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## A 360 degree view of circular RNAs: From biogenesis to functions

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### Abstract

The first circular RNA (circRNA) was identified more than 40 years ago, but it was only recently appreciated that circular RNAs are common outputs of many eukaryotic protein-coding genes. Some circular RNAs accumulate to higher levels than their associated linear mRNAs, especially in the nervous system, and have clear regulatory functions that result in organismal phenotypes. The pre-mRNA splicing machinery generates circular RNAs via backsplicing reactions, which are often facilitated by intronic repeat sequences that base pair to one another and bring the intervening splice sites into close proximity. When spliceosomal components are limiting, circular RNAs can become the preferred gene output, and backsplicing reactions are further controlled by exon skipping events and the combinatorial action of RNA binding proteins. This allows circular RNAs to be expressed in a tissue- and stage-specific manner. Once generated, circular RNAs are highly stable transcripts that often accumulate in the cytoplasm. The functions of most circular RNAs remain unknown, but some can regulate the activities of microRNAs or be translated to produce proteins. Circular RNAs can further interface with the immune system as well as control gene expression events in the nucleus, including alternative splicing decisions. Circular RNAs thus represent a large class of RNA molecules that are tightly regulated, and it is becoming increasingly clear that they likely impact many biological processes.

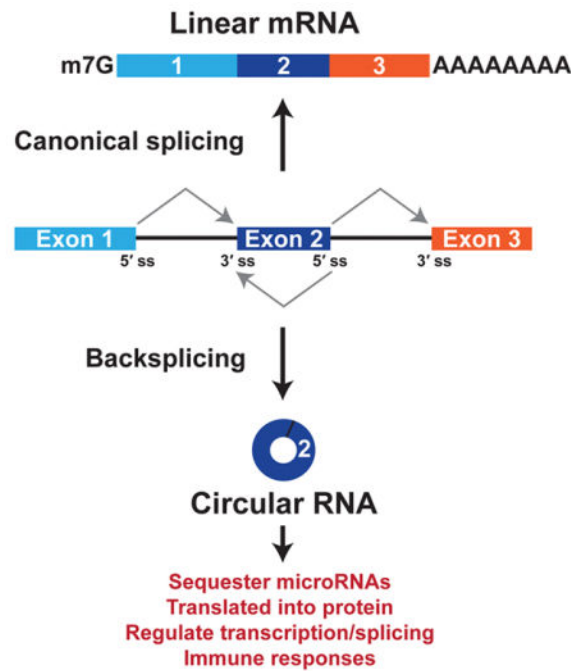
### Graphical Abstract

Besides generating a linear mRNA, many protein-coding genes produce circular RNAs that are tightly regulated and have biological functions.

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## Keywords

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## INTRODUCTION

As most eukaryotic genes are arranged in a split manner, nascent transcripts must be spliced so that each of the noncoding intronic segments are removed and the exonic segments are joined to one another (e.g., exon 1 is joined to exon 2, which is joined to exon 3, etc.). It has long been recognized that this step in gene expression is highly regulated and can be exploited to generate a diverse set of mRNAs from a given gene, with each mature isoform potentially having a unique function, localization pattern, or regulatory role (reviewed in <sup>1, 2, 3</sup>). An extreme case is the *D. melanogaster Dscam* (*Down syndrome cell adhesion molecule*) gene that expresses 38,016 distinct linear mRNAs, and the ability to generate a diverse set of *Dscam* isoforms is critical for proper neural circuit assembly.<sup>4, 5</sup> Broadly speaking, >95% of human genes are alternatively spliced,<sup>6</sup> and these splicing decisions are thought to be regulated in a combinatorial manner by both *cis*-regulatory elements and *trans*-acting factors (reviewed in <sup>7, 8</sup>). Besides generating a variety of linear mRNAs, it is now clear that many eukaryotic genes also surprisingly produce circular RNAs that have covalently linked ends.<sup>9–11</sup>

Circular RNAs are generated when the pre-mRNA splicing machinery “backsplices” to join a splice donor to an upstream splice acceptor (e.g., the end of exon 2 is joined to the beginning of exon 2) (Figure 1). Despite the first circular RNA being identified more than 40

years ago,<sup>12</sup> these transcripts had largely been assumed to be very rare in cells, dismissed as noise, and/or forgotten about. This is, in part, because the prevailing dogma has been that most splicing events occur co-transcriptionally as soon as an intron has been fully transcribed.<sup>13–15</sup> Thus, the intron along with its associated splice acceptor (3' splice site) should be removed before any downstream splice donors (5' splice sites) have been transcribed, making most backsplicing reactions impossible. It is now recognized that some introns are slowly or post-transcriptionally spliced,<sup>16–18</sup> thereby allowing the opportunity for backsplicing reactions to occur. Most circular RNAs are rarely generated and accumulate to low levels,<sup>19</sup> but some are expressed at levels 10-fold higher than their associated linear mRNA.<sup>9, 10</sup> This suggests that the main function of some protein-coding genes may be to generate circular RNAs rather than linear mRNAs or proteins.

Here, I will highlight the tremendous progress that has been made over the last few years to reveal the diversity of circular RNAs present in cells as well as discuss key insights into how these transcripts are made, regulated, and function. High-throughput RNA sequencing (RNA-seq) has identified thousands of circular RNAs across eukaryotes, some of which have clear biological functions that result in organismal phenotypes.<sup>20, 21</sup> Production of circular RNAs is often facilitated by intronic repeat sequences, which base pair to one another to bring the intervening splice sites into close proximity. The efficiency of backsplicing reactions is further regulated by RNA binding proteins, exon skipping events, as well as by the levels of core spliceosomal components, thereby allowing circular RNAs to be expressed in a tissue- and stage-specific manner. Once produced, circular RNAs are naturally resistant to degradation by exonucleases, and thus have long half-lives and some accumulate to high levels. Functions for the vast majority of circular RNAs remain unknown, but some of these transcripts regulate microRNAs, alternative splicing patterns, the immune system, or are bound by ribosomes to produce proteins.

## Early insights into circular RNAs

Sänger and colleagues serendipitously identified the first circular RNA in 1976 as part of an effort to understand how viroids function as plant pathogens.<sup>12</sup> It was previously known that viroids were similar to viruses in that they were infectious and pathogenic, but it was unclear how viroids function as they appeared to be rather small (<400 nt) RNAs that were not protected by protein coats. By characterizing highly purified viroid RNA molecules, Sängers and colleagues demonstrated that viroid RNA was unable to be enzymatically labeled at its 5' or 3' ends nor able to be degraded by snake venom phosphodiesterase.<sup>12</sup> Electron microscopy revealed single-stranded circles of viroid RNA, and subsequent sequencing of the viroid nucleotide sequence unequivocally demonstrated that viroids are indeed true circular RNAs.<sup>22</sup> Hepatitis delta (δ) virus (HDV), a satellite virus of hepatitis B virus, was subsequently shown to be a ~1700 nt circular RNA molecule<sup>23</sup> and, unlike plant viroids, to encode a single open reading frame that is translated to regulate viral replication.<sup>24, 25</sup>

Circular RNAs were further found to be generated from self-splicing introns in unicellular eukaryotes<sup>26</sup> and from ribosomal RNA introns in archaea,<sup>27</sup> but it was not until the 1990s that the first endogenous circular RNAs expressed in higher eukaryotes were identified. In an effort to characterize the *DCC* (*Deleted in Colorectal Carcinoma*) tumor suppressor gene,

Nigro and colleagues performed a number of PCRs to map the exact order of exons in the mature DCC mRNA.<sup>28</sup> Exons were always joined accurately at consensus splice sites, but sometimes in an order different from that present in the nascent transcript (e.g., exon 3 appeared to be upstream of exon 2 in some mature mRNAs). These scrambled transcripts did not appear to be polyadenylated and were highly enriched in the cytoplasm, but were expressed at ~1/1000<sup>th</sup> the level of the normally spliced DCC transcript.<sup>28</sup> It was thus proposed that RNAs containing scrambled exons may simply represent errors in the normal splicing process. By studying the human *ETS-1* gene, Cocquerelle and colleagues similarly identified processed ETS-1 transcripts containing exons in a scrambled order that were expressed at ~1/100<sup>th</sup> the level of the normally spliced transcript.<sup>29</sup> Further characterization of the scrambled ETS-1 transcripts revealed that they were highly stable (half-life >48 h) circular RNAs (with a 3'-5' phosphodiester bond at the junction site) that contained only a subset of the exons that are encoded in the full length *ETS-1* gene.<sup>30</sup> Whereas the DCC and ETS-1 circular RNAs were expressed at very low levels, Capel and colleagues found that the mouse *Sry* gene, which determines sex in mammals, predominately generates a circular RNA in mouse testis.<sup>31</sup> More than 90% of the *Sry* transcripts in the adult testis corresponded to the single-exon *Sry* circular RNA, which accumulated in the cytoplasm, but was not associated with polysomes.

Beyond demonstrating that circular RNAs can be the predominant output of some protein-coding genes, the mouse *Sry* gene provided some of the first mechanistic insights into how specific exons could be selected for circularization. ~50-kb of near perfectly complementary sequences (>99.7%) are present in the introns flanking the *Sry* exon that forms a circular RNA.<sup>31, 32</sup> Capel and colleagues suggested that these flanking sequences may be able to base pair to one another, thereby positioning the splice donor close to its upstream splice acceptor and facilitating backsplicing. Consistent with this model, it was shown using plasmids that *Sry* exon circularization in cells indeed requires the presence of both inverted repeats and that ~400 base pairs were sufficient.<sup>33</sup> Upon examining backsplicing *in vitro* using nuclear extracts, it was likewise shown that intronic inverted repeats promote circular RNA production, especially as exon size increased.<sup>34-36</sup> These *in vitro* experiments further strongly suggested that circular RNAs were generated by the spliceosome, but that backsplicing may be >100-fold less efficient than canonical splicing reactions.

A handful of additional endogenous circular RNAs were identified over the ensuing years.<sup>37-44</sup> For the rat *cytochrome P450 2C24*, human *cytochrome P450 2C18*, and human *dystrophin* genes, alternatively spliced mRNAs lacking the exons present in the mature circular RNA were identified, suggesting that exon skipping and backsplicing could sometimes (but not always) be intimately linked.<sup>37, 38, 40</sup> In addition, the promise of circular RNAs for biomedical applications was recognized (e.g., Ref<sup>45</sup>). However, it was not until the rise of high-throughput RNA-seq methods (**See Sidebar**) and some more serendipity that the widespread nature of circular RNAs was revealed.

## Sidebar

### (Boxed Information)

#### High-throughput identification of circular RNAs

To identify circular RNAs by RNA-seq, it has become standard to deplete ribosomal RNAs (rRNAs) and use random priming. Nevertheless, many circular RNAs are expressed at low levels and rRNA depletion is not sufficient to ensure annotation of all circular RNAs, unless very high depth RNA-seq is used. Therefore, in many protocols, either exonucleases, such as RNase R, or poly(A) selection steps are further employed to deplete linear mRNAs, thereby allowing greater sequencing coverage of circular RNAs.<sup>10, 46–48</sup> Alternatively, commercially available microarrays that include probes to backsplicing junctions are available. On the computational side, multiple pipelines have been developed to identify circular RNAs from RNA-seq datasets, including `find_circ`,<sup>11</sup> MapSplice,<sup>49</sup> CIRCexplorer,<sup>50</sup> `circRNA_finder`,<sup>51</sup> CIRI,<sup>52</sup> KNIFE,<sup>53</sup> NCLScan,<sup>54</sup> Segemehl,<sup>55</sup> DCC,<sup>56</sup> UROBORUS,<sup>57</sup> and PTESFinder.<sup>58</sup> Although all of these algorithms support the idea that circular RNAs are widely expressed, they unfortunately differ significantly in the sets of circular RNAs that they predict.<sup>59–61</sup> When the same RNA-seq dataset was inputted independently into five of the algorithms, many (~40%) putative circular RNAs were predicted by only a single algorithm.<sup>60</sup> The overall number of circular RNAs predicted ranged from 1532 to 4067, and only 854 were identified by all five algorithms.<sup>60</sup> There are thus striking differences in sensitivity and precision among the algorithms, and the field currently lacks a clear gold-standard method to identify circular RNAs from RNA-seq datasets. It is thus strongly recommended that multiple independent pipelines be used to identify circular RNA candidates, and that candidates are subsequently verified using independent approaches, including RT-PCR across backspliced junctions and Northern blotting.<sup>62</sup> With further improvements to the RNA-seq analysis algorithms (reviewed in <sup>63</sup>), it should become easier to computationally eliminate artifacts and identify only true circular RNAs.

## High-throughput sequencing approaches sparked a renaissance for circular RNAs

Beginning ten years ago, many groups started to use RNA-seq to characterize “full” transcriptomes and were able to identify a variety of previously unannotated splice variants and noncoding RNAs.<sup>6, 64–68</sup> These initial analyses missed circular RNAs, however. This is largely due to (i) the widespread use of poly(A) selection steps in library preparation protocols, which depleted circular RNAs and other transcripts that lack poly(A) tails, and (ii) the use of computational algorithms that required RNA-seq reads to align in a linear manner to the genome, thereby causing all reads corresponding to backspliced junctions to be discarded (reviewed in <sup>63, 69</sup>). It was not until Salzman and colleagues used RNA-seq to try to identify cancer-specific transcripts caused by chromosomal rearrangements that it was serendipitously discovered that thousands of circular RNAs are expressed in cells.<sup>9</sup> In that study, hundreds of transcripts with a scrambled exon order were identified in leukemia cells, but the vast majority of these transcripts were surprisingly also observed in normal cells. This indicated that these unusual RNAs were not due to structural rearrangements of the genomic DNA, but from splicing processes that were active in all cells.<sup>9</sup> Consistent with these scrambled transcripts being circular RNAs, they were enriched in poly(A) depleted fractions<sup>48</sup> and were resistant to RNase R, which digests nearly all linear RNAs.<sup>70</sup>

Subsequent RNA-seq studies confirmed these initial observations and identified thousands of circular RNAs that are generated from protein-coding genes across various eukaryotes, including metazoans, plants, fungi, and protists.<sup>10, 11, 50, 51, 71–77</sup> Many of these putative circular RNAs have been assembled into several searchable online databases, including circBase,<sup>78</sup> CIRCpedia,<sup>79</sup> and CircInteractome.<sup>80</sup> In general, circular RNAs can be detected from over 10% of expressed genes in a given cell type, and there is often no clear correlation between levels of circular RNAs and their corresponding mRNAs.<sup>10, 72</sup> Most circular transcripts are tissue-specific and expressed at low levels, but some are expressed in multiple species,<sup>10, 11, 72, 73, 81</sup> and there are hundreds of genes that express circular RNAs at higher levels than the canonical linear mRNA.<sup>9, 73, 82</sup> This is especially true in the nervous system, which is a hotspot for circular RNA expression. For example, in *Drosophila*, >90% of annotated circular RNAs can be detected in heads, and half of these transcripts are not detected in any other tissue.<sup>51</sup> As circular RNAs are often up-regulated during neurogenesis and can be enriched in synapses, it has been suggested that many may respond to and/or regulate neuronal functions.<sup>19, 73, 83, 84</sup>

This review will only focus on the biogenesis and functions of circular RNAs derived from backsplicing of eukaryotic exons, but it should be noted that circular RNAs have been found in archaea<sup>47, 85</sup> and that some circular RNAs in metazoans are generated from tRNA splicing reactions<sup>86</sup> or a failure to debranch certain intron lariats<sup>87, 88</sup> (reviewed in <sup>89–91</sup>).

## Base pairing between intronic repeats facilitates production of many circular RNAs

All internal exons (excluding the first and last exons of genes) have splicing signals at their 5' and 3' ends and theoretically can circularize. However, only a small subset of possible backsplicing events is ever observed in cells, in part because these reactions often occur at an extremely low efficiency.<sup>92</sup> When backsplicing does occur, it can generate a circular RNA that contains a single exon, which is typically longer than the average expressed exon, or one that contains multiple exons. The most commonly observed circular RNAs have 2 to 3 exons with the internal intron(s) removed. As the introns flanking exons that circularize are typically longer than average,<sup>10, 50, 51</sup> Jeck and colleagues searched for intronic motifs that are enriched around human circular RNAs and found that pairs of Alu repetitive elements (~300-nt in length) were commonly observed, especially in an inverted orientation.<sup>10</sup> This arrangement is analogous to the complementary intronic repeats that flank the mouse Sry circular RNA,<sup>31</sup> and suggested that intronic repeats may be a common driver of backsplicing (reviewed in <sup>93</sup>). Indeed, many highly expressed circular RNAs in humans, mice, pigs, *C. elegans*, and *Drosophila* are flanked by complementary repeats [but not always,<sup>51, 94–96</sup> discussed further below], and one can accurately predict many circular RNAs by simply searching for complementary sequences in the flanking introns.<sup>19, 50, 77, 84, 97</sup> Besides Alu elements (which are specific to primates), a variety of complementary sequences can flank circular RNAs, including non-repetitive sequences.<sup>50</sup> It has thus been proposed that most backsplicing events do not require particular sequence motifs (beyond the splice sites), but are instead promoted by base pairing interactions between flanking intronic elements.



Using expression plasmids or by removing intronic repeats from endogenous gene loci using CRISPR-Cas9, we and others have proven that base pairing between intronic repeats can indeed promote circular RNA biogenesis.<sup>19, 50, 97–101</sup> As an example, exon 2 of the *D. melanogaster laccase2* gene generates a 490-nt circular RNA and is flanked by a pair of inverted *DNAREP1\_DM* family transposons (Figure 2A).<sup>97</sup> Mutational analysis revealed that ~100-nt of each repeat (which are highly complementary over a ~55-nt region) is sufficient for exon circularization, even when there is competition between canonical splicing and backsplicing.<sup>97, 102</sup> As expected, disrupting base pairing between the repeats eliminated circular RNA production, while the introduction of compensatory mutations was sufficient to restore backsplicing. Interestingly, the *DNAREP1\_DM* repeats are not present at the *laccase2* gene in other *Drosophila* species, and thus only *D. melanogaster* generates the Laccase2 circular RNA.<sup>97</sup> It is thus important to keep in mind that the genomic repeat landscape varies significantly among species<sup>103</sup> and that distinct circular RNAs can be expressed in different eukaryotes. Even if the protein-coding exons are evolutionarily conserved, the ultimate output and function of a gene may differ across organisms.<sup>93</sup>

At some gene loci, base pairing between short (~30-nt) intronic repeats (including simple repeats) is able to promote circular RNA production.<sup>97, 98</sup> Nevertheless, the presence of intronic repeats is not always sufficient to trigger backsplicing (and, hence, circular RNAs can be expressed in a tissue-specific manner). This is due to the co-transcriptional nature of splicing, thermodynamics, regulation by RNA binding proteins, and the fact that most introns contain multiple repetitive elements that compete for base pairing. Depending on how the repetitive elements base pair to one another, very different spliced isoforms can be generated (Figure 2B).<sup>50</sup> When base pairing occurs across different introns (like at the *laccase2* gene), backsplicing is induced to generate a circular RNA that is composed of the intervening exon(s). In contrast, when base pairing occurs locally within a single intron, canonical splicing occurs to produce a linear mRNA. The number of repetitive elements, the distance between them, and their degree of complementarity all affect which sequences base pair to one another and, hence, the splicing outcome.<sup>50</sup> This competition additionally allows a single protein-coding gene to generate multiple distinct circular RNAs, a process known as alternative circularization.<sup>71, 72, 79, 103, 104</sup>

## Exon skipping can sometimes (but not always) be coupled to circular RNA production

Consistent with earlier studies,<sup>37, 38, 40</sup> a recent global transcriptome analysis suggests that the biogenesis of some circular RNAs can be coupled to exon skipping events, thereby allowing a linear mRNA and a circular RNA to be generated from a single pre-mRNA (Figure 3).<sup>105</sup> In some cases, the exon-skipped mRNA variant is expressed at very low levels and only detectable by RT-PCR, not by RNA-seq.<sup>21</sup> At the *S. pombe mrps16* gene, Barrett and colleagues have beautifully demonstrated a mandatory coupling between exon skipping and circular RNA production.<sup>95</sup> Splicing exon 1 to exon 3 of *mrps16* releases an intron lariat containing exon 2 that is subsequently spliced again to join the beginning and end of exon 2 together. This re-splicing event thereby generates a mature circular RNA that accumulates in cells, whereas the double lariat is rapidly degraded.<sup>106</sup> It remains unclear why the *mrps16*

intron lariat is subjected to a second round of splicing when other lariats are not, but it may be due to topological effects or differences in the speed of lariat debranching. Regardless of the underlying details, coupling exon skipping to circular RNA production provides a mechanism for generating circular RNAs in the absence of intronic repeats.

## Combinatorial control of circular RNA levels by RNA binding proteins

Besides *cis*-acting intronic repeats and exon skipping events, it is now clear that RNA binding proteins are critical regulators of circular RNAs, allowing these transcripts to be expressed in tissue-specific patterns or induced/repressed as cells change their state.<sup>51, 53, 71, 73, 74, 82–84, 96, 107</sup> For example, the intronic repeats themselves can be directly bound by *trans*-acting factors, including NF90/NF110<sup>108</sup> (discussed below) or the RNA helicase DHX9 (DExH-Box Helicase 9) that binds to double-stranded RNAs.<sup>109</sup> DHX9 binding inhibits backsplicing reactions, perhaps by directly unwinding the double-stranded intronic regions or by recruiting ADAR (adenosine deaminase acting on RNA) enzymes that convert adenosines to inosines.<sup>73, 77, 109</sup> As DHX9 is up-regulated during differentiation processes, it has been proposed that these DHX9-driven mechanisms may allow cells to actively suppress production of a wide variety of circular RNAs.<sup>96</sup> On the other hand, the alternative splicing factor Quaking (QKI) promotes the production of hundreds of circular RNAs during human epithelial-mesenchymal transition (EMT).<sup>74</sup> This appears to be because QKI binds to flanking introns and forms dimers, thereby bringing the intervening splice sites into close proximity (analogous to how inverted repeats promote backsplicing). The *Drosophila* Muscleblind (Mbl) protein can likewise bind to intronic elements in its own pre-mRNA to promote production of circular RNAs, thereby auto-regulating host gene expression and ensuring that Mbl protein levels are maintained in a tight range.<sup>94</sup>

Circular RNA production can be further controlled by FUS<sup>110</sup> as well as by multiple hnRNP (heterogeneous nuclear ribonucleoprotein) and SR (serine-arginine) proteins.<sup>97, 102, 111</sup> As discussed above, production of the *Drosophila* Laccase2 circular RNA is promoted by base pairing between intronic repeat elements, but its expression is also inhibited by multiple hnRNPs and SR proteins. Interestingly, simultaneous depletion of these splicing factors resulted in additive increases in Laccase2 circular RNA levels, indicating that each factor plays a non-redundant role to impact splicing decisions.<sup>97</sup> Circular RNAs are thus controlled in a combinatorial manner with intronic repeats generally providing the opportunity for backsplicing to occur, and a variety of *trans*-acting factors acting to fine-tune the efficiency. Considering that each exon or intron has its own unique set of protein-binding sites, one can easily imagine how this code can be used to generate a variety of circular RNA expression patterns.

## The output of genes shifts to circular RNAs when the splicing machinery is limiting

Although mutating the splice sites completely eliminates circular RNA production,<sup>94, 98, 100, 112</sup> we recently showed in *Drosophila* that circular RNAs can become the preferred gene output when core spliceosome or transcription termination factors are depleted from cells.<sup>102</sup> The spliceosome is formed by a stepwise and dynamic assembly of



five snRNAs (small nuclear RNAs) and ~170 proteins (reviewed in <sup>2</sup>). As expected, depletion of core spliceosomal components resulted in decreased expression of spliced linear mRNAs. In stark contrast, we found that the expression of many single-exon circular RNAs, including the Laccase2 circular RNA, increased when factors with very different functions in the spliceosome were depleted from cells. For example, individually depleting snRNP-U1-70K or snRNP-U1-C (which are involved in 5' splice site recognition), Prp8 (the largest spliceosomal protein component, which crosslinks with all the critical sites of chemistry), or Slu7 (which acts in the second catalytic step of splicing) each resulted in significant increases in the expression of many endogenous circular RNAs.<sup>102</sup> Backsplicing was further found to be significantly less sensitive than canonical splicing to pharmacological inhibition of the SF3b complex, a component of the U2 snRNP that is involved in pre-spliceosome assembly.

We propose that the early steps in spliceosome assembly, specifically the mechanism by which exons are initially identified, explain these differences in sensitivity between canonical splicing and backsplicing (Figure 4).<sup>102</sup> During exon definition,<sup>113</sup> U1 and U2 snRNPs bind at opposite ends of each exon and are stabilized by a network of protein-protein interactions that includes additional factors such as SR proteins. These cross-exon interactions then must be converted to cross-intron interactions (to pair splice sites from separate introns) to yield a canonically spliced linear mRNA. However, to generate a circular RNA, these initial exon definition complexes likely do not need to be disrupted and instead may be directly converted into catalytically competent backsplicing complexes. When core spliceosomal components are depleted from cells, we propose that cross-exon interactions are not easily replaced with cross-intron interactions, thereby causing single exon circular RNAs to be the preferred splicing outcome.<sup>102</sup> It remains unclear if backsplicing uses the exact same set of core spliceosomal components as canonical splicing reactions, but this work provides a framework for thinking about how global changes in circular RNA levels (at least those generated from single exons) can be obtained. If a tissue has limiting amounts of spliceosomal components, it would be expected that higher levels of circular RNAs should be produced, especially from highly transcribed genes. It will thus be very informative to determine if this model holds true in neuronal or aging tissues, which produce the highest amounts of circular RNAs.<sup>9, 73, 82</sup>

## Circular RNAs can regulate microRNA activity and levels

Once generated, circular RNAs are naturally resistant to degradation by exonucleases and accumulate as stable transcripts (e.g., the median half-life of 60 human circular RNAs was calculated to be ~24 hours<sup>114</sup>). What then are the molecular functions of circular RNAs? For >99.9% of identified circular RNAs, the answer remains unknown. Nevertheless, some regulate microRNAs, which are ~21-nt small RNAs that function post-transcriptionally by base pairing to mRNAs and repressing protein production and/or triggering mRNA degradation (reviewed in <sup>115</sup>) (Figure 5A). By searching for conserved microRNA target sites within circular RNAs, it was revealed that the mouse Sry and human CDR1as (also known as ciRS-7) circular RNAs represent extreme and notable cases.<sup>11, 99</sup> The Sry circular RNA contains 16 binding sites for the microRNA miR-138, whereas CDR1as contains more than 70 evolutionarily conserved binding sites for miR-7. It was thus proposed that these

circular RNAs may function as decoys or sponges<sup>116, 117</sup> that reduce the number of freely available miR-138 or miR-7 transcripts, respectively.<sup>11, 99</sup>

CDR1as is derived from an antisense long noncoding RNA<sup>44, 106</sup> and is expressed several orders of magnitude higher than the *CDR1* (Cerebellar Degeneration-Related protein 1) gene from the opposite strand.<sup>20, 44</sup> None of the >70 miR-7 binding sites present in CDR1as are perfectly complementary to miR-7, which allows the circular RNA to be densely bound but not sliced by miR-7. Consistent with this idea, depleting CDR1as in cell lines resulted in down-regulation of miR-7 target mRNAs, presumably because miR-7 was no longer sequestered.<sup>11, 99</sup> CDR1as additionally contains a region near perfectly complementary to miR-671, which enables the circular RNA to be endonucleolytically cleaved by Argonaute 2 (AGO2) in a miR-671-dependent manner.<sup>44</sup> This has led to a proposed model in which CDR1as helps store and/or transport miR-7 (bound to an Argonaute protein) until miR-671 slices the circular RNA, releasing the miR-7 cargo so that it can regulate the expression of mRNAs in a specific subcellular location (Figure 5A).

In mice, the CDR1as circular RNA is highly expressed in excitatory neurons, with minimal expression in inhibitory neurons, glial cells, or non-neuronal tissues.<sup>20</sup> Upon deleting the CDR1as genomic region, knockout mice were viable, fertile, and displayed no gross abnormalities in adult brain anatomy. Nevertheless, defects in excitatory synaptic transmission were observed and the knockout mice exhibited impaired sensorimotor gating, a behavioral phenotype associated with neuropsychiatric disorders.<sup>20</sup> These effects are presumably due to loss of the CDR1as circular RNA, but it remains possible that the phenotypes are caused, at least in part, by the long noncoding RNA from which the circular RNA is derived<sup>106</sup> or the overlapping *CDR1* gene. Interestingly, at least in the tissues examined, *CDR1* does not appear to be expressed.<sup>20</sup> On the molecular level, the expression of the vast majority of microRNAs was unchanged in the brains of CDR1as knockout mice.<sup>20</sup> However, miR-7 was markedly down-regulated in the cerebellum, cortex, hippocampus, and olfactory bulb (but unaffected in tissues where CDR1as is not expressed). This resulted in increased expression in the brain of miR-7 target genes, including immediate early genes, such as *Fos*, that serve as markers of neuronal activity. In contrast, miR-671 was consistently up-regulated in the cerebellum, cortex, and olfactory bulb of CDR1as knockout mice.

Considering that expression of the miR-7 and miR-671 passenger (miR\*) strands were unperturbed in CDR1as knockout mice, these specific changes in microRNA expression likely occur post-transcriptionally.<sup>20</sup> Binding to near-perfect target sites (similar to the miR-671 site in CDR1as) can cause tailing and degradation of microRNAs,<sup>118</sup> and thus the observed increase in miR-671 levels is likely directly due to the lack of CDR1as expression in the knockout mice.<sup>20</sup> Likewise, when miR-7 is unable to bind CDR1as, it binds other RNAs in cells, including the long noncoding RNA *Cyano*,<sup>119</sup> which has a near perfect miR-7 target site that may promote miR-7 trimming and degradation.<sup>20</sup>

The CDR1as locus thus beautifully demonstrates how circular RNAs and microRNAs can have a dynamic interplay with important biological effects, but there is still debate as to whether other circular RNAs function in a similar manner. This is because most annotated circular RNAs contain few microRNA binding sites.<sup>72, 114</sup> Nevertheless, there is emerging

evidence that a subset of circular RNAs, including human circHIPK3, human circZNF91, human circHECTD1, and many circular RNAs in *Drosophila*, may function to sponge specific microRNAs.<sup>51, 96, 101, 120</sup> For example, circZNF91, which is induced as human epidermal stem cells differentiate, contains 24 binding sites for miR23b-3p, a microRNA that is known to regulate keratinocyte differentiation.<sup>96</sup> As other circular RNAs up-regulated during this differentiation process also have significant numbers of microRNA binding sites,<sup>96</sup> it has been suggested that a subset of circular RNAs may sponge key microRNAs as cells change their state. Given that microRNAs can confer robustness during development,<sup>121, 122</sup> it will be interesting to determine if circular RNAs also help buffer regulatory networks against perturbation.

## Translation of circular RNAs

Initial experiments suggested that eukaryotic ribosomes can not be loaded onto circular RNAs due to their lack of a 5' cap structure,<sup>123</sup> but it was subsequently demonstrated that the presence of an internal ribosome entry site (IRES) allowed synthetic circular RNAs to be translated *in vitro*<sup>124</sup> and in cells.<sup>97, 108, 112, 125</sup> IRES elements are present in a variety of viruses and directly bind translation initiation factors or the ribosome itself, thereby allowing translation to occur in a cap-independent manner (reviewed in<sup>126</sup>). As initial reports failed to find circular RNAs co-sedimenting with ribosomes<sup>10, 127</sup> or in ribosome profiling libraries,<sup>72, 83</sup> it appeared that endogenous circular RNAs may not be able to recruit ribosomes. However, several recent reports suggest that a subset of endogenous circular RNAs may, in fact, be translated (reviewed in<sup>128, 129</sup>).

Human circ-ZNF609 contains a 753-nt open reading frame that starts at the same AUG codon used to initiate linear mRNA translation and terminates at a stop codon that is encountered three nucleotides past the backsplicing junction (Figure 5B).<sup>130</sup> A small, but significant portion of this circular RNA co-sediments with polysomes, and expression plasmids revealed that circ-ZNF609 could be weakly translated (two orders of magnitude less efficiently than a linear mRNA). Interestingly, knock-down of circ-ZNF609 results in proliferation defects in human and mouse myoblasts,<sup>130</sup> but it remains unknown whether the protein derived from circ-ZNF609 contributes to this phenotype.

Using mass spectrometry, peptides spanning endogenous backsplicing junctions were identified for 19 additional human circular RNAs,<sup>131</sup> and deeply sequenced ribosome profiling datasets revealed 122 *Drosophila* circular RNAs associated with ribosomes.<sup>132</sup> For 40% of these fly circular RNAs, translation is predicted to use the same start codon as the host linear mRNA and to terminate after the backsplicing junction (like circ-ZNF609) (Figure 5B). Many of the putative ORFs have at least one identifiable protein domain, but it must be noted that only a single sequencing read currently supports the vast majority of these translation events. It is near impossible to use ribosome profiling to look for ribosome phasing or start/stop codons on circular RNAs, so a combination of minigene expression plasmids, sucrose gradients, mutational analysis, and mass spectrometry were used to further support the idea that some of these circular RNAs may indeed be translated.<sup>132</sup> Notably, this analysis suggested that circular RNA translation may often occur on membrane-associated ribosomes or in subcellular compartments rich in membranes (e.g. synapses).

Consistent with expectations, the regions upstream of the circular RNA start codons have detectable IRES activity when inserted into bicistronic reporter constructs.<sup>130, 132</sup> For circ-ZNF609, splicing was required for IRES activity, suggesting that deposition of the exon junction complex<sup>133</sup> may enhance translation of some circular RNAs.<sup>130</sup> Yang et al. further demonstrated that N<sup>6</sup>-methyladenosine (m<sup>6</sup>A) residues are enriched in circular RNAs (~13% of circular RNAs contain m<sup>6</sup>A) and that these modified nucleotides can act to promote translation.<sup>131</sup> In fact, a single m<sup>6</sup>A site may be sufficient to initiate translation of synthetic circular RNAs. Upon binding m<sup>6</sup>A, it was proposed that the YTHDF3 reader protein recruits translation initiation factors, including eIF4G2 and eIF3A to the circular RNA.<sup>131</sup>

It remains possible that a number of these recently described translation events represent technical or biological noise as other genome-wide studies failed to identify any evidence for circular RNA translation.<sup>72, 82, 83</sup> Further work is now required to determine how many circular RNAs are truly translated to significant levels. It is tempting to speculate that significant amounts of protein could be made over the long lifetimes of circular RNAs (which could be useful for biotechnology purposes), and that these endogenous proteins may serve as dominant negatives and/or regulate the same processes as proteins generated from their associated host linear mRNAs. Besides being translated themselves, there is emerging evidence that some circular RNAs, including circPABPN1, can regulate translation of their associated linear mRNAs.<sup>134</sup> Mature circular RNAs have also been found to bind to a variety of other proteins,<sup>94, 127, 135, 136</sup> which suggests that these transcripts may impact a variety of other protein activities or functions.

## Circular RNAs in the nucleus regulate transcription and alternative splicing of their host genes

Most characterized circular RNAs predominately localize in the cytoplasm,<sup>9, 10</sup> but there are now emerging examples of circular RNAs that function in the nucleus.<sup>21, 84, 137</sup> Exon-intron circular RNAs are incompletely spliced and have a retained intron that allows them to interact with U1 snRNP and promote transcription of their host gene.<sup>137</sup> In *Arabidopsis*, a circular RNA is generated from exon 6 of *SEPALLATA3* (*SEP3*), a key developmental regulatory gene, and ~15% of these circular transcripts are retained in the nucleus (Figure 5C).<sup>21</sup> Remarkably, plants over-expressing the *SEP3* circular RNA (but not a linear version of exon 6) produced flowers that had fewer stamen and more petals than normal. This appears to be because the ectopically expressed circular RNA forms an R-loop (an RNA:DNA hybrid) at the endogenous *SEP3* genomic loci, which causes increased production of a linear *SEP3* splice variant that has exon 6 skipped (Figure 5C). As over-expression of this *SEP3* splice variant was sufficient to cause the same homeotic phenotype as over-expression of the *SEP3* circular RNA, this circular RNA appears to function solely by modulating alternative splicing of its host gene.<sup>21</sup> It remains to be determined how exactly the circular RNA does this, but it was proposed that the R-loop causes a pause in *SEP3* transcription that allows alternative splicing regulators to be recruited that promote exon skipping. Of note, ~5% of R-loops in *Arabidopsis* are resistant to RNase R treatment,<sup>21</sup> suggesting that other circular RNAs may likewise bind genomic DNA to modulate transcription or alternative splicing patterns.

## Interplay between the immune system and circular RNAs

Considering that some circular RNAs, including viroids and HDV, are pathogens, one would expect that eukaryotic immune systems should somehow be able to sense and destroy these foreign invaders. Indeed, when HeLa cells were transfected with circular RNAs that had been made *in vitro* using self-splicing group I introns<sup>138, 139</sup> or split ligation,<sup>140</sup> a number of well-known pattern recognition receptors, including RIG-I and MDA5, as well as other innate immunity regulators and cytokines were strongly induced.<sup>135</sup> Cells are thus able to mount a significant immune response to foreign circular RNAs, one that was, in fact, stronger than when a linear RNA encoding the same sequence was transfected into cells.

It remains incompletely understood how a foreign circular RNA is sensed, but some important aspects have been revealed (reviewed in <sup>141</sup>). First, RIG-I is required for the immune response and co-localizes with foreign circular RNAs,<sup>135</sup> even though the transcripts lack the 5' triphosphate moiety that RIG-I is thought to require.<sup>142</sup> Second, the mechanism by which the circular RNA is made is the key determinant of whether the transcript is recognized as self or non-self.<sup>135</sup> Whenever a circular RNA was made using self-splicing group I introns (even when it was made in cells from expression plasmids), a robust immune response was observed. In stark contrast, circular RNAs made using the spliceosome were always recognized as self (regardless of the sequence of the mature circular RNA or its flanking introns). This is presumably because the spliceosome deposits a number of RNA binding proteins, such as the exon junction complex or the nuclear export machinery, onto the circular RNA that somehow mark the circular RNA as self. In this manner, cells are able to sense and respond to a diverse set of foreign circular RNAs, irrespective of their primary sequence, while not responding to their many endogenous circular RNAs.<sup>135</sup> Further work is now required to understand how exactly RIG-I recognizes foreign circular RNAs as well as how backsplicing by the spliceosome prevents autoimmune reactions.

Beyond ensuring that foreign circular RNAs are recognized and neutralized, multiple immune regulators, including RIG-I and the dsRNA binding proteins NF90/NF110, appear to regulate the expression of endogenous circular RNAs.<sup>108</sup> In normally growing cells, NF90/NF110 bind to A/U-rich elements (including base paired Alu elements) in the introns flanking many (~30%) exons that yield circular RNAs and act to promote these backsplicing events. However, upon viral infection, NF90/NF110 shuttle to the cytoplasm to bind viral transcripts and suppress viral replication.<sup>143</sup> This causes NF90/NF110 to bind fewer nascent pre-mRNAs in the nucleus, thereby resulting in reduced production of circular RNAs.<sup>108</sup> Interestingly, NF90/NF110 are also able to bind to some mature circular RNAs in the cytoplasm and, in fact, show higher affinity to circular RNAs than linear RNAs. It has thus been proposed that the normal production of circular RNAs allows the establishment of a molecular reservoir of NF90/NF110 in the cytoplasm, which can be released when needed to enable prompt responses to viral infection.<sup>108</sup>

## Circular RNAs in aging and disease

Alternative splicing patterns have been shown to change with aging,<sup>144, 145</sup> and there is a genome-wide trend for increased circular RNA expression in the nervous system as *Drosophila*, *C. elegans*, and mice age.<sup>51, 146</sup> For example, out of 2,513 detected *Drosophila* circular RNAs, 262 were significantly up-regulated >2-fold in the heads of 20 day old animals compared to 1 day old animals.<sup>51</sup> Notably, these increases in expression were largely independent of changes in expression of linear mRNAs from their host genes and were not observed in aging mouse hearts<sup>146</sup> or in rhesus monkey muscles.<sup>147</sup> This suggests that circular RNAs may be preferentially stabilized or generated in the aging nervous system, but it remains unclear whether this accumulation of circular RNAs is protective, detrimental, or an innocuous biomarker of aging. At least in mice, it appears that a circular RNA derived from the *Pwmp2a* gene can help protect from pathological hypertrophy and heart failure.<sup>120</sup>

Compared to normal tissues, cancer cells generally have reduced expression of circular RNAs,<sup>107</sup> which may in part be due to dilution via cell division (reviewed in <sup>148, 149</sup>). Consistent with this idea, it was recently shown that circular RNA levels often increase as cells differentiate and stop proliferating.<sup>96</sup> Down-regulation of some circular RNAs, including circITCH<sup>150–152</sup> and circFOXO3,<sup>136</sup> has been suggested to promote cell growth, whereas over-expression of others appears to promote proliferation.<sup>153</sup> Of particular note, cancer-associated chromosomal translocations can result in production of aberrant fusion circular RNAs when intronic sequences that flank the breakpoint base pair to one another.<sup>154</sup> These fusion circular RNAs can promote transformation, cell viability, and resistance upon therapy, and thus represent promising new therapeutic targets. Furthermore, because some circular RNAs are present in exosomes/extracellular vesicles and bodily fluids,<sup>92, 155–158</sup> circular RNAs may represent promising cancer biomarkers. Packaging of circular RNAs into vesicles may be a way to eliminate circular RNAs from cells and/or a mechanism that enables cell-to-cell communication.

## Perspectives

Thousands of putative circular RNAs have now been identified by RNA-seq, but it remains important to keep in mind that the vast majority have never been verified by orthogonal techniques or studied in any detail. There is thus still much work to do to eliminate sequencing artifacts from these lists, to distinguish backsplicing events from *trans*-splicing events, and to fully characterize circular RNA expression patterns. Nevertheless, a growing number of circular RNAs have now been characterized in sufficient detail such that we have important insights into how they can be generated, regulated, and function. In particular, insights into the biogenesis mechanisms have been translated into efficient methods for expressing circular RNAs in cells,<sup>62, 79, 97, 99, 108, 112</sup> and this has already enabled the functions of some circular RNAs to be revealed.<sup>11, 21, 99</sup> By combining over-expression studies with circular RNA knockouts generated by CRISPR-Cas9,<sup>20</sup> the functions for many more circular RNAs will likely be identified over the coming years. It should be possible to use CRISPR-Cas9 to remove the flanking intronic repeat elements, thereby knocking out the circular RNA of interest while not affecting linear RNA production from the host gene.



Intronic repeats are critical for the biogenesis of many circular RNAs (Figure 2), but it remains incompletely understood how genes can generate circular RNAs in the absence of repeats.<sup>51, 76, 95</sup> For example, most of the circular RNAs up-regulated during human epidermal stem cell differentiation<sup>96</sup> or in *Drosophila*<sup>51</sup> are not flanked by repeats, and it is unclear how these exons (and not others) are selected for backsplicing. Besides exon skipping events (Figure 3), there are likely a number of *cis*- and *trans*-acting regulatory mechanisms that await discovery which enable specific circular RNA expression patterns. Further work is additionally needed to understand the detailed mechanisms of backsplicing reactions and to determine whether all of the canonical core spliceosomal components are involved.<sup>2</sup>

Once a circular RNA is generated, little is known about how they accumulate in the cytoplasm, especially in non-dividing cells. Specific structural features in other classes of RNA are recognized by receptor proteins, enabling those transcripts to be brought to the nuclear pore complex for export to the cytoplasm (reviewed in <sup>159</sup>). It is likely that an analogous mechanism exists for circular RNAs and a better understanding of the proteins that interact with circular RNAs, especially during their biogenesis, may help reveal how circular RNA localization is controlled and can change during development.<sup>84</sup> For those circular RNAs that associate with ribosomes (Figure 5B), how exactly is the ribosome assembled on the transcript and do the generated proteins accumulate to high enough levels to have biological effects? Abundant circular RNAs that are not translated may help form large RNA-protein complexes or otherwise impact the same pathway as the protein produced from its parental gene. Alternatively, production of the circular RNA may itself be the key functional event, as backsplicing reactions can be used to turn down expression of the linear mRNA<sup>94</sup> or terminate readthrough transcription events.<sup>102</sup>

How are endogenous circular RNAs recognized as self, and is it possible to generate circular RNAs *in vitro* that do not invoke an immune response? This avenue could open up many biotechnology applications where treating cells or patients with long-lasting circular RNAs could be ideal. Many circular RNAs have long half-lives, but the mechanisms by which circular RNAs are ultimately degraded remain largely unknown. The CDR1as circular RNA can be cleaved by AGO2 (in complex with miR-671),<sup>44</sup> but this appears to be an isolated mechanism. Other RNA endonucleases may generally facilitate decay of circular RNAs by providing access points for exonucleases (reviewed in <sup>160</sup>), and identification of circular RNAs that are rapidly degraded may provide a starting point for defining such mechanisms. In summary, the last few years have revealed that circular RNAs are not simply rare oddities, but common outputs of eukaryotic genes. Many surprises have been revealed, and the field is well poised to reveal more insights into how these transcripts are regulated and function to impact normal and disease states.

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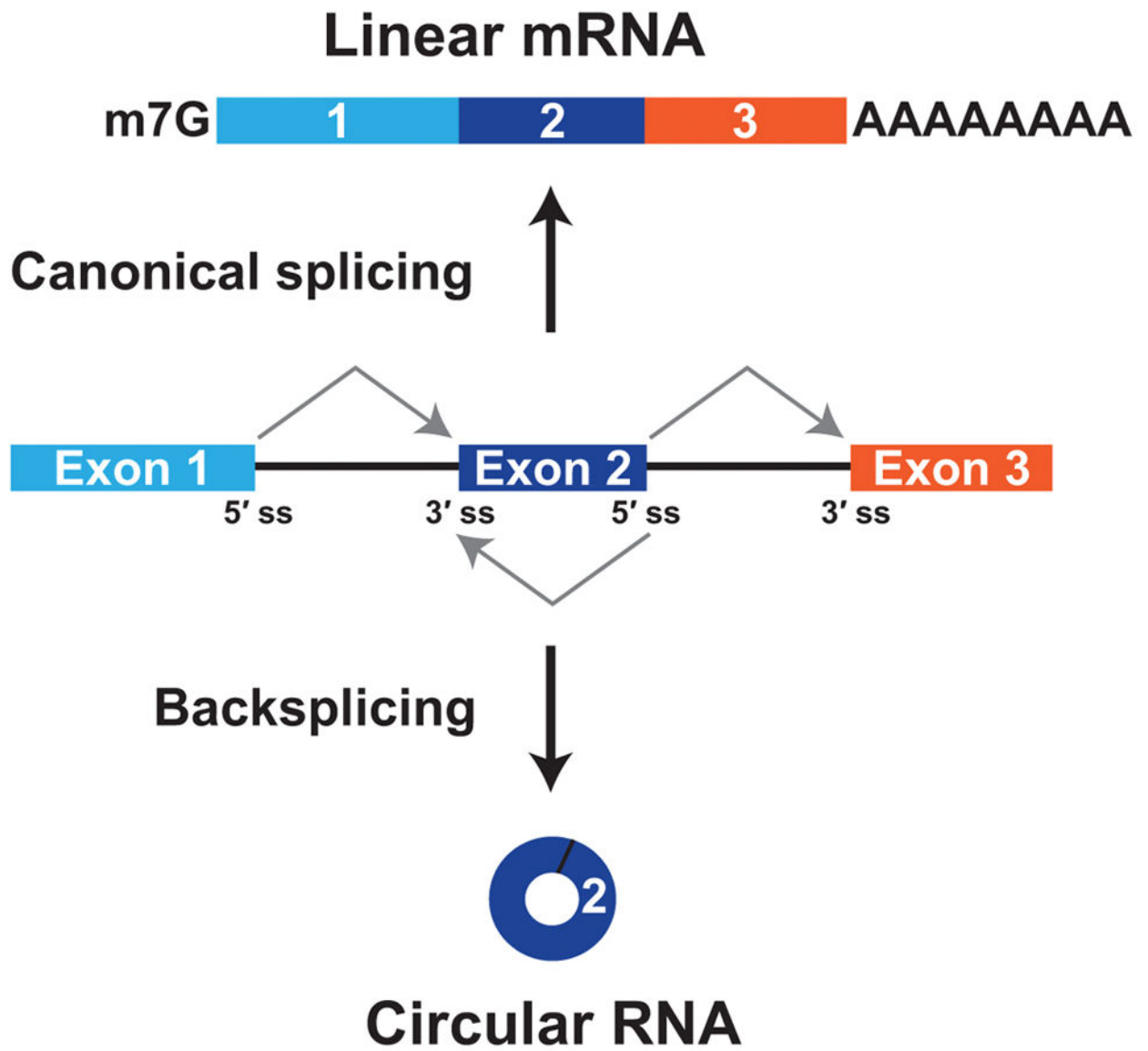
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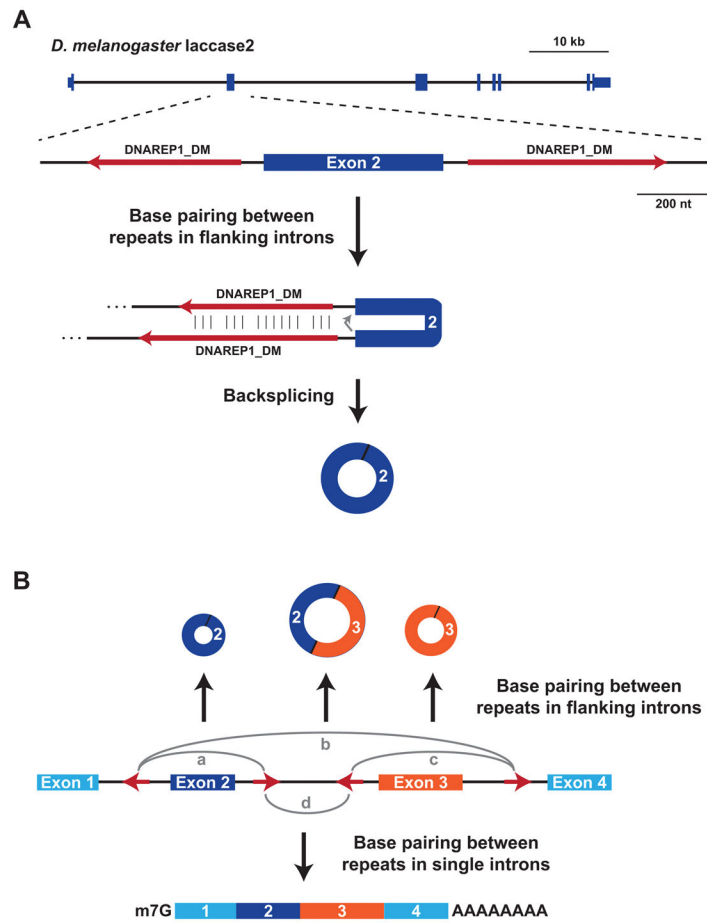
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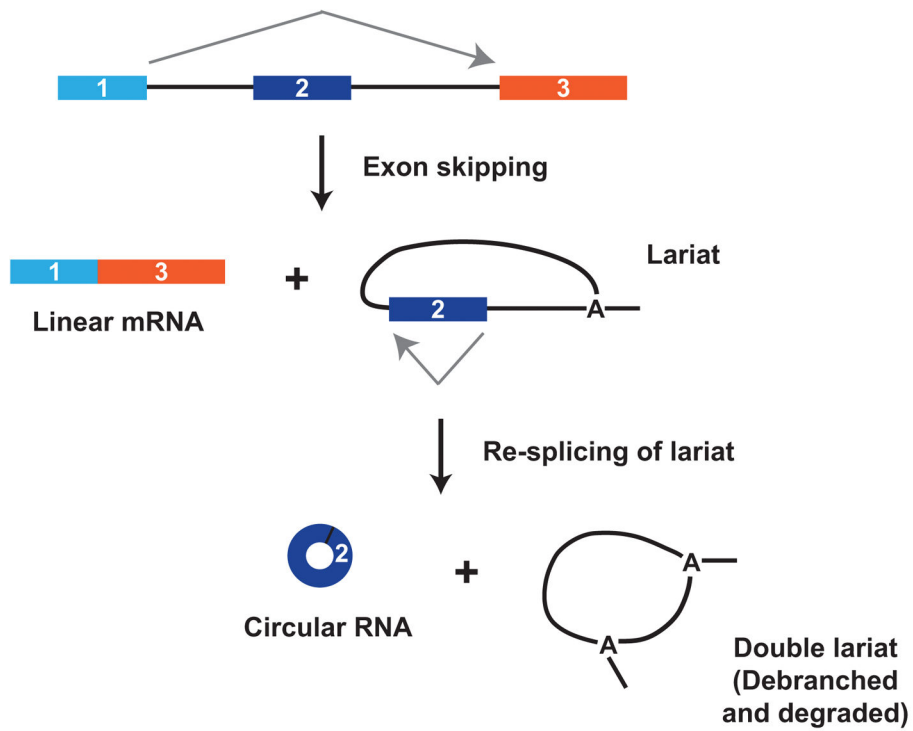
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**Figure 1. Pre-mRNAs can be alternatively spliced to generate a linear mRNA or a circular RNA**  
 If the pre-mRNA splice sites (ss) are joined in a linear order, a mature linear mRNA is generated that is also capped and polyadenylated (top). Alternatively, the pre-mRNA splicing machinery can backsplice and join a 5' ss to an upstream splice acceptor (3' ss), resulting in production of a circular RNA whose ends are covalently linked by a 3'-5' phosphodiester bond (bottom).



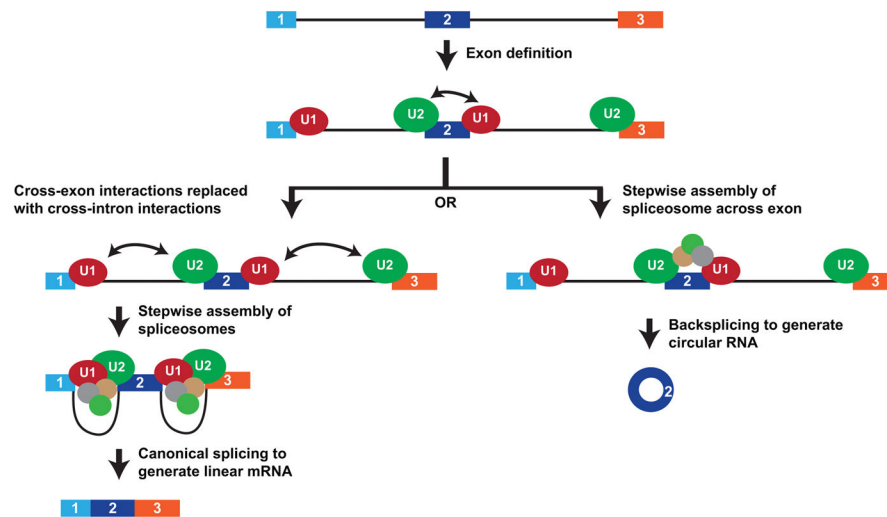
**Figure 2. Base pairing between intronic repeats facilitates many backsplicing events**  
**(A)** Exon/intron structure of the *D. melanogaster laccase2* gene, highlighting exon 2 that can generate a 490-nt circular RNA. A pair of *DNAREP1\_DM* transposons (red arrows) flanks exon 2. Base pairing between these intronic sequences brings the intervening splice sites into close proximity, facilitating backsplicing.<sup>97</sup> **(B)** When multiple intronic repeat elements (red arrows) are present in a pre-mRNA, distinct mature RNAs can be generated depending on which repeats base pair to another (denoted by gray arcs).<sup>50</sup> (a–c) Base pairing between repeats in different introns results in backsplicing and production of a circular RNA that contains a single exon (a and c) or multiple exons (b). (d) In contrast, a linear mRNA is produced when repeats in a single intron base pair to one another.



**Figure 3. Exon skipping can be directly coupled to circular RNA production**

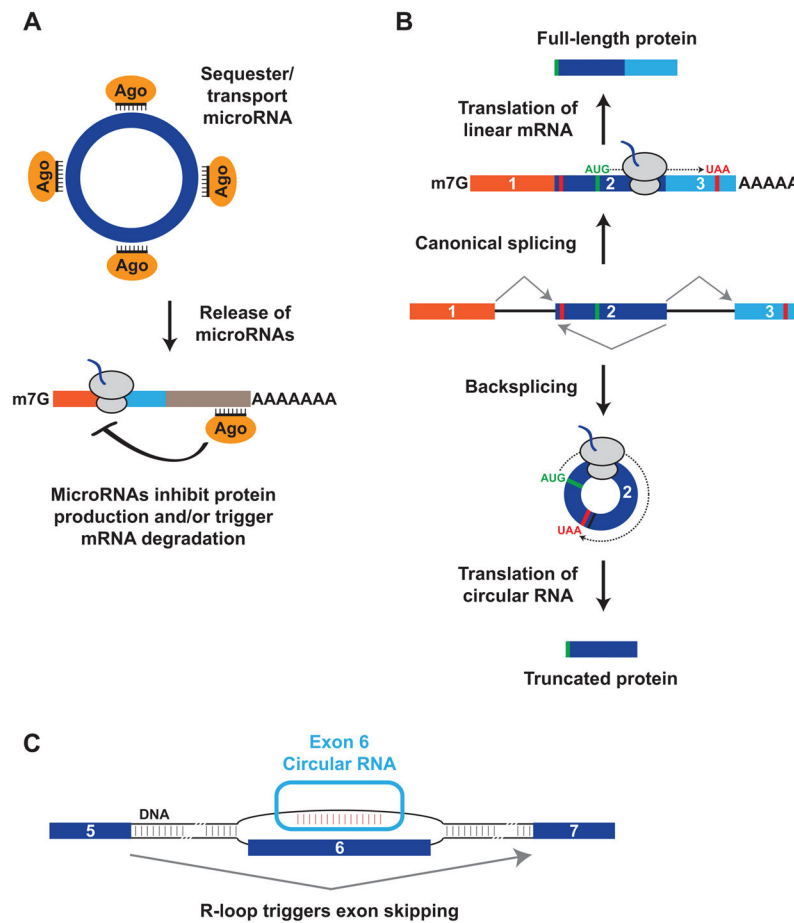
At the *S. pombe mps16* gene, exon skipping results in production of a mature linear mRNA as well as an intron lariat containing exon 2. This lariat is re-spliced to generate a mature circular RNA as well as a double lariat structure that is subsequently debranched and degraded.<sup>95</sup>





**Figure 4. Model for how early steps in spliceosome assembly determine whether canonical splicing or backsplicing occurs**

In pre-mRNAs with long introns, spliceosomal components first assemble across each exon. U1 snRNP (red) and U2 snRNP (green) recognize the 5' and 3' splice sites, respectively, with additional factors, such as SR proteins, serving to stabilize the exon definition complex. These cross-exon interactions must then be replaced with cross-intron interactions in order to generate a mature linear mRNA (left). However, when spliceosome activity is limiting (e.g., due to depletion of core spliceosomal components), recent work suggests that cross-exon interactions may not be easily disrupted and the full spliceosome instead assembles across an exon, resulting in backsplicing.<sup>102</sup>



**Figure 5. Circular RNAs can have a variety of molecular functions**

(A) Some circular RNAs, including CDR1as and Sry, are able to bind many copies of particular microRNAs (in complex with an Argonaute (AGO) protein). In response to a specific stimulus (e.g. cleavage of CDR1as by miR-671), the circular RNA can potentially release these microRNA transcripts, which then bind to mRNAs to down-regulate their expression. (B) Recent work suggests that linear mRNAs and circular RNAs from the same gene locus can be translated to generate distinct protein products. In the example shown, the mature linear mRNA (top) and the circular RNA (bottom) use the same AUG start codon (green), but the circular RNA generates a truncated protein that terminates at a stop codon encountered after the backsplicing junction. (C) Some copies of the *Arabidopsis SEP3* circular RNA are retained in the nucleus, where they form R-loops (an RNA:DNA hybrid) at the endogenous *SEP3* gene loci to impact alternative splicing of its host gene.