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## Biogenesis and Regulatory Roles of Circular RNAs

Li Yang,<sup>1</sup> Jeremy E. Wilusz,<sup>2</sup> and Ling-Ling Chen<sup>3,4,5</sup>

<sup>1</sup>Center for Molecular Medicine, Children's Hospital, Fudan University and Shanghai Key Laboratory of Medical Epigenetics, International Laboratory of Medical Epigenetics and Metabolism, Ministry of Science and Technology, Institutes of Biomedical Sciences, Fudan University, Shanghai, China; email: liyang\_fudan@fudan.edu.cn

<sup>2</sup>Verna and Marrs McLean Department of Biochemistry and Molecular Biology, Therapeutic Innovation Center, Baylor College of Medicine, Houston, Texas, USA; email: jeremy.wilusz@bcm.edu

<sup>3</sup>State Key Laboratory of Molecular Biology, Shanghai Key Laboratory of Molecular Andrology, CAS Center for Excellence in Molecular Cell Science, Shanghai Institute of Biochemistry and Cell Biology, University of Chinese Academy of Sciences, Chinese Academy of Sciences, Shanghai, China; email: linglingchen@sibcb.ac.cn

<sup>4</sup>School of Life Science and Technology, ShanghaiTech University, Shanghai, China

<sup>5</sup>School of Life Science, Hangzhou Institute for Advanced Study, University of Chinese Academy of Sciences, Hangzhou, China

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### Keywords

backsplicing, circRNA, microRNA, noncoding RNA, pre-mRNA splicing, translation

### Abstract

Covalently closed, single-stranded circular RNAs can be produced from viral RNA genomes as well as from the processing of cellular housekeeping noncoding RNAs and precursor messenger RNAs. Recent transcriptomic studies have surprisingly uncovered that many protein-coding genes can be subjected to backsplicing, leading to widespread expression of a specific type of circular RNAs (circRNAs) in eukaryotic cells. Here, we discuss experimental strategies used to discover and characterize diverse circRNAs at both the genome and individual gene scales. We further highlight the current understanding of how circRNAs are generated and how the mature transcripts function. Some circRNAs act as noncoding RNAs to impact gene regulation by serving as decoys or competitors for microRNAs and proteins. Others form extensive networks of ribonucleoprotein complexes or encode functional peptides that are translated in response to certain cellular stresses. Overall, circRNAs have emerged as an important class of RNA



molecules in gene expression regulation that impact many physiological processes, including early development, immune responses, neurogenesis, and tumorigenesis.

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## INTRODUCTION

Unlike other well-known classes of RNAs that were originally named because of their function [e.g., messenger RNA (mRNA)], cellular location [e.g., small nucleolar RNA (snoRNA)], and/or size [e.g., long noncoding RNA (lncRNA)], circular RNAs were so designated because of their unique covalently closed structures. RNA circles without accessible 5'- and 3'-ends were first reported in plant viroids as rodlike structures that were observable on electron micrographs (Sanger et al. 1976). Since then, additional viroid circular RNA genomes have been identified as well as other distinct types of circular RNAs that are classified according to their mechanisms of production. These include circular transcripts that are generated from the processing of cellular noncoding sequences and those that are processed from eukaryotic precursor mRNAs (pre-mRNAs) in a spliceosome-dependent manner.

Pathogenic viroid circular RNA genomes have largely been identified in plants (Flores et al. 1999, Sanger et al. 1976), but hepatitis  $\delta$  virus (HDV), a satellite virus of hepatitis B virus, is also a circular RNA (Kos et al. 1986) (**Table 1a**). These transcripts replicate using rolling-circle RNA polymerization followed by cleavage catalyzed by either a host enzyme or embedded ribozyme sequences. The intermediate sequences are then ligated by host enzymes or intramolecular

**Table 1 A chronological view of circular RNA discoveries**

Different types of RNA circles	Methods to study RNA circles	Reference(s)
<b>A: Covalently closed viral circular RNA genomes</b>		
Circular viroid RNAs	Electrophoresis, thermal denaturation, and electron micrograph	Sanger et al. 1976
Circular RNA genome of hepatitis delta virus	Electron micrograph and Northern blotting	Kos et al. 1986
Self-cleavage and self-ligation of rolling viroid RNA circles in vitro	Primer extension, self-cleavage, and ligation	Côté & Perreault 1997
<b>B: Circular (intermediate) RNAs processed from noncoding sequences</b>		
Yeast mitochondrial RNA circles from cytochrome oxidase gene intron	Electrophoresis, electron micrograph, and RNA–DNA hybridization	Arnberg et al. 1980, Hensgens et al. 1983, Tabak et al. 1987
Circular RNA from self-spliced introns of <i>Tetrahymena</i> rRNA precursors	Electrophoresis, electron micrograph, and endonuclease digestion	Grabowski et al. 1981, Inoue et al. 1986
Circular RNA from introns of archaeal rRNA precursors	Northern blotting, sequencing, and in vitro splicing	Kjems & Garrett 1988
Circular RNA from introns of archaeal tRNA precursors	Northern blotting, in vitro ligation or analysis, and electrophoresis	Clouet d'Orval et al. 2001, Salgia et al. 2003
Various types of circular RNAs from archaeal noncoding transcriptomes	Northern blotting, RT-PCR, and electrophoresis	Tang et al. 2002, Danan et al. 2012
<b>C: Circular RNAs processed from RNA polymerase II-transcribed precursor RNAs (spliceosome dependent)</b>		
Circular RNA in HeLa cytoplasm with unknown origin and function	Electron micrograph	Hsu & Coca-Prados 1979
Abnormally spliced (scrambled) transcripts at consensus splice sites	PCR cloning, sequencing, and nonpolyadenylated RNA isolation	Nigro et al. 1991
Splicing of <i>ets-1</i> exons in an aberrant order at consensus splice sites	PCR and RNase protection	Cocquerelle et al. 1992
Circular transcripts of human <i>ets-1</i> by nuclear pre-mRNA missplicing	PCR and poly(A)-RNA sucrose gradient	Cocquerelle et al. 1993
Circular transcripts of mouse testis-determining gene <i>Sry</i>	RACE, RNase protection, and RNase H digestion	Capel et al. 1993
Inverted repeats required for mouse <i>Sry</i> transcript circularization	Northern blotting, RT-PCR, and molecular cloning	Dubin et al. 1995
Translation of circular RNAs by eukaryotic translational apparatus	In vitro circularization and translation	Chen & Sarnow 1995
Spliceosome-dependent <i>Sry</i> circular RNA formation and recapitulation	Assays of RACE, RNase protection, and RNase H digestion	Pasman et al. 1996

Abbreviations: cDNA, complementary DNA; pre-mRNA, precursor messenger RNA; RACE, rapid amplification of cDNA ends; RNase, ribonuclease; RT-PCR, reverse transcription polymerase chain reaction.

self-ligation to form additional copies of mature circular RNA genomes (Côté & Perreault 1997; Flores et al. 1999, 2011). Beyond viroids, circular RNAs are processed from a variety of noncoding sequences in cells, including the noncoding regions of mitochondrial RNAs, ribosomal RNAs (rRNAs), and transfer RNAs (tRNAs) [see review by Lasda & Parker (2014)]. For example, groups I and II self-splicing introns (Cech 1990, Nielsen et al. 2003) are processed to form circular RNAs in both bacteria and eukaryotes (Inoue et al. 1986, Li-Pook-Than & Bonen 2006, Molina-Sánchez et al. 2006, Tabak et al. 1987) (**Table 1b**). This includes the introns of rRNA precursors

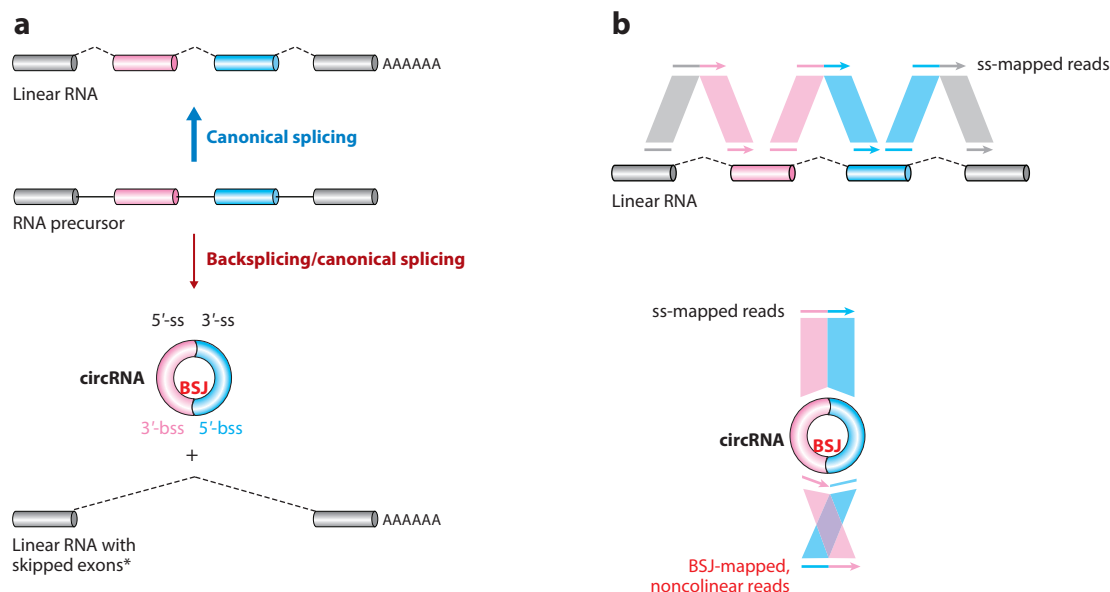
in the protozoa *Tetrahymena* (Grabowski et al. 1981) and the archaeobacterium *Desulfurococcus mobilis* (Kjems & Garrett 1988). Furthermore, the intron of tRNA<sup>Trp</sup> in the euryarchaeote *Haloflex volcanii* forms a stable RNA circle after being cleaved from precursor RNAs and ligated by an RNA ligase (Clouet d'Orval et al. 2001, Salgia et al. 2003, Singh et al. 2004). Circular RNAs have also found to be processed from some snoRNAs, ribonuclease (RNase) P RNA, and 7S RNA in archaea (Danan et al. 2012, Tang et al. 2002).

Another major group of circular RNA (and the focus of this review, named circRNA hereafter) is eukaryotic specific and is produced by the splicing of pre-mRNAs in a spliceosome-dependent manner [for reviews, please see Chen (2016, 2020); Kristensen et al. (2019); Lasda & Parker (2014); Li et al. (2018); Wilusz (2018); and Xiao et al. (2020)]. Eukaryotic circular transcripts were first detected by electron microscopy in the late 1970s, but their origin and function were unknown at that time (Hsu & Coca-Prados 1979) (**Table 1c**). Not until the 1990s was it realized that some of these detected transcripts likely represented exons derived from protein-coding genes that were covalently joined at consensus splice sites to form mature circles (Capel et al. 1993; Cocquerelle et al. 1992, 1993; Dubin et al. 1995; Nigro et al. 1991; Pasman et al. 1996; Zaphiropoulos 1996). Due to their generally low levels of expression (~1/100th the level of the normally spliced transcript generated from the host gene) (Capel et al. 1993, Cocquerelle et al. 1993, Dubin et al. 1995, Pasman et al. 1996, Zhang et al. 2016a), whether these RNA circles represented biological noise of pre-mRNA splicing reactions was obscure. Nonetheless, Capel et al. (1993) found that more than 90% of the transcripts derived from the mouse *Sry* gene, which determines sex in mammals, were circular in adult mouse testis. This was the first indication that circRNAs can be the predominant outputs of some protein-coding genes, suggestive of functionality.

The advent of deep sequencing technologies coupled to methods that enrich for polyadenylated RNAs (mRNA-seq) uncovered pervasive transcription in eukaryotes (Cloonan et al. 2008, Graveley 2008, Mortazavi et al. 2008, Pan et al. 2008, Wang et al. 2008, Wilhelm et al. 2008). However, the lack of a poly(A) tail on circular RNAs caused the expression signals of these transcripts to be limited in mRNA-seq studies (Burd et al. 2010). This situation turned around when deep sequencing of total RNA (depleted of rRNAs) or nonpolyadenylated RNAs came to the fore (Jeck et al. 2013, Memczak et al. 2013, Salzman et al. 2012, Yang et al. 2011). These approaches enabled the genome-wide identification of two major classes of eukaryotic circular RNAs that can be generated when the spliceosome acts on RNA polymerase II (Pol II) transcripts. Circular intronic RNAs (ciRNAs), also known as stable intronic sequence RNAs (sisRNAs), are generated when a subset of intron lariats fails to be debranched (Talhouni & Gall 2014, Zhang et al. 2013). In addition, it was determined that many exons can form circRNAs when pre-mRNAs are backspliced (Ashwal-Fluss et al. 2014, Hansen et al. 2013, Jeck et al. 2013, Liang & Wilusz 2014, Memczak et al. 2013, Salzman et al. 2012, X.-O. Zhang et al. 2014) (**Figure 1a**). Building off these initial observations, a plethora of studies have now characterized the expression patterns, biogenesis mechanisms, and function of these spliceosome-dependent circRNAs. Here, we summarize recent progress in this field that has revealed the tremendous regulatory potential of eukaryotic circRNAs produced from the backsplicing of pre-mRNA exons.

## GENOME-WIDE PROFILING OF CIRCRNAs

Because circRNAs are often coexpressed with their cognate linear (m)RNAs from their host gene loci, the primary sequences of the mature linear RNAs and circRNAs often fully overlap except for the unique backsplicing junction (BSJ) present in circRNAs (**Figure 1a**). To enable efficient circRNA profiling and characterization, it thus has been necessary to develop specific biochemical enrichment strategies for circRNAs as well as computational pipelines that identify sequencing



**Figure 1**

circRNAs can be produced from the backsplicing of precursor (m)RNAs. (*a, top*) An RNA precursor containing multiple exons can be subjected to canonical splicing, leading to the formation of a linear RNA with all exons included. (*Bottom*) In contrast, backsplicing and canonical splicing can lead to the formation of a circRNA with two exons, and an alternatively spliced linear RNA with skipped circle-forming exons. Note that the alternatively spliced linear RNA is only sometimes observed, as indicated by the asterisk. Panel *a* is adapted with permission from X. Li et al.; copyright 2018 Elsevier. (*b*) Short RNA-sequencing reads are mapped to the splicing junction sites of (*top*) a linear RNA and (*bottom*) to the BSJ site of a circRNA. The BSJ-mapped, noncolinear reads are critical for identification of circRNAs from transcriptomic studies. Abbreviations: BSJ, backsplicing junction; bss, backsplice site; circRNA, circular RNA from exon backsplicing; mRNA, messenger RNA; ss, splice site.

reads that specifically map to BSJs (Chen 2016, 2020; Jeck & Sharpless 2014; Kristensen et al. 2019; Szabo & Salzman 2016) (**Figure 1b**).

### circRNA Enrichment Strategies

circRNAs lack poly(A) tails and most are expressed at low levels, so different strategies have been applied to biochemically enrich circRNAs prior to performing short-read deep sequencing (Ma et al. 2021). At a minimum, abundant rRNAs must be depleted prior to sequencing, and this approach (ribo- RNA-seq) has revealed hundreds of circRNAs that are expressed at levels comparable to those of their cognate linear RNAs (Jeck et al. 2013, Memczak et al. 2013, Salzman et al. 2012). Ribo- RNA-seq (Jeck et al. 2013, Memczak et al. 2013, Salzman et al. 2012, X.-O. Zhang et al. 2016) allows a direct quantitative comparison between the expression levels of circRNAs and those of their cognate linear RNAs (Ma et al. 2019), as both nonpolyadenylated and polyadenylated RNAs are sequenced. To enrich for circRNAs, one can select for nonpolyadenylated RNAs by depleting both rRNAs and polyadenylated (m)RNAs (Yang et al. 2011). By comparing the data obtained from poly(A)- RNA-seq libraries to standard oligo(dT)-primed poly(A)+ RNA-seq libraries, many excised introns and exons can be detected that correspond to ciRNAs (Zhang et al. 2013) and circRNAs (Salzman et al. 2012; X.-O. Zhang et al. 2014, 2016), respectively, including some with complex alternative splicing patterns.

To go even further, ribonuclease R (RNase R), an exoribonuclease that degrades most [but not all; see Panda et al. (2017) and Xiao & Wilusz (2019)] linear RNAs (Suzuki et al. 2006), can be used to enrich circRNAs in ribo- or poly(A)- RNA fractions (Jeck et al. 2013, X.-O. Zhang et al. 2014). Indeed, many more individual circRNAs can be identified using the RNase R RNA-seq approach than with ribo- or poly(A)- RNA-seq (Jeck et al. 2013, Memczak et al. 2013, Salzman et al. 2012, X.-O. Zhang et al. 2014), confirming the robustness of RNase R for circRNA enrichment. RNase R treatment additionally serves as a useful means to detect or remove false positive linear RNAs. *Trans*-splicing events and artifacts of reverse transcriptase template switching can result in sequencing reads that resemble BSJs, but such reads should be depleted after RNase R treatment (Chuang et al. 2018, Jeck & Sharpless 2014, Szabo & Salzman 2016, Tang et al. 2018, Yu et al. 2014). RNase R has thus been widely used for circRNA validation (Burd et al. 2010, Hansen et al. 2013, Memczak et al. 2013, Salzman et al. 2012), but note that some circRNAs, such as *cerebellar degeneration-related protein 1 antisense transcript (CDR1as)*, also known as *circular RNA sponge for miR-7, ciRS-7* (Hansen et al. 2011, 2013; Memczak et al. 2013), have been observed to sometimes be partially depleted in RNase R RNA-seq experiments (Jeck et al. 2013). This is because circRNAs, especially long ones, can be partially degraded by prolonged RNase R incubation (Zhang et al. 2016b). Additional tips to ensure robust circRNA studies have been well summarized recently (Dodbele et al. 2021, Li et al. 2018).

### Bioinformatic and Sequencing Approaches for circRNAs

To then identify reads or fragments mapping to BSJs of circRNAs in short-read sequencing data, multiple computational pipelines have been developed that employ distinct strategies (Gao & Zhao 2018; Hansen 2016, 2018; López-Jiménez et al. 2018; Szabo & Salzman 2016). These pipelines have variable efficiencies and accuracies for circRNA profiling, so the application of two or more pipelines has been recommended to reduce circRNA prediction biases (Hansen 2016, 2018; X. Zeng et al. 2017). Nonetheless, all of these pipelines are generally consistent on the identification of highly expressed circRNAs (Hansen 2016, 2018).

Due to the limited read lengths that can be achieved using short-read sequencing data, the exact internal sequences (away from the BSJ) of circRNAs are often ambiguous, especially for long circRNAs. There has thus recently been a significant effort to develop long-read sequencing platforms (Stark et al. 2019) and their corresponding bioinformatic pipelines for genome-wide circRNA profiling, mostly using nanopore technologies (Liu et al. 2021, Rahimi et al. 2021b, Xin et al. 2021, J. Zhang et al. 2021) [for a review, please see Rahimi et al. (2021a) and Zhang & Zhao (2021)]. Compared to short-read sequencing, long-read sequencing approaches have provided more accurate information on the alternative splicing landscape of circRNAs, including the identification of intron retention events, microexons, and exons that are circRNA specific (not observed in the associated cognate linear RNA) (Rahimi et al. 2021b). However, current long-read sequencing approaches can suffer from a number of disadvantages, including high cost, biased enrichment of circRNAs of different lengths, and high sequencing error rates (Rahimi et al. 2021a).

There is also much interest in characterizing the transcriptome, including circRNAs, not just in bulk cell populations but at single-cell or nucleus resolution (Slyper et al. 2020, Stark et al. 2019). Most of these current approaches require the complementary DNA to be primed and amplified with oligo(dT) primers and thus they largely miss circRNA expression. Nonetheless, using random primers, circRNA profiling was obtained at single-cell resolution from mouse preimplantation embryos (Fan et al. 2015). In the future, with improved technologies for circRNA enrichment, long-read sequencing, and advanced computational pipelines, even more precise circRNA expression profiles across cell lines and tissues should be obtained.



## Genomic Expression Patterns and Features of circRNAs

circRNAs have now been profiled in a growing number of eukaryotic cell types and tissues, with several public databases summarizing some of the sequencing data (Dong et al. 2018, Glazar et al. 2014, Wu et al. 2020). These efforts have revealed that circRNAs are typically produced along with their cognate linear RNAs from the host gene loci (**Figure 1a**), but circRNAs accumulate to much lower levels (Jeck et al. 2013, Memczak et al. 2013, Salzman et al. 2012, X.-O. Zhang et al. 2014). A small portion of circRNAs can be detected at higher levels than their cognate linear RNAs (Salzman et al. 2012, 2013), partially due to their natural resistance to degradation by exonucleases. Across tissue types, circRNAs often accumulate to the highest levels in brains, where their levels increase during neuronal differentiation (Zhang et al. 2016a) and are associated with neuronal development and plasticity (Rybak-Wolf et al. 2015, You et al. 2015). These observations suggest that circRNAs can accumulate in slowly dividing cells. Consistent with this idea, decreased circRNA levels have been detected in rapidly dividing colorectal cancer cell lines (Bachmayr-Heyda et al. 2015) and prostate cancers (S. Chen et al. 2019, Vo et al. 2019).

Most circRNAs are derived from previously annotated exons (exons observed in linear RNAs) that are flanked by canonical splice sites, consistent with a critical role for the spliceosome in their biogenesis (Jeck et al. 2013, Liang & Wilusz 2014, Memczak et al. 2013, Salzman et al. 2012, Starke et al. 2015, X.-O. Zhang et al. 2014). circRNAs usually contain two to three exons that are derived from the middle of their host genes (X.-O. Zhang et al. 2014), although they can also sometimes be generated from readthrough transcripts (Liang et al. 2017, Vo et al. 2019). Strikingly, the introns flanking circRNA-forming exons are often much longer than those flanking non-circRNA-forming exons, and this has been observed in multiple species, including flies, pig, rice, and humans (Jeck et al. 2013, Kramer et al. 2015, Lu et al. 2015, Salzman et al. 2013, Veno et al. 2015, X.-O. Zhang et al. 2014). Repetitive elements, such as *Alu* in human and *SINE* in mouse, are often enriched in these long flanking introns that promote circRNA biogenesis (Jeck & Sharpless 2014, Jeck et al. 2013, Liang & Wilusz 2014, X.-O. Zhang et al. 2014).

Interestingly, multiple circRNAs can be generated from individual gene loci in a process known as alternative circularization (X.-O. Zhang et al. 2014). This includes alternative backsplicing events (the use of different splice sites to form the BSJ) as well as all four canonical types of alternative splicing (cassette exon, intron retention, and alternative 5'- and 3'-splice site selection) within circRNA-forming exons (Gao et al. 2016, X.-O. Zhang et al. 2016). For example, two major circRNA transcripts can be produced from the human *CAMSAP1* locus, and they contain the same BSJ but different internal structures: with or without a retained intron (Salzman et al. 2013; X.-O. Zhang et al. 2014, 2016). Likewise, the human *XPO1* locus produces two circRNA transcripts with the same BSJ that differ by the inclusion of a cassette exon (X.-O. Zhang et al. 2016).

## REGULATION OF CIRC RNA PRODUCTION, CONFORMATION, AND TURNOVER

In agreement with the original reports that identified sporadic circRNAs in eukaryotes (Capel et al. 1993; Cocquerelle et al. 1992, 1993; Nigro et al. 1991), genome-wide profiling has now shown that most circRNAs are generated via backsplicing reactions when the spliceosome joins a downstream 5'-splice site to an upstream 3'-splice site (Jeck et al. 2013; Liang & Wilusz 2014; Memczak et al. 2013; Salzman et al. 2012, 2013; X.-O. Zhang et al. 2014). Backsplicing can thus be viewed as a type of alternative splicing (Gao et al. 2016, Jeck & Sharpless 2014, X.-O. Zhang et al. 2016), with circRNA steady-state levels reflecting the dynamic equilibrium of their production and degradation rates (Rabani et al. 2011). Here, we focus on the predominant mechanisms



that regulate circRNA levels, but note that a few circRNAs may be generated by the U12 minor spliceosome (Szabo et al. 2015).

### Coupling with Transcription

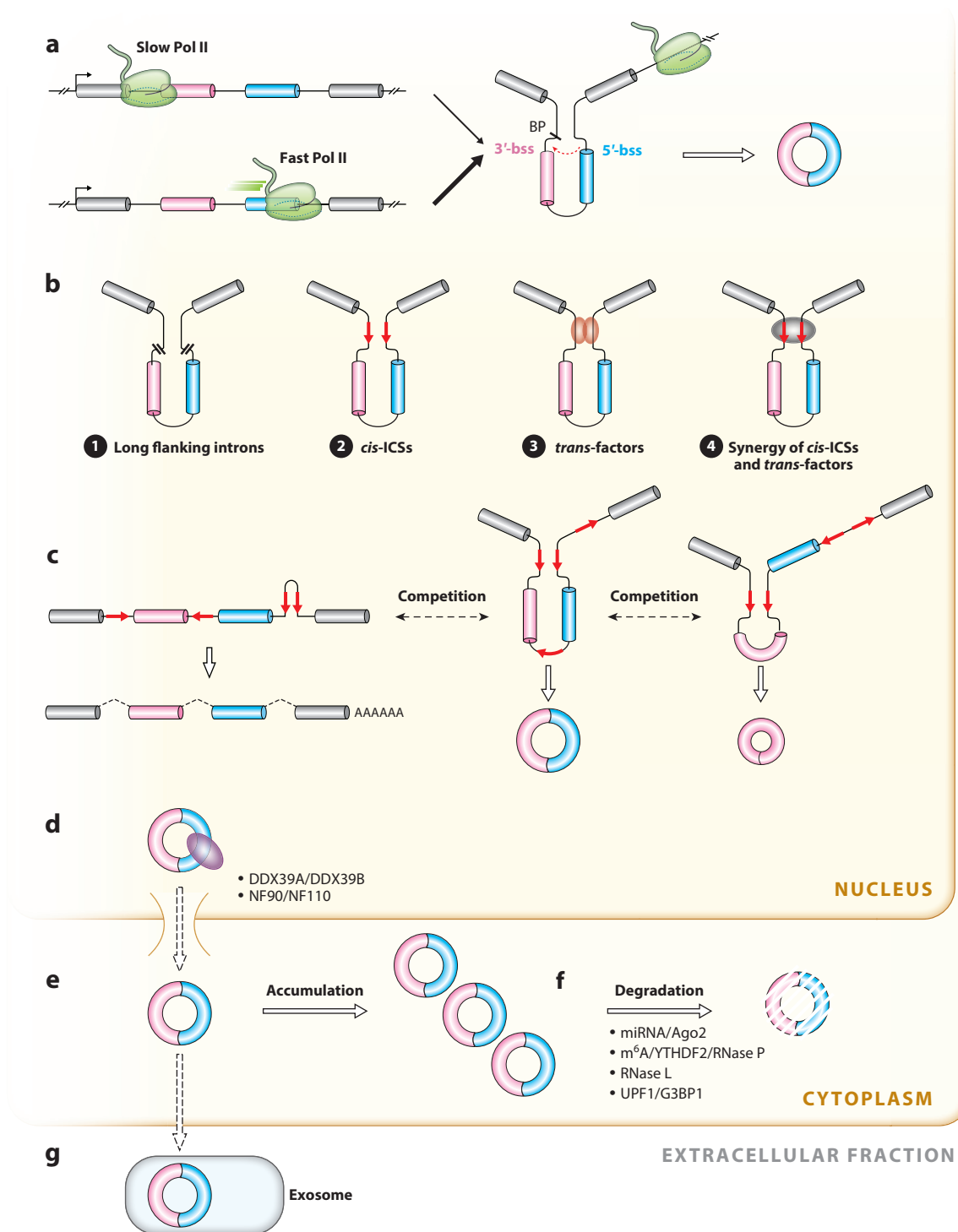
The processes of mRNA capping, splicing, and polyadenylation are closely coupled with Pol II transcription (Bentley 2014). Backsplicing is likewise associated with Pol II transcription, although there is evidence that circRNA biogenesis can occur either co- or posttranscriptionally (Ashwal-Fluss et al. 2014, Zhang et al. 2016a). On the one hand, chromatin-associated circRNAs have been identified from fly heads and mouse liver (Ashwal-Fluss et al. 2014), suggestive of cotranscriptional production. Mutants of the human Pol II large subunit that alter transcription elongation rates (TERs) (Fong et al. 2014) likewise suggest the cotranscriptional nature of circRNA formation, as fast Pol II produces more circRNAs and slow Pol II produces fewer (Ashwal-Fluss et al. 2014, Zhang et al. 2016a) (**Figure 2a**). The mean TER of circRNA-producing gene loci is higher than that of non-circRNA-producing ones (Zhang et al. 2016a), consistent with the notion that fast transcription favors backsplicing. Fast transcription may facilitate cross talk between distal splice sites (both donor and acceptor) as well as RNA pairing between distal complementary sequences in flanking introns (discussed further below in the section titled Regulation of Backsplicing by *cis*-Elements), but the detailed mechanisms remain poorly understood.

On the other hand, analyzing the correlation between the 3'-end processing of pre-RNAs and the production of circRNAs from expression vectors revealed that backsplicing can occur posttranscriptionally (Liang et al. 2017, 2021; Liang & Wilusz 2014). Only when a downstream polyadenylation signal (or an alternative 3'-end processing signal) was present could circRNAs be efficiently generated from expression vectors (Liang & Wilusz 2014). Consistent with this observation, 4-thiouridine (4sU) pulse-chase labeling of nascent RNAs revealed that most BSJ-mapped reads could only be observed after prolonged 4sU treatments when the transcription of almost all circRNA-producing genes has been completed (Zhang et al. 2016a). This suggests most backsplicing events occur posttranscriptionally.

### Interplay with Canonical Splicing

Given that the majority of backsplicing events are produced from exons with canonical splice sites that are also observed in linear RNAs (Ashwal-Fluss et al. 2014; X. Li et al. 2019; Liang & Wilusz 2014; Starke et al. 2015; Vo et al. 2019; Wang & Wang 2015; X.-O. Zhang et al. 2014, 2016), there is inevitably an interplay or competition between backsplicing and canonical splicing events. There are currently two known ways by which the spliceosome generates circRNAs that differ depending on the order of splicing events (Chen & Yang 2015, Jeck & Sharpless 2014, Jeck et al. 2013). In the direct backsplicing model, backsplicing preferentially happens before canonical splicing, resulting in production of a circRNA and a splicing intermediate that is sometimes further processed to a mature linear RNA that lacks the circRNA-forming exon(s) (Chen & Yang 2015, Jeck & Sharpless 2014, Jeck et al. 2013). In contrast, in the lariat intermediate model, a canonical splicing event happens first to produce a mature linear RNA as well as an intron lariat intermediate containing skipped exons. The latter is subsequently backspliced to produce a circRNA consisting of the exons that were originally skipped (Barrett et al. 2017, Chen & Yang 2015, Jeck & Sharpless 2014, Jeck et al. 2013). Stable alternatively spliced linear RNAs lacking the circRNA-forming exon(s) are produced at some circRNA-producing loci (Ashwal-Fluss et al. 2014, Kelly et al. 2015, Zaphiropoulos 1996, X.-O. Zhang et al. 2014), but how cells determine which mechanism is used to generate a given circRNA remains poorly understood.





(Caption appears on following page)

**Figure 2** (Figure appears on preceding page)

The life cycle of circRNAs in cells. (a) Backsplicing is coupled to Pol II transcription. Fast Pol II elongation can lead to enhanced efficiency of backsplicing catalyzed by the spliceosome. (b) Regulation of backsplicing. ① Long introns usually flank circRNA-forming exons. ② ICSs (red arrows) in flanking introns of circRNA-forming exons can facilitate backsplicing by forming transient intronic RNA duplexes that bring the splice sites into close proximity. ③ *trans*-factors bind to intronic ICSs or other sequences in the pre-mRNA to directly bridge distal splice sites to promote backsplicing. ④ *cis*- and *trans*-factors synergistically modulate exon backsplicing. (c) Competition of RNA pairing across introns or within an intron modulates splicing and backsplicing reactions. In this example, when repeats flanking exons 2 and 3 base pair to one another, (middle) a two-exon circRNA is produced. In contrast, when repeats flanking exon 2 base pair to one another, (right) a single exon circRNA is produced. (d) circRNA export is modulated by different proteins and occurs in a length-dependent manner. (e) circRNAs are stable in the cytoplasm and sometimes can accumulate to high levels. (f) Pathways of circRNA degradation in the cytoplasm. (g) Secretion of circRNAs from cells in exosomes. Abbreviations: BP, branch point; bss, backsplicing site; circRNA, circular RNA; ICS, intronic complementary sequence; m<sup>6</sup>A, N<sup>6</sup>-methyladenosine; miRNA, microRNA; Pol II, RNA polymerase II; pre-mRNA, precursor messenger RNA; RNase, ribonuclease.

### Regulation of Backsplicing by *cis*-Elements

Numerous lines of evidence have shown that both *cis*-elements and *trans*-factors, acting in both independent and synergistic manners, can facilitate backsplicing [see reviews by Chen (2020) and Xiao et al. (2020)] (Figure 2b). So far, no specific exonic motifs have been found to be generally required for backsplicing. Instead, circRNA production is typically driven by the flanking intronic sequences. The presence of long introns flanking the circRNA-forming exons appears to be an intrinsic determinant for circRNA biogenesis in some species, including fly (Westholm et al. 2014) and rice (Lu et al. 2015) (Figure 2b). Within these long flanking introns are often orientation-opposite intronic complementary sequences (ICSs) (most often, but not always, from repetitive elements) that can base pair, especially in mammals (Barrett et al. 2015, Capel et al. 1993, Jeck et al. 2013, Kramer et al. 2015, Liang & Wilusz 2014, X.-O. Zhang et al. 2014) (Figure 2b). Consistent with ICSs having a critical function in promoting backsplicing, circRNA formation is impaired when ICSs are disrupted or deleted from endogenous gene loci (Xia et al. 2018, Zhang et al. 2016a, Zheng et al. 2016), and novel circRNAs can be produced in cancer cells when chromosomal translocations cause the formation of new ICS pairs (Guarnerio et al. 2016, Wu et al. 2019).

Cross-species comparisons have shown that a small portion of circRNAs are conserved between human and mouse, and the expression of these circRNAs is correlated with the co-occurrence of ICSs in both species (Dong et al. 2017). In primates, complementary inverted-repeat *Alu* elements in the flanking introns play the largest role in driving circRNA production, while *B1/B2/B4 SINEs* contribute the most in mice (Dong et al. 2017). It nonetheless has been shown that ICSs as short as 30–40 nucleotides are sufficient to promote circRNA production from expression vectors (Liang & Wilusz 2014, Starke et al. 2015).

Most introns contain many repetitive elements, which provides the opportunity for intronic pairing to occur between distinct sets of ICSs and the generation of complex patterns of circRNA expression (Figure 2c). For example, when ICSs in an individual intron (rather than in separate introns) base pair, a linear RNA tends to be produced, and circRNA formation is decreased (X.-O. Zhang et al. 2014) (Figure 2c). In contrast, the base pairing of different combinations of ICSs that bracket alternative backsplice sites can lead to alternative backsplice site selection to produce multiple circRNAs from a single gene locus (Gao et al. 2016; X.-O. Zhang et al. 2014, 2016) (Figure 2c).

### Regulation of Backsplicing by *trans*-Factors

In addition to being regulated by *cis*-elements, backsplicing reactions are further controlled by *trans*-acting factors that often act synergistically with the associated intronic *cis*-elements (Kramer et al. 2015). Modulating components of the spliceosomal machinery can affect the efficiency of

splicing reactions. Notably, the depletion of core spliceosome components, including those in the U1 and U2 small nuclear ribonucleoproteins (snRNPs), results in circRNA upregulation and concomitant downregulation of linear RNAs (Liang et al. 2017). Increased circRNA expression was likewise found in rat neurons treated with the splicing inhibitor isoginkgetin (Wang et al. 2019). Beyond core spliceosome components, many RNA-binding proteins (RBPs) have now been shown to associate with the introns flanking circRNA-producing exons to modulate circRNA expression levels (**Figure 2b**). This includes a number of serine-arginine proteins, heterogeneous nuclear ribonucleoproteins (hnRNPs) (Kramer et al. 2015) (e.g., hnRNP L) (Fei et al. 2017), and other known regulators of alternative splicing such as RNA-binding motif protein 20 (RBM20) (Khan et al. 2016) and FUS (Errichelli et al. 2017). In some cases, it is well understood how RBP binding can lead to physiologically relevant changes in gene outputs. For example, in fly, muscleblind (MBL, also known as MBNL1) directly interacts with the *MBL* pre-mRNA to facilitate *circMBL* biogenesis, thereby limiting linear *MBL* mRNA (and hence MBL protein) expression (Ashwal-Fluss et al. 2014). In humans, the splicing regulator Quaking associates with the introns flanking many circRNAs to promote their production during the epithelial-to-mesenchymal transition (Conn et al. 2015).

A significant number of RBPs directly bind to ICSs through their double-stranded RNA (dsRNA)-binding domains (dsRBDs) to modulate the efficiency of backsplicing reactions (**Figure 2b**). This includes adenosine deaminase acting on RNA 1 (ADAR1), which catalyzes adenosine-to-inosine editing to disrupt the pairing of ICSs and reduce circRNA production in worm and human (Ivanov et al. 2015, Rybak-Wolf et al. 2015). The dsRBD-containing nuclear RNA helicase DHX9 can likewise unwind paired intronic repeat elements that flank circRNA-forming exons to inhibit backsplicing reactions (Aktaş et al. 2017, Ottesen et al. 2019). However, some dsRBD-containing RBPs bind to ICSs to promote circRNA production, most notably nuclear factor 90 (NF90) and NF110 (X. Li et al. 2017) and the splicing factor SFPQ (Stagsted et al. 2021). Considering that all of these RBPs have overlapping binding capacities to bind to ICSs, there must be cross talk with one another, but key details of how they combinatorially dictate circRNA levels remain elusive. A genome-wide screen has further identified several dozen additional RBPs that also likely regulate circRNA production (X. Li et al. 2017). Further investigations are thus warranted to comprehensively understand the mechanisms of how different RBPs collectively modulate circRNA production and the choice between backsplicing versus canonical splicing.

### Modification, Export, and Stability of circRNAs

Once produced, circRNAs can undergo nucleotide modifications, export to the cytoplasm, and ultimately degradation. Genome-wide analyses suggest that N<sup>6</sup>-methyladenosine (m<sup>6</sup>A) modifications occur in circRNAs at levels similar to those observed in linear RNAs (Y.G. Chen et al. 2019, Shi et al. 2019, Zhou et al. 2017), and this can have multiple potential functional consequences. For example, m<sup>6</sup>A modifications may affect circRNA nucleocytoplasmic export, possibly in a manner similar to how linear mRNA export can be promoted by the m<sup>6</sup>A-binding protein YTHDC1 (Roundtree et al. 2017). With the help of YTHDF3, another m<sup>6</sup>A-binding protein (A. Li et al. 2017), a noncanonical eIF4G protein (eIF4G2), can be recruited to m<sup>6</sup>A-circRNAs to initiate cap-independent translation from internal ribosome entry site (IRES) sequences (Y. Yang et al. 2017) (discussed further below in the section titled Translatable circRNAs). It has further been shown that some m<sup>6</sup>A-circRNAs can be recognized by YTHDF2 and HRSP12 (also known as 2-iminobutanoate or 2-iminopropanoate deaminase) (Jarrous 2017), enabling recruitment of the ribonuclease complex RNase P/MRP for circRNA degradation (Park et al. 2019).

Similar to most linear RNAs, fully processed circRNAs are typically exported to the cytoplasm (Jeck et al. 2013, Salzman et al. 2012) (**Figure 2d**), although a small number of incompletely

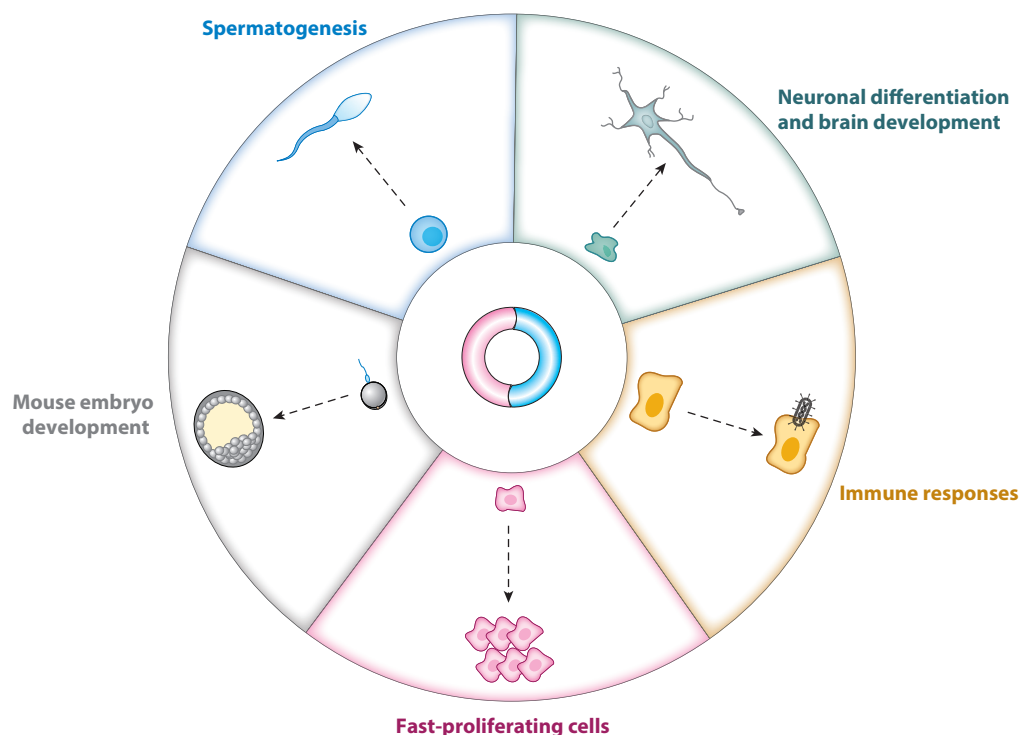


processed circRNAs, such as those with retained introns, remain in the nucleus (Conn et al. 2017, Z. Li et al. 2015, Veno et al. 2015). The detailed mechanism of circRNA nucleocytoplasmic export has yet to be fully characterized, although some key aspects have been revealed. A small scale RNA interference (RNAi) screen in fly cells showed that circRNA export occurs in a length-dependent manner and that the ATP-dependent RNA helicase Hel25E is required for the export of long (>800 nucleotides), but not short, circRNAs (Huang et al. 2018). Similar regulation was observed in human cells: UAP56 (also known as DDX39B), a homolog of Hel25E, controls the export of long (>1,298 nucleotides) circRNAs, whereas URH49 (also known as DDX39A), another homolog of Hel25E, controls the export of short (<356 nucleotides) circRNAs (Huang et al. 2018). Strikingly, the key amino acid motif responsible for the export of long circRNAs is highly conserved between fly Hel25E and human UAP56 (but is absent from URH49), and UAP56 could rescue the functional loss of Hel25E on long circRNA export in fly cells (Huang et al. 2018). Nonetheless, the factors that control the export of medium-length circRNAs (e.g., those between 400 and 1,300 nucleotides in human cells) remain unclear. NF90/NF110, which shuttle between the nucleus and cytoplasm, may mediate the export of some circRNAs, as these factors can interact with both flanking ICSs in the nucleus and fully processed circRNAs in the cytoplasm (X. Li et al. 2017). In addition, other factors, such as m<sup>6</sup>A modifications (Zhou et al. 2017) or RNA duplexes within circRNAs (Liu et al. 2019), may recruit additional proteins to modulate nucleocytoplasmic transport.

Given their lack of free ends, circRNAs are naturally resistant to degradation initiated by exonucleases and typically have much longer half-lives than do linear RNAs (Enuka et al. 2016, Liu et al. 2022, Zhang et al. 2016a). This enables some circRNAs to accumulate to high levels (**Figure 2e**), especially in cells with slow division rates, such as neurons and aging tissues (Rybák-Wolf et al. 2015, Westholm et al. 2014, You et al. 2015, Zhang et al. 2016a). Nevertheless, circRNAs can still undergo decay via several mechanisms that are initiated by endonucleases (**Figure 2f**). For example, the *CDR1as/ciRS-7* circRNA contains a sequence with perfect complementarity to the microRNA (miRNA) miR-671, thereby enabling miR-671 to initiate its decay in an Argonaute 2 (Ago2)-dependent manner (Hansen et al. 2011, Kleaveland et al. 2018). This situation is unique, however, as miRNA target sites in other circRNAs are only partially complementary to miRNAs and are unable to trigger Ago2 endonucleolytic cleavage (Hansen et al. 2013, Jeck & Sharpless 2014, Memczak et al. 2013). Instead, several other endonucleases appear to mediate the degradation of other circRNAs. Upon certain viral infections, activated RNase L can rapidly (within an hour) degrade the vast majority of circRNAs (Liu et al. 2019). A subset of m<sup>6</sup>A-modified circRNAs additionally can be degraded by the ribonuclease complex RNase P/MRP (Park et al. 2019). Finally, a small portion of structured circRNAs can be specifically degraded by the endonuclease G3BP1 when it is in complex with the ATP-dependent RNA helicase upstream frameshift 1 (UPF1) (Fischer et al. 2020). How exactly these endonuclease activities are regulated under physiological conditions remain to be explored; there are likely additional enzymes that degrade circRNAs that await discovery. Nevertheless, the long stability of circRNAs endows these molecules with the potential to carry out important cellular functions (discussed in the section titled Regulatory Roles of circRNAs and Their Modes of Action) as well as be secreted to extracellular fractions, such as exosomes (Y. Li et al. 2015, Memczak et al. 2015) (**Figure 2g**).

## REGULATORY ROLES OF CIRC RNAs AND THEIR MODES OF ACTION

Although most of the initial circRNAs identified in the 1990s were expressed at low levels and thought to have little functional potential, there were hints that these transcripts could be related to certain biological and pathological conditions. For example, *circSRY*, which contains



**Figure 3**

Altered cellular and physiological expression patterns of circular RNAs (circRNAs). Up- and downregulated circRNA expression has been observed in different biological and physiological contexts, including embryo and neuronal development, spermatogenesis, immune responses, and tumorigenesis. Although some specific circRNAs have been shown to participate in these different processes, the regulatory roles of most of these altered circRNAs await further investigation.

16 target sites for *miR-138* (Hansen et al. 2013) and a long open reading frame (ORF) (Capel et al. 1993), was proposed to be required during mouse spermatogenesis (Capel et al. 1993). However, *circSRY* was barely loaded onto polysomes, so its translation potential was of unclear significance (Capel et al. 1993). In another example, an early study found that *circANRIL* is transcribed from a locus associated with atherosclerotic cardiovascular disease on chromosome 9p21, and that the expression of these circRNAs was correlated with atherosclerosis risk (Burd et al. 2010).

### Physiological and Pathological Expression Dynamics of circRNAs

With the advent of large-scale profiling of circRNAs, a growing number of studies have revealed circRNA expression patterns in physiological and pathological contexts (**Figure 3**). In early stage mouse embryos, over 2,800 circRNAs are expressed during the zygote to blastocyst stages (Fan et al. 2015) (**Figure 3**), and CRISPR-Cas13 knockdown approaches have recently revealed that *circMan1a2* is required during mouse embryo preimplantation development (S. Li et al. 2021). A large number of circRNAs are likewise increasingly expressed during murine spermatogenesis (**Figure 3**), specifically at the stage when late pachytene spermatocytes develop into round and then elongating spermatids (Tang et al. 2020). Interestingly, many such circRNAs appear to have translation potential, as they have ORFs with m<sup>6</sup>A-modified start codons (Tang et al. 2020). Although their physiological relevance during spermatogenesis remains to be examined, removal of

a specific noncoding circRNA, *circBoule*, was suggested to dampen male fertility in fly and mouse (Gao et al. 2020).

circRNAs also have been shown to generally accumulate with age (in a manner independent of expression of their linear cognate mRNAs) in *C. elegans* (Cortés-López et al. 2018), fly head (Westholm et al. 2014) and pig brain (Veno et al. 2015), as well as in mouse cortex and hippocampus (Rybák-Wolf et al. 2015, You et al. 2015) and human brain (Szabo et al. 2015). This appears to be because of the long half-lives of circRNAs. In support of this model, nascent RNA-seq has shown that the production rates of circRNAs are largely unchanged between human embryonic stem cells (hESCs) and hESC-derived forebrain neurons, yet the steady-state levels of circRNAs in forebrain neurons are significantly higher (Zhang et al. 2016a) (**Figure 3**). Note that global circRNA accumulation in aged flies can be affected by insulin signaling and that *circSfl* promotes lifespan of insulin mutant flies (Weigelt et al. 2020).

In contrast, a general decrease in circRNA abundance has been reported in several cancers (Bachmayr-Heyda et al. 2015, S. Chen et al. 2019, Vo et al. 2019) (**Figure 3**), likely because circRNAs can be diluted by rapid cell division. Indeed, there is a negative correlation between global circRNA abundance and proliferation (Bachmayr-Heyda et al. 2015). Although circRNAs are generally reduced in fast-proliferating cells, several oncogenic fusion circRNAs (f-circRNAs) have been identified in cells containing cancer-associated chromosomal translocations (Guarnerio et al. 2016, Wu et al. 2019). Such f-circRNAs are required for proliferation of these cancer cells, but the underlying molecular mechanisms remain unknown (Guarnerio et al. 2016, Wu et al. 2019).

Upon infection with some viruses, including the encephalomyocarditis virus, circRNA levels are globally decreased due to activation of the nonspecific RNase L endonuclease (Liu et al. 2019) (**Figure 3**). RNase L is also activated in the autoimmune disease systemic lupus erythematosus and, correspondingly, overall reduced circRNA levels have been observed (Liu et al. 2019). Similar trends have been reported in patients with chronic inflammatory skin diseases (Moldovan et al. 2019, 2021). Note that these altered circRNA expression patterns are independent of changes in cognate mRNA expression, suggesting that at least a subset of circRNAs may play regulatory roles in these diseases. We now summarize the modes of action of some well-studied circRNAs (**Figure 4**), which have largely been characterized using laboratory cultured cell lines.

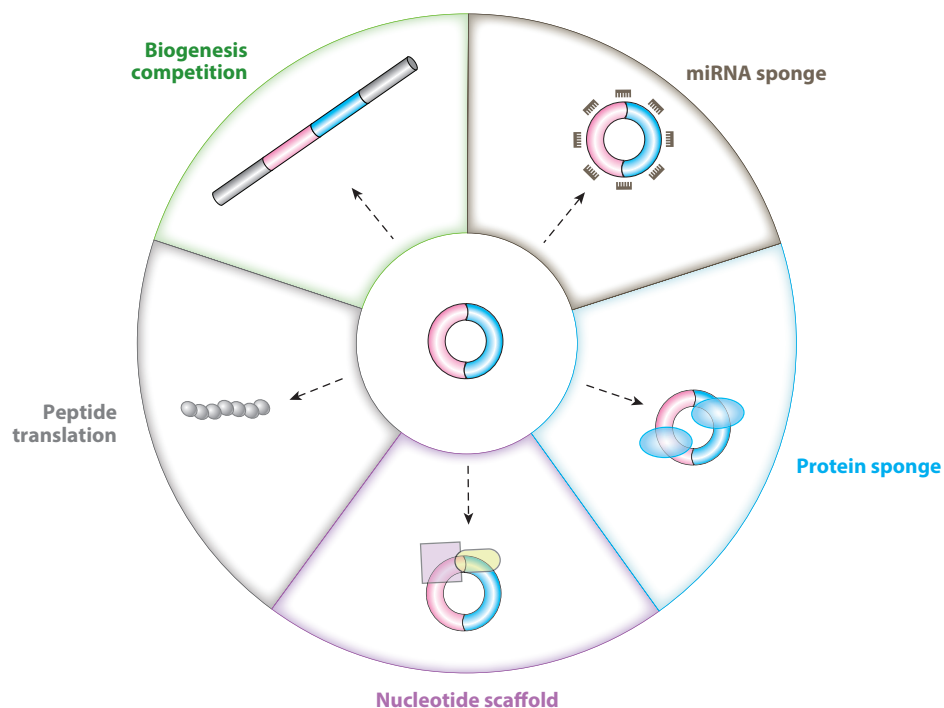
### Modes of circRNA Action

Due to the unique life cycles of circRNAs, the act of their biogenesis, their conformation and binding partners, and their long stability can all contribute to their cellular regulatory potential. Backsplicing is in direct competition with canonical splicing events, and thus just the act of producing a circRNA can sometimes lead to the downregulation of cognate linear RNA levels and/or change the sets of linear isoforms expressed in cells. Alternatively, the mature circRNAs themselves can carry out biological functions, especially by binding to miRNAs, proteins, or other RNA species, sometimes in an inhibitory or competitive manner and other times in a scaffolding role that enables functional complexes to be formed. Other circRNAs appear to be translated to generate peptides with biological functions.

### circRNAs as Decoys or Sponges for microRNAs

Given their largely cytoplasmic localization and cellular stability, many studies have pursued the idea that circRNAs might function as competing endogenous RNAs (ceRNAs) (Salmena et al. 2011) that modulate the bioavailability of miRNAs (Jeck et al. 2013) (**Figure 4**). The most prominent example is *CDR1as/ciRS-7*, a circRNA derived from an lncRNA (*LINC00632*) that is antisense to the *CDR1* protein-coding gene (which is notably not expressed in most tissues) (Barrett et al.





**Figure 4**

Modes of action of circRNAs. A schematic of different modes of action of circRNAs currently annotated in cells. In addition to the interplay between backsplicing and canonical splicing for the same splice site selection that can ultimately affect pre-mRNA splicing, mature circRNAs themselves can act as decoys or sponges for miRNAs and proteins, and as RNA scaffolds. A small subset of circRNAs can serve as templates for protein translation. Abbreviations: circRNA, circular RNA; miRNA, micro RNA.

2017; Hansen et al. 2011, 2013; Memczak et al. 2013). *CDR1as/ciRS-7* is abundantly expressed in mammalian brains (Hansen et al. 2013, Memczak et al. 2013) with a particular enrichment in excitatory neurons (Piwecka et al. 2017). In addition to a perfect *miR-671* target site that can enable cleavage of *CDR1as/ciRS-7* by Ago2 (Hansen et al. 2011), *CDR1as/ciRS-7* harbors 74 conserved binding sites for *miR-7*. Upon reducing *CDR1as/ciRS-7* expression in human cell lines, the expression of mRNAs with *miR-7* binding sites was likewise reduced, suggesting that this circRNA can act as a decoy or sponge for *miR-7* (Hansen et al. 2013, Memczak et al. 2013). Interestingly, when the entire genomic region corresponding to *Cdr1as/ciRS-7* was removed using CRISPR/Cas9, mice had phenotypes resembling neuropsychiatric disorders, including a dysfunction of excitatory synaptic transmission (Piwecka et al. 2017). This was not, however, only due to simple alterations in *miR-7* sponging, as the expression of *miR-7* was also reduced in *Cdr1as/ciRS-7* knockout mice (Piwecka et al. 2017). *Cdr1as/ciRS-7* is thus required for *miR-7* stabilization in vivo and, indeed, a network of lncRNAs, including *Cyranos*, is now recognized to control *miR-7* levels (Kleaveland et al. 2018). *Cyranos* can bind *miR-7* and promote its destruction by inducing 3' tailing and trimming (Kleaveland et al. 2018). Upon deletion of *Cyranos* in mice, increased levels of *miR-7* are observed along with decreased *Cdr1as/ciRS-7* expression, in part due to the slicing of *Cdr1as/ciRS-7* by *miR-671* (Kleaveland et al. 2018). Beyond a role in excitatory synaptic transmission in the brain, *Cdr1as/ciRS-7* has also been implicated in insulin secretion (Xu et al. 2015)

and in cancer (melanoma) progression (Hanniford et al. 2020). The latter notably occurs via an *miR-7*-independent mechanism.

Beyond *Cdr1as/ciRS-7*, additional abundant circRNAs have been proposed to serve as miRNA decoys or sponges. *circHIPK2* modulates astrocyte activation by sponging *miR124-2HG* (Huang et al. 2017), *circZNF1* plays a role in epidermal stem cell differentiation by sponging *miR-23b-3p* (Kristensen et al. 2018), and *circBIRC6* controls hESC pluripotency and differentiation by sequestering both *miR-34a* and *miR-145* (Yu et al. 2017). Nonetheless, most mammalian circRNAs are expressed at low levels, with relatively few miRNA binding sites (Guo et al. 2014). This makes most circRNAs poor candidates for acting as ceRNAs for miRNAs (Bosson et al. 2014, Denzler et al. 2014).

### circRNAs as Decoys or Sponges for Proteins

In addition to binding RNAs, circRNAs can bind to proteins, and some act as decoys that prevent those protein factors from acting elsewhere (**Figure 4**). This mode of regulation is exemplified by *Drosophila circMbl* (Ashwal-Fluss et al. 2014). MBL, the protein encoded by the cognate gene, promotes *circMbl* production by binding to the flanking introns and then subsequently interacts with mature *circMbl* transcripts (Ashwal-Fluss et al. 2014). When MBL protein levels are high, *circMbl* backsplicing is promoted at the expense of canonical splicing, thereby preventing the expression of additional linear *Mbl* mRNAs and MBL proteins. The existing MBL proteins can be further sequestered by the mature *circMbl* transcripts, ensuring an efficient negative feedback loop (Ashwal-Fluss et al. 2014). In another example, *circANKRD1*, which is associated with atherosclerotic cardiovascular disease (Burd et al. 2010), binds the C-terminal lysine-rich domain of pescadillo zebrafish homolog 1 (PES1) (Holdt et al. 2016). PES1 is a key component of the PES1-BOP1-WDR12 (PeBoW) complex that promotes precursor rRNA (pre-rRNA) processing to mature 28S and 5.8S rRNAs. The binding of PES1 to *circANKRD1* can inhibit rRNA maturation, leading to impaired ribosome biogenesis, nucleolar stress, and cell death in vascular smooth muscle cells and macrophages related to atherosclerosis (Holdt et al. 2016).

Just as circRNAs need to be expressed at high levels to function as efficient ceRNAs, the stoichiometry between circRNAs and their binding proteins needs to be evaluated before proposing a decoy or sponge model. Rather than acting alone, recent work has suggested that circRNAs can function as a group to sequester dsRNA-binding proteins, such as NF90/NF110 (X. Li et al. 2017) and PKR (Liu et al. 2019), due to their tendency to form 16–26–base pair imperfect double-stranded (ds) regions. Indeed, a number of dsRNA-binding proteins with degenerate binding motifs, including oligoadenylate-synthetase and ADAR1–150, can bind in vitro–synthesized circRNAs (Liu et al. 2019). In another example, the circRNA *cia-cGAS* contains a dsRNA region that enables strong binding to the DNA-binding domain of the DNA sensor cyclic GMP–AMP synthase (cGAS) in the nucleus. This circRNA thus acts to competitively inhibit the binding of self-DNA to cGAS, thereby preventing inappropriate type I interferon activation and maintaining quiescent hematopoietic stem cells in the bone marrow (Xia et al. 2018).

circRNAs can simultaneously interact with miRNAs and proteins and, therefore, each of these modes of action are not mutually exclusive. Spatial localization, as well as the relative amounts of accessible circRNA and protein molecules, plays an important role in defining the exact function of the transcript. For example, *circFAM120A* is an abundant circRNA that is expressed at more than 20 copies per HeLa and HEK 293 cell (S. Li et al. 2021). This particular circRNA is enriched to monoribosomes, where it binds the translation inhibitor IGF2BP2, preventing it from interacting with the linear cognate *FAM120A* mRNA. Even though IGF2BP2 is present at a level of hundreds of thousands of copies per cell (Hein et al. 2015), this local niche of *circFAM120A* on

monoribosomes enables this circRNA to carry out a critical function: It ensures robust *FAM120A* translation, which is required in the AKT pathway for cell proliferation (S. Li et al. 2021).

### circRNAs as RNA Scaffolds

Some circRNA-protein interactions do not inhibit protein function but instead enable formation of complexes (circRNPs) that are involved in gene regulation. For example, *circFOXO3* can promote cell cycle progression by interacting with cyclin-dependent kinase 2 (CDK2) and cyclin-dependent kinase inhibitor 1 (or p21) to impact CDK2 function (Du et al. 2016). In another example, *circAmotl1* binds AKT1 and phosphoinositide-dependent kinase 1 (PDK1), leading to AKT1 phosphorylation and facilitating the cardioprotective nuclear translocation of pAKT in neonatal human cardiac tissue (Y. Zeng et al. 2017). Additionally, *circACC1* acts as an RNA component of the AMP-activated protein kinase (AMPK) holoenzyme to stabilize and promote the enzymatic activity of AMPK by forming a ternary complex with the regulatory AMPK beta and gamma subunits (Q. Li et al. 2019). As a result, *circACC1* modulates metabolic adaptation during serum deprivation by increasing glycolysis and beta-oxidation.

circRNAs can also act as important scaffolds in the nucleus and the mitochondrion. *circPOK* has been proposed to interact with and activate the ILF2/3 complex in the nucleus, promoting the transcription of ILF2/3-regulated proliferative and pro-angiogenic factors in the context of mesenchymal tumor progression (Guarnerio et al. 2019). *circKent2* inhibits *Batf* expression by recruiting the nucleosome-remodeling deacetylase complex onto the *Batf* promoter, which promotes colitis resolution (Liu et al. 2020). The mitochondrial DNA-encoded *circSCAR* transcript inhibits mitochondrial reactive oxygen species output by forming a complex with ATP synthase 5B (ATP5B) and shutting down the mitochondrial permeability transition pore (Zhao et al. 2020). In each of these examples, the stoichiometry between circRNA and its interacting partners still remains somewhat unclear, along with the mechanistic details of how each circRNP is formed, especially at the structural level.

### Translatable circRNAs

In addition to the multiple noncoding roles that circRNAs can perform, recent work suggests that a subset of endogenous circRNAs may be translated into protein using cap-independent translation mechanisms. Engineered circRNAs that contain strong viral IRESs have long been known to be translated (Chen & Sarnow 1995). Such engineered transcripts can easily be generated in cells using expression vectors (Kramer et al. 2015, X. Li et al. 2017, Wang & Wang 2015) and in vitro using direct (or splint) ligation methods (Liu et al. 2022) or by using self-splicing introns (Chen et al. 2017; Ford & Ares 1994; Wesselhoeft et al. 2018, 2019). Nonetheless, there has been significant debate as to whether endogenous circRNAs are translated. Some ribosome-profiling data sets have provided little evidence for circRNA translation (Capel et al. 1993, Guo et al. 2014, Jeck et al. 2013, Stagsted et al. 2019), while others have suggested a subset of endogenous circRNAs [e.g., *circZNF609* (Legnini et al. 2017), *circMbl* (Pamudurti et al. 2017), *circSfl* (Weigelt et al. 2020), and *circFGFR1* (Chen et al. 2021)] are translatable. Such transcripts have been proposed to often contain regions that are complementary to 18S rRNA as well as a structured RNA element that facilitates IRES-dependent circRNA translation (Chen et al. 2021). Alternatively, m<sup>6</sup>A modifications have been reported to drive circRNA translation by recruiting the translation initiation factor eIF4G2 and the m<sup>6</sup>A reader YTHDF3 (Yang et al. 2017).

Cap-independent translation mechanisms generally have low efficiency, and thus protein products translated from circRNAs are limited under normal conditions (Legnini et al. 2017, Pamudurti et al. 2017, Yang et al. 2017). However, upon cellular stress, cap-dependent mRNA



translation is generally suppressed, thereby allowing products of cap-independent circRNA translation to become elevated during starvation (Pamudurti et al. 2017) and heat shock (Chen et al. 2021, Yang et al. 2017). For example, the *circFGFR1*-encoded protein, which is in frame with full-length FGFR1, can function as an antagonist of the FGFR1 oncoprotein and can suppress FGFR1 signaling and cell growth during heat shock (Chen et al. 2021). In a somewhat analogous manner, *circ-E-Cad* can be translated into an oncogenic secretory E-cadherin protein variant that activates epidermal growth factor (EGF) receptor (EGFR) signaling independently of EGF, leading to glioma stem cell tumorigenicity (Gao et al. 2021). Emerging large-scale analyses of the transcriptome of human hearts (van Heesch et al. 2019), murine spermatogenesis (Tang et al. 2020), and additional cell lines and tissues (Chen et al. 2021) have suggested the existence of other circRNA-encoded proteins. However, these data need to be interpreted cautiously because standard quality control metrics that increase the robustness of ribosome-profiling data cannot be applied to circRNAs, and the robustness of false discovery rate metrics for mass spectrometry of circRNA-derived peptides has been called into question (Hansen 2021).

### EMERGING ROLES OF OTHER TYPES OF CIRCULAR RNAs

This review has focused on the biogenesis and functions of circRNAs produced from backspliced exons, but other types of circular RNAs with distinct properties deserve mention. Hundreds of ciRNAs are generated in a splicing-dependent manner and are derived from intron lariats that fail to be debranched at their 2',5'-phosphodiester bond. Once produced, these transcripts tend to localize in the nucleus where they can promote Pol II transcription (Zhang et al. 2013). For example, *ci-ankrd52* maintains an open conformation that can form a stronger R-loop with its parental locus than can the cognate pre-mRNA. Upon ciRNA removal via RNase H1 cleavage, it has been proposed that this R-loop is resolved, and transcriptional elongation is promoted (X. Li et al. 2021). ciRNAs represent a subset of sisRNAs, which can be linear or circular and were first reported in *Xenopus tropicalis* oocytes (Gardner et al. 2012). Thousands of RNase R-resistant sisRNAs preferentially localize to the cytoplasm, and they can be transmitted to the fertilized egg and persist during early embryogenesis (Talhouarne & Gall 2014). For example, the maternally inherited sisRNA *sisR-4* is produced from the *deadpan* gene, which is essential for *X. tropicalis* development. Female, but not male, mutants for *sisR-4* produce embryos that fail to hatch. This appears to be because *sisR-4* promotes the transcription of its host gene during embryogenesis via a positive feedback loop that activates an enhancer present in the intron where *sisR-4* is encoded (Tay & Pek 2017).

DNA virus genomes can also produce circular RNAs that are likely involved in cell proliferation, but the underlying mechanisms are largely unknown (Huang et al. 2019, Tagawa et al. 2018, Toptan et al. 2018, Ungerleider et al. 2018, Zhao et al. 2019). In other kingdoms, such as Archaea, circular RNAs derived from noncoding sequences have also been suggested to have roles in gene expression regulation (Danan et al. 2012). For instance, the maturation of some tRNAs (Soma et al. 2007) and rRNAs (Tang et al. 2002) in Archaea requires the existence of their circular intermediates. Circular RNAs derived from archaeal tRNA and rRNA introns have additionally been suggested to contain ORFs that encode proteins (Burggraf et al. 1993, Dalgaard & Garrett 1992, Kjems & Garrett 1988) or contain C/D box snoRNAs that can guide RNA modifications (Clouet d'Orval et al. 2001, Singh et al. 2004, Starostina et al. 2004).

### CONCLUSIONS

Recent technological advances have unveiled that circRNAs have diverse expression patterns, unique biogenesis mechanisms, and distinct modes of action. Many significant advances have been



made, but the sequence overlap of circRNAs with their cognate linear mRNA sequences has made it somewhat complicated to precisely define their regulatory roles. Nonetheless, best experimental practices have now been provided to help discriminate these circles from linear cognate mRNAs [see reviews by Dodbele et al. (2021) and Li et al. (2018)]. Hence, we fully expect more regulatory functions and modes of action of this large class of RNA molecules to emerge, especially in physiological in vivo conditions. For example, loss-of-function circRNA studies can now be achieved not only by using RNAi-mediated approaches but also by using different CRISPR-Cas13 systems and guide RNAs targeting the BSJ sites in different contexts (Ai et al. 2022, S. Li et al. 2021, Y. Zhang et al. 2021). In addition, base editors can be used to target backsplice sites of predominantly circularized exons to specifically knock out some circRNAs (Gao et al. 2022). These emerging technologies open new doors to better appreciate circRNA functions in various contexts. In addition, it may enable disease-related circRNAs, such as the steatosis-to-NASH progression-associated circRNA SCAR (Zhao et al. 2020), to serve as potential therapeutic targets.

Beyond revealing how endogenous circRNAs are regulated and function, it is worthwhile to mention that the stability of these transcripts has endowed them with unique capabilities that can be very useful for biotechnological applications. circRNAs can serve as biomarkers (Bahn et al. 2015, Y. Li et al. 2015, Memczak et al. 2015) for cancers (Bachmayr-Heyda et al. 2015, Vo et al. 2019), virus infection (Ungerleider et al. 2018), and autoimmune diseases (Liu et al. 2019; Moldovan et al. 2019, 2021). In addition, circRNAs represent a novel gene expression platform for generating abundant, stable RNAs that contain RNA aptamer sequences (Litke & Jaffrey 2019, Liu et al. 2022) or encode proteins (Chen et al. 2017, Wesselhoeft et al. 2019). In total, the last 10 years of circRNA research has laid a promising foundation for future studies to further understand how these transcripts control key biological processes as well as how circRNAs can be applied as useful technologies in biomedical research.

## DISCLOSURE STATEMENT

J.E.W. serves as a consultant for Laronde.

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