sites. When the sequence of the miRNA differs from that of its homologs, there is usually a compensatory change in the other arm of the fold-back to maintain pairing, which provides phylogenetic evidence for the existence and importance of the fold-back secondary structures. *let-7*, but not *lin-4*, has discernable homologs in more distantly related organisms, including *Drosophila* and human (*31*). At least seven other miRNA genes (*mir-1*, *mir-2*, *mir-34*, *mir-60*, *mir-72*, *mir-79,* and *mir-84*) appear to be conserved in *Drosophila*, and most of these (*mir-1*, *mir-34*, *mir-60*, *mir-72*, and *mir-84*) appear to be conserved in humans (*24*).

The most highly conserved miRNA found, miR-1, is expressed throughout *C. elegans* development (Fig. 3) and therefore is unlikely to control developmental timing but may control tissue-specific events.

The distribution of miRNA genes within the *C. elegans* genome is not random (Table 1). For example, clones for six miRNA paralogs clustered within an 800–base pair (800-bp) fragment of chromosome II (Table 1). Computer folding readily identified the fold-back structures for the six cloned miRNAs of this cluster, and predicted the existence of a seventh paralog, miR-39 (Fig. 1D). Northern analysis confirmed the presence and expression of miR-39 (Fig. 3). The homologous cluster in *C. briggsae* appears to have eight related miRNAs. Some of the miRNAs in the *C. elegans* cluster are more similar to each other than to those of the *C. briggsae* cluster and vice versa, indicating that the size of the cluster has been quite dynamic over a short evolutionary interval, with expansion and perhaps also contraction since the divergence of these two species.

Northern analysis of the miRNAs of the *mir-35–mir-41* cluster showed that these miRNAs are highly expressed in the embryo and in young adults (with eggs), but not at other developmental stages (Fig. 3). For the six detectable miRNAs of this cluster, longer species with mobilities expected for the respective fold-back RNAs also appear to be expressed in the germ line; these longer RNAs were observed in wild-type L4 larvae (which have proliferating germ cells) but not in germ line–deficient mutant animals (Fig. 3) (*30*).

The close proximity of the miRNA genes within the *mir-35–mir-41* cluster (Fig. 1D) suggests that they are all transcribed and processed from a single precursor RNA, an idea supported by the coordinate expression of these genes (Fig. 3). This operon-like organization and expression brings to mind several

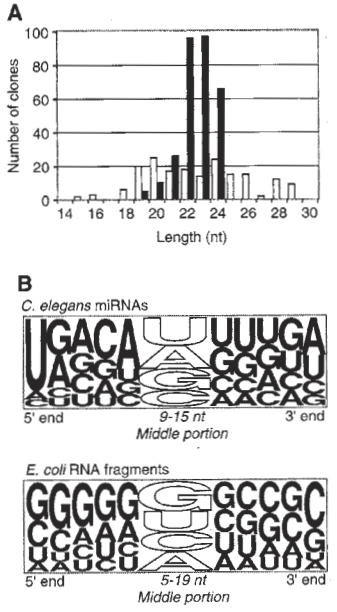

Fig. 2. Unique sequence features of the miRNAs. (**A**) Length distribution of the clones representing *E. coli* RNA fragments (white bars) and *C. elegans* miRNAs (black bars). (**B**) Sequence composition of the unique clones representing *C. elegans* miRNAs and *E. coli* RNA fragments. The height of each letter is proportional to the frequency of the indicated nucleotide. Solid letters correspond to specific positions relative to the ends of the clones; outlined letters represent the aggregate composition of the interior of the clones. To avoid overrepresentation from groups of related miRNAs in this analysis, each set of paralogs was represented by its consensus sequence.

potential models for miRNA action. For example, each miRNA of the operon might target a different member of a gene family for translational repression. At the other extreme, they all might converge on the same target, just as *lin-4* and *let-7* RNAs potentially converge on the 3′ UTR of *lin-14* (*3*).

Another four clusters were identified

among the sequenced miRNA clones (Table 1). Whereas the clones from one cluster were not homologous to clones from other clusters, the clones within each cluster were usually related to each other, as seen with the *mir-35–mir-41* cluster. The last miRNA of the *mir-42–mir-44* cluster is also represented by a second gene, *mir-45*, which is not part of the cluster. This second gene appears to enable more constitutive expression of this miRNA (miR-44/45) as compared with the first two genes of the *mir-42–mir-44* cluster, which are expressed predominantly in the embryo (Fig. 3).

Dicer processing of stRNAs differs from that of siRNAs in its asymmetry: RNA from only one arm of the fold-back precursor accumulates, whereas the remainder of the precursor quickly degrades (*15*). This asym-

Table 1. miRNAs cloned from *C. elegans*. 300 RNA clones represented 54 different miRNAs. Also included are miR-39, miR-65, and miR-69, three miRNAs predicted based on homology and/or proximity to cloned miRNAs. miR-39 and miR-69 have been validated by Northern analysis (Fig. 3), whereas miR-65 is not sufficiently divergent to be readily distinguished by Northern analysis. All *C. elegans* sequence analyses relied on WormBase, release WS45 (*33*). Some miRNAs were represented by clones of different lengths, due to heterogeneity at the miRNA 3′ terminus. The observed lengths are indicated, as is the sequence of the most abundant length. Comparison to *C. briggsae* shotgun sequencing traces revealed miRNA orthologs with 100% sequence identity (+++) and potential orthologs with >90% (++) and >75% (+) sequence identity (*24*, *34*). Five miRNA genomic clusters are indicated with square brackets. Naming of miRNAs was coordinated with the Tuschl and Ambros groups (*25*, *26*).

miRNA gene	Number of clones	miRNA sequence	Length	*C. briggsae* homology	Fold-back arm	Chromosome and distance to nearest gene
lin-4	10	UCCCUGAGAC CUCAAGUGUG A	21	+++	5′	II
let-7	1	UGAGGUAGUA GGUUGUAUAG UU	22	+++	5′	X
mir-1	9	UGGAAUGUAA AGAAGUAUGU A	21	+++	3′	I 3.7 kb from start of T09B4.3, antisense
mir-2	24	UAUCACAGCC AGCUUUGAUG UGC	22–23	+++	3′	I 0.6 kb from start of M04C9.6b
mir-34	3	AGGCAGUGUG GUUAGCUGGU UG	22	+++	5′	X 2.1 kb from end of Y41G9A.4, antisense
mir-35	9	UCACCGGGUG GAAACUAGCA GU	22	+	3′	II 1.3 kb from end of F54D5.12, antisense
mir-36	1	UCACCGGGUG AAAAUUCGCA UG	22	+	3′	II 1.2 kb from end of F54D5.12, antisense
mir-37	2	UCACCGGGUG AACACUUGCA GU	22	++	3′	II 1.1 kb from end of F54D5.12, antisense
mir-38	1	UCACCGGGAG AAAAACUGGA GU	22	+	3′	II 1.0 kb from end of F54D5.12, antisense
mir-39	0	UCACCGGGUG UAAAUCAGCU UG	Predicted	++	3′	II 0.8 kb from end of F54D5.12, antisense
mir-40	2	UCACCGGGUG UACAUCAGCU AA	22	+	3′	II 0.7 kb from end of F54D5.12, antisense
mir-41	2	UCACCGGGUG AAAAAUCAGC UA	22	+	3′	II 0.6 kb from end of F54D5.12, antisense
mir-42	1	CACCGGGUUA ACAUCUACAG	20	+++	3′	II 1.2 kb from end of ZK930.2, antisense
mir-43	1	UAUCACAGUU UACUUGCUGU CGC	23	+++	3′	II 1.1 kb from end of ZK930.2, antisense
mir-44	3*	UGACUAGAGA CACAUUCAGC U	21	+++	3′	II 1.0 kb from end of ZK930.2, antisense
mir-45				+++	3′	II 0.7 kb from end of K12D12.1, antisense
mir-46	2	UGUCAUGGAG UCGCUCUCUU CA	22	+++	3′	III 3.0 kb from end of ZK525.1, antisense
mir-47	6	UGUCAUGGAG GCGCUCUCUU CA	22	+++	3′	X 1.8 kb from end of K02B9.2, antisense
mir-48	11	UGAGGUAGGC UCAGUAGAUG CGA	22–24	+++	5′	V 6.1 kb from start of Y49A3A.4
mir-49	1	AAGCACCACG AGAAGCUGCA GA	22	+++	3′	X 2.7kb from end of F19C6.1, antisense
mir-50	2	UGAUAUGUCU GGUAUUCUUG GGUU	24	++	5′	I in intron of Y71G12B.11a
mir-51	6	UACCCGUAGC UCCUAUCCAU GUU	23	++	5′	IV 0.4 kb from end of F36H1.6, antisense
mir-52	47	CACCCGUACA UAUGUUUCCG UGCU	22–25	+++	5′	IV 4.6 kb from end of Y37A1B.6, antisense
mir-53	2	CACCCGUACA UUUGUUUCCG UGCU	24	–	5′	IV 1.9 kb from end of F36H1.6, antisense
mir-54	2	UACCCGUAAU CUUCAUAAUC CGAG	24	+	3′	X 5.5 kb from end of F09A5.2, antisense
mir-55	5	UACCCGUAUA AGUUUCUGCU GAG	23	+	3′	X 5.3 kb from end of F09A5.2, antisense
mir-56	5	UACCCGUAAU GUUUCCGCUG AG	22	+	3′	X 5.2 kb from end of F09A5.2, antisense
mir-56	2	UGGCGGAUCC AUUUUGGGUU GUA	23	+	5′	X 5.2 kb from end of F09A5.2, antisense
mir-57	9	UACCCUGUAG AUCGAGCUGU GUGU	24	+++	5′	II 0.9 kb from start of AF187012-1.T09A5
mir-58	31	UGAGAUCGUU CAGUACGGCA AU	21–22	+++	3′	I Vin intron of Y67D8A.1
mir-59	1	UCGAAUCGUU UAUCAGGAUG AUG	23	+	3′	IV 1.8 kb from start of B0035.1a, antisense
mir-60	1	UAUUAUGCAC AUUUUCUAGU UCA	23	++	3′	II 1.5 kb from end of C32D5.5
mir-61	1	UGACUAGAAC CGUUACUCAU C	21	+	3′	V 0.4 kb from end of F55A11.3, antisense
mir-62	1	UGAUAUGUAA UCUAGCUUAC AG	22	+++	3′	X in intron of T07C5.1
mir-63	1	UAUGACACUG AAGCGAGUUG GAAA	24	–	3′	X 1.7 kb from start of.C16H3.2, antisense
mir-64	2	UAUGACACUG AAGCGUUACC GAA	23	–	5′	III 0.25 kb from start of Y48G9A.1
mir-65	0	UAUGACACUG AAGCGUAACC GAA	Predicted	+	5′	III 0.10 kb from start of Y48G9A.1
mir-66	10	CAUGACACUG AUUAGGGAUG UGA	23–24	–	5′	III in coding sequence of Y48G9A.1
mir-67	2	UCACAACCUC CUAGAAAGAG UAGA	24	+++	3′	III 4.4 kb from end of EGAP1.1
mir-68	1	UCGAAGACUC AAAAGUGUAG A	21	–	3′	IV 3.3 kb from start of Y51H4A.22
mir-69	0	UCGAAAAUUA AAAAGUGUAG A	Predicted	–	3′	IV 0.6 kb from start of Y41D4B.21, antisense
mir-70	1	UAAUACGUCG UUGGUGUUUC CAU	23	+	3′	V in intron of T10H9.5
mir-71	5	UGAAAGACAU GGGUAGUGA	19, 20, 22	+++	5′	I 7.8 kb from start of M04C9.6b
mir-72	9	AGGCAAGAUG UUGGCAUAGC	20, 21, 23	–	3′	II 0.21 kb from end of F53G2.4, antisense
mir-73	2	UGGCAAGAUG UAGGCAGUUC AGU	23	++	3′	X 2.9 kb from start of T24D8.6, antisense
mir-74	7	UGGCAAGAAA UGGCAGUCUA CA	22	++	3′	X 3.2 kb from start of T24D8.6, antisense
mir-75	2	UUAAAGCUAC CAACCGGCUU CA	22	++	3′	X 3.5 kb from start of F47G3.3
mir-76	1	UUCGUUGUUG AUGAAGCCUU GA	22	++	3′	III 3.0 kb from start of C44B11.3, antisense
mir-77	1	UUCAUCAgGC CAUAGCUGUC CA	22	+++	3′	II 1.5 kb from start of T21B4.9, antisense
mir-78	2	UGGAGGCCUG GUUGUUUGUG C	21	–	3′	IV 2.0 kb from start of Y40H7A.3, antisense
mir-79	1	AUAAAGCUAG GUUACCAAAG CU	22	+++	3′	I 2.3 kb from end of C12C8.2
mir-80	25	UGAGAUCAUU AGUUGAAAGC CGA	23	+++	3′	III 4.7 kb from end of F44E2.2, antisense
mir-81	7	UGAGAUCAUC GUGAAAGCUA GU	22	+++	3′	X in intron of T07D1.2, antisense
mir-82	6	UGAGAUCAUC GUGAAAGCCA GU	22	+++	3′	X 0.11 kb from start of T07D1.2
mir-83	1	UAGCACCAUA UAAAUUCAGu AA	22	++	3′	IV 5.0 kb from start of C06A6.2
mir-84	3	UGAGGUAGUA UGUAAUAUUG UA	22, 24	+	5′	X 0.8 kb from end of B0395.1, antisense
mir-85	1	UACAAAGUAU UUGAAAAGUC GUGC	24	++	3′	II in intron of F49E12.8, antisense
mir-86	6	UAAGUGAAUG CUUUGCCACA GUC	23	+++	5′	III in intron of Y56A3A.7

*Because *mir-44* and *mir-45* encode identical miRNAs, the three clones represent either or both genes.

metry extends to nearly all the miRNAs. For the 35 miRNAs yielding more than one clone, RNAs were cloned from both arms of a hairpin in only one case, miR-56 (Fig. 1C and Table 1). The functional miRNA appears to be miR-56 and not miR-56*, as indicated by sequence conservation between *C. elegans* and *C. briggsae* orthologs, analogy to the other constituents of the *mir-54–mir-56* cluster, and Northern blots detecting RNA from only the 3′ arm of the fold-back (*30*).

We were surprised to find that few, if any, of the cloned RNAs had the features of siRNAs. No *C. elegans* clones matched the antisense of annotated coding regions. Of the 30 *C. elegans* clones not classified as miRNAs, 15 matched fragments of known RNA genes, such as transfer RNA (tRNA) and ribosomal RNA. Of the remaining 15 clones, the best candidate for a natural siRNA is GGAAAACGGGUUGAAAGGGA. It was the only *C. elegans* clone perfectly complementary to an annotated EST, hybridizing to the 3′ UTR of gene ZK418.9, a possible RNA-binding protein. Even if this and a few other clones do represent authentic siRNAs, they would still be greatly outnumbered by the 300 clones representing 54 different miRNAs. Our cloning protocol is not expected to preferentially exclude siRNAs; it was similar to the protocol that efficiently cloned exogenous siRNAs from *Drosophila* extracts (*12*). Instead, we propose that the preponderance of miRNAs among our clones indicates that in healthy, growing cultures of *C. elegans*, regulation by miRNAs normally plays a more dominant role than does regulation by siRNAs.

Regardless of the relative importance of miRNAs and siRNAs in the normal regulation of endogenous genes, our results show that small RNA genes like *lin-4* and *let-7* are more abundant in *C. elegans* than previously appreciated. Results from a parallel effort that directly cloned small RNAs from *Drosophila* and HeLa cells demonstrates that the same is true in other animals (*25*), a conclusion further supported by the orthologs to the *C. elegans* miRNAs that we identified through database searching. Many of the miRNAs that we identified are represented by only a single clone (Table 1), suggesting that our sequencing has not reached saturation and that there are over 100 miRNA genes in *C. elegans*.

We presume that there is a reason for the expression and evolutionary conservation of these small noncoding RNAs. Our favored hypothesis is that these newly found miRNAs, together with *lin-4* and *let-7* RNAs, constitute an important and abundant class of riboregulators, pairing to specific sites within mRNAs to direct the posttranscriptional regulation of these genes (*32*). The abundance and diverse expression patterns of miRNA genes implies that they function in a variety of regulatory pathways, in addition to their known role in the temporal control of developmental events.

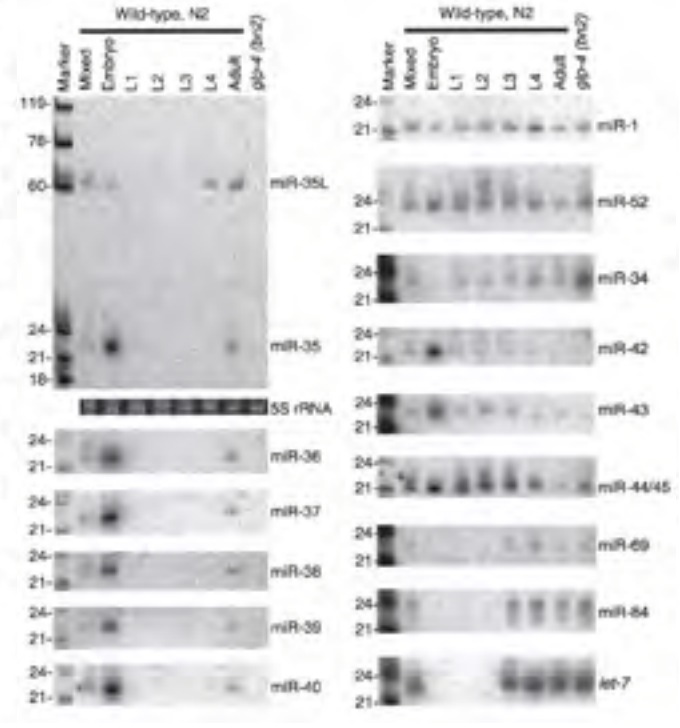

Fig. 3. Expression of newly found miRNAs and *let-7* RNA during *C. elegans* development. Northern blots probed total RNA from mixed-stage worms (Mixed), worms staged as indicated, and *glp-4 (bn2)* adult worms (*24*). Specificity controls ruled out cross-hybridization among probes for miRNAs from the *mir-35–mir-41* cluster (*24*). Other blots indicate that, miR-46 or -47, miR-56, miR-64 or -65, miR-66, and miR-80 are expressed constitutively throughout development (*30*).

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23. Short endogenous *C. elegans* RNAs were cloned using a protocol inspired by Elbashir *et al.* (*12*), but modified to make it specific for RNAs with 5′-terminal phosphate and 3′-terminal hydroxyl groups. In our protocol (*24*), gel-purified 18–26 nt RNA from mixed-stage worms was ligated to a pre-adenylylated 3′-adaptor oligonucleotide in a reaction using T4 RNA ligase but without adenosine triphosphate (ATP). Ligated RNA was gel-purified, then ligated to a 5′-adaptor oligonucleotide in a standard T4 RNA ligase reaction. Products from the second ligation were gel-purfied, then reverse transcribed and amplified by using the primers corresponding to the adaptor sequences. To achieve ligation specificity for RNA with a 5′-terminal phosphate and 3′-terminal hydroxyl, phosphatase and phosphorylase treatments, useful for preventing circularization of Dicer products (*12*), were not included in our protocol. Instead, circularization was avoided by using the preadenylylated 3′-adaptor oligonucleotide and omitting ATP during the first ligation reaction.
24. Supplemental material describing methods and predicted fold-back secondary structures for the miRNAs of Table 1 and some of their homologs in other species is available on *Science* Online at www.sciencemag.org/cgi/content/full/294/5543/858/DC1.
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32. This begs the question as to why more riboregulators have not been found previously. Perhaps they had not been identified biochemically because of a predisposition towards searching for protein rather than RNA factors. They could be identified genetically, which was how *lin-4* and *let-7* were discovered (*1–3*); however, when compared to mutations in protein-coding genes, point substitutions in these short RNA genes would be less likely and perhaps less disruptive of function. Furthermore, mutations that map to presumed intergenic regions with no associated RNA transcript detectable on a standard RNA blot might be put aside in favor of other mutants.
33. WormBase is available on the Web at www.wormbase.org.
34. Sequencing traces (from the Sanger Center) representing 2.5- to 3-fold average coverage of the *C. briggsae* genome were obtained at www.ncbi.nlm.nih.gov/Traces.
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36. We thank C. Ceol for guidance in culturing and staging *C. elegans*; R. Horvitz for the use of equipment and facilities; T. Tuschl, G. Ruvkun, B. Reinhart, A. Pasquinelli, C. Burge, F. Lewitter, A. Ensminger, and C. Mello for helpful discussions; P. Zamore, T. Orr-Weaver, and M. Lawrence for comments on this manuscript; and T. Tuschl, V. Ambros, G. Ruvkin, R. Horvitz, P. Sharp, and J. Hodgkin for discussions on nomenclature.

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An Extensive Class of Small RNAs in *Caenorhabditis elegans*

Rosalind C. Lee and Victor Ambros*

The *lin-4* and *let-7* antisense RNAs are temporal regulators that control the timing of developmental events in *Caenorhabditis elegans* by inhibiting translation of target mRNAs. *let-7* RNA is conserved among bilaterian animals, suggesting that this class of small RNAs [microRNAs (miRNAs)] is evolutionarily ancient. Using bioinformatics and cDNA cloning, we found 15 new miRNA genes in *C. elegans*. Several of these genes express small transcripts that vary in abundance during *C. elegans* larval development, and three of them have apparent homologs in mammals and/or insects. Small noncoding RNAs of the miRNA class appear to be numerous and diverse.

Small RNAs perform diverse functions within cells, including the regulation of gene expression (*1–4*). One class of regulatory RNA includes the small temporal RNA (stRNA) products of the genes *lin-4* and *let-7* in *Caenorhabditis elegans*. The *lin-4* and *let-7* RNAs are ~22 nucleotides (nt) in length, and are expressed stage-specifically, controlling key developmental transitions in worm larvae by acting as antisense translational repressors (*2–4*).

lin-4 and *let-7* were identified by their mutant phenotypes (*2, 3*) and, until recently, were the only known RNAs of their class. However, the phylogenetic conservation of *let-7* RNA sequence and developmental expression (*5*), and the overlap between the stRNA and RNA interference (RNAi) pathways (*6, 7*), suggested that stRNAs are part of an ancient regulatory mechanism involving ~22-nt antisense RNA molecules (*8*).

To identify more small regulatory RNAs of the *lin-4/let-7* class in *C. elegans*, we used informatics and cDNA cloning to select *C. elegans* genomic sequences that exhibited four characteristics of *lin-4* and *let-7*: (i) expression of a mature RNA of ~22 nt in length; (ii) location in intergenic (non–protein-coding) sequences; (iii) high DNA sequence similarity between orthologs in *C. elegans* and a related species, *Caenorhabditis briggsae*; and (iv) processing of the ~22-nt mature RNA from a stem-loop precursor transcript of ~65 nt (*2, 3*).

In an informatics approach to identifying candidate small regulatory RNAs, predicted *C. elegans* intergenic sequences that were also highly conserved in *C. briggsae* (*9, 10*) were analyzed using the RNA folding program "mfold" (*11–13*). Forty sequences were predicted by mfold to form a stem-loop similar in size and structure to *lin-4* and *let-7*. Probes complementary to these sequences were tested against Northern blots of total worm RNA (*13*), and three of them detected small RNA transcripts (Table 1 and Fig. 1A).

In a second approach, a cDNA library (about 1.6×10^6 independent *lambda* clones) was prepared from a size-selected (~22-nt) fraction of *C. elegans* total RNA (*14*) and sequence was obtained for 5025 independent inserts, representing 3627 distinct sequences (*13*). Some 386 of these sequences were represented by multiple (from 2 to 129) clones. Each of these multiple-hit cDNA sequences was compared using BLAST (*15*) to the NCBI database, and to approximately 800,000 raw sequence traces of *C. briggsae* genomic sequence (*16*). Single-copy cDNA sequences that corresponded to no previously known (or previously predicted) transcripts (*17*), and that were conserved in the *C. briggsae* genome, were analyzed using mfold for a predicted stem-loop structure. A total of 38 novel cDNA sequences fit these criteria, of which 13 were tested for expression by Northern hybridization; in all 13 cases, small transcripts (~22 nt and/or ~65 nt) were detected (Table 1 and Fig. 1). (The other 25 sequences have not been tested for expression.)

These 13 new genes identified by cDNA cloning, together with two additional genes from the informatics screen, were named *mir*, for microRNA (*18, 19*). All 15 of these miRNA genes appear to produce ~65-nt stem-loop transcripts (Fig. 2) that may be processed to ~22-nt forms by the same DCR-1/ALG-1/ALG-2 system involved with *lin-4* and *let-7* processing (*6, 7*). For the two RNAs that we tested (*mir-1* and *mir-58*), *dcr-1* activity was required for normal processing of the ~65-nt precursor (Fig. 1E). So in some cases, such as *lin-4* and *let-7*, the ~22-nt form is processed from the 5′ part of the stem (*6, 7*), and in other cases, such as *mir-1* and *mir-58*, from the 3′ part (Fig. 2), suggesting gene-specificity of miRNA processing and/or stabilization. For the three miRNA genes identified in our informatics screen (*mir-60*, *mir-88*, and *mir-89*), the longer stem-loop transcripts were detected by Northern blot, but ~22-nt forms were not detected, suggesting that their processing is inefficient, or is sharply restricted developmentally. For *mir-60*, 20-nt cDNA clones were identified, suggesting that *mir-60* is processed, but the mature form accumulates at levels below threshold for detection by Northern blot.

At least 10 of the 15 miRNAs vary in abundance during *C. elegans* larval development, perhaps reflecting roles for these particular genes in developmental timing (Table 1 and Fig. 1). *mir-1*, *mir-2*, and *mir-87* have apparent orthologs in mammals and/or insects (Table 1 and Fig. 1). *mir-1* is expressed tissue-specifically in humans (heart), and stage-specifically in mouse embryogenesis (Fig. 1D). An evolu-

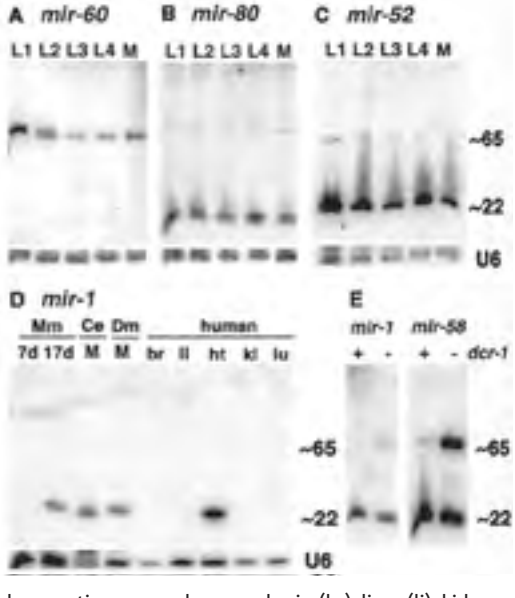

Fig. 1. Northern blots of small RNA transcripts. (A through C) Total RNA from *C. elegans* larvae (stages L1 through L4) or from mixed stage (M) populations were blotted and probed with oligonucleotides complementary to either the 5′or 3′ half of the indicated transcript (*13*). U6 = the same filters were probed with probe to U6 snRNA as a loading control. (**A**) *mir-60* 5′ probe detects a transcript of ~65 nt. The ratio of L1 to L4 *mir-60* signal, normalized to U6, is about 5:1. The *mir-60* 3′ probe (not shown) detects a similar-sized species with a similar developmental profile. (**B**) *mir-80* 3′ probe detects a ~22-nt RNA expressed uniformly at all stages. (**C**) *mir-52* 5′ probe. The normalized *mir-52* signal is threefold greater in the L1 versus the L3. (**D**) *mir-1* 3′ probe detects a transcript of ~22 nt in total RNA from mouse (Mm) 17-day embryos, mixed-stage *C. elegans* (Ce), *Drosophila melanogaster* (Dm) mixture of embryo-larvae-pupae, and in a sample of human heart (ht) tissue. Other human tissue samples were brain (br), liver (li), kidney (ki), and lung (lu). (**E**) *mir-1* and *mir-58* probes to total RNA from mixed populations of wild-type (+) and *dcr-1(ok247)* (–) animals. An increase in the proportion of unprocessed ~65-nt precursor is observed in the *dcr-1* RNA.

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tionarily conserved miRNA such as *mir-1* may have coevolved with its mRNA targets, and hence, could retain a similar developmental or physiological role in diverse taxa (*5*).

lin-4, *let-7*, and the 15 new miRNA genes described here are members of a gene family that could number in the hundreds in *C. elegans* (*18*) and other animals (*19*). To date, approximately 100 miRNA genes have been identified in worms, flies, and human cells (*18, 19*), and it is very likely that the screens conducted so far have not reached saturation. Therefore, additional *C. elegans* miRNAs can be identified using cDNA library sequencing. Also, continued application of whole-genome sequence alignment should identify additional new miRNAs, because this informatics approach complements the cDNA cloning. For example, using only a sample of the worm genome for *C. elegans*/*C. briggsae* alignment, we found two miRNAs (*mir-88* and *mir-89*) that were not represented in the size-selected cDNA library (perhaps due to absent or inefficient processing to the ~22-nt form).

This collection of new miRNAs exhibits a diversity in sequence, structure, abundance, and expression profile. If miRNA genes are as numerous and diverse as they appear to be, they likely occupy a wide variety of regulatory niches, and exert profound and complex effects on gene expression, development, and behavior. The challenge now is to determine the functions of the miRNAs, to identify potential antisense target mRNAs, and to characterize the consequences of their regulatory interactions.

Table 1. MicroRNA gene products in *Caenorhabditis elegans*.

Gene	Source*	Orthologs† Expression‡‖	Development¶
mir-1	cDNA	C‡§‖ Dm‖ Hs‡	Unif
mir-2	cDNA	C‡§‖ Dm‡	L1
mir-42	cDNA	C‡‖	L1
mir-43	cDNA	C‡§‖	L1
mir-52	cDNA	C‡§‖	L1
mir-58	cDNA	C‡‖	Unif
mir-60	cDNA/In	C‖	L1
mir-62	cDNA	C‡§‖	L1
mir-72	cDNA	C‡‖	L1
mir-80	cDNA	C‡§‖	Unif
mir-81	cDNA	C‡‖	L1
mir-87	cDNA	C‡§‖ Dm Hs	L1
mir-88	In	C‖	Unif
mir-89	In	C‖	NT
mir-90	cDNA	C‡‖	L1

*Identified by screening a size-selected cDNA library (cDNA) or by informatics (In). †Predicted by BLAST (*15*) and mfold (*11, 12*). C, *C. elegans*; Dm, *Drosophila melanogaster*; Hs, *Homo sapiens*. ‡Expression of an ~22-nt RNA confirmed by Northern blot. §The ~22-nt transcript confirmed to be single-stranded using probes to both ends of the predicted stem loop. ‖Expression of an ~65-nt predicted stem-loop RNA confirmed by Northern blot. ¶Stage of *C. elegans* larval development (L1 through L4) when the indicated transcript(s) appear most abundant. Unif, uniform expression; less than twofold change in level; NT, developmental profile not tested.

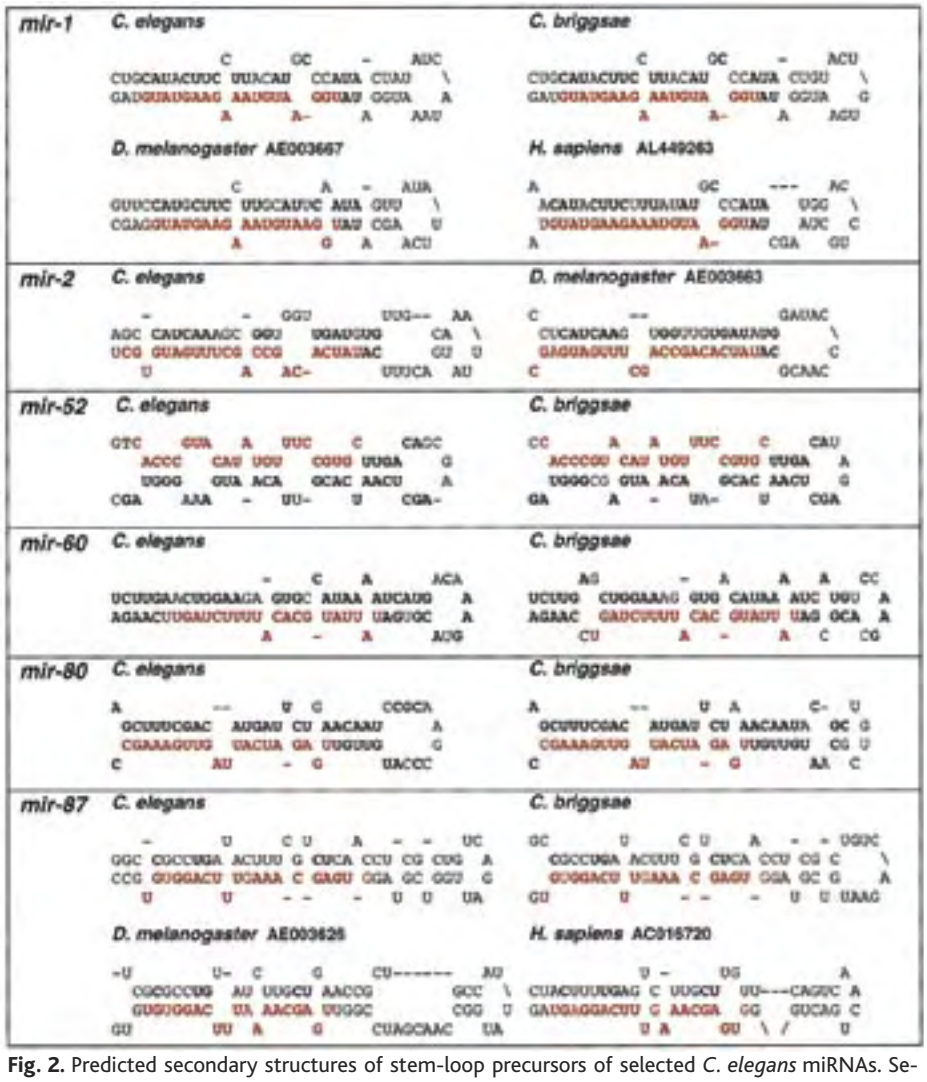

Fig. 2. Predicted secondary structures of stem-loop precursors of selected *C. elegans* miRNAs. Sequences of the ~22-nt mature small RNA are red, and were inferred from cDNA sequence, Northern blots, and/or *C. elegans*::*C. briggsae* homology (Table 1). Phylogenetically conserved nucleotides are bold. The 5′ end is to the upper left.

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20. We thank A. Lee for computer programming, M. McCarthy for worm staging, D. Jewell for helping with BLAST searches, the Sanger Center for *C. briggsae* genomic sequence, and W. J. Kent's Intronerator Web site for presorted worm sequences. We are also grateful to A. Grishok and C. Mello for the *dcr-1* strain and to T. Tuschl and D. Bartel for sharing data prior to publication. This work was supported by NIH grant R01 GM-34028 to V.A.

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MicroRNA Expression in Zebrafish Embryonic Development

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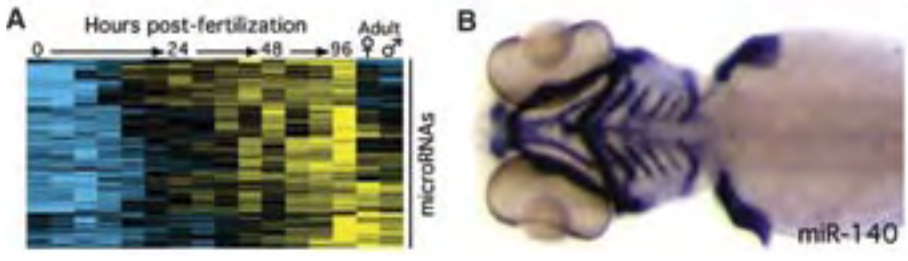
MicroRNAs (miRNAs) are small noncoding RNAs, about 21 nucleotides in length, that can regulate gene expression by base-pairing to partially complementary mRNAs. Regulation by miRNAs can play essential roles in embryonic development. We determined the temporal and spatial expression patterns of 115 conserved vertebrate miRNAs in zebrafish embryos by microarrays and by in situ hybridizations, using locked-nucleic acid–modified oligonucleotide probes. Most miRNAs were expressed in a highly tissue-specific manner during segmentation and later stages, but not early in development, which suggests that their role is not in tissue fate establishment but in differentiation or maintenance of tissue identity.

Current estimates of miRNA gene numbers in vertebrates are as high as 500 (*1*), of which many are conserved, and miRNAs may regulate up to 30% of genes (*2*). The miRNA first discovered, *lin-4*, is involved in developmental timing in the nematode *Caenorhabditis elegans* (*3*). In mammals, miRNAs have been implicated in hematopoietic lineage differentiation (*4*) and homeobox gene regulation (*5*). Zebrafish that are defective in miRNA processing arrest in development (*6*). Recently, miRNAs were shown to be dispensable for cell fate determination, axis formation, and cell differentiation but are re-



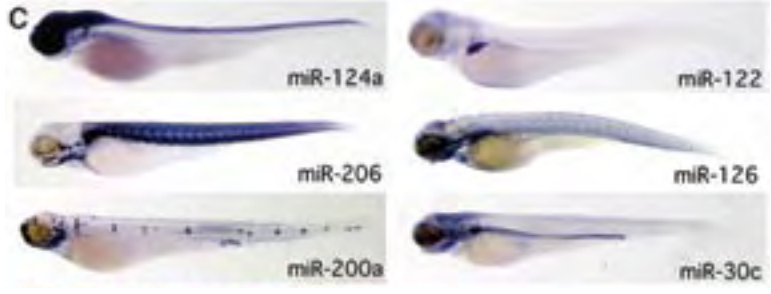


Fig. 1. miRNA expression in zebrafish embryonic development. (**A**) Microarray expression levels of 90 (of the 115) miRNAs during embryonic development. Colors indicate relative and mean-centered expression for each miRNA: blue, low; black, mean; yellow, high. (**B**) Ventral view of miR-140 whole-mount in situ expression in cartilage of pharyngeal arches, head skeleton, and fins at 72 hpf. (**C**) Lateral views of miRNA whole-mount in situ expression in different organ systems at 72 hpf: miR-124a, nervous systems; miR-122, liver; miR-206, muscles; miR-126, blood vessels and heart; miR-200a, lateral line system and sensory organs; miR-30c, pronephros. (**D**) Histological analysis of miRNA in situ expression in the pancreas 5 days after fertilization. Abbreviations: e, exocrine pancreas; i, pancreatic islet; gb, gall bladder; g, gut.

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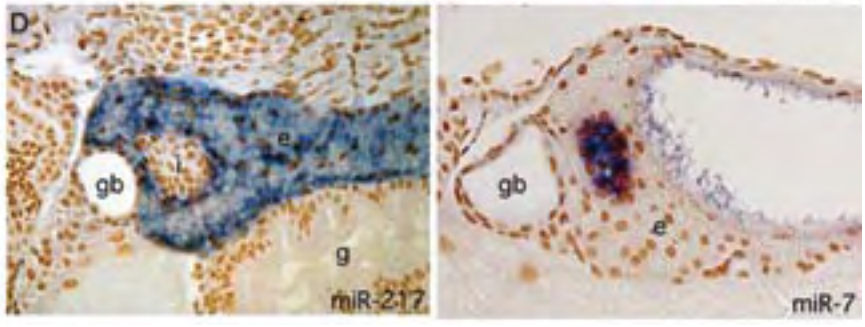

quired for brain morphogenesis in zebrafish embryos (*7*). Together, these findings indicate that miRNAs can play essential roles in development. However, little is known about the individual roles of most miRNAs. To focus future miRNA studies, we determined the spatial and temporal expression patterns of 115 conserved vertebrate miRNAs (see online Material and Methods; table S1; table S2) in zebrafish embryos.

First, we determined the temporal expression of miRNAs during embryonic development by microarray analysis (Fig. 1A and fig. S1A). Up to segmentation [12 hours post fertilization (hpf)], most miRNAs could not be detected. Most miRNAs became visible 1 to 2 days after fertilization and showed strong expression when organogenesis is virtually completed (96 hpf). In adults, the majority of miRNAs remained expressed (Fig.1A). In addition we determined the expression of miRNAs in dissected organs of adult fish. For some miRNAs, a high degree of tissue specificity was observed (figs. S1B and S2, and table S3).

In situ hybridization of miRNAs had thus far not been possible in animals. Recently LNA (locked-nucleic acid)–modified DNA oligonucleotide probes have been shown to increase the sensitivity for the detection of miRNAs by Northern blots (*8*). By Northern blots analysis and in situ hybridization, using LNA probes, we detected predominantly mature miRNAs, which were reduced in Dicer knockout zebrafish (fig. S3). We used these LNA probes for the whole-mount in situ detection of the conserved vertebrate miRNAs in zebrafish embryos and made a catalog of miRNA expression patterns (fig. S4 and database S1).

Most miRNAs (68%) were expressed in a highly tissue-specific manner. For example, miR-140 was specifically expressed in the cartilage of the jaw, head, and fins, and its presence was entirely restricted to those regions (Fig. 1B and database S1). Representative examples are shown (Fig. 1C) of six miRNAs that were expressed in different organ systems: nervous system, digestive system, muscles, circulatory system, sensory organs, and excretory system. Even within organs, there is specificity, as exemplified in Fig. 1D, where miR-217 can be seen to be expressed in the exocrine pancreas, and miR-7 in the endocrine pancreas (Langerhans islets). More than half of the miRNAs (43) were expressed in (specific regions of) the central nervous system (fig. S4). Many miRNA genes are clustered in the genome and, therefore, are probably expressed as one primary transcript, and indeed, we observed that many such clustered genes showed identical or overlapping expression patterns (figs. S4 and S5). We compared the in situ data with microarray data for zebrafish and mammals (fig. S2 and table S3). Up to 77% of the in situ expression patterns were confirmed by at least one of the microarray data sets. In addition, miRNA in situ data showed patterns that cannot easily be detected by microarrays. For example, some miRNAs were expressed in hair cells of sensory epithelia (fig. S6).

In conclusion, we here describe the first comprehensive set of miRNA expression patterns in animal development. We found these patterns to be remarkably specific and diverse, which suggests highly specific and diverse roles for miRNAs. Most miRNAs are expressed in a tissue-specific manner during segmentation and later stages but were not detected during early development. Although we cannot exclude a role for undetectable early miRNAs, this observation indicates that most miRNAs may not be essential for tissue fate establishment but rather play crucial roles in differentiation or the maintenance of tissue identity.

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Supporting Online Material

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Materials and Methods
Figs. S1 to S6
Tables S1 to S3
Database S1

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Erasing MicroRNAs Reveals Their Powerful Punch

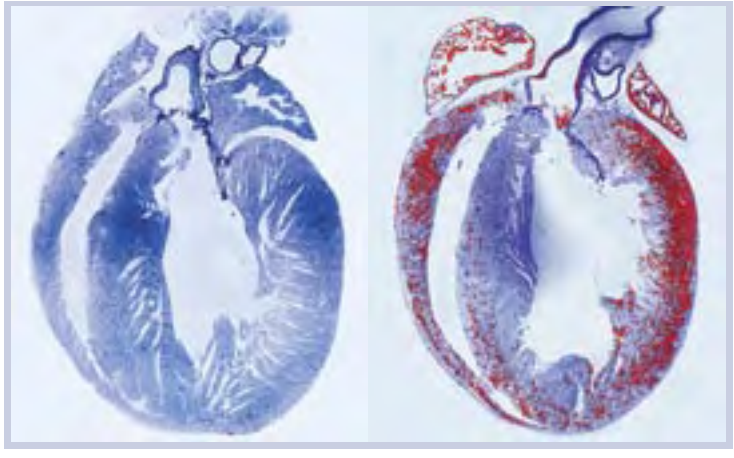
For more than 2 decades, biologists have illuminated the roles of genes by deleting them in mice and studying these "knockout" animals, which lack the proteins encoded by the targeted genes. Now, scientists say they're beginning to uncover an entirely new layer of gene regulation by using the same strategy to erase portions of genes that make snippets of RNA. Just as knockouts of traditional protein-coding genes yielded a treasure trove of knowledge about how different genes govern health and disease, this next generation of knockouts could fill in the gaps that remain.

In a flurry of papers, four independent groups have for the first time deleted mouse genes for microRNAs, RNA molecules that can modulate gene behavior. Each time, the rodents were profoundly affected, with some animals dropping dead of heart trouble and others suffering crippling immune defects.

Since their discovery more than a decade ago, microRNAs have electrified biologists. Geneticists estimate that the human body employs at least 500 during development and adult life. But it wasn't clear, especially in mammals, how important individual microRNAs were, because some evidence suggested that these gene-regulators had backups. In worms, for example, erasing a particular microRNA by deleting the relevant stretch of DNA occasionally had a dramatic effect but more often didn't appear to do much.

"I think there was a fear that nothing could be found" by deleting microRNA genes in mammals one at a time, says David Corry, an immunologist at Baylor College of Medicine in Houston, Texas. As it turns out, the opposite is true. "There's a lot more that the microRNAs are doing that we didn't appreciate until now," says Frank Slack, a developmental biologist at Yale University who studies microRNAs in worms.

Two of the groups that produced the mammalian microRNA knockouts deleted the same sequence, for miR-155, and describe the effects on the mouse immune system on pages 20 and 24 of this booklet. One team was led by Allan Bradley at the Wellcome Trust Sanger Institute and Martin Turner of the Babraham Institute, both in Cambridge, U.K., and the other by Klaus Rajewsky of Harvard Medical School in Boston. The other teams, one whose results were published online by *Science* on 22 March (www.sciencemag.org/cgi/content/abstract/1139089) and one whose work appears in the 20 April issue of *Cell,* eliminated different microRNAs and documented defects in mouse hearts.



Missing molecules. Compared to a normal mouse heart (*left*), one from a mouse with a deleted microRNA (*right*) overexpresses a skeletal muscle gene (in red), among other defects.

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The two groups that deleted miR-155 found that the rodents' T cells, B cells, and dendritic cells did not function properly, leaving the animals immunodeficient. The mutation also cut down the number of B cells in the gut, where the cells help fight infection, and triggered structural changes in the airways of the lungs, akin to what happens in asthma.

Still, left alone in a relatively sterile lab, mice lacking miR-155 survived easily. But when vaccinated against a strain of salmonella, the animals failed to develop protection against the bacterium—as quickly became apparent when most who were exposed to it died within a month. "The animals were no longer able to generate immunity," says Turner, an immunologist.

Biologists typically see a specific defect when they knock out a protein-coding gene, but eliminating a microRNA may pack a bigger punch, because many are thought to control multiple genes. In the case of miR-155, "you get much broader brush strokes … [and] very diverse immunological perturbations," says Corry.

There's a flip side to the promiscuity of microRNAs: A single gene may be the target of many microRNAs. That led some biologists to speculate that built-in redundancy would limit damage caused by deleting individual microRNAs. In the *Cell* study in which miR-1-2 was deleted, the microRNA actually has an identical twin that's encoded by a gene on another chromosome. "We thought that we'd have to delete both of them to see any abnormality in the animal," says Deepak Srivastava of the University of California, San Francisco, who led the work. But half of his group's mice died young of holes in the heart. Others later died suddenly, prompting Srivastava and his colleagues to look for, and find, heart rhythm disturbances.

The heart problems discovered by Eric Olson of the University of Texas Southwestern Medical Center in Dallas and his colleagues [*Science* **316,** 575 (2007)] were more subtle. They erased the microRNA miR-208 and at first thought the mice were normal. Only when they subjected the animals to cardiac stress, by mimicking atherosclerosis and blocking thyroid signaling, did they observe that the animals' hearts reacted inappropriately to such strain.

The four teams that knocked out the various microRNAs still don't know all the gene targets of each molecule. The findings, says Turner, "really do leave open a lot more questions than perhaps there are answers." One is whether these and other microRNAs help explain inherited defects in diseases for which genes have been elusive. Ailments from cancer to Alzheimer's disease, says Carlo Croce of Ohio State University in Columbus, who is studying microRNAs in malignancies, may "have a microRNA component." It's one that scientists are beginning to hunt for in earnest.

–JENNIFER COUZIN

Regulation of the Germinal Center Response by MicroRNA-155

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MicroRNAs are small RNA species involved in biological control at multiple levels. Using genetic deletion and transgenic approaches, we show that the evolutionarily conserved microRNA-155 (miR-155) has an important role in the mammalian immune system, specifically in regulating T helper cell differentiation and the germinal center reaction to produce an optimal T cell–dependent antibody response. miR-155 exerts this control, at least in part, by regulating cytokine production. These results also suggest that individual microRNAs can exert critical control over mammalian differentiation processes in vivo.

MicroRNAs are emerging as key players in the control of biological processes, and the stage-specific expression of certain microRNAs in the immune system suggests that they may participate in immune regulation. One such microRNA is miR-155, produced from the non–protein-coding transcript of the *bic* gene. *bic* was discovered as a recurrent integration site of avian leukosis virus in chicken lymphoma cells (*1*). The hairpin from which miR-155 is processed represents the only evolutionarily conserved sequence of the *bic* gene, indicating that miR-155 mediates *bic* function (*2–4*). *bic*/miR-155 has been shown to be highly expressed in a variety of human B cell lymphomas, including the Hodgkin-Reed-Sternberg cells in Hodgkin's disease, and miR-155 transgenic mice develop B cell lymphomas (*5–8*). In humans, *bic*/miR-155 expression was detected in activated mature B and T lymphocytes (*7, 9*), including germinal center (GC) B cells (*3, 7*), as well as in activated monocytes (*10*). Germinal centers represent sites of antibody affinity maturation and memory B cell generation in T cell–dependent antibody responses *(11)*.

To obtain insights into the physiological function of *bic*/miR-155, we generated two mutant mouse strains. In the first, a major portion of the *bic* second exon, including miR-155, was replaced by a ß-galactosidase (lacZ) reporter (*12*), generating a loss-of-function allele designated *bic*/miR-155$^{-/-}$. The reporter allows one to study the *bic*/miR-155 expression pattern through lacZ expression (fig. S1) (*13*). Northern blots showed that miR-155 expression was completely ablated in activated *bic*/miR-155$^{-/-}$ B cells (fig. S1C). To generate the second mutant strain, we used a previously established knock-in strategy (*14*), to conditionally express miR-155 and an enhanced green fluorescent protein (EGFP) reporter in mature B cells, in a Cre-dependent manner (fig. S2A) (*15*). For simplicity, mice carrying the miR-155 knock-in and the CD21-cre alleles will be referred to as B cell$^{miR\text{-}155}$ mice.

The gut-associated lymphoid tissue (GALT), including Peyer's patches (PPs) and mesenteric lymph nodes (mLNs), contains both B and T cells and activated, proliferating B cells undergoing GC reactions in response to chronic stimulation by gut-derived microbes. We found increased fractions of GC B cells in both PPs and mLNs of B cell$^{miR\text{-}155}$ mice (Fig. 1A and figs. S3 and S4A), and most of these cells, as well as the non-GC B cells, expressed the EGFP reporter. In contrast, in *bic*/miR-155$^{-/-}$ mice, the fraction of GC B cells, determined by fluorescence activated cell sorting (FACS) and immunohistochemistry, was significantly reduced in PPs and mLNs (Fig. 1A and figs. S3 and S4, A and B). In *bic*/miR-155$^{+/-}$ mice, the vast majority of the non-GC B cells were negative for lacZ, whereas ~60% of GC B cells expressed the lacZ reporter (Fig. 1B and fig. S1B). Because the detection of ß-galactosidase activity depends on the sensitivity of the assay as well as the persistence of the enzyme in dividing cells, we conclude that many or perhaps all GC B cells express *bic*/miR-155 in the course of the GC response.

To further characterize miR-155 expression, we isolated spleen B cells from wild-type mice stimulated through the B cell receptor (BCR), CD40, or with mitogens that bind Toll-like receptors (TLRs). Although little *bic*/miR-155 expression was seen in cells before activation, strong up-regulation was detected under each of these activation conditions (Fig. 1, C and D). The signaling requirements were different for BCR versus TLR/CD40-mediated *bic*/miR-155 induction. The former appeared to depend on the calcineurin/NFAT (nuclear factor of activated T cells) pathway, but not NEMO, an essential component of the nuclear factor kB (NF-kB) signaling pathway. The latter required both MyD88 and NEMO (fig. S5) (*16*). A kinetic analysis upon BCR cross-linking showed that both *bic* and miR-155 up-regulation was transient, with a maximum induction of the former at 3 hours and the latter at 24 hours, consistent with a precursor-product relationship (Fig. 1, C and D). Thus, in the GC response, B cells may up-regulate *bic*/miR-155 at its initiation or recurrently during proliferation and selection by antigens.

We have also observed that *bic*/miR-155 expression was absent in nonlymphoid organs (lungs, kidney, brain, liver, and heart) as well as in resting, naïve CD4$^+$ T cells, but strong up-regulation occurs upon activation of these cells by T cell antigen receptor cross-linking (fig. S6), in accord with earlier work in the human (*3, 9*).

The reduced fraction of GC B cells in the GALT of *bic*/miR-155$^{-/-}$ mice, together with its increase in mutants overexpressing miR-155 in B cells, suggests that miR-155 may indeed mediate *bic* function and may also be involved in the control of the GC reaction. To determine whether *bic*/miR-155 is also involved in induced GC responses in the spleen, we immunized mice with alum-precipitated 3-hyroxy-4-nitro-phenylacetyl (NP) coupled to chicken gamma globulin (CGG), which normally initiates a GC response accompanied by the production of antigen-specific antibodies detectable at day 7 after immunization and reaching a peak 1 week later (*17*). Antigen-specific immunoglobulin G1 (IgG_1) antibody titers and the fractions of GC B cells were compared between immunized mutant and wild-type mice. In mice overexpressing miR-155, the antibody response was marginally enhanced at both time points (Fig. 2A). In contrast, the *bic*/miR-155$^{-/-}$ mice produced about one-fifth as much NP-specific antibody titers as their littermate controls (Fig. 2A). The percentages and numbers of spleen GC B cells were higher in the miR-155–overexpressing mice, but reduced in the knockouts, compared to controls, most notably on day 14 after immunization (figs. 2B and S7). Furthermore, *bic*/miR-155$^{-/-}$ spleens displayed reduced numbers of GCs that appeared smaller than those of controls and B cell$^{miR\text{-}155}$ mice (Fig. 2, C and D). Together, these results complement those obtained for GC formation in the GALT and indicate that

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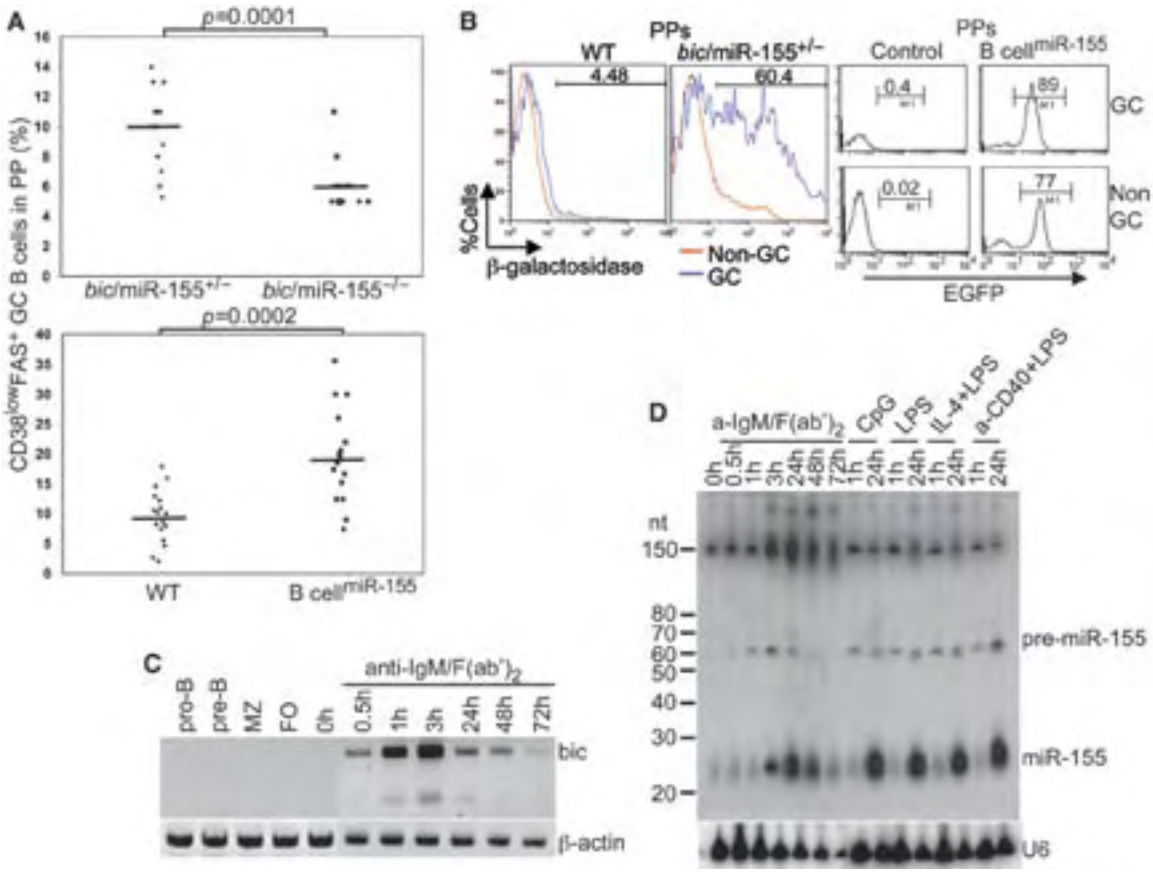
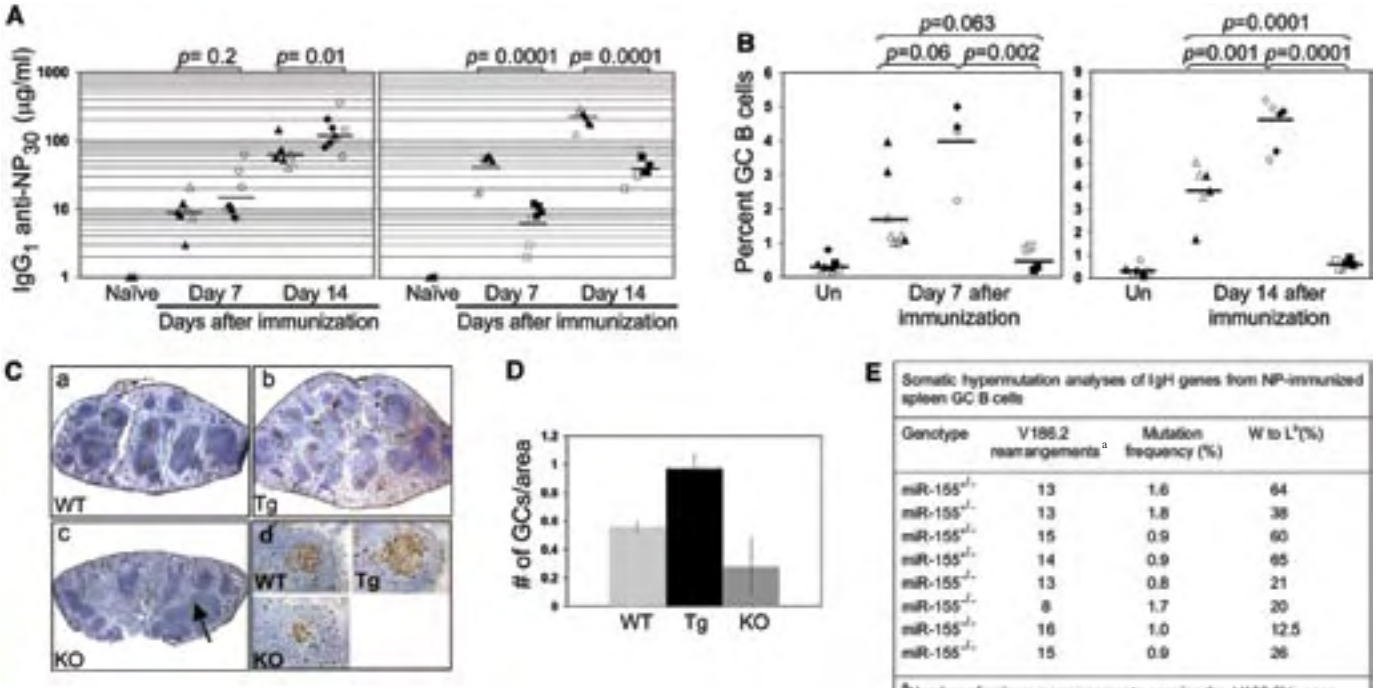


Fig. 1. *bic*/miR-155 regulates the GC response and is induced upon activation. (**A**) The percentage of PP GC B cells was determined by FACS in *bic*/miR-155$^{-/-}$ mice (n = 13) and controls (n = 15), and in B cell$^{miR\text{-}155}$ mice (n = 16) and controls (n = 17). (**B**) (Left panels) *bic* promoter activity was measured by LacZ staining in GC B cells with the use of FACS. (Right panels) In B cell$^{miR\text{-}155}$ mice, *bic*/miR-155 expression in mature B cells is reported by EGFP expression. (**C**) RT-PCR was used to detect *bic* in progenitor, resting B cells and anti–IgM-(Fab´)$_2$–stimulated mature spleen B cells (10 μg/ml). The smaller transcript represents the spliced form of *bic*. (**D**) miR-155 expression was detected by Northern blots in the same samples as in (C). LPS, lipopolysaccharide.



E

Somatic hypermutation analyses of IgH genes from NP-immunized spleen GC B cells

Genotype	V186.2 rearrangements[a]	Mutation frequency (%)	W to L[b](%)
miR-155$^{+/-}$	13	1.6	64
miR-155$^{+/-}$	13	1.8	38
miR-155$^{+/-}$	15	0.9	60
miR-155$^{+/-}$	14	0.9	65
miR-155$^{-/-}$	13	0.8	21
miR-155$^{-/-}$	8	1.7	20
miR-155$^{-/-}$	16	1.0	12.5
miR-155$^{-/-}$	15	0.9	26

[a]Number of unique rearrangements carrying the V186.2V$_H$ gene
[b]Percent of rearrangements carrying the tryptophan to leucine substitution at position 33
Mutation frequency in non-GC B cells: 0.05%

Fig. 2. *bic*/miR-155$^{-/-}$ mice show impaired T cell–dependent antibody responses. (**A** and **B**) Mice were immunized intraperitoneally with NP-CGG/Alum and analyzed on days 7 and 14 after immunization. Open and closed symbols: experiments 1 and 2; triangles: controls; diamonds: B cell$^{miR\text{-}155}$; squares: *bic*/miR-155$^{-/-}$. (A) NP-specific IgG$_1$ levels were measured by enzyme-linked immunosorbent assay. (B) The percentage of spleen CD38^{lo}Fas^{hi} GC B cells was determined by FACS. Un: unimmunized. (**C**) Immunohistochemistry was performed on day 14 NP-immunized spleen sections from wild-type (a), B cell$^{miR\text{-}155}$ (b), and knockout mice (c) to detect GCs (brown, PNA$^+$; blue, hemotoxylin). High-magnification image is shown in (d). Images are representative of three mice per group. (**D**) Number of GCs (±SEM) was determined from sections in (C); n = 3 mice per group. (**E**) The frequency of W33L replacement was determined by sequence analyses with spleen GC B cells 12 or 14 days after NP-CGG immunization.

miR-155 plays a specific role in the control of the GC reaction in the context of a T cell–dependent antibody response.

We next investigated a possible molecular basis for the effects of miR-155. With no obviously relevant predicted miR-155 targets in hand, we focused on basic features of the GC response; namely, B cell proliferation, the generation of somatic antibody mutants, and selection of mutated B cells that bound antigen with high affinity. We also examined the production of tumor necrosis factor (TNF) and lymphotoxin-α (LT-α) and LT-β by mutant and control B cells, because it is known that TNF and LT-α, produced by B cells, are critical for GC formation (*18–20*). When *bic*/miR-155$^{-/-}$ B cells were induced to proliferate in vitro by a variety of stimuli, their proliferation profile, determined by dilution of the cell-bound carboxyfluorescein diacetate succinimidyl ester (CFSE) label, was indistinguishable from that of control cells (fig. S8). There was thus no indication from these experiments that miR-155 expression is directly involved in the control of B cell proliferation. The anti-NP response is characterized by the preferential usage of the V_H186.2 gene segment of the IgH^b haplotype. Furthermore, high-affinity anti-NP antibodies acquire a tryptophan-to-leucine mutation at position 33 (W33L) (*17*). GC B cells were thus isolated from *bic*/miR-155$^{+/-}$ and *bic*/miR-155$^{-/-}$ mice on day 12 or 14 after immunization with NP-CGG, and rearranged V_H186.2 gene segments were sequenced (*17*). Although there were no notable differences in overall mutation frequency between control and mutant cells, the selection for the W33L mutation was compromised in *bic*/miR-155 knockout cells (Fig. 2E). Therefore, although miR-155 is not required for somatic hypermutation of antibody genes in GC B cells, it contributes to an optimal selection of cells acquiring high-affinity antibodies. A possible clue to an understanding of the defective GC reaction in *bic*/miR-155 knockout mice came from the analysis of cytokine production by activated B cells from knockout and control mice. Two days after in vitro activation by BCR cross-linking, TNF production by *bic*/miR-155$^{-/-}$ B cells was noticeably reduced when compared with that of the controls (Fig. 3A). Consistent with this, the concentration of TNF in culture supernatants of the mutant B cells was about one-third of that in control supernatants (Fig. 3B). The differences in TNF production between knockout and wild-type cells were also apparent at the level of gene expression, as demonstrated by reverse transcription–polymerase chain reaction (RT-PCR) analysis of TNF-specific transcripts (Fig. 3C). We further showed, by RT-PCR, that *lt*-α but not *lt*-β expression is also compromised in the mutant cells. These defects were also observed in ex vivo sorted GC and non-GC B cells from mLNs of the knockout mice, where B cells may be chronically activated by exposure to bacterial antigens (Fig. 3D). Together, these data suggest that miR-155 controls the GC response at least in part at the level of cytokine production. Although this control may follow pathways of posttranscriptional gene silencing, we note that fragile-X mental retardation–related protein 1 (FXR1) and Argonaute-2, an RNA-binding protein involved in the microRNA pathway, were shown

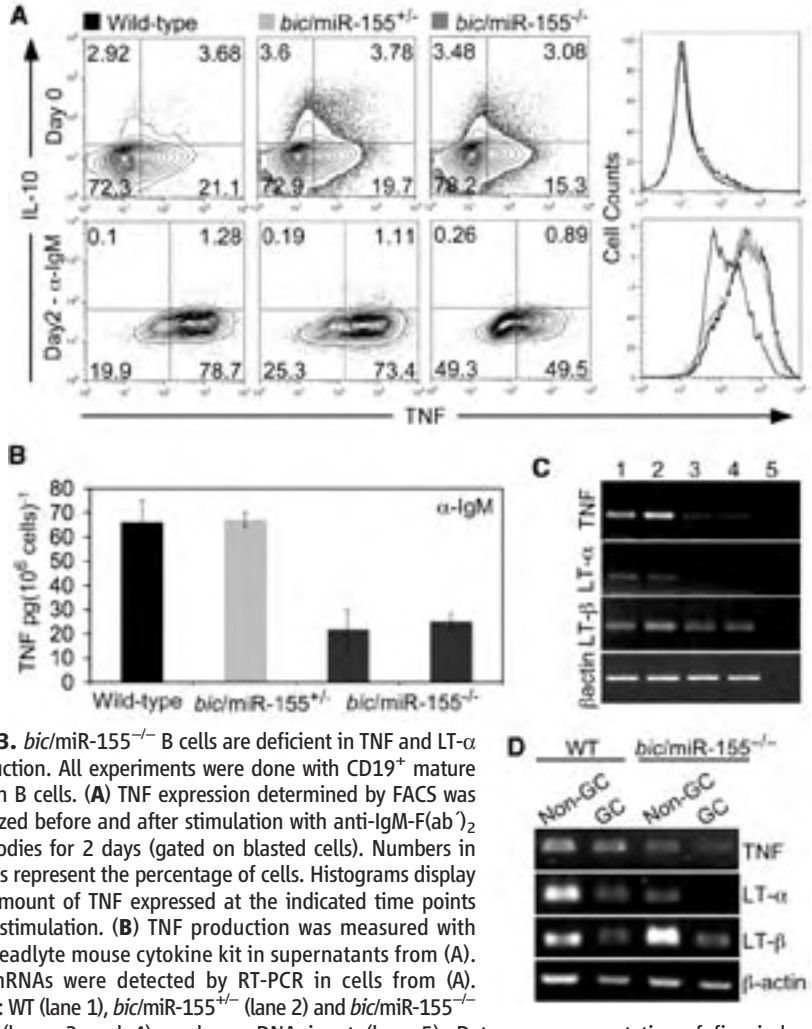


Fig. 3. *bic*/miR-155$^{-/-}$ B cells are deficient in TNF and LT-α production. All experiments were done with CD19$^+$ mature spleen B cells. (**A**) TNF expression determined by FACS was analyzed before and after stimulation with anti-IgM-F(ab´)$_2$ antibodies for 2 days (gated on blasted cells). Numbers in panels represent the percentage of cells. Histograms display the amount of TNF expressed at the indicated time points after stimulation. (**B**) TNF production was measured with the Beadlyte mouse cytokine kit in supernatants from (A). (**C**) mRNAs were detected by RT-PCR in cells from (A). Lanes: WT (lane 1), *bic*/miR-155$^{+/-}$ (lane 2) and *bic*/miR-155$^{-/-}$ mice (lanes 3 and 4), and no cDNA input (lane 5). Data are representative of five independent experiments. (**D**) mLNs non-GC and GC B cells were sorted, and RT-PCR was performed as in (C).

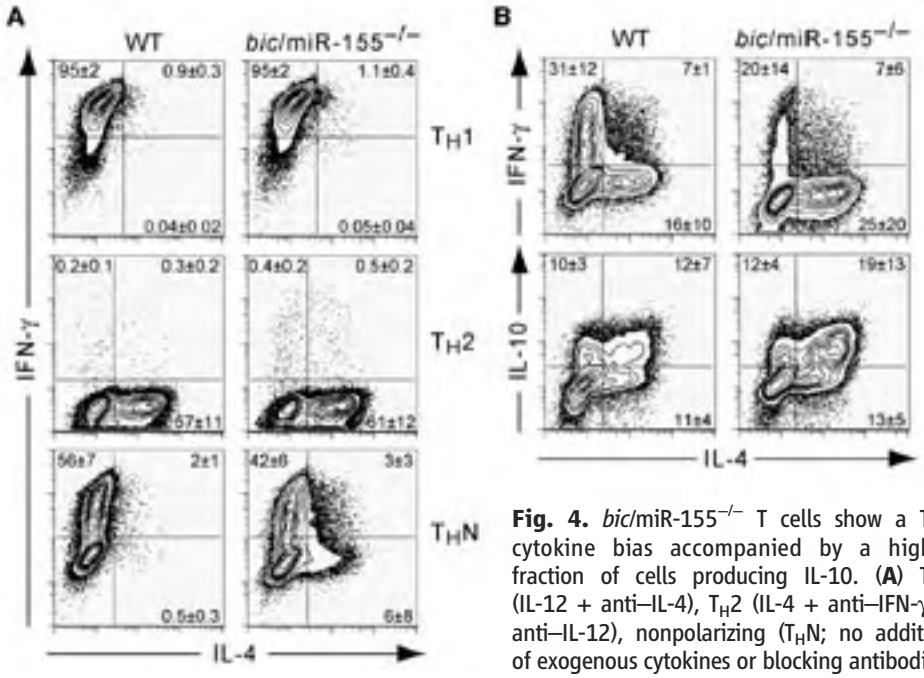


Fig. 4. *bic*/miR-155$^{-/-}$ T cells show a T_H2 cytokine bias accompanied by a higher fraction of cells producing IL-10. (**A**) T_H1 (IL-12 + anti–IL-4), T_H2 (IL-4 + anti–IFN-γ + anti–IL-12), nonpolarizing (T_HN; no addition of exogenous cytokines or blocking antibodies) conditions were used to study T cell differentiation with purified CD4$^+$ T cells from peripheral lymph nodes. On day 5, intracellular IL-4 and IFN-γ production was measured by FACS (mean ± SD; five knockout and four wild-type mice from three independent experiments). (**B**) Cells prepared as in (A) were differentiated under the influence of a limited quantity of IL-4 (12.5 U/ml) (mean ± SD, four knockout and three wild-type mice, from two independent experiments). Numbers in panels represent the percentage of cells.

to associate with an AU-rich element in the 3′ untranslated region (UTR) of the TNF mRNA during translation activation *(21)*. A conserved miR-155 binding site (AGCGUUA) downstream of this element could contribute to the targeting of Argonaute-2 and FXR1 to the TNF 3′ UTR.

Because *bic*/miR-155 is also expressed in T cells upon activation and differential cytokine production is a hallmark of T cell differentiation into T helper cell 1 (T_H1) and T_H2 effector cells, we tested T_H1 and T_H2 differentiation of knockout and control T cells in vitro (*22, 23*). We found that T cell differentiation proceeded normally in both cases (Fig. 4A). However, when T cells were cultured under conditions that promote neither differentiation pathway or suboptimally promote T_H2 differentiation, *bic*/miR-155$^{-/-}$ cells produced more interleukin-4 (IL-4) and less interferon-γ (IFN-γ), suggesting that they were more prone to TH2 differentiation than controls (Fig. 4 and fig. S9). In addition, mutant T cell cultures generated more cells producing IL-10, a cytokine known to dampen immune responses (*24, 25*).

Although it remains to be seen whether these observations relate to the impaired GC response in the mutants, the present experiments establish, through a combined genetic loss- and gain-of-function approach, that miR-155 is critically involved in the in vivo control of specific differentiation processes in the immune response and that it exerts its functions at least partly at the level of control of cytokine production.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/316/5824/604/DC1
Materials and Methods
Figs. S1 to S9
References

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Requirement of *bic/microRNA-155* for Normal Immune Function

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MicroRNAs are a class of small RNAs that are increasingly being recognized as important regulators of gene expression. Although hundreds of microRNAs are present in the mammalian genome, genetic studies addressing their physiological roles are at an early stage. We have shown that mice deficient for *bic/microRNA-155* are immunodeficient and display increased lung airway remodeling. We demonstrate a requirement of *bic/microRNA-155* for the function of B and T lymphocytes and dendritic cells. Transcriptome analysis of *bic/microRNA-155*–deficient $CD4^+$ T cells identified a wide spectrum of microRNA-155–regulated genes, including cytokines, chemokines, and transcription factors. Our work suggests that *bic/microRNA-155* plays a key role in the homeostasis and function of the immune system.

MicroRNAs (miRNAs) posttranscriptionally regulate gene expression by forming imperfect base pairing with sequences in the 3′ untranslated region (3′ UTR) of genes to prevent protein accumulation by repressing translation or by inducing mRNA degradation (*1, 2*). More than 500 miRNAs have been identified in mammals, although their functions are only now being elucidated (*3*). In the immune system, the enzyme responsible for regulatory RNA biogenesis, Dicer, is required for T cell function, which suggests regulatory roles for miRNAs in lymphocytes (*4, 5*). One miRNA, miR-155 (*6*), maps within, and is processed from, an exon of the noncoding RNA known as bic (7, 8), its primary miRNA precursor (9). *bic/miR-155* shows greatly increased expression in activated B and T cells (9–*11*), as well as in activated macrophages and dendritic cells (DCs) (*12, 13*). Overexpression of *bic/miR-155* has been reported in B cell lymphomas and solid tumors (*14*), and transgenic miR-155 mice have also been shown to develop B cell malignancies in vivo (*15*), indicating that the locus may also be linked to cancer.

To define the in vivo role of *bic/miR-155* (*bic*), we generated mutant alleles in embryonic stem cells (*16*) to obtain *bic*-deficient ($bic^{m1/m1}$ and $bic^{m2/m2}$) mice (fig. S1, A and B). *bic*-deficient mice were viable and fertile but developed lung pathology with age. At 320 to 350 days, 56% (5 out of 9) of $bic^{m1/m1}$ mice displayed significant remodeling of lung airways, with increased bronchiolar subepithelial collagen deposition and increased cell mass of sub-bronchiolar myofibroblasts (Fig. 1, B, D, and F), relative to age-matched control mice ($n = 8$) (Fig. 1, A, C, and E). A statistically significant increase in the ratio of collagen thickness/bronchiolar diameter and smooth muscle cell area/bronchiolar diameter could be measured in *bic*-deficient mice, compared with wild-type controls (Fig. 1, G and H). Increased airway remodeling in aged $bic^{m1/m1}$ mice was accompanied by a significant increase in the numbers of leukocytes in bronchoalveolar lavage fluids (BAL) (Fig. 1, I) but not the lung interstitium. These changes are reminiscent of the lung fibrosis that often complicates systemic autoimmune processes with lung involvement (*17, 18*). We also noted that many $bic^{m1/m1}$ mice developed enteric inflammation, a trait we have not investigated further. Thus, the phenotype we observed suggested that *bic/miR-155* may participate or play a role in regulating the homeostasis of the immune system.

The pathology observed in *bic*-deficient mice prompted us to examine the requirement of *bic/miR-155* in immunity. Although no gross defect in myeloid or lymphoid development in *bic*-deficient mice was observed (tables S1 and S2), protective immunity did appear to be impaired. Thus, after intravenous immunization with the live attenuated form of the enteric pathogen *Salmonella typhimurium* (*aroA* mutant strain), mice were assessed for their ability to resist oral challenge with virulent *S. typhimurium* bacteria (*19, 20*). Both unvaccinated $bic^{m2/m2}$ and wild-type control mice (5 out of 5; $n = 5$) died within 7 days after infection (Fig. 2A). However, unlike their wild-type counterparts, $bic^{m2/m2}$ mice were less readily protected by *aroA* vaccination, and the majority of mice (5 out of 6; $n = 6$) succumbed to challenge with the virulent strain by 33 days after infection (Fig. 2B). Thus, immunized *bic*-deficient mice, unlike wild-type mice, could not be protected by immunization to this pathogen.

Protective immunity requires the function of T and B lymphocytes. Therefore, we next examined the in vivo B and T cell responses of *bic*-deficient mice immunized with the T-dependent antigen, tetanus toxin fragment C protein (TetC). Immunized $bic^{m1/m1}$ mice produced significantly reduced amounts of immunoglobulin M (IgM) and switched antigen-specific antibodies (Fig. 2C), indicative of impaired B cell responses. For examination of T cell function, splenocytes from mice immunized with TetC were restimulated in vitro, and the levels of interleukin (IL)–2 and interferon (IFN)–γ cytokines were measured. As expected, splenocytes from wild-type mice immunized with TetC produced significantly increased levels of IL-2 and IFN-γ relative to naive mice (Fig. 2D). In contrast, $bic^{m1/m1}$ immunized mice failed to produce significant levels of these cytokines (Fig. 2D). Thus, B and T cell responses were diminished in *bic*-deficient mice, possibly contributing to their impaired enteric immunity.

To understand the nature of defective immune responses in vivo, we explored the possibility of an intrinsic requirement for *bic/miR-155* in B cells and T cells. Dendritic cell (DC) function was also tested, because these cells act as professional antigen presenting cells (APCs) with the ability to influence T cell activation and differentiation. Production of IgG1 by lipopolysaccharide (LPS)– and IL-4–stimulated $bic^{m2/m2}$ B cells was significantly reduced (Fig. 2E), although this defect did not appear to correspond with abnormal proliferation (fig. S2). After encountering antigen, DCs increase their immunostimulatory capacity (*21*) through a process that is mimicked in vitro by stimulation with LPS. After treatment with LPS $bic^{m2/m2}$, bone marrow–derived DCs expressed levels of major histocompatibility complex–II and costimulatory molecules similar to those seen on identically treated matured wild-type DCs (fig. S3, A and B), which indicates that *bic/miR-155* is not required for maturation. Nevertheless, $bic^{m2/m2}$ DCs failed to efficiently activate T cells, consistent with defective antigen presentation or costimulatory function (Fig. 2F). Collectively, these results suggest that the effects of *bic/miR-155* may operate in part on T cells through its influence on DC function.

To establish whether there is also an intrinsic requirement for *bic/miR-155* in T cell function, the response of receptor-stimulated naïve $bic^{m2/m2}$ $CD4^+$ T cells was tested. Despite normal proliferation, uncommitted $bic^{m2/m2}$ $CD4^+$ cells showed a significant reduction of the T helper (Th)–1 cytokine, IFN-γ, after stimulation with antibodies to CD3 and CD28 (fig. S4). A reduction by a factor of 5 in the number of IFN-γ–producing cells was also

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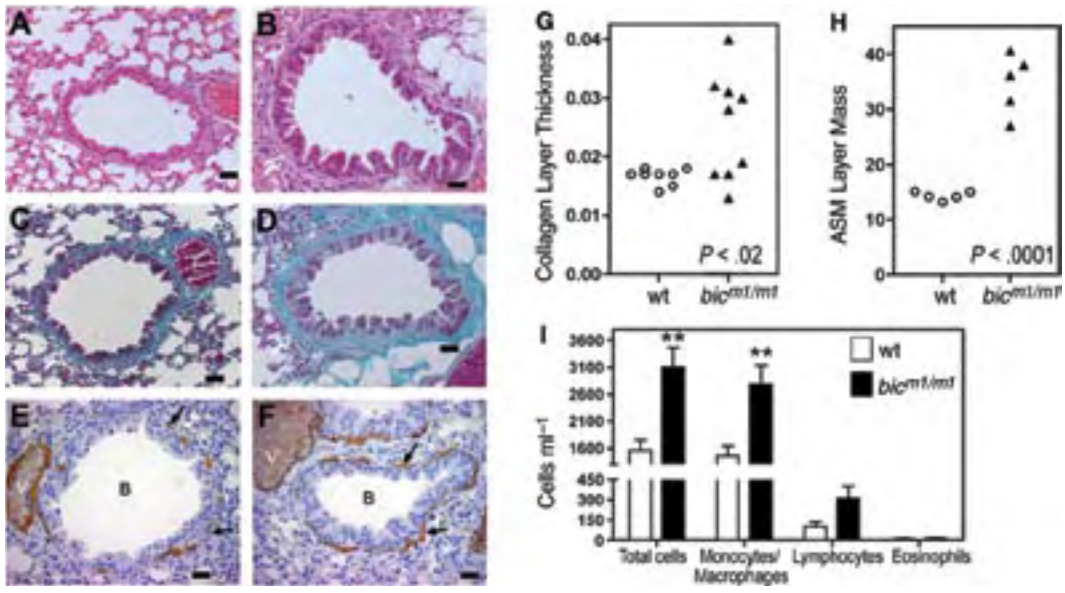

Fig. 1. Mice deficient for *bic/miR-155* show increased lung airway remodeling. (**A** to **F**) Histological examination of sections of lung bronchioles from control wild-type (A, C, and E) and $bic^{m1/m1}$ mice (B, D, and F). Scale bar, 100 μm. (A and B) Haematoxylin and eosin stain; (C and D) Masson Trichrome stain; (E and F) Immunohistochemical staining for smooth muscle actin. Collagen layer (white arrows), lung myofibroblasts (black arrows), bronchioles (B), and blood vessels (V) are indicated. (**G**) Quantitation of peribronchiolar collagen thickness or (**H**) airways smooth muscle cell (ASM) mass in $bic^{m1/m1}$ mice compared with that of wild-type mice. (G) $P <$ 0.02 or (H) $P < 0.0001$, in comparison with wild-type group, Student's two-tailed *t* test. Open circles, control mice; filled triangles, $bic^{m1/m1}$ mice. Notably, $bic^{m1/m1}$ mice with increased collagen layer thickness also had increased ASM mass. (**I**) Total and differential cell counts in BAL from the indicated mice. Data are the mean + SE from seven *bic*-deficient mice and six control mice. $^{**}P < 0.01$ in comparison with wild-type group, Student's two-tailed *t* test.

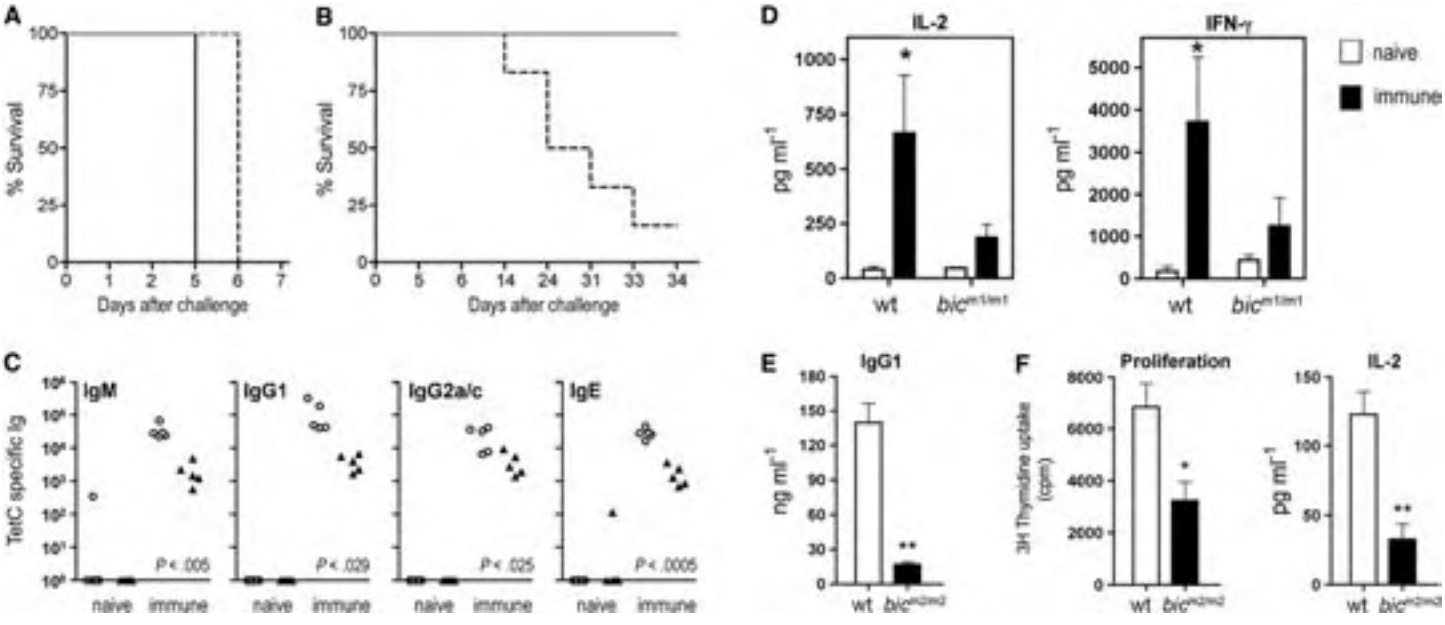

Fig. 2. Defective adaptive immunity by *bic*-deficient mice. (**A**) Survival curve for mice ($n = 5$ in each group) infected orally with 1×10^8 colony-forming units (CFU)—virulent *S. typhimurium* strain SL344. As expected for mice of this genetic background, all failed to survive challenge. (**B**) Survival of mice ($n = 6$ in each group) infected intravenously with 1×10^4 CFU of *S. typhimurium* aroA strain followed by oral challenge *with S. typhimurium* SL344 6 weeks after prime. In contrast with control mice, $bic^{m2/m2}$ mice demonstrate reduced survival after challenge. (A and B) Line, control C57BL/6] (wild-type) mice; dashed line, N5 C57BL/6] backcross $bic^{m2/m2}$ mice. (**C**) TetC-specific Ig levels from control mice (open circles) or *bic*-deficient mice (filled triangles) immunized with TetC at days 1 and 21 and analyzed 13 days after secondary immunization. *P* values denote significant differences; Student's two-tailed *t* test. (**D**) Production of IL-2 and IFN-γ by splenocytes isolated from wild-type or $bic^{m1/m1}$-naïve mice (open bars) or immunized with TetC as in (C) (closed bars) and cultured for 48 hours in the presence of TetC. Data are the mean ± SE from four mice. $^{*}P <$ 0.05 versus naïve mice; Student's two-tailed *t* test. (**E**) Reduced IgG1 production by $bic^{m2/m2}$ B cells cultured in the presence of LPS and IL-4 for 4 days. Data are the mean + SE from 3 mice. $^{**}P < 0.01$ versus wild-type; Student's two-tailed *t* test. (**F**) Significantly reduced proliferation and IL-2 production by ovalbumin T cell receptor transgenic (OT-II) cells cultured with LPS-matured, bone marrow–derived, *bic*-deficient DCs in the presence of cognate (2.5 μM) ovalbumin protein. Cell proliferation was determined by [^{3}H]-thymidine incorporation at 72 hours. IL-2 was measured from supernatants by enzyme-linked immunosorbent assay (ELISA) at 48 hours. Data are the mean + SE from five mice of each genotype. $^{*}P < 0.05$ versus wild-type; Student's two-tailed *t* test.

observed after restimulation of *bic*$^{m2/m2}$ CD4^{+} T cells cultured under conditions designed not to polarize Th responses (Fig. 3A); and was accompanied by a doubling in the number of IL-4 single-producing cells (Fig. 3A). In light of the expression of bic/miR-155 in both Th1 and Th2 cell lineages (fig. S5, A and B), we next examined the phenotype of *bic*$^{m2/m2}$ CD4^{+} T cells after culture in conditions that promote Th1 or Th2 cell differentiation. The levels of IFN-γ, as well as the number of *bic*$^{m2/m2}$ Th1 cells secreting cytokine, were similar to controls, which indicates that *bic/miR-155* is not required for Th1 differentiation (Fig. 3, A and B). However, phenotypic alterations were observed as *bic*$^{m2/m2}$ Th1 cells produced elevated levels of CCL-5 (Fig. 3B and table S3). By contrast, increased commitment to the Th2 pathway was evident in *bic*$^{m2/m2}$ Th2 cell cultures as higher numbers of IL-4–producing cells were observed (Fig. 3A). In support of this result, enhanced levels of the Th2 cytokines IL-4, IL-5, and IL-10 were generated by *bic*$^{m2/m2}$ cells after culture in Th2 polarizing conditions (Fig. 3C). Taken together, these data demonstrate that *bic*-deficient CD4^{+} T cells are intrinsically biased toward Th2 differentiation. Moreover, Th1 cells may have altered function despite normal production of IFN-γ.

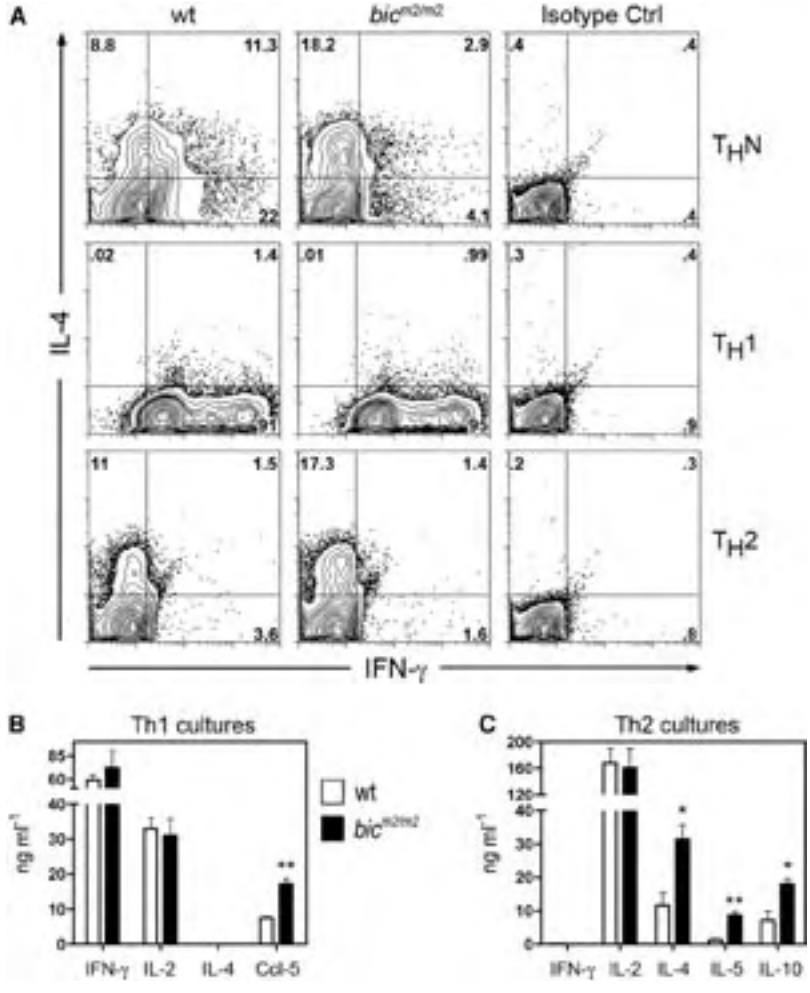

Fig. 3. Increased Th2 polarization and amplified Th2 cytokine production by *bic*-deficient CD4^{+} T cells. CD4^{+}CD62L^{+} cells of indicated genotypes were cultured under (**A**, middle panel, and **B**) Th1 conditions, (**A**, lower panel, and **C**) Th2 in vitro differentiation conditions, or (**A**, upper panel) nonpolarizing (ThN) conditions and restimulated with immobilized antibody to CD3 (10 μg/ml) and soluble 2 μg/ml antibody to CD28 on day 6. (A) Intracellular cytometric analysis for IFN-γ and IL-4 production (*16*). The panel shows a representative result of three mice of each genotype analyzed in the same experiment. Data are representative of two independent experiments (n = 3 per genotype). Numbers in each quadrant are percentages of cells of indicated phenotype. (B and C) Cytokine levels were assayed by ELISA 21 hours after restimulation of cells cultured under (B) Th1 or (C) Th2 polarizing conditions. Data are the mean + SE from three individual mice. *P < 0.05 or **P < 0.01 versus wild-type; Student's two-tailed t test.

To understand how *bic/miR-155* regulates Th2 commitment and to gain a more global insight into the extent of deregulation in Th1 cells, we analyzed gene expression in *bic*$^{m2/m2}$ Th1 or Th2 cells using microarray analysis. In addition, because the 5′ region of miRNAs (referred to as the "seed" region) is believed to be crucial for target mRNA recognition (*1, 2*), we searched the 3′ UTRs of significantly up-regulated genes in microarrays for the presence of seed matches specific for miR-155. In *bic*-deficient Th1 cells, we identified 46 of 53 up-regulated transcripts as potential miR-155 targets (table S3 and fig. S6). In *bic*-deficient Th2 cells, 53 out of 99 up-regulated transcripts were predicted targets (table S4 and fig. S7). To confirm these genes as likely targets of miR-155, we then searched the 3′ UTRs for seed matches specific for all of the known mouse miRNAs in the miRbase public database (*3*). miR-155 seed sequences were significantly overrepresented over all other tested mouse miRNAs, indicating a significant probability that these genes are direct targets of miR-155 (Fig. 4, A and B). This computational data strongly suggests that miR-155 represses a wide assortment of genes in CD4^{+} T cells and lends support for the hypothesis that miRNA targets are generally abundant in mammals (*22*).

A wide spectrum of miR-155 target genes with diverse molecular roles, such as T cell costimulation (e.g., *Tnfsf9*), chemotaxis (e.g., *Ccl-5*), and signaling (e.g., *Ikbke*), were identified. Among these, we noted that the transcription factor *c-Maf* contains phylogenetically conserved miR-155 seed matches in the 3′ UTR (fig. S8). c-Maf is a potent transactivator of the *IL-4* promoter, and ectopically expressed c-Maf is sufficient to cause increased IL-4, IL-5 and IL-10 production by Th2 cells (*23–25*). In concordance with the microarray results, a significant induction of *c-Maf* mRNA was detected in *bic*$^{m2/m2}$ Th2 cells, and the levels of c-Maf protein were correspondingly increased (Fig. 4, C and D). By contrast, levels of *Gata3* transcript, which does not contain a miR-155 seed, were not elevated (Fig. 4C). Increased expression of *c-Maf* may thus contribute, at least in part, to the increased Th2 cytokine production phenotype observed in *bic*$^{m2/m2}$ Th2 cells. To further confirm whether *c-Maf* is a direct target of miR-155, we cloned its 3′ UTR into a luciferase reporter plasmid. The wild-type *c-Maf* reporter exhibited significant miR-155–dependent repression relative to the reporter with a mutant seed sequence, which indicates that this is a direct target for miR-155 (Fig. 4E). We conclude from these experiments that *bic/miR-155* modulates levels of *c-Maf* in CD4^{+} T cells and this is likely

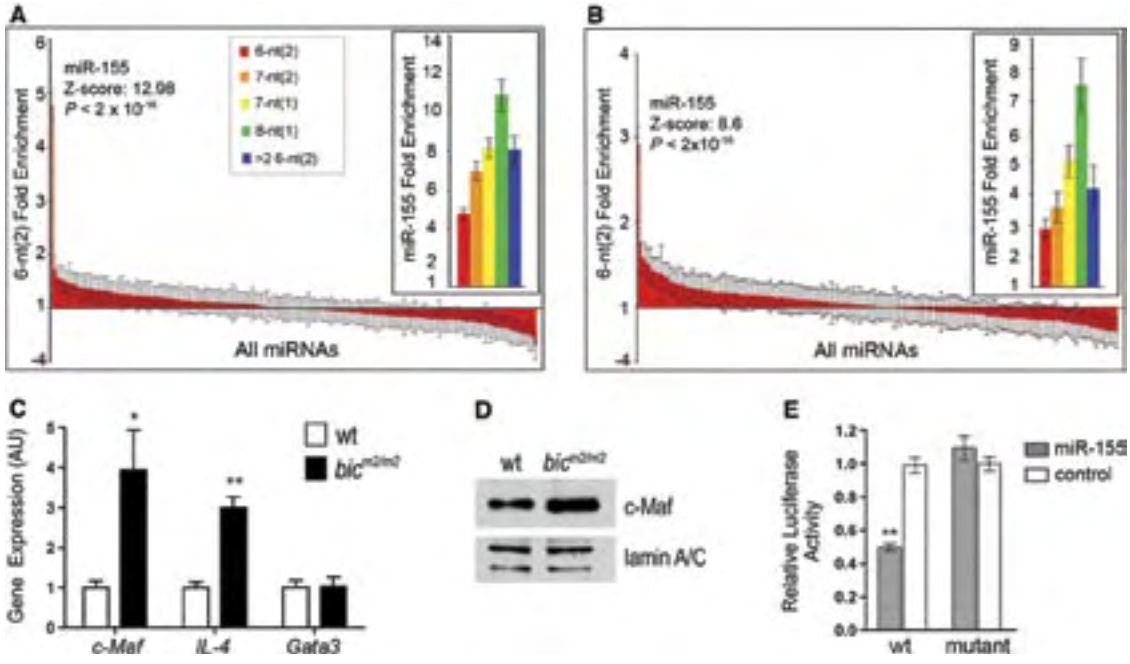

Fig. 4. miR-155 pattern sequences are enriched in the Th1 and Th2 cell up-regulated genes, and *c-Maf* is a bona fide target of miR-155. (**A** and **B**) Fold enrichment of 5′ miRNA pattern sequences of the indicated types contained in the 3′ UTRs of the (A) Th1 or (B) Th2 cDNA microarray significantly up-regulated gene sets. The standard deviation, *Z* score, and *P* value were calculated by sampling 1000 random sets of 53 (for Th1 set) or 99 (for Th2 set) genes from the mouse genome (*16*). Data are fold enrichment ± SD. (**C**) Quantitative PCR analysis for *Gata3*, *c-Maf*, and *IL-4* transcript levels from Th2 cells restimulated with antibodies to CD3 and CD28. Data are the mean + SE from three mice. **P* < 0.05 versus wild-type; Student's two-tailed *t* test. (**D**) c-MAF protein levels were assessed by Western blot of nuclear extracts of Th2 cells isolated from the indicated genotypes. Expression of lamin A/C was used as loading control. (**E**) miR-155–dependent repression of c-Maf reporter in vitro. A luciferase (Rluc) reporter was used to validate *c-Maf* as a direct target of miR-155. Wild-type (wt) or mutant plasmids (mut) were contransfected with the indicated duplex miRNA for miR-155 (open bars) or control Cel-miR-64 (filled bars) into HeLa S3 cells. Data are mean ± SE from three experiments. ***P* < 0.0001 in comparison with wild-type plasmid treated with nonspecific RNA duplex, Cel-miR-64; Student's two-tailed *t* test.

to contribute to the attenuation of Th2 cell responses in vivo.

Our data demonstrate that mice carrying a null mutation in the *bic/miR-155* gene display altered immune responses. Thus, along with an increase in airways remodeling suggestive of altered homeostasis, we observed that *bic/miR-155* regulates the function of both lymphocytes and DCs, leading to an overall diminution of immune responses. The identification of multiple novel potential targets of miR-155 supports the view that *bic/miR-155* is a core regulator of gene expression in multiple cell types, with a "targetome" optimized to modulate the immune response. Interestingly, *bic*-deficient mice share some of the cellular features observed in CD4-*Cre/Dicer*^{FL} mice, including defects in CD4$^+$ T cell cytokine production and immune homeostasis (*3*, *4*). It will now be important to define the pathophysiology of *bic*-deficient lymphocytes and further test the role of miR-155–dependent repression of *c-Maf* on immune responses in vivo. The strength of the *bic/miR-155* mutant phenotype more generally suggests critical roles for miRNAs in vivo, with potentially severe loss-of-function phenotypes directly relevant to human disease. In this regard, it is intriguing that the human *BIC/miR-155* gene maps to an asthma, pollen sensitivity, and atopic dermatitis susceptibility region on chromosome 21q21 (*26*–*28*). Given the severe phenotypes noted in these mice, BIC/miR-155 should be investigated as a potential immune disease locus in humans.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/316/5824/608/DC1
Materials and Methods
Figs. S1 to S8
Tables S1 to S4
References

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miRNAs in Neurodegeneration

Sébastien S. Hébert and Bart De Strooper

Noncoding microRNAs are necessary for the survival of postmitotic cells such as neurons that die in Parkinson's and other brain diseases.

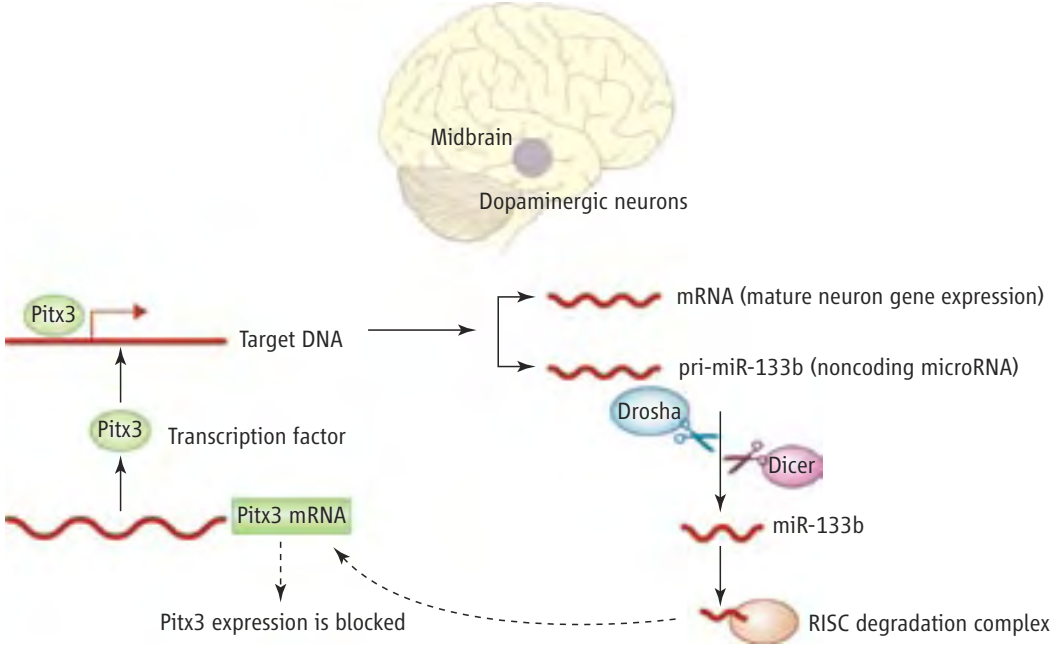
The human genome sequencing effort has taught us that it takes relatively few genes to build a human being. Complexity arises from the combination of these building blocks into genetic programs that are finely tuned in space and time during cell and tissue differentiation. A major part of this regulation is performed by microRNAs (miRNAs), small RNA molecules encoded by the genome that are not translated into proteins; rather, they control the expression of genes. Deregulation of miRNA function has been implicated in human diseases including cancer and heart disease (*1*, *2*). Kim *et al.* (*3*) suggest that miRNAs are essential for maintaining dopaminergic neurons in the brain, and thus could play a role in the pathogenesis of Parkinson's disease.

Similar to classical genes, regions of the genome that encode miRNAs are transcribed in the cell nucleus. Nascent miRNA transcripts are initially processed into long (up to several kilobases in length) precursor miRNAs that are then sequentially cleaved by two enzymes, Drosha and Dicer, into small functional RNAs (~22 nucleotides). These miRNAs are subsequently incorporated into an RNA-induced silencing complex (RISC), which suppresses the translation and/or promotes the degradation of target messenger RNAs (mRNAs)—RNA molecules that encode proteins—by binding to their 3′-untranslated regions (3′-UTRs) (*4*). miRNAs are abundant in the brain and are essential for efficient brain function. In this regard, expression of a brain-specific miRNA (miR-124a) in nonneuronal cells converts the overall gene-expression pattern to a neuronal one (*5*, *6*). Another brain-specific miRNA, miR-134, modulates the development of dendritic spines—neuronal protrusions that connect with other neurons—and therefore probably controls neuronal transmission and plasticity (*7*).

Recent evidence suggests that miRNAs and transcription factors work in close concert. For instance, the RE1 silencing transcription factor can inhibit transcription of miR-124a, thereby suppressing cell differentiation into neurons (*8*). Kim *et al.* observe a similar relationship between miR-133b and the transcription factor Pitx3. The pair forms a negative-feedback loop that regulates dopaminergic neuron differentiation (see the figure). Pitx3 transcribes miR-133b, which in turn suppresses Pitx3 expression.

Although Kim *et al.* provide insights into current concepts in the miRNA field and in neuronal differentiation, the implication that miRNA dysfunction could underlie certain cases of sporadic Parkinson's disease is profound given that after Alzheimer's disease, Parkinson's disease is the second most prevalent age-associated neurodegenerative disorder. The gradual loss of dopaminergic (and eventually other) neurons results in severe mobility problems and occasionally evolves into full-blown dementia. As with Alzheimer's disease, gene mutations can result in inherited forms of Parkinson's disease (*9*). Although the study of these rare familial forms has helped enormously in understanding their molecular pathogenesis, the real challenge for future research in the field is the vast number of nonfamilial cases.

The hypothesis that alterations in miRNA networks in the brain contribute to neurodegenerative disease is appealing and has been tested to a certain extent by Kim *et al.* along with previous work in mice (*3*, *10*), flies (*11*), and cultured neurons (*3*), in which the enzyme Dicer was genetically inactivated. Loss of Dicer leads to the complete absence of miRNAs and is lethal (*12*). However, Kim *et al.* show that mice lacking Dicer in specific dopamine neurons are born alive but develop a progressive loss of neurons later in life, displaying a Par-



Neuronal survival in the brain. An autoregulatory feedback loop composed of the transcription factor Pitx3 and miR-133b is implicated in dopaminergic neuron maturation and survival in the brain. miR-133b is deficient in the midbrain of Parkinson's disease patients and in mouse models of dopamine neuron deficiency.

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kinson's disease–like phenotype. Thus, Dicer is essential for neuronal survival and loss of miRNAs may be involved in the development and/or progression of Parkinson's disease (*3*, *10*). Given that the transfer of cellular-derived small RNAs (including miRNAs) partially preserved the dopaminergic phenotype in cell culture (*3*), it is likely that the absence of miRNAs, and not the lack of other potential Dicer-related functions, is involved in the neurodegenerative process.

The next important steps are to determine the specific miRNAs responsible for neuronal cell death, the particular genetic programs and biological processes regulated by these miRNAs, and the extent to which these miRNAs play a relevant role in the neurodegenerative phenotype. The evidence presented by Kim *et al.* is somewhat ambiguous as far as relevance to neurodegeneration. Screening the expression of 224 different miRNAs obtained from brain samples of patients with Parkinson's disease and control subjects revealed notable changes in a small number of miRNAs, including miR-133b. Normally, miR-133b is enriched in the midbrain; however, it was surprisingly deficient in the brains of patients with Parkinson's disease. The relative number of patients investigated in this study is too small to draw definite conclusions about the clinical relevance of this observation. The finding that miR-133b suppresses the full differentiation of dopaminergic neurons in cell culture, whereas its expression is down-regulated in the brain of Parkinson's disease patients, is, however, puzzling. This observation suggests that miR-133b might have additional functions in dopaminergic neuronal differentiation beyond suppressing Pitx3 expression. Further work is necessary, not only to elaborate the clinical importance of these findings, but also to elucidate the full genetic program that miR-133b modulates.

Apart from the possibility that an overall loss of miRNA function could be associated with aging and could contribute to the age-related increased risk for Parkinson's and Alzheimer's disease, very specific molecular mechanisms should also be envisaged. Thus, polymorphisms in the genetic regions encoding specific miRNAs and alterations in molecular machinery (such as miRNA-processing enzymes) should be investigated. In particular, the 3′-UTR of the mRNAs encoding proteins such as α-synuclein or amyloid precursor protein should be scrutinized. Because dosage effects of these proteins are sufficient to induce Parkinson's disease (*13*) and Alzheimer's disease (*14*), respectively, further alterations that control their expression might also contribute to pathogenesis. Indeed, the neurological disorder Tourette's syndrome is associated with a variation in the binding site for a specific miRNA in the 3′-UTR of mRNAs encoding the neuronal proteins Slit and Trk-like 1 (SLITRK1) (*15*).

The work by Kim *et al*. and other recent studies (*7*, *11*) herald a new area of exciting research in the field of neurodegenerative diseases. Clinical studies will rapidly determine the extent to which miRNAs contribute to the pathogenesis of sporadic Parkinson's and Alzheimer's disease; however, the role of miRNAs as a potential therapeutic target remains a challenging question.

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MicroRNA studies using miRCURY™ LNA products

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MicroRNAs (miRNAs) are short non-coding RNA molecules that affect stability and/or translation of messenger RNAs. MicroRNAs have been shown to be involved in the regulation of many biological processes, including development, differentiation and apoptosis, and are implicated in the pathogenesis of several human diseases including cancer.

Here we present a range of unique products for the detection, profiling and inhibition of microRNAs. These products are part of the miRCURY™ LNA product line from Exiqon based on the Locked Nucleic Acid (LNA™) technology.

MicroRNA *in situ* hybridization - subcellular resolution

In recent years expression profiling techniques such as microarrays or northern blots have generated fruitful insights into the tissue and/or developmental specificity of microRNAs.[1-4] However, these methods do not provide the opportunity to study microRNA expression with cellular resolution, thus making it impossible to localize the specific expression of microRNAs in non-homogenous tissues.

Using Exiqon's miRCURY™ LNA technology, detection and localization of microRNAs are possible with a sensitivity and specificity not obtainable using standard detection technologies. The miRCURY™ LNA microRNA detection probes have proven their ability to detect microRNAs in numerous important research papers all listed on www.exiqon.com. A few papers are discussed here.

Kloosterman et *al.*[5] clearly showed that miRCURY™ LNA microRNA detection probes are superior in visualization of microRNAs compared to other kinds of probes (figure 1).

Wienholds et *al.*[6] reported highly tissue specific expression of most microRNAs during the embryonic development of zebrafish. The detection of microRNAs in specific tissues of zebrafish embryos was made possible by the miRCURY™ LNA technology. The paper showed additionally that approximately 30% of all microRNAs are expressed at a given time point in a given tissue (termed "call rate"). The 20-30% microRNA call rate has recently been validated in a paper by Landgraf et *al.*[1]

Figure 1

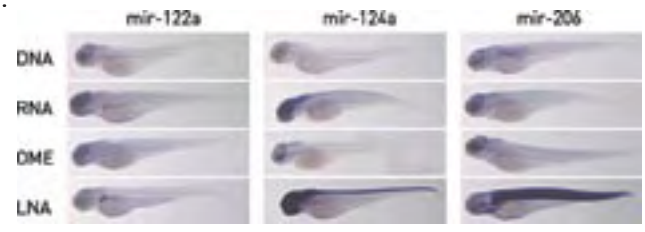

Figure 1. Only LNA probes give clear staining. Detection of miR-122a (liver specific), miR-124a (brain specific), and miR-206 (muscle specific) with DIG-labeled DNA, 2'-OMet and miRCURY™ LNA microRNA detection probes in 72h zebrafish embryos.
Lowering the hybridization temperature for DNA, RNA or 2'-OMe probes resulted in higher background staining. Image kindly provide by Dr. Ronald Plasterk, Hubrecht Laboratory, The Netherlands.[2]

Obernosterer et *al.*[2], used *in situ* hybridization and northern blot experiments to show that the expression of mammalian microRNAs can be regulated at the post-transcriptional level. They concluded that miRCURY™ LNA microRNA detection probes are making it possible to detect the different microRNA maturation stages with subcellular resolution.

Schratt et *al.*[7] showed that exposure of neurons to extracellular stimuli such as brain-derived neurotrophic factor reduces the brain specific miR-134 that contributes to synaptic development, maturation and/or plasticity. Figure 2 shows the unique images of microRNA expression in the dendritic spines of hippocampal neurons using a miRCURY™ LNA microRNA detection probe.

Ason et *al.*[8] showed that temporal expression and localization of microRNAs in vertebrates are not strictly conserved, and that variation in microRNA expression is more pronounced with increasing differences in physiology. This study was performed by comparing the expression of >100 microRNAs in medaka, chicken, zebrafish and mouse. Figure 3 shows the specific detection of miR-206 in chicken using a miRCURY™ LNA microRNA detection probe.

Figure 2

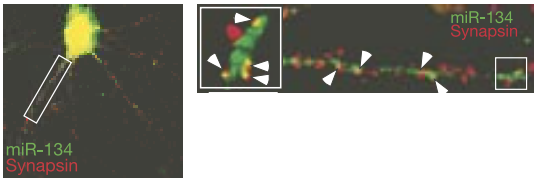

Figure 2. microRNA expression in dendritic spines of hippocampal neurons. Re-printed with permission from Nature. Images from Schratt et *al.*[7] showing the unique subcellular detection of miR-134 using a specific miRCURY™ LNA microRNA detection probe for miR-134 (green). Presynaptic marker protein synapsin (red) in hippocampal neurons is also shown. The boxed area in the left panel shown at greater magnification in the right panel.

Figure 3

Figure 3. Specific detection of mir-206 in *Gallus gallus* embryo using a miRCURY™ LNA microRNA detection probe. Mir-206 is expressed in all skeletal muscle cells, appearing at the onset of myogenic cell differentiation. At the pictured stage in embryonic development, mir-206 is detected in the myotomal muscle cells.[8]

These and many other papers confirm that miRCURY™ LNA microRNA detection probes are uniquely able to achieve precise visualization of microRNAs with subcellular resolution. Furthermore, they provide superior specificity and sensitivity.

MicroRNA profiling using microarrays – sensitive and specific

Microarrays represent one of the fastest and most comprehensive methods for determining the microRNA profile of a given sample.

The miRCURY™ LNA microRNA Arrays provide researchers with the ability to conduct genome-wide profiling of microRNAs in various samples including tissue, blood and FFPE samples, and to identify microRNA signatures associated with development, differentiation and metabolism, providing valuable diagnostic and prognostic indicators of disease.

MicroRNA profiling differs from global messenger RNA expression profiling in several important aspects. First of all, it is extremely difficult to target RNA molecules as short as microRNAs (16-29 nt). Standard DNA probes are not always able to discriminate sequences with single nucleotide differences.[4] This can result in many nonspecific signals.[9] Another difference between global mRNA expression profiling and microRNA profiling is the number of expressed targets at a given time point in a given tissue (call rate). Messenger RNA gene expression arrays contain thousands of different capture probes and in most cases the call rate is higher than 50%. In contrast, the call rate for microRNA samples is usually low as only 20-30% are expressed per cell type.[1, 6]

The miRCURY™ LNA microRNA Arrays are optimal for detection of microRNAs with superior sensitivity and specificity. The LNA capture probes are intelligently designed and Tm-normalized ensuring that all microRNA targets hybridize to the array with equal affinity under high stringency hybridization conditions. Without prior knowledge of the microRNA content in the sample, it is recommended to use around 250 ng total RNA to facilitate the most robust expression profiles. However, the high sensitivity of the miRCURY™ LNA microRNA Arrays enables reliable microRNA expression profiles from only 30 ng of total RNA. Figure 4 demonstrates that reliable microRNA profiling results can be obtained from only 30 ng of total RNA.

Figure 4

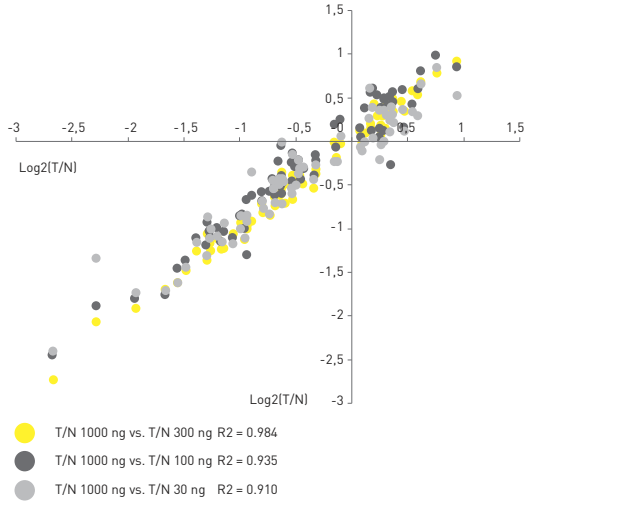

Figure 4. Excellent correlation of 91% between 30 ng and 1000 ng total RNA samples. Log2 ratios of Tumour vs. Normal (T/N) adjacent tissue of a oesophagus cancer using 1000 ng total RNA material is plotted against Log2 ratios in identical experiments using 30 ng, 100 ng, and 300 ng total RNA. The correlations to the microRNA profile from 1000 ng total RNA were 91%, 93% and 98% for 30 ng, 100 ng and 300 ng, respectively.

The specificity of the miRCURY™ LNA microRNA Array platform has previously been illustrated by a microRNA profile

of breast cancer tissue.[10] The study revealed many differentially expressed microRNAs, including those reported earlier to be associated with cancer, such as several members of the let-7 family and miR-21.[3] Some of these microRNAs may represent new molecular biomarkers with diagnostic and prognostic promise for patients with cancer (figure 5). Additionally, a randomly selected subset of the thousands of samples that have been analyzed at Exiqon's microRNA profiling service department shows an average call rate of 29% (data from 187 human samples from a variety of different tissues (figure 6). This is in line with published *in situ* hybridization and sequencing data, and thus further confirms the high level of specificity of the miRCURY™ LNA microRNA Array platform.[5,6]

The list of papers published using the miRCURY™ LNA microRNA Arrays is continuously growing. A few are mentioned below.

Suárez et *al*.[11] made a microRNA profile on Dicer knockdown in human endothelial cells (EC) using the miRCURY™ LNA microRNA Arrays. This study revealed 25 highly expressed microRNAs in human EC and using microRNA mimicry, miR-222/221 regulate endothelial nitric oxide synthase protein levels after Dicer silencing. Collectively, these results indicate that maintenance and regulation of endogenous microRNA levels via Dicer mediated processing is critical for EC gene expression and functions *in vitro*.

Valadi et *al*.[12] used the miRCURY™ LNA microRNA Array to show that exozomes do contain microRNAs (Exosomes are vesicles of endocytic origin released by many cells), and that exozomes contain both messenger RNA and microRNA that can be delivered to another cell and can be functional in this new location.

A global microRNA profiling study by Muralidhar et *al*.[13] showed that Drosha over-expression in cervical squamous cell carcinomas (SCCs) appears to be of functional significance. Unsupervised principal component analysis of a mixed panel of cervical SCC cell lines and clinical specimens showed clear separation according to Drosha over-expression. The microRNAs most significantly associated with Drosha over-expression are implicated in carcinogenesis in other tissues, suggesting that they regulate fundamental processes in neoplastic progression.

These data, as well as many other papers published using the miRCURY™ LNA microRNA Arrays, clearly confirm that they produce sensitive, specific and reliable data. An updated list of published papers using the miRCURY™ LNA arrays can be found at www.exiqon.com/array

Figure 5

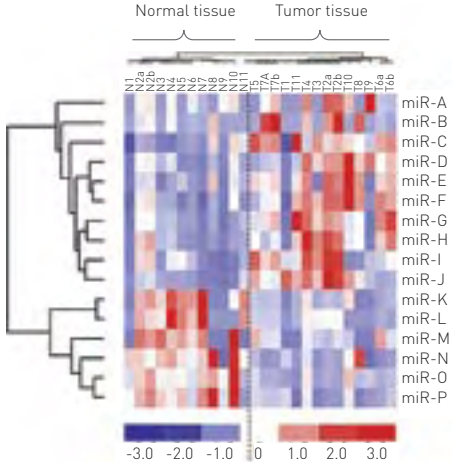

Figure 5. microRNA profiles differentiate breast tumors from normal tissue. The unsupervised hierarchical clustering and heatmap shows that primary tumors (T) and normal tissue (N) clearly segregated from one another based on their microRNA expression profiles. Samples consisted of paired samples from eleven patients. Both downregulated (blue) and upregulated (red) microRNAs were identified in breast cancer. The microRNA nomenclature does not refer to specific microRNAs.

Figure 6

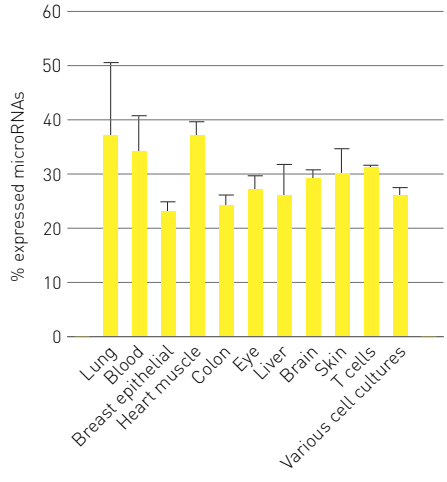

Figure 6. The call rate in various human samples using miRCURY™ LNA Arrays is shown. The call rate of the microRNA present in a given sample is shown. Samples are from human tissues and the number of samples from a given tissue is between 3 and 21. The average call rate from 187 different samples is 29% (StDev 6.5%). This is in line with *in situ* hybridization and sequencing data. [1, 6]

Knockdown – determine microRNA function

Inhibition of microRNAs represents one of the most popular methods to determine microRNA function and to validate putative microRNA targets. As microRNAs reduce protein levels by causing translational inhibition or degradation of the target mRNA, inhibition of a microRNA will typically cause an increase in target protein expression, thus ultimately providing a useful tool for the identification of predicted microRNA targets.

miRCURY™ LNA microRNA Knockdown probes are more effective at inhibition of microRNA than standard competing methods such as DNA-based probes and 2'-O-methyl (2'-OMe)-based probes (figure 7). The miRCURY™ LNA microRNA Knockdown probes provide researchers with an effective tool for determining microRNA function as evidenced by the peer-reviewed articles.

Figure 7

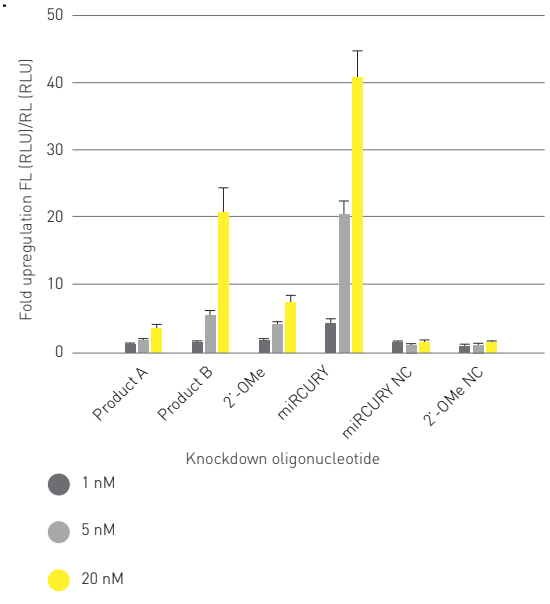

Figure 7. Comparison of the effectiveness of different knockdown methods. The diagram shows results of a representative experiment (n = 3), indicating fold upregulation, relative to the untreated control of the pMIR-21 reporter (firefly luciferase) when cotransfected with various hsa-mir-21 knockdown products. Expression levels in MCF7 cells were measured 60 h after transfection.
The negative control (NC) oligonucleotides are 2'-OMe or LNA-DNA mixmers with a random sequence showing no significant complementarity to any known microRNAs. Product A and B are not based on the LNA™ technology.

Fazi et *al.*[14] reported that knockdown of miR-223 using a miRCURY™ LNA microRNA Knockdown probe, produced a 40% reduction of CD11b while maintaining the level of control CD14, thus demonstrating that hsa-mir-223 is an important modulator of human myeloid differentiation.

Triboulet et *al.*[15] provided evidence for a physiological role of the microRNA-silencing machinery in controlling HIV-1 replication. This was shown by the inhibition of miR-17-5p and miR-20a using miRCURY™ LNA microRNA Knockdown probes, resulting in increased PCAF expression and HIV-1 replication.

The use of miRCURY™ LNA microRNA Knockdown probes has allowed researchers to elucidate the function of microRNAs in development as well as human diseases and to provide specific knockdown/inhibition of microRNAs with much higher potency than traditional antisense technologies.[16]

Summary

The miRCURY™ LNA microRNA product portfolio has enabled significant studies of microRNAs by providing researchers with highly sensitive, specific, and potent products. Several peer-reviewed publications demonstrate the effectiveness and validity of the miRCURY™ LNA microRNA products and show how the products are enabling microRNA science to advance.

Additional information about the miRCURY™ LNA microRNA product portfolio from Exiqon is available at www.exiqon.com

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Lars Kongsbak, President & CEO of Exiqon

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