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And Now Introducing Mammalian Mirtrons

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Mirtrons are short hairpin introns recently found in flies and nematodes that provide an alternative source for animal microRNA biogenesis and use the splicing machinery to bypass Drosha cleavage in initial maturation. The presence of mirtrons outside of invertebrates was not previously known. In the October 26 issue of *Molecular Cell*, Berezikov et al. expose a number of short mammalian introns as mirtrons.

microRNAs (miRNAs) are noncoding small RNAs of ~22 nucleotides (nts) that negatively regulate gene expression by binding to the 3′ untranslated region (3′UTR) of messenger RNA transcripts, triggering translational repression or cleavage of the target (reviewed by Bartel, 2004). miRNAs are generally transcribed by RNA polymerase II as primary miRNAs (pri-miRNAs) that range from hundreds to thousands of nts in length and contain one or more extended hairpin structures (reviewed by Du and Zamore, 2005). In animals, the nuclear RNase III enzyme Drosha, collaborating with DGCR8/Pasha/PASH-1, cleaves both strands near the base of the primary stem-loop and yields the precursor miRNA (pre-miRNA), an ~65-nt stem-loop that harbors the miRNA in the 5′ or 3′ half of the stem (Lee et al., 2003). The cleavage by Drosha defines one end of the mature miRNA and generates a 5′ phosphate and an ~2-nt 3′ overhang. After being exported to the cytoplasm by Exportin-5/RAN, pre-miRNAs are further cleaved by the RNase III Dicer to define the other end of the mature miRNA and produce the double-stranded miRNA/miRNA* duplexes (Du and Zamore, 2005). One strand of the miRNA/miRNA* duplex is then preferentially incorporated into the RNA-induced silencing complex (RISC) and can function to negatively regulate target genes (Du and Zamore, 2005).

In animals, most miRNAs either originate from their own transcription units, or derive from the exons or introns of other genes (Du and Zamore, 2005). All of them seem to require both Drosha and Dicer for the two sequential cleavage events in maturation. Recently, the Bartel and Lai laboratories found that short hairpin introns in flies and nematodes can be alternative sources of miRNAs (Okamura et al., 2007; Ruby et al., 2007). By high-throughput pyrosequencing, Bartel and colleagues identified 14 introns in *D. melanogaster* with a sequence and predicted stem-loop structure of a pre-miRNA, but without the lower stem structure and flanking single-stranded segments of the pri-mRNA (Figure 1), which mediate the recognition and cleavage by the DGCR8/Drosha complex (Han et al., 2006). In these cases, the 5′ and 3′ portions of the intron were inversely complementary to each other and the base-pairing capacity abruptly ended at the borders of the intron. The small RNAs obtained by pyrosequencing originated from the outer edges of the intron, and the most abundant ones, annotated as the mature miRNAs, derived from the 3′ arm of the predicted stem-loop structure (Ruby et al., 2007). Because of their pre-miRNA and intronic characteristics, the Bartel laboratory named these introns "mirtrons." They also found three *C. elegans* mirtrons and reclassified a previously annotated miRNA gene, *mir-62*, as a mirtron due to its intron-edge location and lack of pri-miRNA structures (Ruby et al., 2007). Lai and colleagues detected expression of *D. melanogaster* mirtrons in various tissues and developmental stages (Okamura et al., 2007). Both groups demonstrated that mirtrons are processed initially by the splicing machinery, enter the miRNA-processing pathway without Drosha-mediated cleavage, and produce functional small regulatory RNAs (Okamura et al., 2007; Ruby et al., 2007).

Mirtrons have only been found in flies and nematodes to date, and it was suggested that mirtrons might be more common in these species because they have a higher proportion of short introns with lengths typical of pre-miRNA hairpins than mammals do (Ruby et al., 2007). Now, in the October 26 issue of *Molecular Cell*, Berezikov et al. (2007) report the discovery of mammalian mirtrons. They found three mirtrons, sblock2, sblock3, and sblock4, that generate orthologous small RNAs in human, macaque, chimpanzee, rat, and mouse by analyzing 25,935 introns, 50–200 nt in length, from the UCSC Genome Browser Database (Kuhn et al., 2007). Small RNAs from sblock4 were recently cloned by Tuschl and colleagues independently and annotated as the canonical miRNA *mir-877* (Landgraf et al., 2007).

To identify additional mammalian mirtrons, Berezikov et al. (2007) sequenced a set of small RNA libraries from human and rhesus macaque brains. Brains were chosen since high-throughput pyrosequencing of *D. melanogaster* head samples has given the highest diversity of mirtrons and canonical miRNAs, possibly to satisfy the high complexity of neuronal translational regulation. They identified 16 primate-specific mirtrons, and classified 46 additional hairpin introns from human (23 loci), macaque (16 loci), chimpanzee (3 loci), and mouse (4 loci) as mirtron candidates. Several lines of evidence argued that these

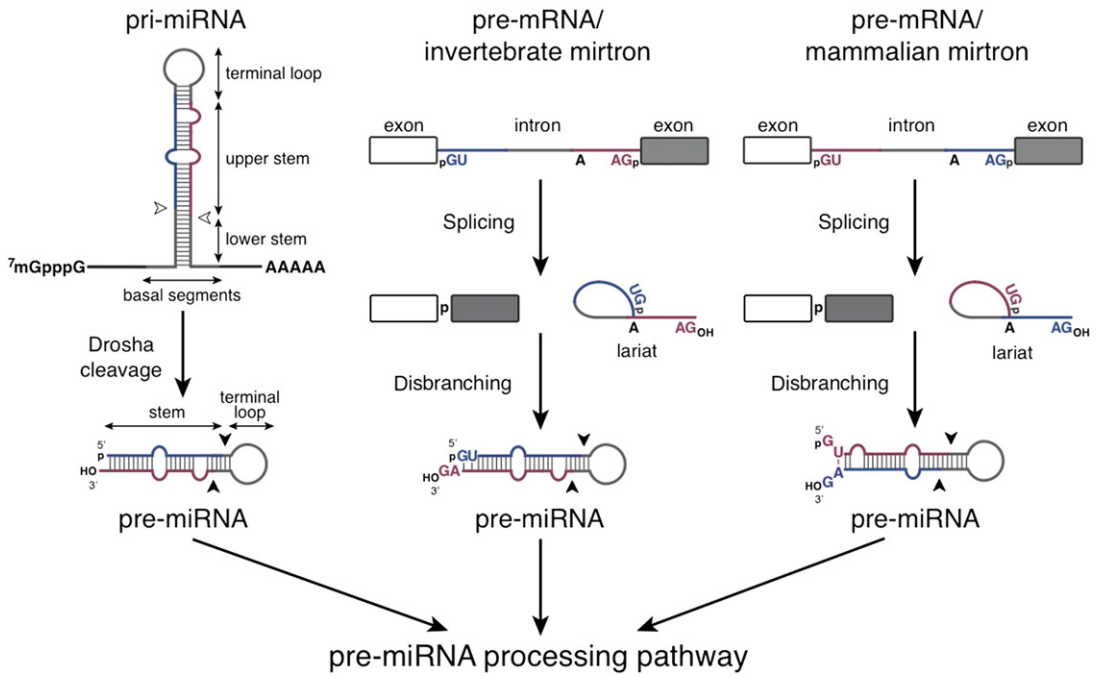

Figure 1. Model for Mirtonic miRNA Biogenesis Pathway

Mirtrons are short introns with hairpin potential that are spliced and disbranched into pre-miRNAs and bypass the Drosha cleavage of the canonical miRNA pathway. Mirtrons lack a lower stem and basal single-stranded segments, which are typical of a pri-miRNA structure and mediate recognition and cleavage by the DGCR8/Drosha complex. Invertebrate mirtrons exhibit a stem structure with an "AG" 3′ overhang, and the 3′ hairpin product (shown in red) is preferentially selected as the functional miRNA and transferred into an RISC. However, mammalian mirtrons predominately exhibit a stem structure with single nt overhangs at both ends, and the 5′ hairpin product (shown in red) is preferentially selected. In the canonical pathway, either 5′ or 3′ hairpin product of the canonical pre-miRNA could be preferentially selected, depending on its specific characteristics. Unfilled and solid arrowheads indicate Drosha and Dicer cleavage sites, respectively.

sequences are not intron degradation products, but rather, mammalian mirtron products that are likely generated via the miRNA-processing pathway, as in flies and nematodes.

There are several differences between mammalian and invertebrate mirtrons (Figure 1). Invertebrate mirtrons predominantly generate small RNAs from their 3′ portion, and these 3′ species preferentially start with a 5′ "U" residue, consistent with the bias for the 5′ nt of canonical miRNAs (Lau et al., 2001). Indeed, the Bartel and Lai groups have shown that several of these species are functional miRNAs (Okamura et al., 2007; Ruby et al., 2007). In contrast, several of the most highly expressed mammalian mirtrons predominantly produce small RNAs from the 5′ portion, which carries a 5′ "G" residue from the conserved splice site. Furthermore, these mirtrons exhibit a stem structure with single nt overhangs at both ends, instead of the typical 2-nt 3′ overhang found in most highly expressed invertebrate mirtrons. To explain this structural constraint, Berezikov et al. (2007) surveyed miRbase and showed that there are many deduced pre-miRNA hairpins lacking perfect 3′ 2-nt overhangs. It is possible that the pre-miRNA processing pathway accepts a range of hairpin structures, or that other factors participate. In addition, unlike in invertebrates, where mirtrons and bulk short introns exhibit similar GC content, in mammals mirtrons exhibit higher GC content than bulk short introns. The GC content of mammalian mirtrons is also higher than those of invertebrate and mammalian canonical miRNAs.

The existence of several well-conserved mirtrons among diverse mammals, as well as in Drosophilids and nematodes (Okamura et al., 2007; Ruby et al., 2007), indicates their relatively ancient incorporation into regulatory pathways, and their retention for beneficial reasons. However, since flies, nematodes, and mammals have completely different sets of mirtrons, it is possible that different animals evolved mirtrons independently. On the other hand, most *D. melanogaster* mirtrons are preserved within species of the melanogaster subgroup (Okamura et al., 2007; Ruby et al., 2007), suggesting that mirtrons are possibly fast-evolving in nature. This idea is supported by the observation that a number of mammalian mirtrons are restricted to the primates, with some presenting conserved hairpin structures restricted to one or few primate subsets. The observation of many newly-evolved mirtrons suggests an easy way for the birth of new regulatory RNAs along with the pre-existing canonical pre-miRNA pathway and highlights their likely contribution to animal evolution.

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A Flip-Flop Switch in Polarity Signaling

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The Rho GTPase Cdc42 is essential for polarized growth of budding yeast. Temporal control of Cdc42 depends partly on the activity of its GTPase-activating proteins (GAPs). In this issue of *Developmental Cell*, Saito et al. report that Cdc42 GAP activity is regulated by the phospholipid composition of the bud-tip membrane, under control of the phospholipid flippases Lem3-Dnf1 and Lem3-Dnf2.

Internal spatial cues generated early in the G1 phase of the cell cycle cause yeast cells to polarize their growth to a unique site on the plasma membrane and initiate bud formation (Lew and Reed, 1995; Chang and Peter, 2003; Park and Bi, 2007). The site of new bud growth is specified by the bud scar, a remnant of the previous cell division cycle, and by protein landmarks that recruit the Ras family GTPase Rsr1. Activated Rsr1 forms a complex with the GDP-bound form of the Rho family GTPase Cdc42 and its GTP exchange factor (GEF), Cdc24. When Rsr1 hydrolyzes GTP, it triggers Cdc24 to convert Cdc42(GDP) to a GTP-loaded "active" state. The production of Cdc42(GTP) at the bud site ultimately leads to polarization of the actin and microtubule cytoskeleton and localization of elements of the secretory apparatus to the bud tip. Vesicles deliver new plasma membrane and cell wall components to the bud tip, ensuring polarized "apical growth." As the bud elongates and the cell enters G2, Cdc42 disperses from the bud tip and the pattern of secretion switches so that vesicles are delivered uniformly over the bud membrane instead of only at its tip. This is termed the apical/isotropic switch.

Cdc42 cycles between GDP- and GTP-bound forms, and signals its effectors from its GTP-loaded active state. GTP hydrolysis by Cdc42 is regulated by three dedicated GTPase-activating proteins (GAPs) in *S. cerevisiae*: Bem3, Rga1, and Rga2, and an additional promiscuous GAP, Bem2. While Cdc42's sole GEF, Cdc24, has been extensively analyzed, studies of the GAPs are not as far along. Now, Saito et al. (2007) report in this issue of *Developmental Cell* that the activity of the Rga GAPs, and to a lesser extent that of Bem3, is controlled by the composition of the bud tip membrane. They suggest that in the absence of functional phospholipid flippases that translocate lipids across membranes, phospholipid asymmetry at the bud tip is altered and GAP activity is low. This slows dispersal of Cdc42 from the bud tip and delays the apical/isotropic switch.

The transbilayer distribution of phosphlipids in the plasma membrane of mammalian cells is strongly asymmetric, such that the aminophospholipids phosphatidylserine (PS) and phosphatidylethanolamine (PE), as well as the phosphoinositides (PIs), are largely confined to the cytoplasmic leaflet. Although the phospholipid asymmetry of the yeast plasma membrane is not as well defined, it is clear that it also has little detectable surface PE. An exception occurs during a limited period of the cell cycle. Using Bio-Ro, a PE-binding peptide, Iwamoto et al. (2004) discovered that a hot spot of PE develops at the surface of incipient bud sites and at the tip of small buds, and disappears in G2 as cells depolarize growth within the bud.

How does PE come to be exposed at the cell surface and in such a spatially and temporally restricted fashion? Since lipid mobility in the outer leaflet of the yeast plasma membrane is anomalously low (Greenberg and Axelrod, 1993), PE may be kinetically polarized (Valdez-Taubas and Pelham, 2003) after it is locally delivered by tip-directed vesicular traffic or by translocation from the inner leaflet. Both delivery scenarios require a cell cycle-regulated change in the balance of phospholipid flip-flop controlled by ATP-driven lipid translocators (flippases and floppases) that flip (out→in) or flop (in→out) lipids