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Review

The Biogenesis, Functions, and Challenges of Circular RNAs

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Covalently closed circular RNAs (circRNAs) are produced by precursor mRNA back-splicing of exons of thousands of genes in eukaryotes. circRNAs are generally expressed at low levels and often exhibit cell-type-specific and tissue-specific patterns. Recent studies have shown that their biogenesis requires spliceosomal machinery and can be modulated by both *cis* complementary sequences and protein factors. The functions of most circRNAs remain largely unexplored, but known functions include sequestration of microRNAs or proteins, modulation of transcription and interference with splicing, and even translation to produce polypeptides. However, challenges exist at multiple levels to understanding of the regulation of circRNAs because of their circular conformation and sequence overlap with linear mRNA counterparts. In this review, we survey the recent progress on circRNA biogenesis and function and discuss technical obstacles in circRNA studies.

A variety of circular RNAs have been reported to be generated by distinct mechanisms. For example, they were identified as circular RNA genomes in plant viroids (Sanger et al., 1976) and hepatitis delta virus (Kos et al., 1986). Housekeeping non-coding RNAs, including small nucleolar RNAs (snoRNAs) and RNase P RNA, were found in circular formats in archaea (Danan et al., 2012). Circular RNA intermediates can also be generated during rRNA processing (Danan et al., 2012; Tang et al., 2002) or permuted tRNAs with rearranged segments in archaea and algae (Soma et al., 2007). RNA intermediates escaped from intron lariat debranching can form circular RNAs as well (Box 1; Gardner et al., 2012; Kopczynski and Muskavitch, 1992; Qian et al., 1992; Talhouarne and Gall, 2014; Zhang et al., 2013). Despite these different forms of circular RNAs, most currently studied circular RNAs (circRNAs) are produced from precursor mRNA (pre-mRNA) back-splicing of exons in which a downstream 5′ splice site (ss) is joined with an upstream 3′ ss, and the resulting RNA circle is ligated by a 3′-5′ phosphodiester bond at the junction site (Figure 1A; reviews by Chen, 2016; Lasda and Parker, 2014; Wilusz, 2018).

circRNAs were discovered more than 25 years ago. Only a handful of circRNAs were found at that time, and they were often considered aberrant splicing byproducts with little functional potential (Capel et al., 1993; Cocquerelle et al., 1992, 1993; Nigro et al., 1991; Pasman et al., 1996). Because of their non-linear conformation and lack of polyadenylated (poly(A)) tails, circRNAs are rarely seen in the next-generation RNA sequencing (RNA-seq) profiling that is usually enriched for poly(A)$^+$ RNAs. Only profiling with non- poly(A) RNAs or enrichment of circular RNAs with RNase R, which is an enzyme that preferentially digests linear RNAs, has uncovered widespread expression of circRNAs from pre-mRNA back-splicing. For example, over 10,000 circRNAs have been found in metazoans, from worm and fruit fly (Ivanov et al., 2015; Westholm et al., 2014) to mouse, monkey, and human (Dong et al., 2017; Fan et al., 2015; Guo et al., 2014; Jeck et al., 2013; Memczak et al., 2013; Salzman et al., 2012; Yang et al., 2011; Zhang et al., 2014), and widespread expression of circRNAs was also found in plants, fungi, and protists (Barrett et al., 2015; Broadbent et al., 2015; Lu et al., 2015; Wang et al., 2014a).

Recent research into circRNA biogenesis has shown that back-splicing is catalyzed by the canonical spliceosomal machinery and modulated by both intronic complementary sequences (ICSs) and RNA binding proteins (RBPs). Emerging studies have revealed that some circRNAs are implicated in neuronal function, innate immune responses, cell proliferation, and pluripotency. At the molecular level, they are involved in gene expression by titrating microRNAs, sequestering proteins, modulating RNA polymerase II (Pol II) transcription, and interfering with pre-mRNA processing. Furthermore, a few endogenous circRNAs are translatable, and some others can act as sources of pseudogene derivation. Despite these encouraging advances, it is worthwhile noting that the circular conformation and almost complete sequence overlap with their linear mRNA counterparts have made the precise evaluation of circRNA expression and function challenging.

In this review, we survey the most recent progress regarding the regulation of circRNA biogenesis and function. We also discuss experimental designs and their challenges in circRNA studies.

Box 1. Circular Intronic RNAs

Circular intronic RNAs (ciRNAs) represent another class of circular RNA molecules. They are derived from lariat introns of Pol II transcripts and depend on a consensus RNA motif containing a 7-nt GU-rich motif near the 5′ splice site and an 11-nt C-rich motif at the branchpoint site to escape debranching. The resulting RNA circles are covalently ligated by 2′ 5′-phosphodiester bonds at the joining sites and lack the 3′ linear sequences from the 3′ end of the introns to the branchpoint sites (Zhang et al., 2013). Interestingly, stable lariat intronic RNAs (named sisRNAs) were also found in oocytes of *Xenopus tropicalis* (Gardner et al., 2012; Talhouarne and Gall, 2014), and maternally inherited sisRNAs could trigger expression of their host genes via a positive feedback loop during embryogenesis (Tay and Pek, 2017). In human cells, ciRNAs have little enrichment for miRNA target sites but, rather, largely accumulate in the nucleus to regulate gene transcription in *cis* by promoting Pol II transcription of their parental genes through unknown mechanisms (Zhang et al., 2013).

Complexity of circRNA Expression

In general, circRNAs are expressed at low levels (Guo et al., 2014; Jeck et al., 2013; Memczak et al., 2013; Salzman et al., 2013; Zhang et al., 2014). However, some circRNAs are more abundant than their linear transcripts, and their expression is independent of related linear isoforms (Conn et al., 2015; Rybak-Wolf et al., 2015; Salzman et al., 2013; You et al., 2015; Zhang et al., 2016a). In addition, the expression patterns of circRNAs are diverse among cell types and tissues in mammals. For example, a significant enrichment of circRNAs was observed in the brain (Rybak-Wolf et al., 2015; You et al., 2015) and during certain biological processes, such as human epithelial-mesenchymal transition (EMT) (Conn et al., 2015).

Comparison of circRNA expression from human and mouse revealed that only a small portion (10%∼20%) of human circRNAs could be observed in parallel mouse samples (Dong et al., 2017; Guo et al., 2014; Rybak-Wolf et al., 2015). This is in part due to the predominant contribution of *cis* ICSs across circRNA-forming exons in circRNA formation (Dong et al., 2017). Of note, back-splicing often requires ICSs residing in introns flanking circularized exons (Jeck et al., 2013; Zhang et al., 2014; Liang and Wilusz, 2014) (discussed in detail below). It has been shown that repetitive element sequences, which contribute the most to the formation of ICSs, evolve fast in time, leading to increased complexity of circRNA expression in evolution (Dong et al., 2017; Rybak-Wolf et al., 2015). For instance, the conservation of circRNA-forming exonic sequences in the *GCN1L1* locus is high between human and mouse. However, a pair of ICSs exists in human flanking introns but not the corresponding mouse locus (Figure 1B, left). Correspondingly, *circGCN1L1* is only detected in humans (Figure 1B, right), although the exonic sequences are conserved between human and mouse (Zhang et al., 2014).

At the individual gene level, it has been found that one gene locus can produce multiple circRNAs (Gao et al., 2016; Jeck et al., 2013; Memczak et al., 2013; Salzman et al., 2013; Zhang et al., 2014, 2016a) with mechanisms related to alternative back-splicing and alternative splicing site selection. Alternative back-splicing selectively uses different downstream 5′ splice donors or upstream 3′ splice acceptors, leading to alternative 5′ or 3′ back-splicing choices to generate multiple circRNAs from a single gene locus (Figure 1C; Zhang et al., 2016a). For example, multiple highly expressed circRNAs from human *DNMT3B* and *XPO1* gene loci are generated through the alternative back-splicing mechanism. Importantly, these alternatively back-spliced circRNAs could be experimentally validated by northern blotting (NB) (Zhang et al., 2014). Alternative splicing also occurs within circRNAs that contain multiple exons. All four basic types of canonical alternative splicing have been found in circRNAs: cassette exon, intron retention, alternative 5′ splicing, and alternative 3′ splicing (Zhang et al., 2016a). It is worthwhile noting that circRNAs produced from a single gene locus in this manner have the same back-ss but contain distinct canonical ss within each circRNA (Figure 1C; Gao et al., 2016; Zhang et al., 2016a). For example, NB assays confirmed that the *CAMSAP1* locus produces two major circRNA isoforms with or without a retained intron (Salzman et al., 2013; Zhang et al., 2014) and that the human *XPO1* locus contains a circRNA-predominant cassette exon (Zhang et al., 2014).

Alternative back-splice selection and/or circRNA-specific alternative splice selection leads to thousands of new exons (previously unannotated in NCBI Reference Sequence Database (RefSeq), University of California Santa Cruz [UCSC] Known Genes, or Ensembl) included within circRNAs. For instance, in the human *MED13L* locus, several previously unannotated exons were identified in multiple non-poly(A) RNA-seq datasets from various cell lines and could be experimentally validated (Zhang et al., 2016a). Among them, three are alternatively back-spliced, and one is a new cassette exon mainly alternatively spliced within circRNAs. In contrast, these novel exons were barely detected in the linear *MED13L* mRNA (Zhang et al., 2016a). Although less conserved, these circRNA-predominant novel exons have similar sequence signatures as annotated ones. In this case, it remains unclear how the spliceosome could specifically recognize these exons for circular but not linear RNAs.

Regulation of circRNA Biogenesis

By a broader definition, back-splicing circularization is an additional type of alternative splicing. Recent data have shown that back-splicing requires spliceosomal machinery and that the regulation of circRNA formation depends on both *cis*-regulatory elements and *trans*-acting factors. Despite general commonalities, emerging lines of evidence have identified unique features of circRNA formation.

Generally speaking, the level of steady-state circRNA expression in cells can be regulated at three levels (Figure 2A). First, regulation of circRNA biogenesis initiates from and is coupled with the transcription of circRNA-producing pre-mRNA by Pol II. Second, *cis* and *trans* regulatory factors can further

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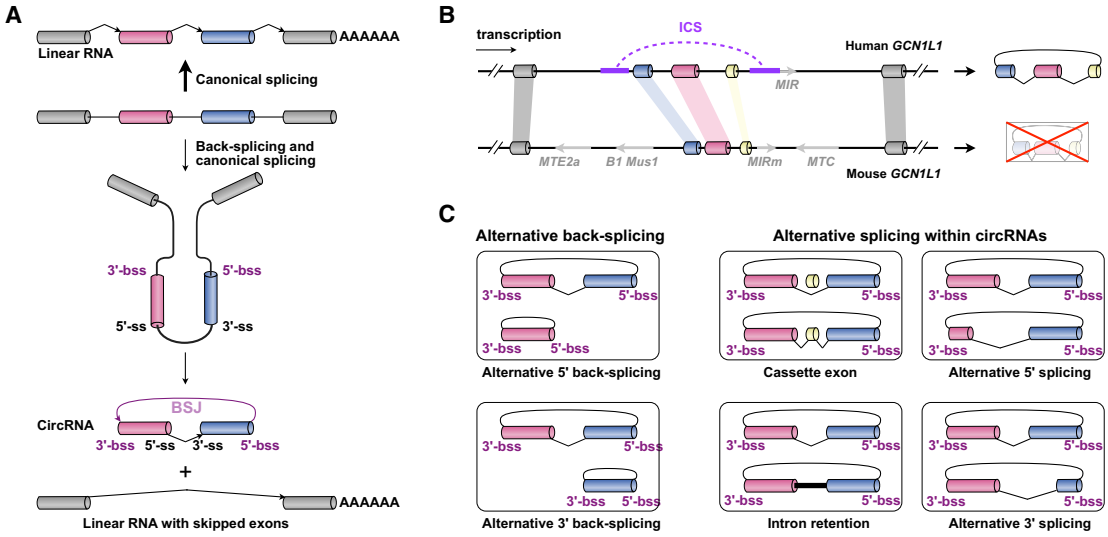

Figure 1. Complexity of circRNA Expression

(A) Back-splicing for circRNA biogenesis. Pre-mRNA undergoes either canonical splicing to generate a linear RNA (top) or back-splicing with low efficiency to produce a circRNA and an alternatively spliced linear RNA with exon exclusion (bottom). ss, splice site; bss, back-splicing site.

(B) Conservation of circRNA expression. Although the conservation of circRNA-forming exonic sequences in the GCN1L1 locus is high between human and mouse, circGCN1L1 is clearly detected in humans but barely in mice, largely because of the existence of intronic complementary sequences (ICSs) in the human but not the mouse locus.

(C) Alternative back-splicing and alternative splicing in circRNAs. There are two types of alternative back-splicing and four basic types of alternative splicing in circRNAs. See text for details.

influence the efficiency of back-splicing, which is catalyzed by spliceosomal machineries. These factors include ICSs flanking circle formation exons, core spliceosomal components, and other regulatory RBPs. Third, circRNA turnover also plays a role in their expression levels.

Coupling Back-Splicing with Pol II Transcription

It is known that Pol II transcription and pre-mRNA processing are tightly coupled, and the transcription elongation rate is an important factor in determining the outcome of splicing events (Bentley, 2014). A recent study began to uncover the link between parental gene transcription elongation, pre-mRNA splicing, and circRNA back-splicing at individual gene loci by using metabolic tagging of newly transcribed RNAs with 4-thiouridine (4sU) to quantitatively measure circRNA processing kinetics globally in human cells (Figures 2A and 2I; Zhang et al., 2016b).

Back-splicing of circRNA-forming exons could occur both co- and post-transcriptionally. On one hand, some abundantly expressed nascent circRNA formation events were detected co-concurrently with Pol II transcription. This observation was in agreement with an earlier study showing that some circRNAs were found to be associated with chromatin by measuring the relative abundance of circRNAs in chromatin-bound RNA from fly heads and that flies having a slow polymerase have less circRNAs (Ashwal-Fluss et al., 2014). Interestingly, it was also found that the average Pol II transcription elongation rate (TER) of nascent circRNA-producing genes is higher than that of non-circRNA genes (Zhang et al., 2016b). Similar to alternative splicing (Braunschweig et al., 2013), a relatively modest increase or decrease in TER had a measurable effect on circRNA formation (Zhang et al., 2016b). Because back-splicing is facilitated by RNA pairing across introns (Jeck et al., 2013; Liang and Wilusz, 2014; Starke et al., 2015; Zhang et al., 2014), such a positive correlation indicates that fast elongation may allow non-sequential complementary sequences across introns (rather than within introns) to pair, facilitating back-splicing. It is also possible that the parental gene TER may influence alternative back-splicing selection by perturbing the competition of putative RNA pairs across different sets of intron pairs. Alternatively, higher processivity might result in less efficient canonical splicing (or read-through polyadenylation signal [PAS] signal) (Bentley, 2014) and, hence, increased back-splicing (Liang et al., 2017).

On the other hand, a significant proportion of nascent circRNAs were detected only after transcriptional completion of their host pre-mRNAs (Zhang et al., 2016b), suggesting that circularization largely occurs post-transcriptionally. This view was also in line with studies of individual circRNA-producing genes. For example, mutation of the polyadenylation signal of circRNA-producing linear genes in manipulatable vectors could eliminate circRNA production, suggesting that circularization in expression vectors occurs post-transcriptionally (Liang and Wilusz, 2014). Furthermore, although a functional 3′ end processing signal is required for the generation of upstream circRNAs (Liang and Wilusz, 2014), suppression of co-transcriptional 3′ end processing resulted in enhanced circular RNA levels from reporter plasmids and several endogenous loci because of increased readthrough transcription past the poly(A) signals by Pol II (Liang et al., 2017).

Regulation of circRNA Biogenesis by the Core Spliceosomal Machinery

Mutagenesis analyses in vectors that express circRNAs have shown that exon circularization efficiency was dependent on the presence of canonical ss bracketing circRNA-producing

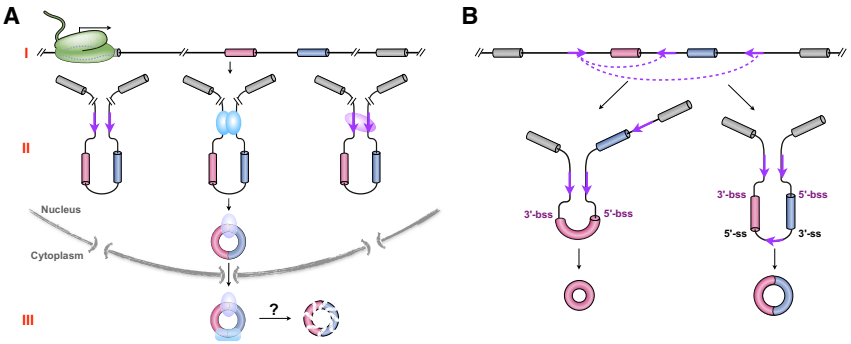

Figure 2. Regulation of circRNA Expression
(A) Regulation of the steady-state level of circRNA expression. Endogenous circRNA expression is regulated at three major levels, including precursor RNA transcription (I), post- or co-transcriptional processing (II), and turnover (III).
(B) The competition of ICSs leads to alternative back-splicing for multiple circRNAs in a single gene locus.

exons (Ashwal-Fluss et al., 2014; Starke et al., 2015; Wang and Wang, 2015). Although only the steady-state level of circular and linear RNAs was examined in most studies, it was readily seen that the efficiency of back-splicing was lower than that of canonical splicing from these expression vectors. Analysis of nascent circRNAs has further confirmed inefficient back-splicing at most endogenous loci in human cells (Zhang et al., 2016b). The low catalytic efficiency was likely due to the sterically unfavorable ligation of a downstream 5′ ss with an upstream 3′ ss by the spliceosome. Despite inefficiency, how do cells modulate the relative amounts of circular versus linear RNAs that are expressed from one gene locus? Using minigene expression plasmids based on the *Drosophila melanogaster laccase2* gene, combined with RNAi screening, a recent study has found that depletion of many core spliceosomal components, including the SF3b and SF3a complexes, resulted in preferred circRNA expression (Liang et al., 2017). This suggested a shift from canonical splicing to back-splicing under spliceosome starving conditions by depleting spliceosomal factors or chemically inhibiting spliceosomal activities (Liang et al., 2017). In addition to core spliceosomal factors, in human cells, a genome-wide siRNA screen has identified that a group of splicing regulators are required for circRNA expression (Li et al., 2017). Although these recent studies have provided new insights into differences between back-splicing and canonical splicing, how exactly the spliceosome is involved in back-splicing still remains incompletely understood. *In vitro* back-splicing assays carried out in nuclear extracts may provide additional mechanistic insights into back-splicing circularization.

Regulation of circRNA Biogenesis by cis Elements

In general, there is no specific exonic motif required for circularization beyond ss, but most endogenous human circRNAs comprise several exons, usually two or three (Zhang et al., 2014). Back-splicing often requires regulatory elements residing in introns flanking circularized exons. Most circRNAs in mammals (Jeck et al., 2013; Zhang et al., 2014) and worms (Ivanov et al., 2015) are processed from internal exons with long flanking introns usually containing ICSs (Figure 2A, II). RNA pairing formed across introns that flank back-spliced exons is expected to bring the distal ss into close proximity to facilitate circRNA biogenesis (Jeck et al., 2013; Liang and Wilusz, 2014; Zhang et al., 2014). Correspondingly, eliminating RNA pairs significantly reduces and, sometimes, even removes circRNA production, as revealed by mutagenesis analysis in circRNA expression vectors (Liang and Wilusz, 2014; Zhang et al., 2014, 2016a) as well as at the endogenous gene locus (Zhang et al., 2016b). For example, when the intronic RNA pairing was disrupted by removal of one ICS flanking the *circGCN1L1*-forming exons by CRISPR/Cas9, *circGCN1L1* was completely lost in human cells (Zhang et al., 2016b). Furthermore, the identification of fusion-circRNAs (f-circRNAs) that are derived from aberrant chromosomal translocations in cancers also supported the view that intronic RNA pairing across circle-forming exons is critical for enhanced circRNA formation (Guarnerio et al., 2016). *f-circM9* is produced from the *MLL/AF9* fusion gene that joins *MLL* gene exon 1–8 with *AF9* gene exon 6–11. After translocation, the originally in *trans* intronic sequences of the *MLL* and *AF9* genes are juxtaposed in *cis*, which subsequently facilitates circRNA generation by forming newly paired ICSs flanking the translocation breakpoint (Guarnerio et al., 2016).

RNA pairs are often derived from repetitive elements, such as *Alu*s in primates, which pair to form inverted repeated *Alu*s (IR*Alu*s) (Jeck et al., 2013; Zhang et al., 2014) but sometimes form from non-repetitive complementary sequences (Zhang et al., 2014; Liang and Wilusz, 2014). Short repeats (~30- to 40-nt) are also sufficient to induce circRNA formation in expression vectors (Liang and Wilusz, 2014). In humans, *Alu* elements are highly abundant and constitute 11% of the reference human genome, and nearly half of the *Alu* elements are located in human introns (Lander et al., 2001). A recent analysis has indicated that repetitive elements are responsible for most circRNA formation in human (Dong et al., 2017).

RNA pairing can regulate the dynamics of circRNA expression at multiple levels. RNA pairing formed across flanking introns generally enhances circRNA formation (Ashwal-Fluss et al., 2014; Dong et al., 2017; Jeck et al., 2013; Liang and Wilusz, 2014; Starke et al., 2015; Zhang et al., 2014). Furthermore, RNA pairing within individual introns generally facilitates canonical splicing in linear RNA, competing with RNA pairing across flanking introns and leading to reduced circRNA formation from the same gene locus (Ashwal-Fluss et al., 2014; Kelly et al., 2015; Zhang et al., 2014). Moreover, multiple RNA pairs can be formed in a single gene locus across different sets of introns flanking distinct circRNA-forming exons. Competition of these putative RNA pairs across different sets of flanking introns can lead to alternative back-splicing from the same gene (Figure 2B; Zhang et al., 2014, 2016a). How the distinct pattern of alternative (back-) splicing between circRNAs and linear RNAs is regulated and whether alternatively spliced circRNAs retain any functions remains to be studied.

Of note, because repetitive elements are preferentially enriched in mammals during evolution (Lander et al., 2001), the flanking intronic complementarity may not be a critical feature for circRNA formation in lower organisms, such as *Drosophila melanogaster* (Dong et al., 2017; Westholm et al., 2014) and *Oryza sativa* (Lu et al., 2015). For instance, abundant circRNAs in the fruit fly are preferentially generated from exons with long flanking introns like those in mammals (Westholm et al., 2014). However, the great majority of circRNA formation in fruit fly seems not to be driven by flanking ICSs (Westholm et al., 2014), although RNA pairing by inverted DNAREP1_DM family transposons promotes circRNA production in the *Drosophila melanogaster Laccase2* gene locus (Kramer et al., 2015). Furthermore, circRNAs were also detected in expression vectors in human cells when the inserted flanking intronic region was as short as 20 nt in length or had no inverted repeats (Starke et al., 2015). This indicated that other mechanisms or regulatory elements for circRNA biogenesis could exist. For example, circular RNAs were found to be produced through an exon-containing lariat precursor in *Schizosaccharomyces pombe* (Barrett et al., 2015).

Regulation of circRNA Biogenesis by RBPs

Despite having the same *cis* elements, the expression levels of circRNAs from the same loci differ between cell lines and tissues (Rybak-Wolf et al., 2015; Salzman et al., 2013; Starke et al., 2015; Zhang et al., 2016a), suggesting the involvement of *trans* factors in circRNA regulation (Figure 2A, II).

Double-stranded RBPs (dsRBPs) can facilitate circRNA production by binding to and then probably stabilizing back-splicing, transiently formed intronic paired RNAs across circle-forming exons. The immune factors NF90 and/or NF110, each of which contains two dsRNA binding domains (dsRBDs), promote circRNA formation in general by directly binding to IR*Alu*s formed in nascent pre-mRNA (Li et al., 2017). In the endogenous NF90 knockdown condition, re-introduction of wild-type NF90, but not NF90 mutants with dsRBD truncations, rescued circRNA expression, further supporting that both dsRBDs of NF90 are required for circRNA production (Li et al., 2017). However, dsRBPs can also inhibit circRNA formation by destabilizing RNA pairing. The enzyme adenosine deaminase 1 acting on RNA (ADAR1) suppresses circRNA expression by A-to-I editing of RNA pairs flanking circularized exon(s), which diminishes the complementarity and stability of these RNA pairs (Ivanov et al., 2015; Rybak-Wolf et al., 2015). Another example is DHX9, which is a nuclear RNA helicase containing both a dsRBD and an RNA helicase domain (Aktaş et al., 2017). Loss of DHX9 led to an increase in the amount of circRNAs in general. Because DHX9 can bind to IR*Alu*s and possesses RNA helicase activity, it was speculated that DHX9 may inhibit circRNA expression by unwinding RNA pairs flanking circularized exon(s). Interestingly, there seems to be a functional link between DHX9 and ADAR1 regulation of circRNA, possibly because of their association with *Alu*s (Aktaş et al., 2017). Additional RNA helicases and dsRBPs have been identified as potential regulators in circRNA production in a genome-wide screen with an efficient circRNA expression reporter containing flanking ICSs (Li et al., 2017). Their detailed mechanisms of action still need to be explored.

RBPs without a dsRBD domain can also participate in the regulation of circRNA levels by directly binding to specific RNA motifs. For instance, many circRNAs are upregulated during human EMT via the splicing factor Quaking (QKI), which lacks a dsRBD (Conn et al., 2015). QKI was found to enhance circRNA formation by binding to its consensus target single-stranded RNA (ssRNA) motif in introns flanking circRNA-forming exons. Consequently, insertion of synthetic QKI-binding sites into introns was sufficient to produce circRNAs. Because QKI forms dimers, it was suggested that QKI could bring the circularized exons closer together via dimerization, resulting in augmented circRNA formation (Conn et al., 2015). Other splicing factors have been found to regulate back-splicing in different biological settings. FUS was found to regulate circRNA generation by binding introns flanking back-splicing junctions in mouse embryonic stem cell (ESC)-derived motor neurons (Errichelli et al., 2017). The heterogeneous nuclear ribonucleoprotein L (HNRNPL) is involved in circRNA regulation in human prostate cancer (Fei et al., 2017). RNA-binding motif protein 20 (RBM20) is critical for the formation of a subset of circRNAs originating from the *titin* gene, which is known to undergo complex alternative splicing in mammalian hearts (Khan et al., 2016). Splicing factor-regulated back-splicing events also occur in other species. In *Drosophila melanogaster*, the splicing factor Muscleblind (Mbl) regulates circRNA production from its own pre-mRNA by binding to multiple Mbl-binding sites in introns flanking the circularized exon in the *Mbl* pre-mRNA (Ashwal-Fluss et al., 2014).

In addition to the regulation of circRNA production in cells by individual RBPs, back-splicing of the *Drosophila melanogaster Laccase 2* gene is regulated by both intronic repeats and multiple hnRNP (heterogeneous nuclear ribonucleoprotein) and SR (Ser/Arg) proteins functioning in a combinatorial manner (Kramer et al., 2015). Of note, the complicated regulation of circRNA by *trans* factors is consistent with the observation that circRNA formation largely occurs post-transcriptionally (Liang and Wilusz, 2014; Zhang et al., 2016b).

Together, these recent findings reveal that circRNA production is highly dependent on biological settings and tightly regulated in cells using different *cis* elements and *trans* factors that are specific for back-splicing.

Turnover of circRNAs

Circular RNAs are speculated to be stable because of their circular structures being resistant to degradation by most RNA decay machineries. Although back-splicing is inefficient, some circRNAs can accumulate to high levels post-transcriptionally (Zhang et al., 2016b). For example, examined circRNAs in PA1 cells gradually accumulated within 16 hr, whereas their corresponding linear transcripts had an average half-life of 8 hr (Zhang et al., 2016b). Another study has shown that the median half-life of 60 circRNAs in mammary cells was 18.8–23.7 hr compared with 4.0–7.4 hr for their corresponding linear transcripts (Enuka et al., 2016). The steady-state levels of circRNAs were observed to be positively correlated with their nascent levels in cells with similar mitotic cycles, indicating that the detection of steady-state circRNAs in a cell- and/or tissue-specific manner likely reflects the endogenous synthesis of circRNAs: the more nascent circRNAs are produced, the higher the steady-state levels of circRNAs detected (Conn et al., 2015; Salzman et al., 2013; Starke et al., 2015; Zhang et al., 2016b). Interestingly, in cells with a slow division rate, such as neurons and aging neural

tissues, highly expressed circRNAs are the dominant RNA isoforms produced from some genes (Liang et al., 2017; Rybak-Wolf et al., 2015; Westholm et al., 2014; You et al., 2015; Zhang et al., 2016b). Such a high expression of circRNAs in the brain is independent of their linear transcripts (Rybak-Wolf et al., 2015; You et al., 2015), likely because of discrepant decay rates observed between circRNAs and their linear counterparts (Zhang et al., 2016b).

How circular RNAs are ultimately degraded remains poorly understood (Figure 2A, III). One hypothesis is that endonucleases may initiate cleavage of circRNAs, but such enzymes have not yet been identified. Alternatively, circRNA binding by miRNAs may initiate circRNA decay by Ago2-mediated cleavage. Degradation of the circRNA *CDR1as* was shown to be dependent on miR-671-mediated Ago2 cleavage (Hansen et al., 2011). The miR-671 binding site on *CDR1as* is perfectly complementary to this microRNA (miRNA) and also conserved among vertebrates, indicating that miR-671-mediated *CDR1as* degradation is an evolutionarily conserved mechanism for *CDR1as* turnover (Hansen et al., 2011). However, it should be noted that conserved miRNA target sites on circRNAs can also be miRNA lodging sites for transport when these sites are only partially complementary to miRNAs (Hansen et al., 2013; Memczak et al., 2013; Piwecka et al., 2017; You et al., 2015). Although the perfect complementarity causes Ago2 cleavage (Hansen et al., 2011), whether other circRNAs undergo similar miRNA-mediated cleavage remains unknown. Finally, it has been recently shown that N^6-methyladenosines (m^6A), one of the most abundant internal RNA modifications that occur on adenosine bases, is widely distributed on circRNAs (Zhou et al., 2017). m^6A-modified circRNAs were found to be frequently derived from exons that are not methylated in mRNAs, whereas mRNAs that are methylated on the same exons that compose m^6A circRNAs exhibit less stability, indicating a link between m^6A circRNAs to their corresponding linear mRNA stability (Zhou et al., 2017). However, whether m^6A modification could affect the stability of circRNAs remains unclear. Future studies are warranted to uncover the mechanism of circRNA turnover.

Functional Implications of circRNAs

Although the generally low expression of circRNAs suggests the possibility that they are spurious members of the eukaryotic transcriptome, merging studies have begun to reveal that at least some circRNAs play potentially important roles in physiological and pathological conditions by distinct modes of action at the molecular level.

Emerging Roles of circRNAs in Physiological and Pathological Conditions

circRNAs are preferentially back-spliced from neural genes (Ashwal-Fluss et al., 2014; Westholm et al., 2014) and accumulate to high levels in the mammalian brain (Rybak-Wolf et al., 2015; You et al., 2015), neuronal cell lines (Rybak-Wolf et al., 2015; Zhang et al., 2016b), and aging neural tissues in flies (Westholm et al., 2014). Many circRNAs are also upregulated during neurogenesis (Rybak-Wolf et al., 2015; Zhang et al., 2016b) and are significantly more enriched in synaptogenesis than their linear isoforms (You et al., 2015). Although the high expression of circRNAs in the brain is largely attributed to their post-transcriptional accumulation, expression of some brain-specific circRNAs is conserved from human to mouse and even fly (Rybak-Wolf et al., 2015), indicating that they have potential neuronal functions. This view has been recently supported by the generation of *Cdr1as* knockout mice that showed dysfunction of excitatory synaptic transmission associated with neuropsychiatric disorders (Piwecka et al., 2017).

circRNAs have also been implicated in cancer development. Hundreds of circRNAs are regulated during human EMT, indicating that certain circRNAs may affect EMT-related cellular functions, such as migration, invasion, and cancer metastasis (Conn et al., 2015). A global reduction of circular RNA abundance in colorectal cancer cell lines and a negative correlation of global circular RNA abundance and proliferation were reported (Bachmayr-Heyda et al., 2015). A more recent study has further shown that reducing *circPVT1* levels in proliferating fibroblasts could trigger senescence (Panda et al., 2017a). Moreover, circRNAs have also been implicated in the etiology of acute promyelocytic leukemia (APL). *PML/RARα*, the most recurrent translocation in patients with APL, induces the formation of cancer-specific f-circRNAs that can promote transformation and cell survival (Guarnerio et al., 2016).

circRNAs are associated with innate immune responses. Transfection of *in vitro*-generated circRNA into mammalian cells led to potent induction of innate immunity genes that confer protection against viral infection (Chen et al., 2017b). How the exogenous circRNA elicits an immune response remains unclear, but it has been shown that endogenous circRNAs are associated with a diverse set of RNA-binding proteins that allow cells to distinguish self from non-self circRNAs (Chen et al., 2017b). In addition, the expression level of endogenous circRNAs can be modulated by immune factors such as NF90/ NF110, and reduced circRNA formation and decreased association of circRNAs with NF90 and/or NF110 were found to be involved in viral infection (Li et al., 2017). It was also recently reported that a highly expressed circRNA, *cia-cGAS*, could protect dormant long-term hematopoietic stem cells (LT-HSCs) from cGAS-mediated exhaustion to avoid autoimmunity (Xia et al., 2018).

Despite the above-mentioned biological settings, additional circRNAs derived from important genomic loci have been reported to be associated with human diseases. For example, the mouse testis-specific circRNA sex-determining region Y (*circSry*) is a most abundant transcript in the testis and is associated with testes development (Capel et al., 1993; Hansen et al., 2013). circRNAs produced from the *ANRIL* locus on chromosome 9p21 are positively correlated with atherosclerosis risk (Burd et al., 2010). *Cdr1as*, one of the best-known circRNAs, acts as a sponge for miRNA-7 in neuronal tissues (Hansen et al., 2013; Memczak et al., 2013) and is dramatically reduced in sporadic Alzheimer's disease (AD) (Lukiw, 2013), indicating a mis-regulated "ciRS-7-miRNA-7" system in AD. Finally, a group of circRNAs were found to be regulated by FUS in an amyotrophic lateral sclerosis (ALS)-associated cellular model, indicating yet another potential link of circRNA to neuronal diseases (Errichelli et al., 2017).

The Processing of circRNAs Naturally Affects Splicing of Their Linear Cognates

Because most circRNAs are derived from middle exons of protein-coding genes (Zhang et al., 2014), the processing of

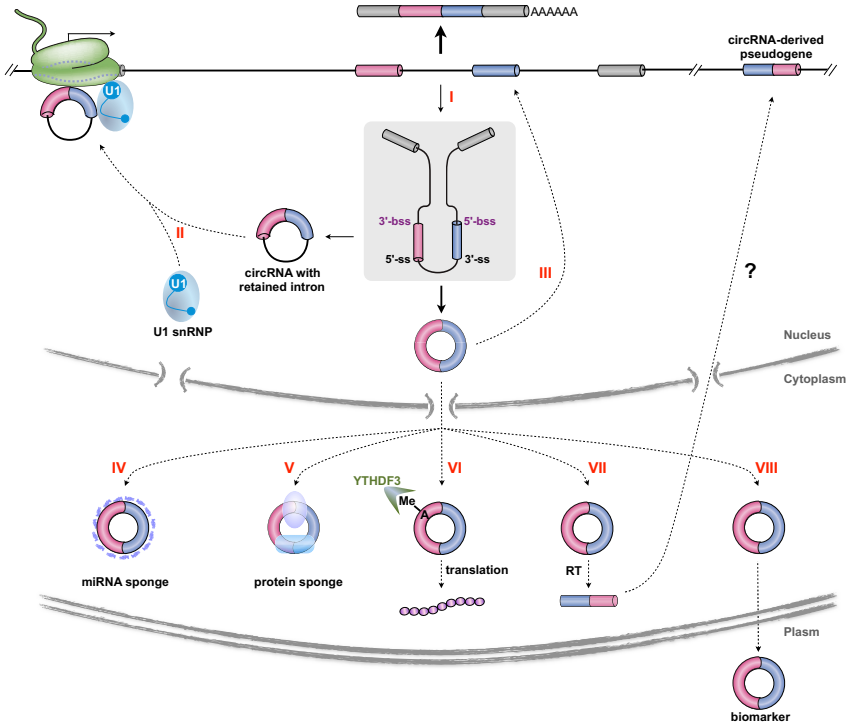

Figure 3. Potential Functions of circRNAs
Endogenous circRNAs are involved in gene expression regulation through distinct mechanisms. I: the processing of circRNAs affects splicing of their linear mRNA counterparts. II: circRNAs can regulate transcription of their parental genes. III: circRNAs can regulate splicing of their linear cognates. IV: circRNAs can act as miRNA sponges. V: circRNAs can act through associated proteins. VI: circRNAs can be translated. VII: circRNAs are resources for derivation of pseudogenes. VIII: circRNAs are promising biomarkers. See text for details.

circRNAs can affect splicing of their precursor transcripts, leading to altered gene expression outcomes (Figure 3, I). Although back-splicing is less favorable than normal splicing, it has been shown that the usage of 5′ and 3′ ss in circRNA biogenesis can compete with pre-mRNA splicing, resulting in lower levels of linear mRNAs with exon inclusion (Ashwal-Fluss et al., 2014; Kelly et al., 2015; Zhang et al., 2014). In general, the more an exon is circularized, the less it presents in the processed mRNA (Kelly et al., 2015). However, not all skipped exons could produce circRNAs (Kelly et al., 2015), suggesting that additional regulators could affect exon circularization or skipping in linear isoforms. It will be of interest to identify the degree to which exon circularization correlates with exon-skipped splicing in endogenous conditions as well as whether such events would result in observable biological effects.

Nuclear Retained Circular RNAs Can Regulate Transcription and Splicing

Although most circular RNAs are located in the cytoplasm (Jeck et al., 2013; Salzman et al., 2012), circular RNAs produced from processed intron lariats (ciRNAs) (Zhang et al., 2013; Box 1) or from back-splicing with retained introns (EIciRNAs) (Li et al., 2015b) are restricted in the nucleus in human cells.

Nuclear retained circRNAs were found to be involved in transcription regulation (Figure 3, II). Knocking down a couple of EIciRNAs could reduce the transcription of their parent genes. EIciRNAs could interact with U1snRNPs (U1 small nuclear ribonucleoproteins) and the EIciRNA-U1 snRNP complexes associate with Pol II at the promoters of their parent genes to enhance gene expression (Li et al., 2015b). Blocking such RNA-RNA interactions impaired the interaction of EIciRNAs with Pol II and, subsequently, reduced the transcription of their parental genes (Li et al., 2015b). It remains to be explored whether additional nuclear retained circRNAs could act in a similar manner.

CircSEP3 is a nuclear retained circRNA derived from exon 6 of *SEPALLATA3* (*SEP3*) in *Arabidopsis* and has been reported to regulate splicing (Figure 3, III) of its linear counterpart, a homeotic MADS box transcription factor required for floral homeotic phenotypes (Conn et al., 2017). *CircSEP3* bound strongly to its cognate DNA locus, forming an RNA:DNA hybrid, whereas the linear RNA with the same sequence bound to the DNA much more weakly. Presumably, such circRNA:DNA formation resulted in transcriptional pausing, leading to the formation of alternatively spliced *SEP3* mRNA with exon skipping. These studies together suggest that some nuclear localized circular RNAs can modulate gene expression at both transcription and splicing levels (Conn et al., 2017; Li et al., 2015b; Zhang et al., 2013).

circRNAs Can Act as miRNA Sponges

It has been proposed that the competing endogenous RNAs (ceRNAs) can act as sponges for miRNAs through their binding sites and that changes in ceRNA abundance from individual genes can modulate the activity of miRNAs on other target genes (Salmena et al., 2011). Recent studies have shown that several abundant circRNAs can function as miRNA sponges (Figure 3, IV).

CDR1as is a single-exon circularized, highly conserved, and abundant circRNA in the mammalian brain (Hansen et al., 2011, 2013; Memczak et al., 2013). *CDR1as* contains over 60 binding sites for miR-7. Decreased expression of *CDR1as* caused reduced expression of mRNAs containing miR-7 binding sites, suggesting that *CDR1as* competes for miR-7 targeting as miR-7 sponges to participate in the gene expression network (Hansen et al., 2013; Memczak et al., 2013). In mice, *Cdr1as* is highly expressed in excitatory neurons. *Cdr1as* knockout mice were viable and fertile but exhibited dysfunction of excitatory synaptic transmission associated with neuropsychiatric disorders (Piwecka et al., 2017). Interestingly, miR-7 expression was also decreased in *Cdr1as* knockout mice (Piwecka et al., 2017), which may contribute to the observed dysfunction of excitatory synaptic transmission with a different mechanism.

In addition to *CDR1as*, several other circRNAs in mammals have been suggested as potential miRNA sponges. For example, the testis-specific circRNA *circSRY* contains 16 target sites for miR-138 in the mouse (Hansen et al., 2013). *CircHIPK2* may act as a sponge for miR124-2HG to regulate astrocyte activation via cooperation of autophagy and endoplasmic reticulum (ER) stress (Huang et al., 2017). *CircBIRC6* was found to modulate human ES cell pluripotency and differentiation by sequestering miR-34a and miR-145 (Yu et al., 2017). Furthermore, many miRNA binding sites have been predicted in fly circRNAs (Westholm et al., 2014), but the regulatory roles of these RNA circles on their potentially bound miRNAs are largely unexplored. It should be noted that the majority of circRNAs in mammals are expressed at low levels and that they rarely contain multiple binding sites for the same miRNAs (Guo et al., 2014); thus, it seems unlikely that many circRNAs can function as miRNA sponges.

circRNAs Can Act through Associated Proteins

circRNAs can interact with different proteins to form specific circRNPs that subsequently influence modes of action of associated proteins (Figure 3, V). The multifunctional protein MBL could promote the biogenesis of *circMbl* that is produced from the same gene locus; MBL was also found to be associated with *circMbl* (Ashwal-Fluss et al., 2014). Thus, it has been speculated that there is a feedback loop between MBL and *circMbl* production. When the protein is in excess, it decreases the production of its own mRNA by promoting *circMbl* production. This circRNA could then sponge out the excess MBL protein by binding to it (Ashwal-Fluss et al., 2014). Such a model of action of circRNA was also observed in the highly expressed *circFOXO3* in the mammalian heart, which could promote cardiac senescence by enhancing its interaction with the anti-senescent protein ID-1 and the transcription factor E2F1 as well as the anti-stress proteins FAK and HIF1α (Du et al., 2017). Another example is the atherosclerotic cardiovascular disease-associated *circANRIL*. This circRNA bound to the essential 60S-pre-ribosomal assembly factor pescadillo homolog 1 (PES1) and suppressed ribosome biogenesis in vascular smooth muscle cells and macrophages, resulting in nucleolar stress and cell death, which are key cellular events in atherosclerosis (Burd et al., 2010; Holdt et al., 2016).

Despite these interesting findings, one general and unanswered question is to what extent lowly expressed circRNAs can confer detectable regulation on their sequestered or bound proteins. circRNAs can indeed act as a group to modulate protein function, which should be advantageous considering their low copy numbers in cells. For example, accumulation of intronic lariats likely acts to sequester TDP43 and suppress TDP43 toxicity in an ALS disease model (Armakola et al., 2012). A recent study has shown that many circRNAs together likely serve as a molecular reservoir for NF90 and/or NF110 in a sequence-independent manner in normal cells prior to viral infection (Li et al., 2017).

circRNAs Can Be Translatable

The vast majority of annotated circRNAs produced from back-splicing are primarily located in the cytoplasm (Jeck et al., 2013; Salzman et al., 2012), raising the intriguing question of whether they are translatable. Linear mRNA translation normally requires a 5′ end 7-methylguanosine (m^7G) cap structure and a 3′ poly(A) tail. Because circRNAs lack both caps and poly(A) tails, it has been proposed that translation of circRNAs should occur in a cap-independent manner. One way to achieve circRNA translation is via sequences that act as internal ribosome entry sites (IRESs) to promote direct binding of initiation factors or the ribosome to the translatable circRNAs. Engineered circRNAs can express proteins when containing IRESs (Chen and Sarnow, 1995; Conn et al., 2015; Li et al., 2017; Wang and Wang, 2015). Although most endogenous circRNAs have been reported not to associate with ribosomes for translation (Guo et al., 2014; Jeck et al., 2013), three recent studies have shown that a small subset of endogenous circRNAs are translatable (Legnini et al., 2017; Pamudurti et al., 2017; Yang et al., 2017; Figure 3, VI).

The human *circZNF609* was found to be regulated during muscle differentiation and plays a role in controlling myoblast proliferation. This circRNA encodes a protein, as evidenced by its association with heavy polysomes and translation of tagged circular transcripts from both vectors and the endogenous locus (Legnini et al., 2017), but it remains unknown whether this polypeptide produced from *circZNF609* contributes to myoblast proliferation. Furthermore, ribosome footprinting from fly heads revealed that a group of circRNAs was associated with translating ribosomes and that *circMbl* was able to produce a protein (Pamudurti et al., 2017). Of note, an IRES is embedded within *circMbl* and *circZNF609* to allow the cap-independent translation. Because ribosome-associated circRNAs use the same start codons as their hosting mRNAs (Legnini et al., 2017; Pamudurti et al., 2017), it raises the possibility that such circRNA-derived polypeptides may have similar functions or act as dominant-negative competitors to their mRNA-encoded proteins. In addition to IRESs, m^6A modification can also drive circRNA translation (Yang et al., 2017). circRNA translation derived from both a reporter and endogenous loci was promoted by m^6A modification, as evidenced by the fact that the translation efficiency was reduced by m^6A demethylase fat mass and obesity-associated (FTO) protein and promoted by the adenosine methyltransferase complex METTL3 and/or 14 (Yang et al., 2017).

Although the function of circRNA-encoded peptides remains unknown, circRNA translation was altered in cells under stress conditions. For instance, cellular starvation led to augmented circMbl translation (Pamudurti et al., 2017), and heat shock promoted the translation of GFP from the m^6A-containing circRNA plasmid (Yang et al., 2017). These observations imply that cap-independent circRNA translation may play a role under stress conditions. However, because only a small proportion of circRNAs are associated with polysomes (Guo et al., 2014; Jeck et al., 2013; Yang et al., 2017), and the initiation efficiency in a cap-independent manner is low, products translated from circRNAs may be limited.

Pseudogenes Derived from circRNAs

Pseudogenes are usually derived from the integration of reverse-transcribed (linear) mRNAs into their host genomes. Thousands of near-full-length processed pseudogenes have been estimated to be created from mRNAs that are located in about 10% of known gene loci in human and mouse (Zhang et al., 2003, 2004). Dozens of circRNA-derived pseudogenes have been identified by retrieving non-colinear back-splicing junction

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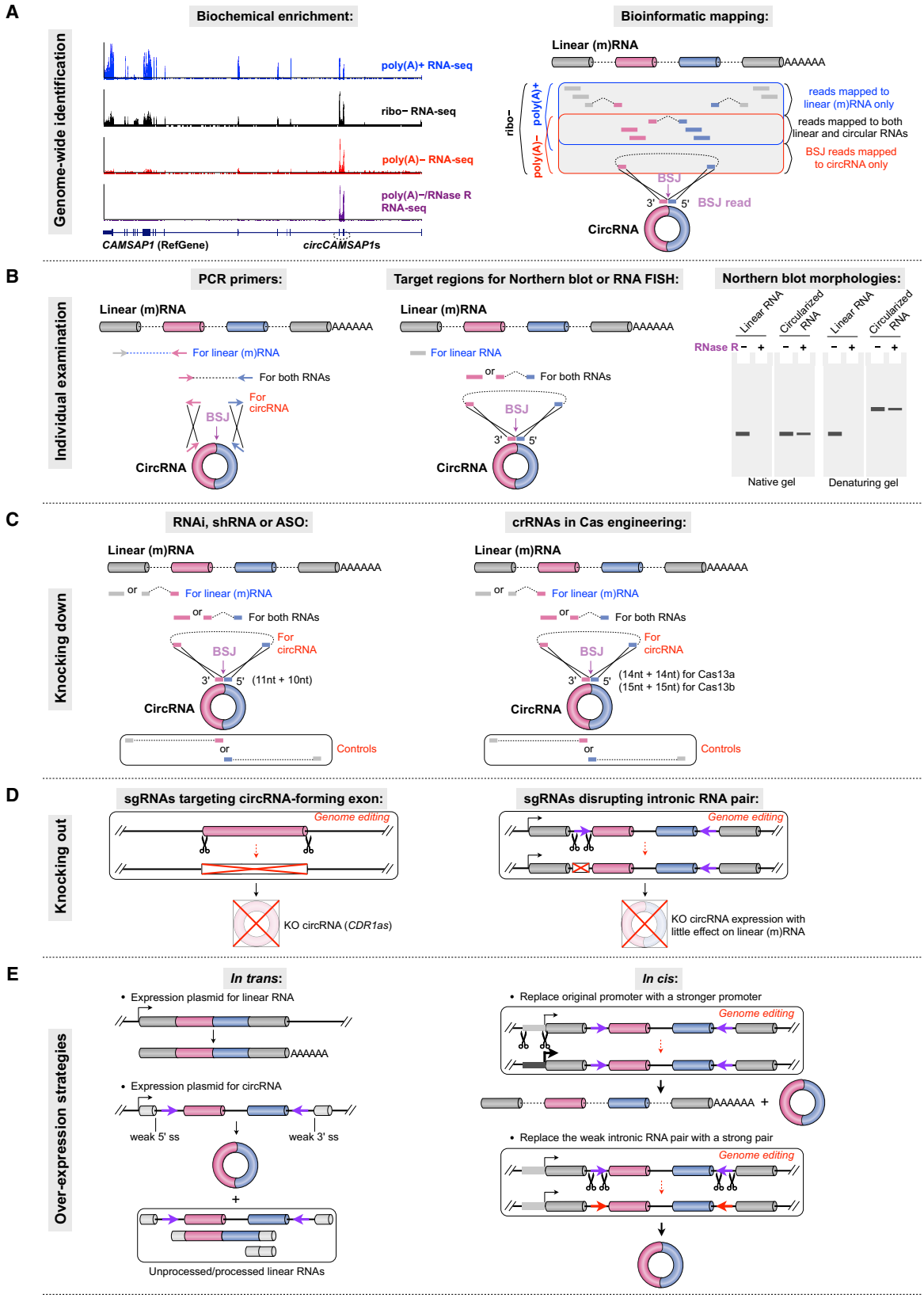

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sequences existing in both mouse and human reference genomes (Figure 3, VII; Dong et al., 2016). Among them, dozens of *circRFWD2*-derived pseudogenes were found in different mouse strains. The high density of long terminal repeats (LTRs) retrotransposon sequences in their flanking regions indicate that the retrotransposition processing of *circRFWD2* is associated with LTRs. Interestingly, the insertion of retrotransposed circRNAs could potentially interrupt host genome integrity. For example, the *circSATB1*-derived pseudogene locus overlapped with CCCTC binding factor (CTCF) and/or Rad21-binding sites in several mouse cell lines. Such CTCF binding was specific in the *circSATB1*-derived pseudogene region but not in its original *SATB1* region (Dong et al., 2016). The molecular mechanism of circRNA retrotransposition is unclear.

Circulating circRNAs as Biomarkers

The intrinsic circular characteristics render circRNAs unusually stable both inside cells and in extracellular plasma, including blood and saliva (Bahn et al., 2015; Li et al., 2015a; Memczak et al., 2015). circRNAs were also reported to be transported by exosomes from the cell body to extracellular fluid (Li et al., 2015a). Although it remains to be determined whether circRNAs could regulate gene expression at distant tissues and cells other than where they are produced, the existence of circulating circRNAs nevertheless suggests that disease-associated circRNAs are promising diagnostic biomarkers (Figure 3, VIII).

Current Approaches in circRNA Studies

So far, the functional implications of circRNAs have only been explored preliminarily, which is, at least in part, due to limitations of the tools used to study them. Because sequences of individual circRNAs fully overlap with their cognate linear RNA isoforms processed from the same pre-mRNAs, dissecting the functional significance of circRNAs has been a challenge. Resolving the contribution of a circRNA from its residing gene into an observable effect remains difficult. Here we discuss both existing and additional potential methods that can be used to address circRNA functions and their limitations.

Genome-Wide Annotation of circRNAs

Unlike most linear RNAs, circRNAs feature neither 5′ to 3′ polarity nor a 3′-poly(A) tail. This intrinsic characteristic has led to failed genome-wide identification of circRNAs in poly(A)$^+$ RNA-seq. Recently, distinct non-poly(A) RNA fractions were collected from total RNAs prior to RNA-seq profiling, enabling the discovery of widespread expression of circRNAs (Fan et al., 2015; Guo et al., 2014; Jeck et al., 2013; Memczak et al., 2013; Salzman et al., 2012; Westholm et al., 2014; Yang et al., 2011; Zhang et al., 2013, 2014). Specific RNA fractionations that allow circRNA characterization include rRNA-depleted total RNAs (ribo− RNA-seq), rRNA-depleted and non-poly(A) RNAs (p(A)− RNA-seq), and circRNA-enriched RNAs by RNase R, which digests linear RNAs and preserves circular RNAs (ribo− and RNase R or p(A)− and RNase R RNA-seq). Compared with ribo− RNA-seq, which profiles both poly(A) (linear) and non-poly(A) (circular) RNAs, p(A)− RNA-seq is better suited to examine poorly expressed circRNAs (Zhang et al., 2016a; Figure 4A, left) because overlapping sequences between circular and linear RNAs cannot be easily discriminated in ribo− RNA-seq. By contrast, most poly(A) RNAs are discarded during p(A)-RNA sample preparation prior to sequencing; thus, circRNAs can be specifically enriched in such samples.

Because circRNA sequences almost completely overlap with their cognate linear RNAs, genome-wide detection of circRNAs mainly depends on the identification of RNA-seq reads uniquely mapped to back-splice junctions (BSJs) (Figure 4A, right). A number of algorithms have been developed to globally detect circRNA expression from different RNA-seq datasets (Fan et al., 2015; Guo et al., 2014; Jeck et al., 2013; Memczak et al., 2013; Salzman et al., 2012; Zhang et al., 2014, 2016a). Most of these algorithms largely rely on mapping unique BSJs to locate circRNAs. Notably, different circRNA prediction outcomes among algorithms have been observed because of various strategies used for back-splicing prediction. Discrepancies can also be derived from the low expression of most circRNAs in examined samples and the low coverage of RNA-seq reads mapped to BSJs. Thus, circRNA annotation should be handled with care, and several algorithms should be combined to achieve reliable predictions (Hansen et al., 2016).

Experimental Validation of circRNAs

Considering the high false-positive rates of the available computational methods (Hansen et al., 2016), experimental approaches are required to validate results of computational prediction and select high-confidence circRNAs for further study. A simple method is to use divergent PCR to amplify speculated BSJ sites, followed by Sanger sequencing to confirm these sites (Figure 4B, left). "Divergent primers" flank the BSJ site, which are "tail-to-tail"-oriented toward the outside of the BSJ site compared with the regular

Figure 4. Approaches for circRNA Studies

(A) Biochemical enrichment and high-throughput methods for circRNA annotation. Compared with those in p(A)+ and ribo− RNA-seq, circRNAs are enriched in p(A)− RNA-seq, and additional RNase R treatment of the poly(A)− samples prior to RNA-seq can further enrich circRNAs (left). Also shown is a schematic drawing to illustrate an RNA-seq read that uniquely maps to the back-splicing junction (BSJ) site for circRNA annotation (right).

(B) Experimental validation of circRNAs. With distinct primers and probes, linear and/or circular RNAs can be further examined by (semiquantitative) PCRs after reverse transcription (left), by northern blot and by RNA fluorescence *in situ* hybridization (FISH) (center). Notably, circRNA migrates much more slowly than its linear counterpart by denaturing PAGE (right).

(C) Knockdown strategies for circRNAs. By targeting BSJ sites, circRNAs can be specifically knocked down by the RNAi machinery (left) or the RNA-guided RNA-targeting Cas13 system (right).

(D) knockout strategies for circRNAs. Shown are direct deletion of circRNA-forming exons by the CRISPR/Cas9 genome engineering system (left) and disruption of the pairing between intronic ICS flanking circRNA-forming exons to minimize circRNA generation without affecting the expression of residing protein-coding gene (right).

(E) Overexpression strategies for circRNAs. Similar to linear protein-coding transcripts, overexpression of circRNAs can be achieved in *trans* by overexpression plasmids (left). Of note, unprocessed and processed linear RNAs are inevitably co-expressed. Manipulation of the endogenous promoter with the CRISPR/Cas9 genome engineering system can lead to overexpression of both circular and linear RNAs from a gene locus in *cis* (top right). Theoretically, in *cis* overexpression of circRNAs can be specifically obtained by replacing the weak intronic RNA pair with a strong one (bottom right).

"convergent primers," which are "head-to-head"-oriented. Of note, a set of "convergent primers," when located within circRNA-producing exons, detects both circular and linear RNAs because of their sequence overlap. Another set of convergent primers, located within linear RNA-producing exons or spanning linear and circular RNA-producing exons, only detects linear RNAs (Figure 4B, left).

However, such PCR-based validation is not a guarantee of circRNA existence because any linear RNA with the same sequences as those across the BSJ site can be amplified by PCR. However, such signals could be generated by other mechanisms, including template switching by reverse transcriptase, tandem duplication, and *trans*-splicing (Chuang et al., 2018; Jeck and Sharpless, 2014; Shen et al., 2017).

A more direct and accurate way to confirm circRNA existence is using NB assays. A probe that targets sequences within circRNA-producing exons can detect both circular and linear RNAs; a probe that recognizes sequences in linear RNA-specific exons only detects linear transcripts (Figure 4B, center). By denaturing PAGE, circRNAs migrate much more slowly than their linear counterparts having the same length of nucleotides (Figure 4B, right; Memczak et al., 2013; Zhang et al., 2013, 2016c).

In both NB and PCR methods, isolated RNAs can be pretreated with RNase R to further validate the presence of circRNAs. Although some circRNAs were found to be unstable *in vitro* by RNase R digestion, and some abundant linear RNAs were still detectable even after prolonged RNase R treatment (Zhang et al., 2016c), isolation of highly pure circRNA populations has been achieved using RNase R treatment followed by polyadenylation and poly(A)$^+$ RNA depletion (RPAD) (Panda et al., 2017b).

Suppression Strategies for circRNAs

Loss of function (LOF) and gain of function (GOF) are commonly used to annotate a gene's function. Different approaches have been developed to target specific linear RNAs or their corresponding genomic locations (i.e., RNAi; Boutros and Ahringer, 2008) and CRISPR/Cas9-mediated genome editing (Shalem et al., 2015)]. Recent studies have applied these existing methods to deplete specific circRNAs in cells and animals; however, it remains a challenge to alter the level of circRNAs without affecting their residing genes.

First, RNAi-mediated degradation has been used to disrupt circRNA expression. It was originally found that *CDR1as* is associated with the Ago complex for its degradation in an miR-671-dependent manner (Hansen et al., 2011), suggesting that circRNAs can be digested by the Ago complex. To discriminate overlapping sequences between a circRNA and its cognate linear RNA, a specific small interfering RNA (siRNA) or short hairpin RNA (shRNA) must target the BSJ site uniquely present in the circRNA to achieve a circRNA-specific knockdown effect (Figure 4C, left). Such a requirement imposes a limitation in that it is impossible to design multiple RNAi molecules with distinct coverages to exclude potential off-target effects. Moreover, the partial complementarity of a half-RNAi sequence (~10 nt) to its cognate linear RNA may have an effect on the linear parental RNA expression. To tackle this shortcoming, strict control RNAi molecules with half-sequence (i.e., ~10 nt) replacements should be used to exclude an influence on linear RNAs (Figure 4C, left).

So far, existing methods appear to be inadequate to achieve specificity or high efficacy in targeting circRNAs, which may inevitably generate artifacts in interpreting experimental results. The recently developed RNA-guided, RNA-targeting Cas13 system (Abudayyeh et al., 2017; Cox et al., 2017) represents a promising tool for selective degradation of circRNAs. The Cas13 enzymes belong to the class 2 type VI CRISPR/Cas effectors. They have RNA cleavage activities and can degrade ssRNA targets when guided by CRISPR RNAs (crRNAs). Efficient Cas13 knockdown requires 28- to 30-nt long spacers and is intolerant to mismatches in spacers. Thus, crRNAs carrying spacers that specifically target and span the BSJ site, in principle, should be able to discriminate circular and linear RNAs (Figure 4C, right).

Strategies for circRNA knockout are similarly tricky. Linear mRNAs can be routinely depleted by the classical Cre-LoxP system or CRISPR/Cas9 tools to introduce out-of-frame mutations, which lead to un- or mis-translatable products for LOF at the protein level. However, such strategies theoretically will not work for circRNAs because most circRNAs do not encode functional proteins. Alternatively, circRNA knockout can be achieved by large fragment deletion by CRISPR/Cas9, but it will inevitably affect linear RNA expression because sequences of circRNAs totally overlap with linear RNAs. In this scenario, the knockout experiment with genome editing tools should be managed with caution. Nevertheless, knockout at the *CDR1as* locus has been successfully achieved by deleting the entire *CDR1as*-producing genomic region (Figure 4D, left), generating the first circRNA KO animal model (Piwecka et al., 2017). However, it should be noted that using such a strategy to study circRNA function at the *CDR1as* locus is more likely to be an exception than a general rule because *CDR1as* is the major RNA produced from this locus in most examined samples (Piwecka et al., 2017). Furthermore, even at the *CDR1* locus, *Cdr1as* is also embedded within a long noncoding RNA (Barrett et al., 2017). Moreover, complete removal of genomic sequences may have an effect on neighboring gene expression (Joung et al., 2017; Liu et al., 2017).

LOF of a circRNA may be achieved by targeting ICSs (Figure 4D, left) because back-splicing is dramatically enhanced by RNA pairing of ICSs (Liang and Wilusz, 2014; Zhang et al., 2014). Thus, removal of the intronic unilateral ICS by the CRISPR/Cas9 system to disrupt the formation of RNA pairing, in principle, was able to reduce circRNA expression or, in some circumstances, to completely knock out a circRNA. Nearly complete knockout has been achieved for *circGCN1LI* in human PA1 cells (Zhang et al., 2016b). However, as the regulation of RNA pairing by ICSs for circRNA formation is complicated, the targeted impairment of RNA pairing to suppress circRNA expression should be well designed and evaluated.

Finally because both knockdown and knockout methods can be combined for high-throughput screening (Abudayyeh et al., 2017; Boutros and Ahringer, 2008; Shalem et al., 2015), future studies with well-characterized RNAi and single guide RNA (sgRNA) or crRNA libraries designed for circRNAs may facilitate the process to annotate their functions.

Overexpression Strategies for circRNAs

Overexpression of a circRNA to obtain the GOF effect is also challenging. Similar to linear RNA overexpression, a number of plasmids containing circRNA-producing exons and their flanking intronic sequences with ICSs have been used to introduce circRNAs to cells by transfection (Figure 4E, left; Conn et al., 2015; Kramer et al., 2015; Li et al., 2017; Liang et al., 2017; Liang and Wilusz, 2014; Zhang et al., 2014, 2016a). Although back-splicing is more than 100-fold less efficient than canonical splicing reactions at the endogenous locus (Zhang et al., 2016b), well-designed circRNA expression vectors with appropriate ICSs could produce circRNAs at levels comparable with linear RNAs in cell lines (Li et al., 2017; Liang et al., 2017; Liang and Wilusz, 2014; Zhang et al., 2014) as well as *in vivo* in flies (Pamudurti et al., 2017). However, circRNA overexpression in *trans* from a plasmid construct is naturally accompanied by abundant pre- and mature linear RNA isoforms. Carefully designed circRNA expression vectors are needed to minimize the generation of linear RNAs, and additional sets of controls should be set up to separate the contribution of these RNA isoforms to measured effects. Recent studies have developed circRNA vectors without flanking exons to produce minimal linear RNAs (Kramer et al., 2015; Pamudurti et al., 2017).

Gene overexpression can also be accomplished in *cis*. Replacing the original weak promoter with a strong one by genome-editing tools would enhance RNA products, including both linear and circular ones (Figure 4E, top right). Such an in *cis* strategy provides a precise way to investigate the function of genes of interest (Xiang et al., 2014). However, both linear and circular RNAs would be increased after manipulating promoters of circRNA-producing genes. In theory, although it can be time-consuming, insertion of a perfect pair of ICSs across the circle-forming exons should be able to promote in *cis* overexpression of circRNAs (Figure 4E, bottom right).

Imaging circRNAs

Like proteins, functions of regulatory RNAs depend on their subcellular localization patterns, and circRNAs are no exception. However, imaging circRNAs by RNA fluorescence *in situ* hybridization (FISH) is difficult because their low copy numbers in cells and the largely indistinguishable signal interferences from their cognate linear RNAs (Figure 4B, center). To avoid this, RNA FISH combined with RNase R treatment has been used to impair linear RNA signals in fixed cells (Li et al., 2015b). However, because the majority of circRNAs are expressed at low levels compared with their linear isoforms, such an enrichment of circRNAs by RNase R may not be able to completely destroy linear RNAs and should be used with caution. Alternatively, catalytically inactive CRISPR/Cas9 (Batra et al., 2017) or Cas13 (Abudayyeh et al., 2017; Cox et al., 2017) systems for programmable RNA targeting combined with an enhanced fluorescent protein tagging system, such as SunTag, which can recruit up to 24 copies of GFP (Tanenbaum et al., 2014), represent additional future tools for circRNA visualization and tracking in living cells.

In sum, technological obstacles for understanding the physicochemical properties and mechanisms of action of circRNAs occur at multiple levels. Further challenges exist in assays to identify circRNA binding proteins. In such experiments, *in vitro*-circularized RNAs rather than linear RNAs with the same sequences should be used as bait for RNA precipitation in cell lysates (Chen et al., 2017b; Li et al., 2017). In addition to the above-mentioned concerns regarding circRNA studies, attentions that are generally applicable to many other experiments used to study linear regulatory RNAs (Chu et al., 2015) also apply to circRNAs. Future employment of improved experimental assays will be able to provide new insights into the regulation and function of circRNAs.

Perspectives

circRNAs' dynamic expression patterns, complicated regulatory networks, and emerging roles at multiple cellular levels together suggest that they are not simply byproducts of aberrant splicing but, rather, emerging regulatory RNA molecules. Despite these recent advances in our understanding of circRNA biogenesis and functions, many questions concerning their post-transcriptional regulation remain to be explored. For example, we lack an understanding of how they are ultimately degraded and how their structures might confer functional differences from their linear RNA counterparts. As the expression and functions of regulatory RNAs are often coupled and coordinated to some extent, an in-depth annotation of circRNA biogenesis and regulation will undoubtedly enhance our understanding of their functions. In addition, future studies of circRNAs in the nervous system, cancer development, innate immune response, and other biological settings and diseases will further unveil the mystery of circRNAs. Improvements in methods to study these RNA circles without affecting their residing genes will be the key to understanding what they do in cells.

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