# Multiple products from microRNA transcripts

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## Abstract

**A single transcript sometimes codes for more than one product. In bacteria, and in a few exceptional animal lineages, many genes are organized into operons: clusters of open reading frames that are transcribed together in a single polycistronic transcript. However, polycistronic transcripts are rare in eukaryotes. One notable exception is that of miRNAs (microRNAs), small RNAs that regulate gene expression at the post-transcriptional level. The primary transcripts of miRNAs commonly produce more than one functional product, by at least three different mechanisms. miRNAs are often produced from polycistronic transcripts together with other miRNA precursors. Also, miRNAs frequently derive from protein-coding gene introns. Finally, each miRNA precursor can produce two mature miRNA products. We argue, in the present review, that miRNAs are frequently hosted in transcripts coding for multiple products because new miRNA precursor sequences that arise by chance in transcribed regions are more likely to become functional miRNAs during evolution.**

## Background

It has long been known that a single gene may produce multiple functional products. For example, alternative splicing generates different mature protein-coding mRNAs from the same pre-mRNA (precursor mRNA) (reviewed in [1]). Some transcripts, called polycistrons, have multiple ORFs (open reading frames) that encode multiple proteins. Genes producing polycistronic transcripts are generally known as operons, and they are widely represented in bacteria [2] and in a few animal lineages [3]. The potential to generate multiple functional products from a single transcript also applies to non-protein-coding RNAs. For example, snoRNAs (small nucleolar RNAs), a class of RNA sequences that guide modifications of other RNAs are often encoded within introns of protein-coding genes in animals [4].

miRNAs (microRNAs) are small (~22 nt) RNA molecules found in the genomes of animals and plants. miRNAs regulate protein expression at the post-transcriptional level, and are produced from long mRNA-like transcripts [5,6], and sometimes from the introns of protein-coding mRNA transcripts. Mature miRNAs are generated from long primary transcripts by a multi-step biogenesis pathway, with a number of variants (Figure 1). We discuss, in turn, several mechanisms by which a miRNA transcript can produce multiple products, and the evolutionary and functional consequences of these linked products.

**Key words:** arm switching, genomics, intronic microRNA (intronic miRNA), linkage, mirtron, polycistronic transcript.

**Abbreviations used:** DGCR8, DiGeorge syndrome critical region 8; miRNA, microRNA; ORF, open reading frame.

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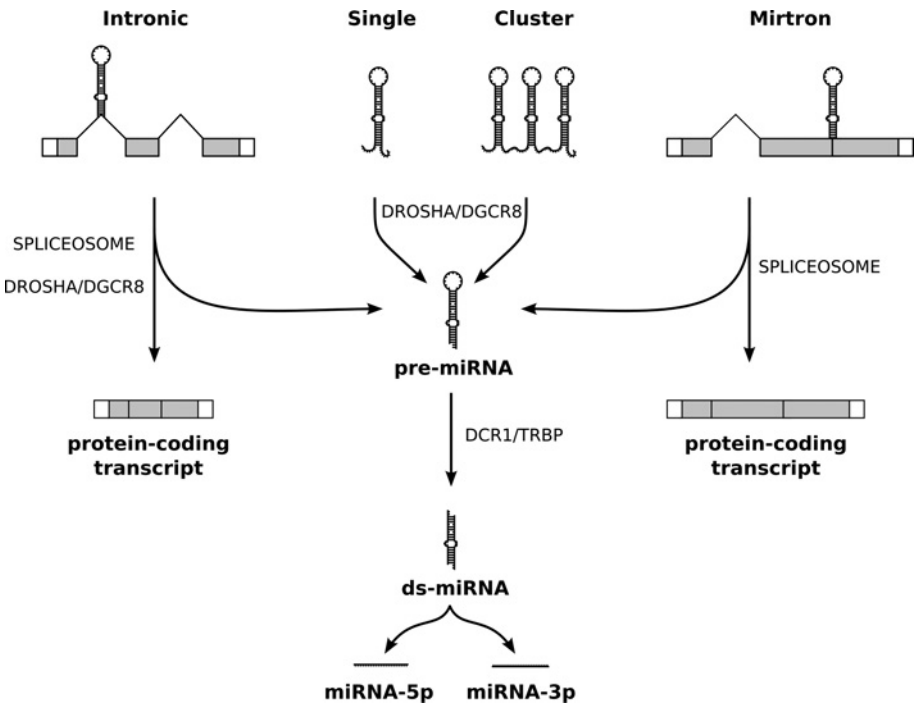
## miRNA products from the same hairpin precursor

Mature miRNAs are produced from RNA precursor hairpin structures by the action of the endonucleases DROSHA [with its partner DGCR8 (DiGeorge syndrome critical region 8)/PASHA] and DICER (Figure 1). The processing of these hairpins generates a duplex of two small RNA products [5]. It has long been believed that one of the products (the mature miRNA) is functional, whereas the other (the so-called star sequence or miRNA*) is a non-functional by-product of the biogenesis process. This is supported by the observation that one of the strands of the duplex accumulates at a higher concentration in cells. This is particularly obvious in small RNA deep sequencing experiments, where we usually see many more reads mapping to one arm of the hairpin than to the other (Figure 2). However, several lines of evidence suggest that both mature products from a hairpin have the potential to function as miRNAs. For example, miRNA* molecules have shown targeting activity *in vivo* and *in vitro* [7–9]. Work in our laboratory and others has also shown that the arm of the hairpin precursor that produces the dominant mature miRNA can change across different tissues [10,11] and also throughout evolution in a process known as arm switching [12–15]. In particular, members of the *miR-10* family of miRNAs have undergone multiple arm-switches during evolution of the family [15]. This strongly suggests that both *miR-10* mature products have the ability to regulate targets. A recent computational analysis showed that miRNAs from the same precursor not only target different genes, but also generally target genes involved in different cellular processes [16]. This suggests that arm switching is likely to be associated with a change in function. These data strongly suggest that, as a general rule, a single miRNA precursor encodes two mature miRNAs, with distinct targeting properties.

miRNAs are produced from RNA transcripts by the action of multiple RNases. The position of cleavage by

**Figure 1** | **Multiple products from a miRNA-encoding transcript**

An intronic miRNA (left-hand panel) is processed from a protein-coding transcript by the miRNA-processing machinery (DROSHA/DGCR8 in animals). Precursor mirtrons (right-hand panel) are produced by the spliceosome. Single and clustered miRNA hairpins (middle) are processed by the Microprocessor complex comprising DROSHA and DGCR8. Precursor miRNAs (pre-miRNAs) are further cleaved by the DICER RNase (DCR-1–TRBP complex in animals) to produce a double-stranded RNA (dsRNA), which subsequently yields two mature miRNA products: *miRNA-5p* and *miRNA-3p*; one from each arm of the precursor.



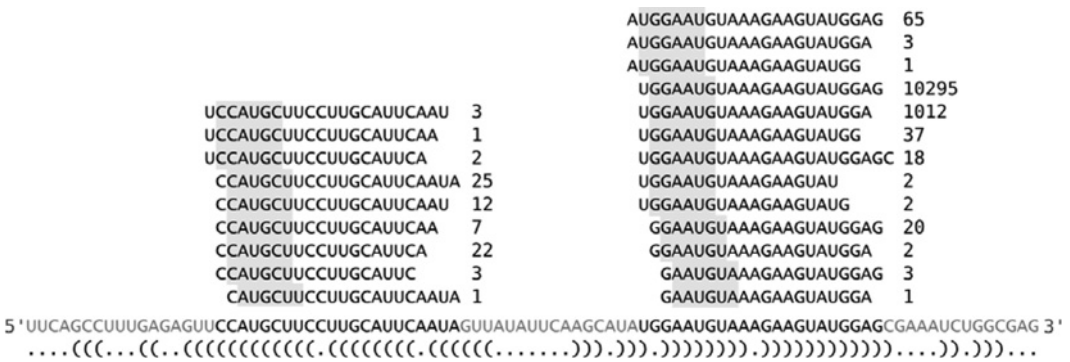

these enzymes appears to be subject to some variation, producing multiple offset variants of the mature miRNAs. These miRNA variants, produced from the same arm of the precursor, are collectively called isomiRs (reviewed in [17]), and they are frequently detected in high-throughput sequencing of small RNAs (Figure 2). Deep sequencing data suggests that the 3′ end of the mature miRNA is subject to more variation than the 5′ end. The 3′ exonuclease nibbler has been associated with the heterogeneity at the 3′ end [18,19]. miRNA target specificity is thought to be determined, in large part, by the six nucleotides in positions 2–7 of the mature sequence, known as the miRNA seed sequence [20]. Target specificity may therefore be determined by the exact cleavage point during miRNA biogenesis. Consequently, different isomiRs have, in principle, different targeting properties. This phenomenon is known as seed-shifting [21]. The level of complexity of isomiR sequences is often ignored, as it is difficult to distinguish between real isomiRs and unknown biases and artefacts from deep-sequencing experiments and analysis. However, isomiRs are likely to be functionally relevant [17,22]. As high-throughput technologies and analysis methods become more robust, the contribution of isomiRs to differential gene regulation will be better understood.

## Multiple miRNAs precursors from a single transcript

As soon as miRNAs were characterized as a widespread class of molecules, it became apparent that their loci were often close to each other in genome sequences [23–25]. According to miRBase [26], approximately one-third of miRNAs are closer than 10 kb to another miRNA in animal genomes. miRNAs that are clustered are usually assumed to be co-expressed from a single transcript [27–33]. For instance, Ryazansky et al. [33] have shown that, in *Drosophila melanogaster*, highly co-expressed miRNAs are most likely to be separated by less than 1 kb. Polycistronic miRNA transcripts have also been cloned and characterized [28,34,35]. For example, the ancient miRNAs *miR-100*, *let-7* and *miR-125* (a paralogue of *miR-100*) are clustered in the genomes of deuterostomes and most protostomes [15,36,37]. There is only one copy of the cluster in most invertebrates studied and it has an important role in regulating larva-to-adult transition and neural development [38]. Interestingly, the individual functions of each miRNA in the cluster seem to be different; *let-7* is suggested to be the functionally most important miRNA in the cluster [38]. In mammals, the *let-7* cluster has undergone several duplications and rearrangements [36,37], and the functional profiles of the miRNAs may have

**Figure 2 | miRNAs and deep sequencing**

Reads from high-throughput sequencing of small RNAs from *Drosophila* adult males (NCBI/GEO accession number GSM286602) mapped to the *dme-miR-1* precursor. Both arms of the precursor produce multiple isomiRs, the numbers on the right-hand side represent the number of reads associated with each sequence. Shaded areas in reads indicate the predicted miRNA 'seed' region (see miRNA products from the same hairpin precursor section). The secondary structure of the miRNA precursor is shown in dot-bracket notation (bottom).




diversified. Another example of a conserved cluster is the invertebrate *miR-71/miR-2* cluster. It is formed by *miR-71* and several (usually five) tandem copies of *miR-2* family members [39]. In the *Drosophila* lineage, this cluster has been duplicated and rearranged [39]. The deep conservation of miRNA clusters shows that they tend to be evolutionarily stable, although in some lineages they may be significantly re-arranged, often after whole duplication of the original cluster.

It is striking that polycistronic miRNA transcripts are common, whereas other types of polycistronic transcripts are phylogenetically restricted. One possible explanation is that the actions of functionally related miRNAs may be more efficiently regulated if they are under the control of a single regulatory region, and this co-regulation of related products may be favoured by natural selection [40]. On the other hand, many clusters may be the product of tandem duplications, which generate clusters of evolutionarily related miRNAs [36]. The latter mechanism appears to explain the origin of most plant miRNA clusters [30], but cannot account for most animal clusters, which are mainly composed of members of different families [6]. We have recently described the evolutionary origin of *D. melanogaster* miRNA clusters. We find that polycistronic miRNAs are mostly generated by the emergence of new miRNAs within existing miRNA transcripts [41]. The emergence of miRNAs in clusters appears to occur at a high rate, which may explain why clusters of miRNAs are much more common than those of other gene products.

## miRNAs hosted in other transcripts

miRNA loci often overlap with known protein-coding genes; the overwhelming majority of these sequences are found in the sense orientation within introns [42]. In addition, a few miRNAs overlap with annotated protein-coding exons or untranslated regions (Table 1). In animals, 30–60 % of miRNA loci reside within a protein-coding gene (Table 1). Intronic miRNA–host gene associations are often stable over broad evolutionary times [43,44]. In addition, intronic miRNAs may be retained even after the host gene is inactivated or lost, as illustrated by the case of *mir-190* in humans [45], suggesting that the number of miRNAs that originally emerged in introns may be higher. Introns are thus an important source of novel miRNAs [45,46].

There are two classes of intronic miRNAs, defined by their mechanism of biogenesis (Figure 1). Canonical intronic miRNAs are localized in large introns and they are cleaved by the DROSHA–DGCR8 RNase during transcription and before splicing [42,47]. A second type of intronic miRNAs, known as 'mirtrons', derive from small introns with hairpin structure and do not require DROSHA–DGCR8 processing; instead the ends of the precursor miRNA are defined by splicing of the host gene transcript [48,49]. The expression of intronic miRNAs and their host genes is, in general, correlated [29,50], although a few counter-examples have been described. For instance, the intronic miRNA *miR-7* and its host gene *bancal* have different expression patterns in *Drosophila* [51]. miRNAs can also derive from long non-coding RNA transcripts. For example, *miR-675* is produced from the H19 transcript, one of the most abundant long-non-coding RNA transcripts in the mammalian placenta [52]. It is important to note that in many such cases, the annotated long non-coding transcript is simply the miRNA primary transcript, and may have no other function. For example, the miRNA *iab-4* was named after it was characterized as the only product of the previously annotated long non-protein-coding transcript *iab-4* [34,53].

## Evolutionary origins of miRNAs

In the present review, we have described three mechanisms by which miRNA transcripts can produce multiple products

**Table 1** | **miRNAs within protein-coding gene loci in different genomes**

*C. elegans, Caenorhabditis elegans; D. rerio, Danio rerio; H. sapiens; Homo sapiens; M. musculus, Mus musculus.*

| | | | Sense† | | | Antisense† | | |
|---|---|---|---|---|---|---|---|---|
| **Species** | **Total*** | **Overlapping** | **Intron** | **Exon** | **UTR‡** | **Intron** | **Exon** | **UTR‡** |
| *D. melanogaster* | 238 | 137 | 98 | 9 | 13 | 19 | 1 | 3 |
| *C. elegans* | 223 | 100 | 64 | 2 | 2 | 32 | 0 | 2 |
| *D. rerio* | 344 | 84 | 66 | 0 | 2 | 12 | 2 | 0 |
| *H. sapiens* | 1600 | 936 | 675 | 37 | 70 | 161 | 17 | 18 |
| *M. musculus* | 855 | 475 | 311 | 23 | 33 | 70 | 12 | 13 |

*miRNAs were retrieved from miRBase version 19 and transcript annotations from Biomart/ENSEMBL. A single miRNA can overlap different transcripts in different contexts.

†Sense and antisense orientation with respect to the host gene transcript.

‡Overlapping either 3′ or 5′ untranslated regions (UTRs).

both RNA and protein. First, a single hairpin precursor can produce a pair of mature sequences. Secondly, a single polycistronic transcript can be processed to produce multiple hairpin precursors. Thirdly, miRNA hairpins can be processed from protein-coding mRNAs, usually from introns. In *D. melanogaster*, for example, close to 80 % of all known miRNA precursors are produced from polycistronic transcripts, either encoding another miRNA precursor or an ORF. In some cases, a single transcript can even produce a protein and several clustered miRNAs, such as the *Gmap* locus in *Drosophila* whose transcripts code for the GMAP (Golgi microtubule-associated protein) protein and the clustered miRNAs *miR-283*, *miR-304* and *miR-12* [26]. An obvious question arises: why are miRNAs often produced from polycistronic transcripts?

In the case of protein-coding genes, novelty arises primarily by duplication and divergence [54,55]. The generation of ORFs *de novo* is a slow process, mostly leading to non-functional products. However, in some cases, a functionally relevant product may emerge and, eventually, be transcribed by the acquisition of a transcription initiation site. The origin of novel protein coding genes has been reviewed recently by Guerzoni and McLysaght [56].

However, the evolutionary emergence of a miRNA *de novo* is much more likely. A novel miRNA must be transcribed and the transcribed RNA must have a hairpin structure recognizable by the small RNA-processing machinery. Unlike ORFs, hairpin structures are easily formed out of random sequences. For instance, it is predicted that the relatively small *Drosophila* genome has approximately 760 000 sequences that could form hairpin structures with miRNA-like features [57]. Potential hairpins can therefore arise randomly at a high rate in already transcribed regions, so these regions become hotspots for miRNA emergence [45,46]. Indeed, we have shown recently that the emergence of miRNA clusters probably follows a non-adaptive dynamics driven by the emergence of new miRNAs in existing miRNA precursors [41]. Formally, miRNA emergence can occur by hairpin formation in pre-transcribed regions, or novel transcription of pre-existing random hairpin sequences. The contexts in which miRNAs are found all support a model whereby miRNA hairpins arise by chance in pre-existing transcripts.

## Acknowledgements

We thank Ana Kozomara (University of Manchester) for helping with miRNA genome annotation data from miRBase.

## Funding

This work was supported by the Wellcome Trust [grant number 097820/Z/11/Z] and the Biotechnology and Biological Sciences Research Council [grant number BB/G011346/1]. M.N. is funded by a Wellcome Trust Ph.D. studentship.

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Received 20 March 2012
doi:10.1042/BST20130035