Emerging roles and context of circular RNAs

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Circular RNAs (circRNAs) represent a large class of noncoding RNAs (ncRNAs) that have recently emerged as regulators of gene expression. They have been shown to suppress microRNAs, thereby increasing the translation and stability of the targets of such microRNAs. In this review, we discuss the emerging functions of circRNAs, including RNA transcription, splicing, turnover, and translation. We also discuss other possible facets of circRNAs that can influence their function depending on the cell context, such as circRNA abundance, subcellular localization, interacting partners (RNA, DNA, and proteins), dynamic changes in interactions following stimulation, and potential circRNA translation. The ensuing changes in gene expression patterns elicited by circRNAs are proposed to drive key cellular processes, such as cell proliferation, differentiation, and survival, that govern health and disease. Published 2016. This article is a U.S. Government work and is in the public domain in the USA.

INTRODUCTION

Circular RNAs (circRNAs) comprise a family of noncoding RNAs (ncRNAs) that have drawn intense interest in the last few years. Although they were first discovered in 1979 by electron microscopy, they were thought to be byproducts of splicing and did not receive much attention due to their low abundance and lack of known functions. However, recent advances in RNA sequencing, quantitative PCR, and computational analysis revealed that circRNAs constitute a vastly abundant and heterogeneous class of RNAs, often expressed in a tissue-specific manner. Given their long half-life, circRNAs are emerging as critical posttranscriptional regulators of gene expression by binding microRNAs and buffering their repression of mRNA targets. However, they may influence gene expression at other levels, such as transcription and splicing, as discussed below. The biogenesis of circRNAs is not fully understood, but most circRNAs are believed to arise from the canonical spliceosomal machinery via head-to-tail backsplicing. About 14% of actively transcribed genes can produce circRNAs in fibroblasts. A substantial fraction of spliced transcripts generate circRNAs, suggesting that RNA circularization is a conventional cellular feature. In addition, more than one type of circRNA containing exon(s), intron(s), or both can be produced from a single gene. Recent studies and reviews have covered several aspects of circRNA metabolism, including biogenesis, expression, regulation, and function. Here, we will briefly discuss their known functions and focus the discussion on considerations that are essential for elucidating the functions of circRNAs on key cellular processes. For instance, circRNAs may bind transcription factors (TFs) and RNA-binding proteins (RBPs), forming ribonucleoprotein complexes with specific functions. CircRNAs may also form RNA–RNA complexes with long noncoding RNAs (lncRNAs) and mRNAs. These hybrid RNA complexes may alter the functions or the stability of both RNA molecules, while circRNA–mRNA complexes may alter mRNA stability or translation. The context in...
which circRNAs are found, including circRNA concentration, subcellular distribution, and dynamic interaction with molecules, will be proposed and discussed.

Biogenesis and Types of circRNAs

Circularization of RNAs through backsplicing occurs by covalent end-joining of the 5’ and 3’ ends of the spliced RNA. Another mechanism that has been proposed for biogenesis is through an exon-containing lariat precursor; in this case, an exon-skipping event creates a lariat containing an exon, and the internal splicing of the lariat removes the intron sequence and produces a circRNA. Despite low circRNA-splicing efficiency, circRNA synthesis correlates with high transcription of the nascent mRNAs. Long introns may play a role in backsplicing as they usually contain complementary inverted repeats such as Alu sequences that form double-stranded RNA (dsRNA) structures and theoretically bring the splice sites in close proximity. Along with RNA cis-elements that act on RNA circularization, transacting RBPs have also been found to regulate circRNA biogenesis. For instance, the RNA-editing enzyme adenosine deaminase acting on RNA-1 (ADAR1) is able to melt the dsRNA hairpin structure when inverted complementary sequences are present in the flanking introns of circularized exons and thus inhibits circRNA biogenesis. As ADAR1 also functions as a dsRNA-binding protein, it may regulate circularization nonenzymatically. Two splicing regulators were recently identified as positive regulators of circRNA biogenesis, the proteins muscleblind (MBL) and quaking I 5 (QKI5). MBL regulates circularization of exons derived from its own gene when bound to conserved binding sequences on both the exonic and flanking intronic sequences of MBL pre-mRNA. Further studies showed that the addition of MBL binding sequences into a minigene was sufficient to induce circRNA biogenesis. In the same study, circRNA splicing was found to compete with the canonical linear splice sites. QKI5 is another RBP and splicing regulator that has been shown to regulate circRNA biogenesis. QKI5 is upregulated during endothelial-to-mesenchymal transition, indicating that circRNA biogenesis could be cell type-specific. Similar to MBL, QKI5 binding sites in intronic sequences flanking the circularized exons regulate circularization. Furthermore, in Drosophila, it has been proposed that SR proteins and hnRNPs play a role in the formation of specific circRNAs.

One of the first circRNAs discovered comprised a single exon of the mouse sex-determining gene, SRY. CircRNAs may encompass just one or multiple exons, which indicates the potential for alternatively spliced isoforms. They may also consist of intronic RNA sequences that are formed when intronic lariats fail to debranch. Another subset of circRNAs exists, which contains both exons and introns (known as exon-intron-circRNAs or EIciRNAs). The circular intronic RNAs (ciRNAs) class is derived from lariat introns during canonical splicing; failure to debranch at the branch point site and trimming of the lariat tail leads to the formation of a stable ciRNA. CircRNAs have been reviewed in detail, focusing on their biogenesis, classification, and possible role in diseases, and have been cataloged in databases (Figure 1). In this review, we will highlight these aspects but will concentrate our discussion on new potential functions and considerations of these regulatory RNAs.

REPORTED circRNA FUNCTIONS

miRNA Sponging

There are accumulating examples of circRNAs acting as miRNA sponges, thereby influencing the posttranscriptional actions of miRNAs as suppressors of the translation and/or stability of target mRNAs. The circRNA CiRS-7 bears more than 70 miR-7 binding sites and thus acts as an miR-7 antagonist, in turn limiting the impact of miR-7 on target mRNAs. Indeed, CiRS-7 promoted myocardial infarctions by sponging miR-7a and thus controlled the expression of the miR-7a targets PARP mRNA and SP1 mRNA in myocardial cells. Sex-determining region Y (SRY), another circRNA expressed in murine testis, encompasses 16 binding sites for miR-138, a microRNA implicated in several physiological and pathological processes. Cir-ITCH (Itchy E3 ubiquitin protein ligase) was reported to sponge miR-7, miR-17, and miR-214, leading to the upregulation of ITCH and the inhibition of WNT signaling in esophageal squamous cell carcinoma. circHIPK3, generated from the second exon of HIPK3 (homeodomain-interacting protein kinase 3) mRNA, can sponge miR-124 and other miRNAs; accordingly, silencing circHIPK3 reduced cell growth, suggesting a role of circHIPK3 in cell proliferation through changes in the availability of miR-124 to target miRNAs. Several circRNAs generated from cattle casein (CSN) genes, which are highly expressed in bovine mammary glands, can sponge microRNAs in the miR-2284 family, which target CSN1S1 and CSN2 mRNAs. Taken together, these reports suggest that the general ability of circRNAs to sponge
miRNAs could be an internal cellular mechanism used to fine-tune miRNA actions (Table 1).

**Transcription**

Recently, circRNAs were shown to regulate transcription. ElciRNAs in the nucleus were found to associate with RNA polymerase II and modify its transcriptional activity. However, ElciEIF3J interacts with the small nuclear ribonucleoprotein U1 and the promoter of EIF3J to enhance EIF3J transcription. CircRNA Cdr1as (also known as CiRS-7) was found to regulate insulin transcription by sponging miR-7 and its targets, although the exact mechanism is not yet known (Table 1). Future studies will likely reveal other examples of transcriptional regulation by circRNAs.

**Splicing**

The impact of circRNAs on splicing is just emerging. Recently, the possibility that circRNA biogenesis is competing with splicing has begun to develop. The second exon of the MBL pre-mRNA mentioned above can circularize to form circMbl, with flanking introns that strongly bind to MBL. circMbl–MBL interaction modulates the splicing activity of MBL and regulates MBL pre-mRNA splicing by competing...
with the canonical splicing machinery (Table 1). As our understanding of circRNAs continues to grow, additional examples of circRNAs influencing splicing events are also likely to arise.

**Protein Decoy**

Protein decoy or antagonist is another rising function of circRNAs. For instance, circ-Foxo3 is downregulated in cancer cells and is associated with cell cycle progression. circ-Foxo3 binds cyclin-dependent kinase 2 (CDK2) and p21 (CDKN1A), forming an RNA–protein complex that disrupts the interactions of CDK2 with cyclins A and E, required for cell cycle progression (Table 1). Circ-Foxo3 also interacts with proteins ID1, E2F1, FAK, and HIF-1α (HIF1A), retaining them in the cytoplasm and thus promoting cardiac senescence. These findings indicate that circRNAs may function as decoys that modify the cellular destination and/or function of bound factors.

**CONSIDERATIONS FOR FUTURE circRNA ANALYSIS**

**Determine Abundance and Localization**

One of the critical issues to consider regarding circRNA function is their abundance compared to their linear RNA counterparts (mRNAs or lncRNAs). Although generally, circRNAs are less abundant than linear RNAs, in some instances, circRNAs are in higher concentrations. CircRNAs are usually more stable due to the lack of free ends, but may be downregulated in certain disease conditions, like colorectal cancer and sporadic Alzheimer’s disease (as shown for ciRS-7), and during physiological changes, such as skeletal muscle aging in monkeys. Thus, it is imperative to determine the underlying mechanisms that govern circRNA levels under various conditions in different tissues. A related major consideration for circRNA function is their localization. It has been shown that exonic circRNAs are predominantly cytoplasmic, but it will be essential to measure if intronic circRNAs and EIciRNAs are nuclear, cytoplasmic, or both. This knowledge will set the stage for understanding circRNA function and possibly for devising circRNA-based therapeutic approaches.

**Test Potential Interplay of circRNAs with mRNAs, ncRNAs, and Proteins**

The association of circRNAs with miRNAs can impact mRNA translation and/or stability. In addition, circRNAs may interact directly with other RNA molecules, such as mRNAs and lncRNAs. These interactions may influence the stability, translation, and localization of mRNAs and lncRNAs. For example, as translation efficiency is higher when an mRNA forms a loop, it is conceivable that circRNAs pairing with the 5' and 3' ends of target mRNAs brings the two ends close enough to form a loop, influencing translation of the mRNA. In addition, given that circRNAs may also interact with RBPs and even act as RBP sponges, the interaction of circRNAs with HuR, KSRBP, TTP, AUF1, and other potent regulatory RBPs could affect the fate of their respective target mRNAs, in turn affecting their...

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**TABLE 1 | Reported Circular RNA Functions**

<table>
<thead>
<tr>
<th>circRNA Name</th>
<th>Parent Gene</th>
<th>Function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CiRS-7/CDR1as</td>
<td>Cerebellar degeneration-related protein 1 (Cdr1) antisense transcript</td>
<td>miR-7 antagonist, myocardial infarction, insulin transcription</td>
<td>10,34,39</td>
</tr>
<tr>
<td>circSRY</td>
<td>Sex-determining region Y (SRY)</td>
<td>miR-138 sponging</td>
<td>9</td>
</tr>
<tr>
<td>circ-ITCH</td>
<td>Itch E3 ubiquitin protein ligase (ITCH)</td>
<td>miR-7, miR-17, and miR-214 sponging; inhibition of WNT signaling in esophageal squamous cell carcinoma</td>
<td>36</td>
</tr>
<tr>
<td>circHIPK3</td>
<td>Homeodomain-interacting protein kinase 3 (HIPK3)</td>
<td>miR-124 sponging, cell proliferation</td>
<td>37</td>
</tr>
<tr>
<td>Cattle casein circRNAs</td>
<td>Cattle casein genes CSN1S1, CSN1S2, CSN2, and CSN3</td>
<td>miR-2284 family sponging</td>
<td>38</td>
</tr>
<tr>
<td>ElciEIF3J</td>
<td>Eukaryotic translation initiation factor 3 subunit J (EIF3J)</td>
<td>Upregulates EIF3J transcription by interacting with small nuclear ribonucleoprotein (snRNP) U1 on EIF3J promoter</td>
<td>29</td>
</tr>
<tr>
<td>circMbl</td>
<td>Muscleblind (Mbl)</td>
<td>Biogenesis interferes with canonical splicing</td>
<td>20</td>
</tr>
<tr>
<td>Circ-Foxo3</td>
<td>Forkhead box 3 (Foxo3)</td>
<td>Protein decoy for CKD2, p21, ID1, E2F1, FAK, and HIF-1α, cell cycle progression, cardiac cell senescence</td>
<td>40,41</td>
</tr>
</tbody>
</table>

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splicing, transport, storage, turnover, and translation. Reciprocally, binding of RBPs to mRNAs may impact circRNA processing, function, abundance, and/or subcellular localization. It is important to note that despite the fact that circRNAs are considered to be highly stable, their turnover has not been studied after altering the levels of interacting RBPs. Similarly, circRNAs interacting with TFs may form circRNA–TF complexes that influence TF translocation into the nucleus and/or transcriptional activity. Such influence has been observed in IncRNAs such as GASS, which acts as a decoy for the TF glucocorticoid receptor and prevents it from binding to DNA to elicit transcriptional activation. As mentioned above, circ-Foxo3 is considered to be a decoy for TFs. Future analysis of the dynamic associations of circRNAs with RBPs, TFs, and RNAs will undoubtedly uncover rich and versatile mechanisms by which

![Diagram of circRNA interactions](image)

**FIGURE 2** Emerging activities, proposed functions, and dynamic interactions of circular RNA (circRNAs). (From top clockwise) circRNAs may influence transcription factor (TF) function by influencing TF localization and/or activity. CircRNAs may also associate with traditional RNA-binding proteins (RBPs) or multi-RBP complexes and influence the fate of the circRNAs themselves (e.g., localization, stability), impact the mRNAs that the RBPs interact with (e.g., mRNA stability or translation), or perhaps serve as a platform for the assembly of multiprotein complexes. Partial interaction of circRNAs with mRNAs can similarly lead to altered mRNA turnover and/or translation. CircRNAs may also interact with single-stranded (as in the schematic) or double-stranded DNA, forming double or triple helices, respectively, with a potential impact on DNA metabolism (e.g., transcription, replication). The interaction of circRNAs with linear long noncoding RNAs (lncRNAs) could directly affect lncRNA functions (e.g., localization, folding, etc.) or impact the target molecules (e.g., RNAs or proteins) with which the lncRNA associates. The ability to sequester or ‘sponge’ microRNAs is another recognized function of circRNAs. As several circRNAs comprise exonic 5’ UTR segments that bear internal ribosome entry sites (IRESs), circRNAs may also serve as templates for translation. Finally, circRNA interaction with splicing factors may affect splicing of pre-mRNAs. Shaded text boxes, additional considerations critical for the study of circRNAs, including the aforementioned interactions with DNA in the nucleus and with the translation machinery via IRESs in the cytosol. CircRNA levels change as a function of development, in disease states and in response to stress, immune, hormonal or other stimuli (left). CircRNA interactions with RNA, protein, and DNA may change depending on the cell context, and these interactions may be competitive, cooperative, sequential, and so on (center). The localization of circRNA intracellularly and possibly even outside should be examined as it directly impacts its function (right).
circRNAs control gene expression programs (Figure 2).

Evaluate Dynamic Interactions of circRNAs Under Basal and Stimulated Conditions
While the association of circRNAs with other cellular factors (RNAs or proteins) can occur in unstimulated conditions such as those mentioned here, the expectation is that circRNA functions can be altered in response to changing conditions in the cell. Exposure to various stimuli or pathological conditions could alter circRNA interactions with DNA, RNA, or protein due to changes in the abundance, type, structure, localization, or function of the interacting factors. In turn, the new circRNA-associated factors could impact the components of the ribonucleoprotein (RNP) complex and the circRNA in similar ways by changing their abundance, localization, function, and structure. In particular, regarding structure, it is important to note that changes in circRNA folding may expose or hide binding sites of other RNAs or proteins and thus influence circRNA function. As circRNAs are generated in the nucleus during splicing, it is expected that many of the intron-containing circRNAs are retained in the nucleus and may influence the splicing of pre-RNA counterparts by competing with splicing regulators.

Potential circRNA–DNA Interaction
During DNA replication, two strands of the genomic DNA separate to form single-stranded DNA. CircRNAs in the nucleus may interact with the opposite strand of its genomic DNA via sequence complementarity, which may form a DNA–RNA triple helix affecting DNA replication. Linear ncRNAs residing in the nucleus were found to interact with DNA-forming RNA–DNA duplexes and triplexes in association with proteins to control gene expression. For instance, promoter-associated RNA forms a triplex with the binding site of ITTF1 and simultaneously recruits DNMT3b to repress RNA expression, suggesting a transcription-associated interaction with the genomic locus. It has been suggested that IncRNA ANRASSF1 interacts with a DNA-forming RNA/DNA hybrid at the transcription start site that facilitates the recruitment of the chromatin-modifying PRC2 complex, leading to a specific reduction in RASSF1A transcriptional activity. In addition, mapping of the IncRNA HOTAIR identified enriched DNA-interacting sequences. These and other examples have been reviewed elsewhere. In a manner akin to linear ncRNAs, the interactions between DNA and nucleus-residing circRNAs, particularly those comprising intronic sequences, is another area that warrants exploration.

Assess Possible circRNA Translation
General translation of mRNAs begins when the translation machinery recognizes the 5' UTR cap, scans for the start codon, and generates a polypeptide until it encounters a stop codon. Cap-independent translation can also occur and requires an internal ribosomal entry site (IRES). Interestingly, circular mRNAs have been engineered to generate large proteins in vitro by removing the stop codon. A limited screen of circRNAs indicated that they are not associated with polyribosomes, suggesting the possibility that circRNAs are not translated. However, circRNAs may not require large polyribosomes, and instead, just a few ribosomes can generate small proteins or micropeptides. In this regard, a micropeptide was found to be encoded by a linear ncRNA that regulates muscle performance, and the viral circRNA covalently closed circular (CCC; 220-nt long) was found to be translated into a 16-kDa protein in infected rice plants. Bioinformatic analysis uncovered several circRNAs that encompass IRESs, indicating that they could putatively be translated into small proteins or micropeptides. An in vitro synthesized circRNA with an IRES was shown to bind the 40S ribosomal subunit and initiate translation. Another report suggested the translation of an artificial circRNA with a green fluorescent protein (GFP) open-reading frame in Escherichia coli, and a recent study suggested that mRNAs with an N6-methyladenosine (m6A) modification in the 5' UTR can be translated in a cap-independent manner through an m6A interaction with the eukaryotic initiation factor 3 (eIF3). Thus, it will be interesting to investigate whether IRESs and/or m6A modifications occur in circRNAs and if they have an impact on cap-independent circRNA translation. We hypothesize that some circRNAs that contain short open reading frames (ORFs), like linear IncRNAs, could be translated to generate small proteins, micropeptides, and even truncated or chimeric proteins. It will be particularly important to establish more comprehensively whether subsets of circRNAs associate with polysomes and might be translated.

CLOSING REMARKS
Intense efforts are underway to elucidate the functions of circRNAs. Some examples of circRNAs affecting gene expression are already becoming
apparent. Given that they can bind microRNAs and proteins involved in transcription and splicing, circRNAs are widely believed to influence mRNA metabolism on many levels (transcription, splicing, mRNA turnover, translation) (Figure 2). Based on the same ability to bind nucleic acids and proteins, additional functions are hypothesized for circRNAs, such as their translation via IRES elements and interaction with DNA. However, as highlighted in this review, these interactions must be investigated in the context of: (1) the levels of circRNAs and associated factors, which may change with development, cell stimuli, disease states, and other conditions; (2) the dynamic interactions among the circRNA-associated factors, which can compete, cooperate, and otherwise influence the composition of the circRNA molecular complex; and (3) the localization of the circRNA in distinct cellular locales (nucleus, cytosol, endoplasmic reticulum, mitochondria, extracellular vesicles, etc.) as specific sets of circRNA-interacting molecules reside in each subcellular compartment. The ongoing efforts to elucidate circRNA function must include these considerations as they will illuminate more fully the rich and versatile impact of circRNAs in physiology and pathology.

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REFERENCES


