NON-CODING RNA

# Evolution of microRNA diversity and regulation in animals

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Abstract | In the past decade, microRNAs (miRNAs) have been uncovered as key regulators of gene expression at the post-transcriptional level. The ancient origin of miRNAs, their dramatic expansion in bilaterian animals and their function in providing robustness to transcriptional programmes suggest that miRNAs are instrumental in the evolution of organismal complexity. Advances in understanding miRNA biology, combined with the increasing availability of diverse sequenced genomes, have begun to reveal the molecular mechanisms that underlie the evolution of miRNAs and their targets. Insights are also emerging into how the evolution of miRNA-containing regulatory networks has contributed to organismal complexity.

Although the first microRNA (miRNA) gene was discovered in 1993 (REFS 1,2), it was the realization 7 years later that miRNAs are widespread through the animal kingdom[3] and that they represent an entire novel level of gene regulation that forced scientists to "reorganize [their] view of the universe" (REF. 4). Tremendous advances in the understanding of miRNA biology have since been made, including: the identification of hundreds of miRNA genes; the dissection of miRNA biogenesis pathways; the identification of numerous miRNA targets; the establishment of principles of target gene regulation; and an understanding of the key role of miRNAs in reducing noise in gene expression.

Here, I summarize our current knowledge about evolution of miRNA systems in animals. First, an overview of the phylogenetic distribution of miRNAs is provided, highlighting the ancient origin of miRNAs and several waves of miRNA expansion during animal evolution. Next, genomic sources of novel miRNAs are discussed, followed by an overview of molecular mechanisms that underlie diversification of the miRNA repertoire. The principles of miRNA targeting are then outlined, and co-evolution of miRNAs and their targets is discussed. Finally, I provide the current view of how the incorporation of miRNAs in gene-regulatory networks influences the evolution of increasingly complex organisms.

Although miRNA systems in plants and animals have many common features, there are also substantial differences between them (reviewed in REF. 5). The specifics of miRNA evolution in plants can be found in REF. 6.

An understanding of the evolution of miRNA genes requires knowledge of the structure of these genes and of how their products are processed to produce mature miRNAs. As such, a brief overview of these topics is provided in FIG. 1; more detailed information can be found elsewhere (for example, reviewed in REFS 7,8).

## Phylogenetic distribution of miRNAs

The first miRNA gene that was discovered, *lin-4* in *Caenorhabditis elegans* (REFS 1,2), appeared to be specific to worms. However, the second miRNA that was discovered in *C. elegans*, *let-7* (REF. 9), was conserved between worms, flies and humans[3]. This discovery jump-started the miRNA field, and soon many additional miRNA genes were found in *C. elegans*, *Drosophila melanogaster* and human genomes[10–12].

With the development of next-generation, high-throughput sequencing methods, the so-called 'deep sequencing' of small RNAs followed by computational analysis became the *de facto* approach for miRNA discovery (BOX 1). Using small RNA sequencing, as well as northern blot and *in situ* hybridization approaches, a number of studies on the distribution of miRNAs across the animal kingdom have revealed several characteristic features of miRNA distribution in animals (BOX 2). First, miRNAs as a class of gene regulators were present early on in the evolution of animals; for example, *mir-100* is shared between eumetazoans[13]. Second, a large set of 34 miRNAs that are conserved between protostomes and deuterostomes[14] suggest a burst of innovation at the base of bilaterian lineage[15,16] ('bilaterian expansion'). Additional miRNA expansions are observed at the base of vertebrate lineage[15,17] and in the lineage leading to placental mammals[15,17] (BOX 2). Finally, the distribution

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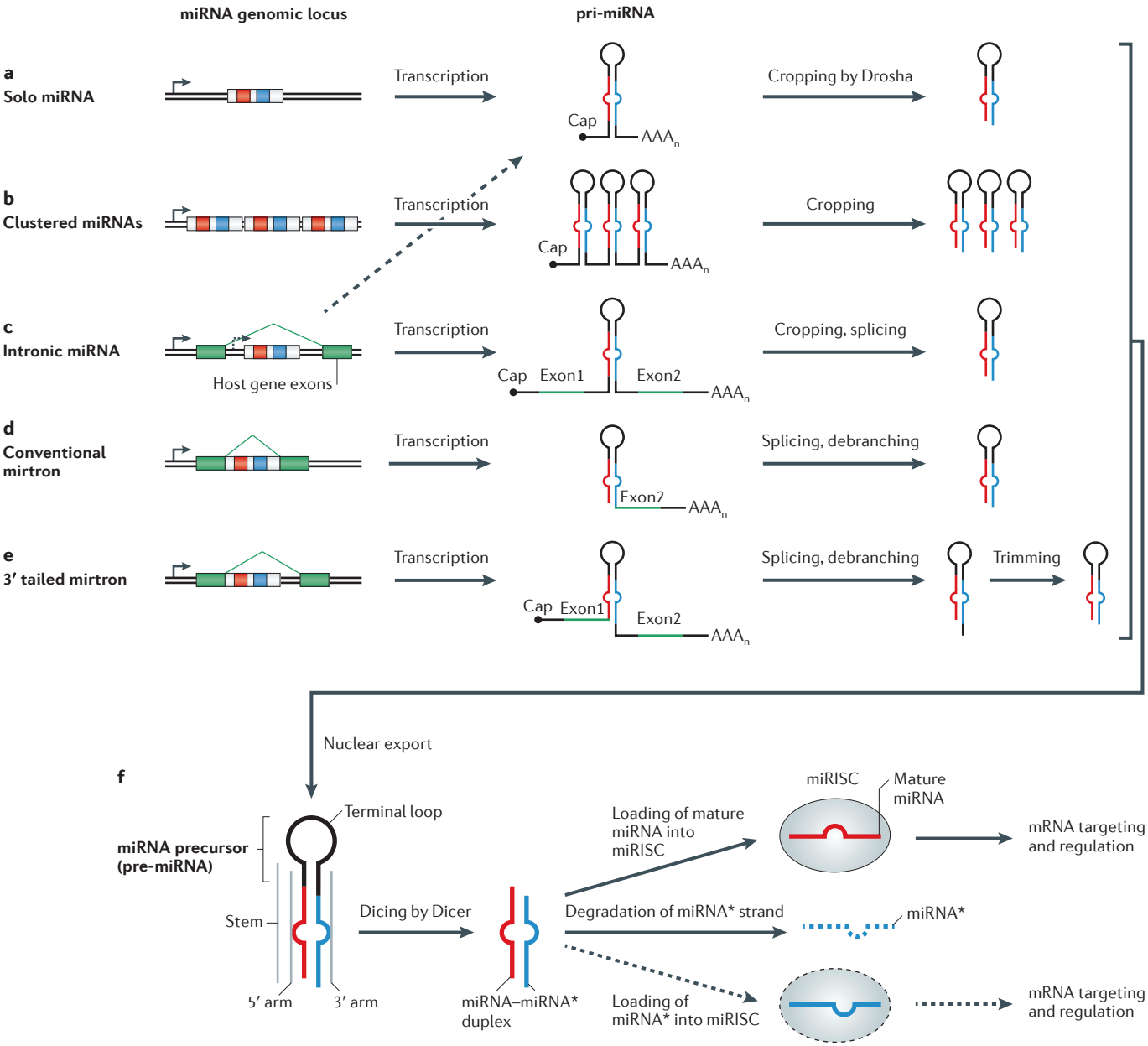
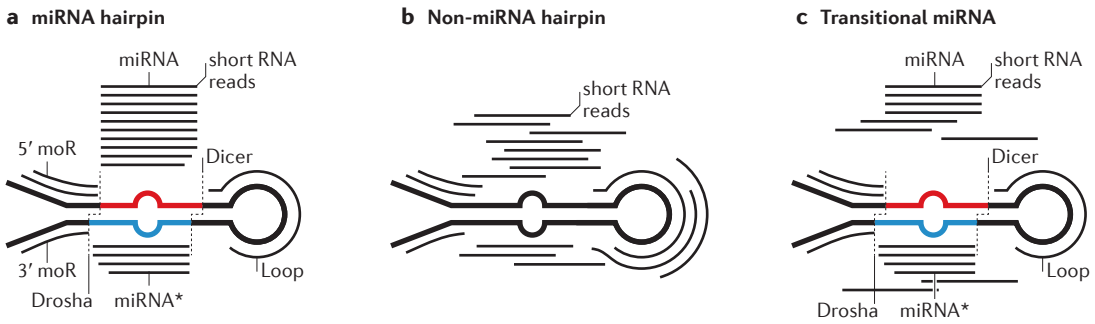



Figure 1 | **RNA gene structure and biogenesis.** The distinguishing feature of microRNA (miRNA) genes is the folding of their intermediate RNA transcripts into hairpin structures that are specifically recognized and processed by the miRNA biogenesis machinery. These primary miRNA transcripts (pri-miRNAs) are otherwise typical RNA polymerase II products with 5′ cap structures and polyA tails[157,158]. miRNAs are encoded in genomes either as independent transcriptional units with their own promoters (solo miRNAs) (**a**) or as clusters of several miRNA genes transcribed as a single pri-miRNA (**b**). A substantial fraction of animal miRNA genes are located in introns of protein-coding genes[32] (**c**). Whereas some intronic miRNAs have antisense orientation relative to their host genes and thus do not directly depend on host gene transcription, sense-oriented intronic miRNAs are thought to be processed as part of the host-gene mRNA and their expression correlates with that of their hosts[75,159]. However, there is increasing evidence that many sense-oriented intronic miRNA genes also have their own promoters located in upstream intronic regions[33–35]. For all miRNAs, the hairpin regions in pri-miRNAs (also called stem–loop structures) are recognized and processed by the microprocessor complex. This complex contains the RNase III type endonuclease Drosha, which cleaves the double-stranded stem region[160,161]. The result of this cropping process is a precursor miRNA (pre-miRNA) with a characteristic 2-nucleotide (nt) overhang at the 3′ end[162]. One class of unconventional miRNAs called mirtrons, which are encoded in introns, do not rely on Drosha processing and instead use the splicing machinery to generate pre-miRNAs[163–165] (**d**). For most mirtrons, the spliced intron is debranched and refolded into a pre-miRNA, such that the intron boundaries correspond to the pre-miRNA boundaries. In some cases, splicing can result in tailed mirtrons, which require additional trimming by the exosome to produce a functional pre-miRNA[166] (**e**). Processed pre-miRNAs are exported into the cytoplasm, where they are recognized and further processed by another RNase III enzyme, Dicer[167–170] (**f**). The dicing of pre-miRNA cleaves off the loop region of the hairpin and results in a ~22–23 nt double-stranded RNA called the miRNA–miRNA* duplex. The mature miRNA strand of this duplex is next loaded into an Argonaute-containing miRNA-induced silencing complex (miRISC), whereas the so-called miRNA* sequence is degraded[75]. The mature miRNA within miRISC serves as a guide for recognizing target mRNAs by partial base-pairing, which leads to a block in the translation of the mRNA target and/or its degradation[171].

Box 1 | **Identification of miRNA genes**




Transcripts that are generated from microRNA (miRNA) loci can be experimentally identified by cloning and sequencing of size-fractionated small RNA libraries (reviewed in REF. 154). Initially, miRNA discovery relied on conventional Sanger sequencing of ~22-nucleotide (nt) small RNAs, but with the development of high-throughput sequencing technologies, the task of small RNA sequencing has been greatly simplified, making the investigation of small RNA repertoires of cells possible at the unprecedented resolution of hundreds of millions of reads. The flood of small RNA data has provided an opportunity for miRNA gene discovery but also poses the challenge of distinguishing miRNAs from other small RNA types, as the origin of a small RNA from a predicted hairpin region, although required, is not sufficient alone for its classification as an miRNA[21,155]. However, additional 'signatures' of miRNA biogenesis can be inferred from the abundance and distribution of small RNA reads across the predicted hairpin[53,155]. Indeed, if a given hairpin is a true miRNA (part **a** in the figure), then it will have consistent Drosha and Dicer processing sites, which will result in the production of phased small RNA reads. Among these reads, the most abundant form will correspond to the 22 nt long mature miRNA, and the less abundant ones will correspond to miRNA isoforms, miRNA* sequences, loop sequences and miRNA offset reads (moRs). Importantly, there should be little variation in the RNA ends corresponding to Drosha and Dicer cleavage sites and, in particular, the 5′ ends of the reads should have a nearly uniform position. The second important miRNA signature is the 2 nt overhang at the 3′ ends of miRNA duplexes that are generated by Drosha and Dicer processing. The length of the 3′ overhangs can only be determined if the reads that correspond to both mature and miRNA* sequences are detected, which is usually the case provided that there is sufficient sequencing depth[37].

On the other hand, if small RNAs are derived from a hairpin by a process that is different from the precise excision by Drosha and Dicer (part **b** in the figure), the distribution of the small RNA reads over the hairpin will not give a clear phased pattern and will instead look more random, and the 3′ overhang signatures will be not evident.

Hairpin loci that are in the process of evolving towards an miRNA function — so-called transitional miRNAs[37] (part **c** in the figure) — may exhibit 'relaxed' signatures, in which more variation in read distribution over the hairpin is seen. In such cases, additional experimental evidence for miRNA biogenesis is required: for example, by demonstrating association of the corresponding small RNAs with Argonaute proteins[37].

For species with available sequenced genomes, the deep sequencing of small RNAs allows the identification of miRNA genes, regardless of their conservation in other species. For species without available genome sequence, deep sequencing can still be useful for the identification of conserved miRNA genes by comparing their small RNA sequences to known mature miRNAs from other species. Alternatively, northern blot and *in situ* hybridization analysis can be used to probe for the presence of conserved miRNAs across species, albeit with less sensitivity than sequencing-based methods, and with a larger degree of uncertainty that is due to potential non-specific annealing inherent for hybridization-based methods.

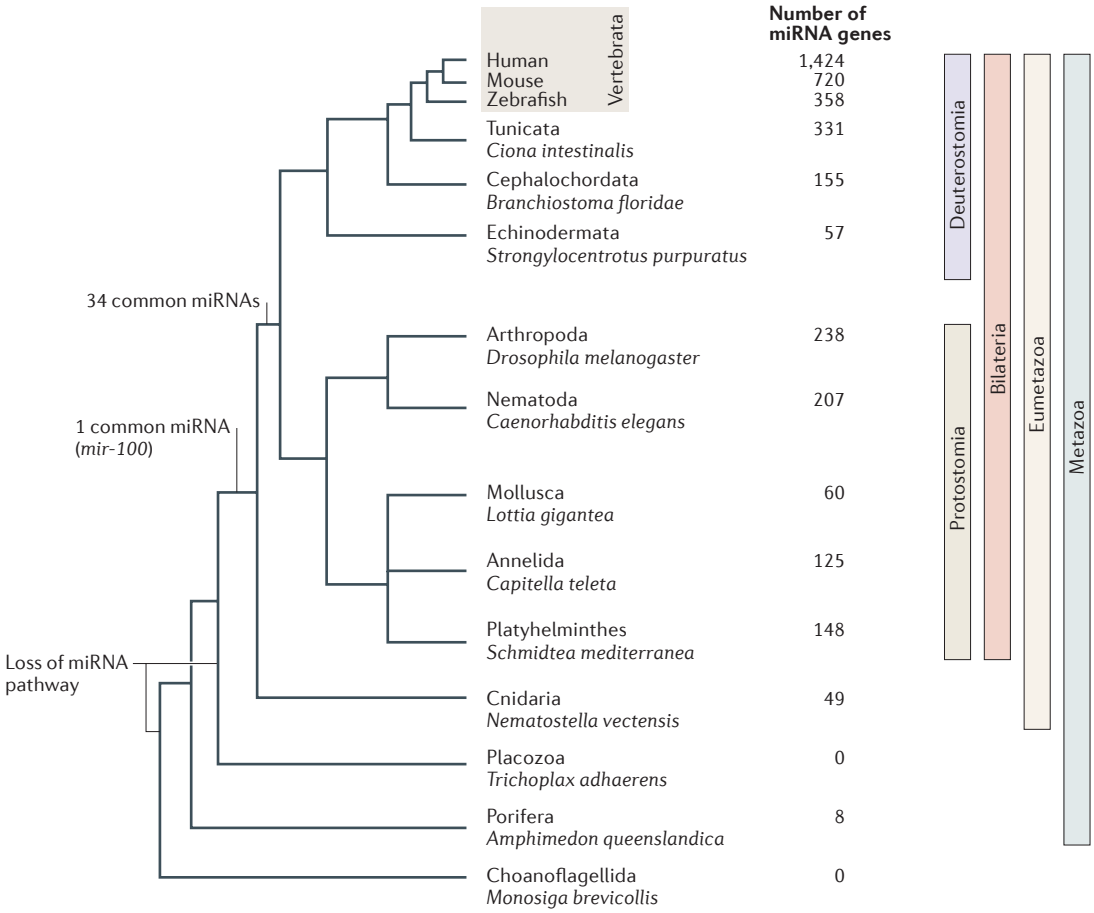
of conserved miRNAs indicates that, after an miRNA gene emerges in a particular lineage, it is rarely lost in the descendant lineages[16–19]. For example, comparison of miRNA repertoires of the key representatives of deuterostomes (that is, humans, lampreys, amphioxi, sea urchins and acorn worms) identified just 12 miRNA gene losses during deuterostome evolution[20]. Similarly, only nine ancestral miRNAs distributed in four operons were confidently identified as having been lost in the *obscura* species group of *Drosophila*[21]. Combined with this low rate of secondary loss, continuous acquisition of novel miRNAs has led to the ever-increasing repertoire of miRNAs during evolution. For example, in the same study of the key deuterostome representatives[20], approximately one-third of the miRNAs in each lineage seem to have been specifically acquired in that lineage. Remarkably, there is a direct correlation between the number of miRNAs and morphological complexity, suggesting that miRNA innovation may be a key player in the emergence of increasingly complex organisms[13–26].

## Genomic sources of novel miRNA genes

How do new miRNA genes emerge in a genome? From the viewpoint of miRNA biogenesis, the central requirement for an miRNA gene is the correct secondary structure of its RNA product, as only hairpins that are consistently recognized and processed by Drosha and Dicer can result in a functional miRNA-induced silencing complex (miRISC) (FIG. 1). Thus, the prerequisite for the emergence of a novel miRNA is a transcribed genomic locus that can be evolved to produce an RNA fold that is recognizable by the miRNA machinery. Because RNAs easily form non-perfect foldback structures, the evolution of a new miRNA gene appears to be more likely than the emergence of a novel protein-coding

## Box 2 | **Distribution of miRNA genes in the animal kingdom**

The analysis of small RNA libraries from early branching animal phyla has revealed that miRNAs were present at the dawn of metazoan evolution[13]. Within the Eumetazoa, there is one miRNA, *mir-100*, that is present both in all bilaterians and in *Nematostella vectensis*, a member of the Cnidaria — an outgroup to bilateria. In the demosponge *Amphimedon queenslandica*, a representative of the even more deeply branching Porifera phylum, eight miRNA genes were identified[13,18] that were largely conserved in different sponge lineages[18]. However, none of these miRNAs shared sequence homology with eumetazoan miRNAs[13,18]. Together with the unusually long structure of precursor miRNAs (pre-miRNAs) in demosponges, this finding raised the possibility that sponge miRNAs emerged independently from those in eumetazoans[16,18]. However, the presence of the core miRNA-processing proteins Drosha and Pasha in demosponges suggest the single origin of miRNA machinery in animals and, by inference, the presence of common miRNA substrates in an early animal ancestor[13]. The possible loss of ancient metazoan miRNAs in Porifera and the evolution of miRNAs that are specific to this clade are supported by the apparent absence of miRNAs in *Trichoplax adherens*[13] — a representative of another basal lineage, the Placozoa. Based on genome sequence data, placozoans — which are morphologically among the simplest metazoans — are derived from a more complex ancestor that is shared with cnidarians and bilaterians, but then they lost a number of features[156]. Pasha — the only protein that exclusively functions in the miRNA-processing pathway — was not identified in the *T. adherens* genome, suggesting that all miRNAs have been lost in this lineage[13]. Similarly, neither miRNA genes nor miRNA- and RNAi-pathway proteins were identified in the single-celled organism *Monosiga brevicollis*, which belongs to the choanoflagellates, the closest known outgroup to the Metazoa[13]. However, the presence of RNAi-pathway proteins in the common ancestor of *M. brevicollis* and metazoans suggests these components might have been lost in *M. brevicollis*. Thus, although miRNAs clearly emerged very early in the evolution of Metazoa as a class of gene regulators, the precise timing remains uncertain[13]. The most recent analysis of miRNAs that are conserved between the two major superphyla of bilaterian animals, protostomes and deuterostomes, revealed 34 miRNA families that are common to both groups[14], supporting earlier observations that a substantial number of miRNAs are conserved in nephrozoans (protostome and deuterostome clades combined)[15,24,26]. Because only one of these miRNAs is also present in cnidarians (*mir-100*), it appears that there was a burst of miRNA innovation at the base of the bilaterian lineage[15,16]. The next expansion of the miRNA repertoire is observed at the base of vertebrate lineage[15,17,19]. Finally, within vertebrates, there is a further increase in the miRNA numbers in the lineage leading to eutherian (placental) mammals[15,17] (for example, note the doubling of miRNA numbers between zebrafish and mice). The number of miRNA genes in a given clade shown in the figure is taken from miRBase version 17 (REF. 29) and, as such, represent a snapshot on the current knowledge about miRNAs in different phyla. The actual number of miRNA genes is likely to be higher in the less studied groups (for example, *Lottia gigantea*). Branch lengths in the cladogram are not to scale.



gene[27]. As discussed below, there is accumulating evidence for several distinct genomic sources from which a novel miRNA gene can evolve (FIG. 2).

***Gene duplication.*** miRNA genes that have significant sequence homology to each other and identical (or almost identical) seed regions in their mature miRNA sequences are grouped into miRNA families[28]. For example, of the 1,424 human miRNA genes listed in the miRNA repository miRBase[29] (v17), one-third (456) can be grouped into 141 families of two or more miRNA genes based on miRNA naming conventions[28]. Similarly, 38% of miRNA genes in mice, 10% in *C. elegans* and 13% in *D. melanogaster* belong to families. These miRNA gene families, which represent paralogous sequences, arise through gene duplication, and two types of duplication events can be distinguished[15]. In local or tandem duplications, the duplicated gene typically remains located in the same transcript, whereas non-local duplications result in a new miRNA in a remote location, often on a different chromosome[15]. The majority of non-local miRNA duplications that have been analysed in the vertebrate lineage are associated with the genome duplication events that have occurred during vertebrate evolution[15,17,20,30]. Gene duplication events, followed by subfunctionalization and neofuctionalization processes[31], are considered to be a major source for emergence of novel miRNA genes (FIG. 2a,g).

***Introns.*** Many miRNAs reside in introns of protein-coding genes[32]. For example, almost half of human miRNAs[20] and 20% of *C. elegans* miRNAs[33] are located in introns in the same orientation as the host gene. It appears that introns are a 'sweet spot' for the emergence of novel miRNAs (FIG. 2b), as they readily provide material for the evolution of a hairpin structure from an RNA that is already transcribed and do not require the prior evolution of a new promoter unit[20]. Consistently with this hypothesis, younger, species-specific miRNAs are more often located in introns than ancient miRNAs are[20]. Traits that initially evolve to serve one function but later come to serve another function are called exaptations. Accordingly, the evolution of miRNAs in intronic sequences was recently termed 'intronic exaptation' (REF. 20).

As previously mentioned, there is accumulating experimental evidence that a substantial fraction of intronic miRNAs have their own promoter regions[33–35]. Thus, it is essential to distinguish between 'true' host-dependent intronic miRNAs and autonomous host-independent intronic miRNAs genes, and further research is required to improve our understanding of the differences in evolution between these two types of intronic miRNAs.

The presence of miRNAs not only in introns but also in the protein-coding exons of genes has been suggested[36]. However, the annotation of some of these loci as miRNAs has been challenged based on patterns of small RNA heterogeneity[21] (BOX 1). In a subsequent large-scale effort to annotate small RNAs in *D. melanogaster* based on more than one billion raw sequencing reads, only nine miRNA loci were identified in the coding sequences of genes, and two were identified within untranslated regions (UTRs)[37]. As such, protein-coding exons are not likely to be important sources of novel miRNA genes.

***Pseudogenes, snoRNAs and tRNAs.*** As well as introns of protein-coding genes, other sources of RNA transcripts can, in principle, be shaped by evolution into miRNA hairpins (FIG. 2e), and a number of miRNAs that are derived from small nucleolar RNAs (snoRNAs), tRNAs and pseudogenes have been reported in the literature[38–43]. However, because snoRNAs and tRNAs themselves can be processed by the RNAi and miRNA machinery, resulting in a wide range of small RNAs, it is challenging to distinguish a bona fide miRNA gene that has emerged from a snoRNA or tRNA ancestor as opposed to by-products of normal snoRNA or tRNA biogenesis[21] (BOX 1). Nevertheless, several snoRNA- and tRNA-derived small RNAs have been identified in Argonaute complexes[39,44], some of which have been shown to induce gene silencing in luciferase reporter assays[39], indicating miRNA-like properties.

***Transposable elements.*** Once thought of as 'genome parasites', transposable elements are now recognized as an important source of genomic innovation (reviewed in REFS 45,46), including a contribution to the emergence of novel miRNA genes[15,47–52] (FIG. 2d). A recent survey by Yuan *et al.* (REF. 52) identified 278 human miRNA genes that are either definitely or possibly repeat-derived and that have originated from both DNA transposons and retrotransposons[52]. Similarly, an improved algorithm for miRNA identification from small RNA reads revealed 16 high-confidence, repeat-derived miRNAs in humans[53]. Transposon-derived miRNAs are significantly enriched for younger, less conserved and lineage-specific miRNAs compared with miRNAs that have evolved from other origins[49,52], as would be expected of a source of continuous miRNA emergence.

There is no question about the authenticity of some of the repeat-derived miRNAs[49]. However, complications that are similar to those concerning the annotation of novel miRNAs from snoRNAs and tRNAs apply to repeat-derived miRNAs (BOX 1). Specifically, in many cases, the origin of a small RNA cannot be unambiguously assigned to a single genomic locus owing to the repetitive nature of the sequence and owing to potential overlap with small RNAs that are produced from repeats by the PIWI-interacting RNA (piRNA) pathway (reviewed in REF. 54).

***Antisense miRNA transcripts.*** Because miRNA hairpins are essentially imperfect palindromes, antisense transcription of miRNA loci often results in transcripts that fold into hairpins and can be processed by miRNA machinery. Importantly, as mature miRNAs and miRNA* sequences are often not fully complementary, antisense transcription of miRNA loci can result in miRNAs with novel mature and miRNA*. Production of small RNAs from antisense miRNA loci has been observed for a number of miRNAs[31,37,55–57], and thus antisense transcription of existing miRNAs is indeed one of the sources for the evolution of novel miRNA genes (FIG. 2f).

Paralogous
The homology between two genomic segments in the same organism that arose from a duplication event.

Subfunctionalization
The division of the ancestral function of a gene following gene duplication, in which different copies of the duplicated gene retain different aspects of the original function.

Neofunctionalization
The acquisition of a novel function by one of the copies of a duplicated gene, which comes about through mutational changes.

DNA transposons
Transposable elements that rely on a transposase enzyme to excise themselves from one region of the genome and insert themselves into a different region without increasing in copy number.

Retrotransposon
Transposable element that replicates via an RNA intermediate, which is converted by reverse transcriptase to cDNA. The cDNA can be inserted into genomic DNA, increasing the number of copies of the retrotransposon in the genome.

PIWI-interacting RNAs
(piRNAs). Single-stranded RNAs in the range of 25–35 nucleotides that form complexes with the PIWI protein. piRNAs are involved in transposon silencing.

miRNA*
One of the strands in the imperfect double-stranded intermediate RNA duplex that is generated after processing of the primary miRNA precursor RNA by Drosha and Dicer. The other strand — mature miRNA — is predominantly loaded into the miRNA-induced silencing complex (miRISC), whereas miRNA* is degraded.

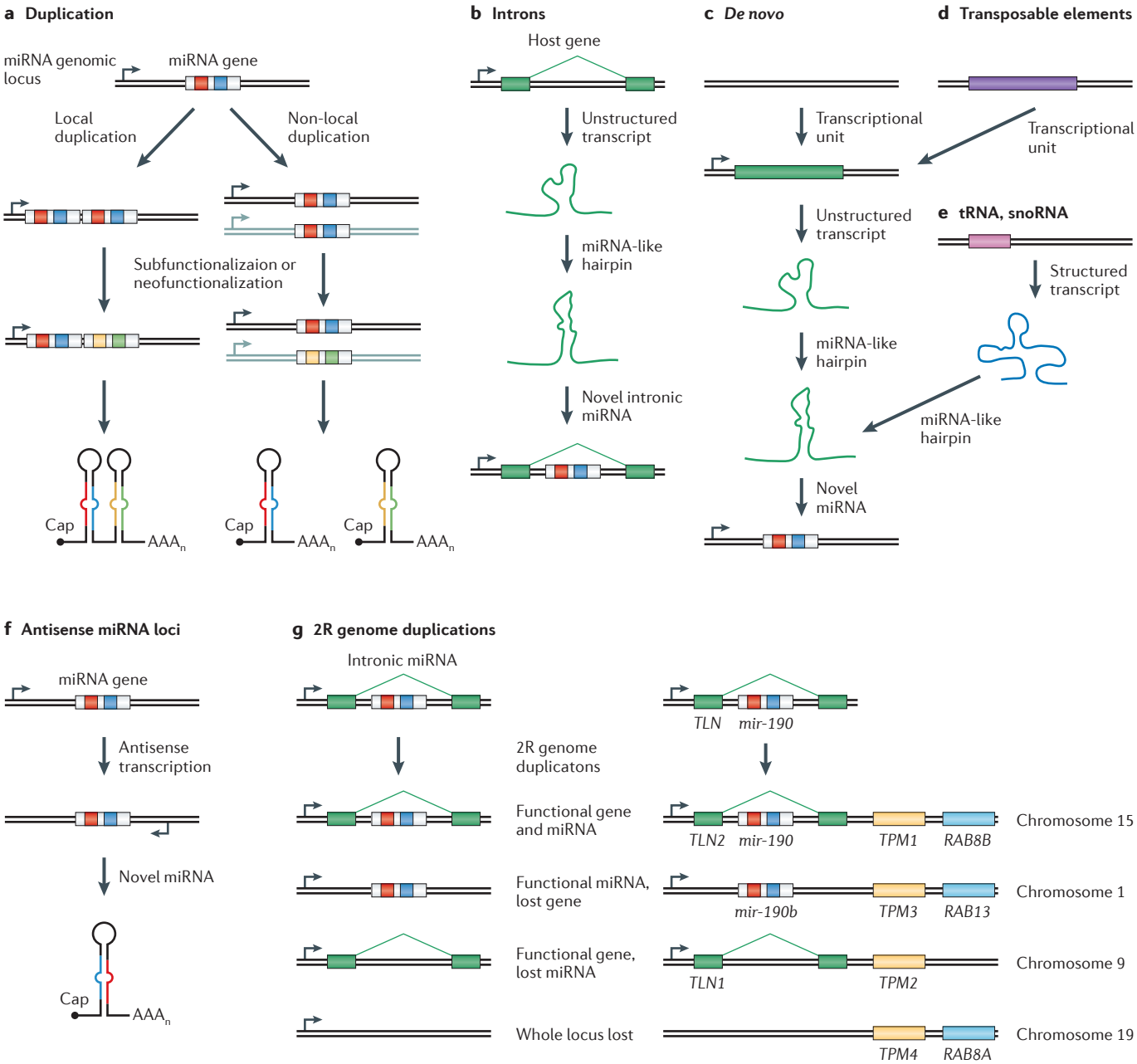
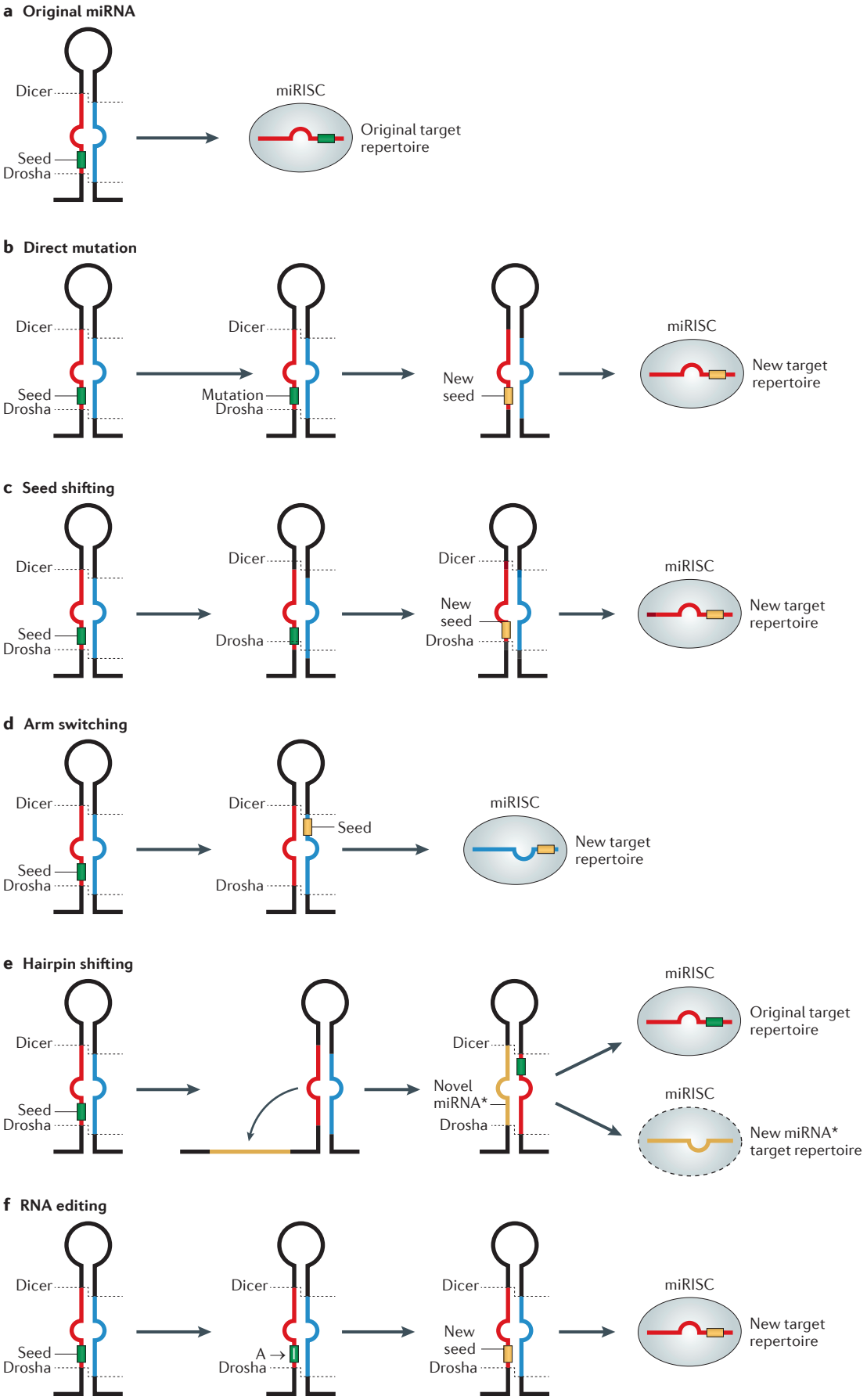



Figure 2 | **Genomic sources of novel miRNA genes. a** | Novel miRNA genes can emerge by local or non-local duplication of existing miRNA genes. In local duplication, the new copy of the gene is located in close proximity to the original, and the two copies are transcribed as a single primary transcript. In non-local duplication, the new copy is located remotely from the original and is transcriptionally independent. Processes of subfunctionalization or neofunctionalization drive the evolution of the duplicated genes and can eventually lead to sequence changes in mature miRNA and miRNA* regions (indicated by red, blue, yellow and green) and emergence of novel miRNA sequences. **b** | Introns are a frequent source of unstructured transcripts that can gradually evolve into novel intronic miRNA through a stage of miRNA-like hairpin. **c** | In *de novo* emergence of miRNAs, a transcriptional unit is first evolved that provides a source of initially unstructured transcript that transitions through the miRNA-like hairpin stage and evolves into a novel miRNA gene. **d** | Transposable elements can provide novel transcriptional units for the evolution of miRNA-like hairpins into novel miRNA genes. **e** | Structured transcripts, such as tRNA and small nucleolar RNA (snoRNA), can evolve into novel miRNAs through the miRNA-like hairpin stage. **f** | Antisense transcription of existing miRNA loci can lead to the formation of miRNA hairpins with novel mature miRNA and miRNA* (blue and red, respectively) sequences. **g** | Theoretical fates of intronic miRNAs and their host genes after two rounds (2R) of genome duplication. After 2R genome duplication, the whole locus can retain both functions (gene and miRNA) or either host gene or miRNA, or the entire locus can be lost. The human *mir-190/TAL* locus is an example of all four possible outcomes. *RAB8B*, member RAS oncogene family; *TLN*, talin; *TPM1*, tropomyosin 1. Part **g** is modified from REF. 20 © (2011) Wiley.



◀ Figure 3 | **Mechanisms of miRNA sequence diversification. a** | The original microRNA (miRNA) hairpin is processed by Drosha and Dicer. The mature miRNA (red) is loaded into miRNA-induced silencing complex (miRISC), and its sequence — in particular the seed region (green) — determines the target repertoire. The miRNA* (blue) usually does not have an important role in target regulation. **b** | Mutations that affect the mature miRNA sequence can directly lead to changes in the target repertoire: specifically, if they give rise to a new seed sequence (yellow). **c** | Mutations anywhere in the miRNA precursor region can result in changes in hairpin structure and hairpin processing. In seed shifting, the dominant mature miRNA form is shifted owing to changes in Drosha and Dicer processing. As a consequence of the changed 5′ end of the mature miRNA, a new seed sequence (yellow) comes into play, resulting in novel target repertoire. **d** | In arm switching, changes somewhere in the miRNA precursor lead to a shift in the ratio of miRNA to miRNA* loading into miRISC, and miRNA* (blue) is preferentially incorporated, providing a novel miRNA sequence and thus a new target repertoire. **e** | In hairpin shifting, a novel hairpin is evolved from an adjacent region, keeping the same mature miRNA (red) but introducing a novel miRNA* sequence (yellow), which then could acquire regulatory function through loading into miRISC. **f** | RNA editing results in post-transcriptional conversion of adenosines into inosines (A to I) in the double-stranded parts of an miRNA precursor. This can affect processing of the precursor and can introduce changes in to mature miRNA sequence; in particular, editing of the seed region (yellow) can result in a changed target repertoire.

**De novo *miRNA emergence.*** Hundreds of thousands of miRNA-like hairpins have been predicted to be encoded in the human genome[58], and a large fraction of the genome is known to be transcribed in mammals (so-called pervasive transcription[59]). As such, it appears that there are many RNA substrates in a cell that could be shaped by evolution into miRNAs. Thus, the *de novo* emergence of miRNAs from unstructured transcripts is a plausible source of novel miRNAs[36,60,61] (FIG. 2c). The precision of hairpin processing by Drosha and Dicer depends on the secondary structure of the hairpin, and there are a limited number of genomic loci for which there is experimental evidence for such precise processing, all of which are true miRNAs. Thus, it seems unlikely that the *de novo* emergence of an miRNA can result from a single evolutionary event[36]. Instead, gradual evolution of a hairpin towards a 'perfect' miRNA structure represents a more likely scenario, in which case evolutionary transitional forms of hairpins should exist[60]. Indeed, such intermediates — where small RNA reads show a semi-confident signature for processing by Drosha and Dicer (BOX 1) — are found among hundreds of putative hairpins with matching small RNA reads[37].

As a newly emerging miRNA is initially likely to target many genes simply by chance — because only short sequences are required for target recognition — such targeting can often have deleterious consequences[27]. To explain how novel miRNAs can evolve in this context, Chen and Rajewsky[27] put forward a model of transcriptional control of new miRNAs . According to this model, novel miRNAs are initially expressed at low levels and in specific tissues to limit deleterious effects of accidental targeting. Gradually, deleterious targets are purged from the transcriptome by natural selection, and the level and the spatial range of miRNA expression can be increased[27]. In support of this model, less-conserved miRNA genes are indeed generally expressed at lower levels compared with broadly conserved miRNAs[22,36]. Furthermore, deleterious effects of high expression of 'newly emerged' miRNAs on fitness were convincingly demonstrated by overexpression of heterospecific miRNA in *D. melanogaster*[62]. One of the implications of this model is that many miRNAs that show low levels of conservation and low levels of expression might still be in the 'purging' phase of evolution and might not yet have acquired any biological targets that they regulate to any substantial degree[27,36].

## Modes of miRNA sequence diversification

After an miRNA gene has emerged and acquired a regulatory function, can it evolve further? Comparative genomics analyses of small RNA data sets from a large array of species have revealed a number of ways in which an existing miRNA gene can be further diversified (FIG. 3). As the sequence of the mature miRNA molecule that is loaded in the miRISC complex is the primary determinant of the target repertoire for a given miRNA gene, mechanisms that lead to changes in the mature miRNA sequence are of central importance when considering evolution of established miRNAs. These mechanisms include both mutations that arise directly in the mature sequence region (FIG. 3b) and also include mutations elsewhere in the precursor miRNA, which lead to changes in hairpin structure and, as a consequence, changes in Drosha and Dicer recognition and miRISC loading. As well as 'hardwired' changes at the DNA level, post-transcriptional mechanisms, such as RNA editing, also contribute to the diversification of mature miRNA sequences.

***IsomiRs and seed shifting.*** Depending on the precision of hairpin processing by Drosha and Dicer, several different small RNAs can be produced from the same hairpin[31,37,63–67]. Mature miRNAs that are produced from the same hairpin but that differ in their termini are called isomiRs[68]. In canonical miRNAs, there is usually only one prominent isomiR sequence (which is annotated as the mature miRNA), and all other isomiRs are produced at a much lower level[31,64,67,69]. IsomiRs can be classified using the predominant form as a reference.

3′ and 5′ isomiRs are distinguished based on their varying termini. 3′ isomiRs are seen in small RNA-sequencing data for most miRNAs and can be the result of variation in Drosha or Dicer processing and non-templated 3′ nucleotide addition[31,37,64,67,69,70]. The 3′ end of a mature miRNA is believed to be a minor contributor in determining miRNA target specificity, and 3′ isomiRs are thought to have largely the same targets as the predominant miRNA, although formation of 3′ isomiRs is developmentally regulated and can be biologically relevant[71]. The situation is completely different for 5′ isomiRs. According to mutational profiles of mature miRNAs, positions 2 to 7 represent a particularly conserved region[18,72]. This so-called seed region is the largest contributor in determining miRNA target specificity (reviewed in REF. 73). As such, changing the 5′ terminus leads to a shift in the seed region and can result in extensive changes in target repertoire. Presumably because of this, 5′ isomiRs are less common than 3′ isomiRs and represent only a minor fraction of reads in small RNA deep sequencing data[37,67]. However, for some miRNA genes

(for example, *mir-79*, *mir-193* and *mir-210* in *D. melanogaster*[31,37], and *mir-124*, *mir-133a* and *mir-223* in mice[67]) two or more 5′ isomiRs accumulate to levels that are comparable to the major form.

The preference for the dominant 5′ isomiR can change during evolution, leading to so-called 'seed shifting' (REF. 18) (FIG. 3c). For duplicated miRNA genes, seed shifting is one mechanism for acquiring divergent function. Examples include *mir-281* and *mir-2* families in *D. melanogaster*, where uniquely mapping miRNA* reads revealed production of different 5′ isomiRs from different paralogues in these families[31]. Seed shifting in miRNA orthologues is often observed between distant species. For example, 13% of miRNAs that are conserved between *D. melanogaster* and the red flour beetle *Tribolium castaneum* have undergone seed shifting[74]. The most ancient metazoan miRNA, *mir-100*, has also experienced seed shifting and has a one-base shift at the 5′ end of its mature miRNA in the sea anemone *Nematostella vectensis* compared to all bilaterians[13].

***miRNA* sequence and arm switching.*** The miRNA* strand was initially thought to be biologically inert (which is why it is sometimes called the 'passenger strand'). This notion came from the fact that miRNA* species were rarely detected in the early, low-coverage efforts to clone small RNAs[75]. However, deep-sequencing data revealed that, although miRNA* molecules are generally less abundant compared with their mature miRNA counterparts, they can still accumulate in cells to physiological levels[31], associate with Argonaute proteins and regulate target mRNAs[76] with biologically relevant consequences[77]. As miRNA* sequences compete with the miRNA strand for loading into miRISCs, they present an evolutionary substrate for the diversification of miRNA functions[60,76]. Indeed, it is possible that evolutionary drift in duplicated miRNA genes can change the ratio of miRNA versus miRNA* species that are loaded into miRISC from one of the gene copies, eventually leading to the predominant expression of the miRNA*. This process of switching the dominant arm is termed 'arm switching' (REF. 76) and has been documented for multiple miRNA families in flies[74,76] and worms[78]. Arm switching (FIG. 3d) is a frequent phenomenon (for example, it is found in 11% of orthologous miRNAs in insects[74]) and has been proposed to be one of the fundamental mechanisms of miRNA diversification[76,78,79].

The molecular mechanisms that underlie miRNA arm usage and that lead to arm switching are not fully understood. The relative thermodynamic stability of the ends of the intermediate dsRNA duplex can be one of the determinants[80,81]. However, arm usage can also switch between tissues and developmental time points[67,82], suggesting additional determinants in the form of accessory proteins or within the miRNAs precursor sequence (reviewed in REF. 7). The evidence that the arm usage can be encoded in the primary miRNA sequence is provided by Griffiths-Jones *et al.* [79]. They demonstrated that arm switching occurred for *mir-10* of *Tribolium castaneum* compared to the homologous miRNA in *D. melanogaster*. Although the sequence of *mir-10* dsRNA duplex is identical between the two species, expression of the *T. castaneum* precursor *mir-10* sequence in *D. melanogaster* S2 cells leads to the selection of the *T. castaneum* arm as mature miRNA.

***Hairpin shifting.*** Whereas in arm switching the hairpin sequences remain overall the same and only the relative abundances of mature and miRNA* species change, in hairpin shifting[78] the entire fold is changed into new configuration (FIG. 3e). In some miRNA families, mature miRNAs originate from one arm of a hairpin for some members and from the opposite arm in other members[78]. As miRNA families arise through duplication events, changing the hairpin arm while keeping the same mature sequence can be explained by the evolution of a new hairpin from one of the arms of the old hairpin and adjacent upstream or downstream sequence (FIG. 3e). This process of hairpin shifting was identified in eight out of 15 miRNA families in nematodes[78] and thus appears to be a common mechanism in the evolution of miRNA families. Importantly, owing to the shift in the genomic sequence that is used as a template, an entirely new part of the miRNA precursor — including novel miRNA* sequence — comes into play through hairpin shifting, providing further ways for miRNA diversification.

***Editing.*** RNA editing is carried out by adenosine deaminases (ADARs), which convert adenosines at specific sites in dsRNA into inosines (reviewed in REF. 83). Because miRNAs transition through a double-stranded form, they can be modified by ADARs, and indeed cases of adenosine-to-inosine (A-to-I) editing in miRNAs have been reported[84–87]. RNA editing in mature miRNAs can have important consequences, as it will lead to changes in targeting[87] (FIG. 3f). However, in general, the frequency of RNA editing in mature miRNAs appears to be low and difficult to distinguish from the background level of sequencing errors[67]. The few miRNAs that have robust RNA-editing signals were primarily identified in neuronal tissues[37,67], a finding that is consistent with the predominantly neuronal expression of ADARs. As well as causing changes in the sequence of mature miRNAs, RNA editing of precursor forms can also influence hairpin processing and miRNA loading[86,88]. Thus A-to-I editing can have a role in miRNA diversification, but its extent appears to be limited to a few cases (reviewed in REF. 89).

## Evolution of miRNA expression patterns

Regulation of gene expression by miRNAs requires direct interaction between the mature miRNA and its target mRNAs, implying that, at least under particular circumstances, the miRNA and its target are expressed in the same cell. Changes in the expression pattern of an miRNA can expose novel sets of messages to its regulatory influence, leading to formation of novel regulatory circuits. Comparisons of miRNA expression patterns between representatives of protostome and deuterostome branches of bilateria, as well as in the bilaterian outgroup species, revealed that expression patterns of conserved miRNAs remain conserved overall — even over large evolutionary distances — when comparing

Drift
Random fluctuations in allele frequencies as genes are transmitted from one generation to the next.


Figure 4 | **Principles of miRNA targeting.** Canonical miRNA target sites show perfect matching between both the mRNA and nucleotides 2–7 of the mature miRNA and either nucleotide A at position 1 in the target or an additional match at position 8 (REF. 73). Mutations in target genes (highlighted in red) can easily lead to the creation of novel miRNA target sites or to the disruption of existing ones.

orthologous tissues and organ systems[14]. This comparison allowed reconstruction of the minimal set of cell types and tissues that existed in the protostome–deuterostome ancestor[14] (reviewed in REF. 90). Similarly, fundamental conservation of miRNA expression patters has been shown between jawless and jawed vertebrates[19]. At the same time, comparison of miRNA expression patterns in closely related species, such as between zebrafish and medaka, revealed variation in the timing and location of expression of many orthologous miRNAs[91]. Notably, the most prominent differences were observed for miRNAs expressed in the head and sensory epithelium, which correspond to the most physiologically different systems between zebrafish and medaka — the structure of the head and jaw and the lateral line[91].

As well as changes in the expression patterns of orthologous miRNAs between species, divergence of expression patters between duplicated miRNAs is a crucial mechanism for miRNA subfunctionalization and neofunctionalization[60]. Multiple studies in both invertebrates and vertebrates demonstrated that, in many miRNA families, individual members differ in their spatio-temporal expression patterns[31,64,91,92]. Changes in miRNA expression patterns can thus be an important factor in the evolution of novel phenotypes.

## Evolution of miRNA targets

Evolution of miRNAs is intimately intertwined with the evolution of miRNA targets. Advances in understanding the mechanisms of regulation by miRNAs have shed light on the dynamics of miRNA–target coevolution.

***Principles of miRNA targeting.*** The identification of biologically relevant targets is the major challenge in dissecting regulatory pathways that involve miRNAs. Tremendous methodological progress in this direction has been made during the last decade (reviewed in REFS 73,93), and general rules of miRNA target recognition are now well understood (FIG. 4). As described above, the seed region that comprises nucleotides 2–7 of mature miRNAs is the most important determinant of miRNA targeting[94]. A requirement for perfect Watson–Crick pairing between this region and target mRNAs improves miRNA target prediction reliability, particularly when conservation of the target site is also required[94–96]. Further refinements to miRNA target predictions included requirements for adenosine at position 1 or for an extra matching nucleotide at position 8 of the mature miRNA[97,98]. However, although seed matching is a good indicator of miRNA targeting, it is not an absolute requirement, and alternative target pairing configurations have been recognized, including 3′ compensatory sites[95,99] and centred pairing[100]. However, such targets are relatively rare compared to seed targets[73].

A combination of experimental and computational approaches has revealed that 3′UTR regions are the preferred location of miRNA target sites[73]. However, there is growing evidence that a significant number of biologically relevant targets can be located in the 5′UTRs and coding region of genes[101–112]. Although the regulation effect of such sites on their targets is generally small[73], genes with multiple target sites in their open reading frames (ORFs), which are derived from repeat-rich regions, are particularly amenable to such targeting[111].

As well as the target sequence that pairs with the miRNA, the local context of the target site can influence the efficiency of miRNA regulation[73,99], as indicated by the fact that the same target site does not always mediate equally efficient regulation in different UTRs[95,113,114]. In particular, the location of the target site relative to the ends of the UTR and the local content of A and U appear to be important determinants of targeting efficiency[73]. Importantly, different target sites for different miRNAs within the same message act independently of each other, providing an additive effect of simultaneous repression by several miRNAs[99,115,116]. However, for target sites that are located 8–40 nucleotides (nt) from each other, repression effects that are greater than the sum of the effects from two sites have been observed, suggesting the cooperative action of miRNAs[99,117]. Both additive and cooperative modes of miRNA actions provide a basis for the development of sophisticated combinatorial regulatory circuits.

***Conserved and non-conserved targeting.*** Although the conservation of an miRNA target site between species provides strong evidence for miRNA regulation, messages with non-conserved seed targets can also be regulated by miRNAs[113]. In fact, there are ten times more predicted non-conserved seed targets than conserved ones[73]. Analyses of expression patterns of miRNAs and their predicted target genes have revealed two distinct trends in the expression of conserved and non-conserved targets relative to their cognate miRNAs[113,118,119]. mRNAs with conserved miRNA target sites tend to be expressed in the same tissue as the cognate miRNA but are separated in time and have initially high levels of target mRNA that decrease with accumulation of the miRNA regulator[113]. As a result, conserved targets are co-expressed with their cognate miRNAs, but at much lower levels compared to their expression levels in tissues that do not express the miRNA[113,118,119]. In addition, tissues with high levels of miRNA expression and high levels of conserved target mRNA expression tend to be located adjacent to each other[118]. These relationships between the expression patterns of miRNAs and their conserved targets emphasize the role of miRNAs in development and maintenance of tissue identity[73].

By contrast, analyses of expression patterns of mRNAs with non-conserved miRNA target sites revealed that they are often expressed in tissues in which the cognate miRNA is not expressed, and that mRNAs that are highly co-expressed with the miRNA have significantly fewer predicted non-conserved target sites[113,118]. Thus, there are pools of genes called 'anti-targets' (REF. 120), which specifically avoid targeting by particular miRNAs. This process of selective targeting avoidance is estimated to be as widespread as target conservation and is thus a major driving force in UTR evolution[113,118].

***miRNA targeting and 3′UTR length.*** Disadvantageous miRNA target sites that have arisen by chance are removed from the population by selective target avoidance. However, if such sites appear too frequently, this could theoretically have a detrimental effect on fitness, as many individuals would carry these disadvantageous sites[27]. One solution to this problem is to shorten the length of 3′UTRs, thus decreasing the sequence space in which an miRNA target site can evolve. This is indeed the case for broadly expressed housekeeping genes; for example, ribosomal protein-coding genes are exposed to a large number of different miRNAs and tend to have much shorter 3′UTRs compared to neuronal genes[118]. At the same time, genes with complex and tissue-specific expression patterns — for example, neuronal genes and transcription factors — tend to have longer 3′UTRs and a higher density of predicted miRNA target sites[118].

3′UTRs are subject to an alternative cleavage and polyadenylation process, which has been associated with the proliferation and differentiation states of the cells[121,122]. Notably, 3′UTR shortening through alternative polyadenylation can lead to the exclusion of miRNA target sites and has been linked to oncogenic transformation[123]. By contrast, lengthening of 3′UTRs was observed in mouse development and was attributed to the use of alternative polyadenylation sites owing to weakening of mRNA polyadenylation activity. This weakening of activity occurs when proximal polyadenylation signals are recognized less effectively, which is presumably due to lowered expression of key polyadenylation machinery genes[124]. Thus, 3′UTR length can be dynamically affected by cellular physiology and is likely to be an important factor in miRNA–target co-evolution.

***Target polymorphism and miRNA regulation.*** Genetic variation in target genes can influence their regulation by miRNAs by creating novel miRNA target sites or destroying existing ones. A prominent example of the phenotypic consequences of target site polymorphism is the muscular hypertrophy that is seen in Texel sheep owing to creation of a target site for miR-1 and miR-126 by a G-to-A transition in the 3′UTR of the myostatin gene[125]. A systematic study of the effects of SNPs on miRNA regulation demonstrated that, for a substantial fraction of polymorphic miRNA target sites, the extent of miRNA-mediated repression is affected by the polymorphism, providing evidence that many genes can be differentially expressed owing to variations in miRNA target sites[126]. Although there is strong negative selection against changes in miRNA target sites[127,128], there are many SNPs in predicted human miRNA targets[125,127,128], some of which have potentially causal roles in human disease. Indeed, there is growing evidence for roles of SNPs in miRNA target sites in cancer[129–132].

Interestingly, an analysis of polymorphisms in cichlid fish, which represent a remarkable model for understanding genetic mechanisms of speciation processes (reviewed in REF. 133), revealed that predicted miRNA target sites have higher than expected densities of single-nucleotide changes between species[134]. This finding suggests that there is positive selection for diversification of miRNA targets. Thus, changes in miRNA target sequences appear to be an important evolutionary mechanism of phenotypic diversification and speciation[134].

## miRNA networks and organismal complexity

Organism development, homeostasis and response to external factors rely on the coordinated expression of genes. This spatio-temporal coordination is implemented through gene-regulatory networks, and inclusion of miRNAs in such networks appears to increase the precision of network functionalities (reviewed in REF. 135). This extra precision is needed because gene expression processes are intrinsically noisy (reviewed in REF. 136), and gene networks composed of only transcription factors and their targets can result in large phenotypic variation. Studies of the many experimentally established miRNA targets have revealed several scenarios in which miRNAs can contribute to gene regulation at the network level, and they include positive and negative feedback loops, as well as coherent feedforward loops and incoherent feedforward loops. The details of the organization and functions of miRNA networks have been extensively reviewed elsewhere [73,120,135,137], so here I briefly outline the current views on the role that miRNA networks have in the evolution of complex systems.

Although there are examples of miRNAs that act as genetic switches by fully downregulating their targets (*lin-4* and *let-7* miRNAs targeting heterochronic genes in *C. elegans*[1,2,9,138]), the accepted view is that most miRNAs only partially downregulate their targets[139–142] and thus act as micromanagers of gene expression[120]. Depending on the configuration of the miRNA circuit, different modes of micromanagement are recognized[16,23,143,144]. In the expression-tuning (threshold-setting) mode, miRNAs act to set the mean expression level of a gene[23,144], whereas in the expression-buffering mode, miRNAs act to reduce the variance in the expression level of the target[23,144]. Regardless of the particular structure of an miRNA network (for example, involving coherent versus incoherent feedforward loops), the end result is increased network robustness, which ensures an invariant phenotype in the face of perturbations[16,23,144]. For example, miR-9a in *D. melanogaster* sets a threshold level for the expression of the gene *senseless*, which is required for the commitment of cells in a proneuronal cluster to become sense organ precursor cells[145]. Although *mir-9a* mutant flies are viable and fertile, they sporadically have extra sense organs owing to uncontrolled fluctuations in the expression of *senseless*[145]. miRNAs also have roles in stabilizing gene expression against the influence of external factors. This function is demonstrated by miR-7, which is involved in several networks that determine the fates of sensory organs in *D. melanogaster* and is required to maintain normal determination of these fates under fluctuating temperature conditions[146]. In both the miR-9a and miR-7 examples, the loss of miRNA function leads to an increase in the variability of the phenotype. The reduction in the variability of a trait is called canalization (reviewed in REF. 147), and miRNAs have been recognized as prominent contributors to this process[16,23,144]. Importantly, although canalization restricts the variability of a trait, by doing so, it in fact contributes to the evolvability of the trait[16]. This is because the heritability of a trait is important for its evolution by natural selection. If heritability or phenotypic resemblance between parents and offspring for a given trait is low (for example, owing to imprecision in underlying gene networks), the beneficial traits are not efficiently acted on by selective pressure. Conversely, if the heritability of a trait is high (for example, owing to canalization by an miRNA network), it is more responsive to natural selection[16]. Based on these considerations, it was proposed that by conferring robustness to gene networks, miRNAs increase the heritability of important phenotypes and thus have a role in the evolution of lineages[16]. This canalizing function of miRNAs can explain why a loss of an miRNA often does not result in a visible phenotype[148,149] and why studies on the population level and in sensitized genetic backgrounds are required to reveal the biological roles of the miRNA[150] (reviewed in REF. 151).

The lack of correlation between organismal complexity and genome size or between organismal complexity and the number of protein-coding genes are known as the C-value and G-value paradoxes, respectively (reviewed in REF. 152). As organismal complexity is built upon increasing the number of traits and the continuous addition of novel miRNAs to gene networks is thought to have allowed canalization of an increasing number of traits, miRNAs appear to have been major players in the evolution of organismal complexity[13–26] and thus can be part of the solution of the C-value and G-value paradoxes.

## Concluding remarks

The introduction of miRNAs as post-transcriptional regulators of gene expression was a major innovation at the dawn of multicellular life. Because the requirements for a functional miRNA gene are less demanding than for a protein-coding gene, miRNAs can easily evolve from various sources of unstructured transcripts. As interaction with target mRNAs requires only a short sequence, miRNA target sites can easily be acquired and lost in the transcriptome. miRNAs themselves are easily diversified through changes in miRNA precursor sequences. Because the impact on overall gene expression from individual miRNA targeting events is modest, miRNAs can easily be incorporated in existing gene-regulatory networks. This flexibility in miRNA-regulatory systems provided widespread adoption of miRNAs through evolution and hundreds of miRNA genes exist in extant animals.

Integration of miRNA regulation provides robustness to gene-regulatory networks, and development of this robustness facilitates canalization of traits and evolution of ever-increasing organismal complexity. However, the causality between emergence of miRNAs, rewiring of transcriptional programmes because of changes in *cis*-regulatory regions and evolution of organismal complexity remains unclear, and detailed dissection of gene-regulatory networks on all levels will be required in order to address this question. Some miRNA genes are ancient and present in all bilaterians, but only a small number of deeply conserved miRNA regulatory circuits have been uncovered[153], suggesting rapid divergence in miRNA–target relations through evolution.

Coherent feedforward loop
A gene network motif in which a regulator gene controls a target gene directly, as well as indirectly, through another regulator, and both regulation paths act in the same direction on the target.

Incoherent feedforward loop
A gene network motif where a regulator gene controls the target gene directly as well as indirectly through another regulator, and the two regulation paths act in opposite directions on the target.

The challenge is to identify those specific miRNA–target combinations that were crucial for evolution of particular phylogenetic clades. Another challenge is to assess the impact of non-conserved, lineage-specific and lowly expressed miRNAs — are most of them transient miRNAs without biological roles, or have some of them acquired lineage-specific functions? It is this rewiring and build-up of miRNA circuitry that will be the subject of extensive research in the quest to comprehend molecular underpinnings of today's diversity of life.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### Competing interests statement
The author declares competing financial interests: see Web version for details.

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