Circular RNAs in Eukaryotic Cells

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Abstract: Circular RNAs (circRNAs) are now recognized as large species of transcripts in eukaryotic cells. From model organisms such as *C. elegans*, *Drosophila*, mice to human beings, thousands of circRNAs formed from back-splicing of exons have been identified. The known complexity of transcriptome has been greatly expanded upon the discovery of these RNAs. Studies about the biogenesis and physiological functions have yielded substantial knowledge for the circRNAs, and they are now more likely to be viewed as regulatory elements coded by the genome rather than unavoidable noise of gene expression. Certain human diseases may also relate to circRNAs. These circRNAs show diversifications in features such as sequence composition and cellular localization, and thus we propose that they may be divided into subtypes such as cytoplasmic circRNAs, nuclear circRNAs, and exon-intron circRNAs (EIciRNAs). Here we summarize and discuss knowns and unknowns for these RNAs, and we need to keep in mind that the whole field is still at the beginning of exciting explorations.

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INTRODUCTION

Early days of molecular biology guided under the so called central dogma had led to the identification of RNA molecules such as rRNA, mRNA, and tRNA between 1950s and 1960s [1, 2]. These RNAs are essential to all the cells in fulfilling the task of expressing information coded by genomic DNA into functional proteins. They also had set up one of the general features of RNA as linear molecule with a 5' end and a 3' end. As a special form of RNA comparing to the "common" linear form, circular RNAs (circRNAs) were firstly described in 1976 that several plant viroids were covalently closed and single-stranded circular molecules without any end [3].

The decade following the discovery of plant viroids as circRNAs had witnessed the identification of RNA genomes of several plant and then animal viruses as circular molecules [4, 5]. The possible existence of circRNAs with covalent linkages in the cytoplasm of mammalian cells was indicated by electron microscopy in 1979 [6]. It was also reported that some yeast mitochondrial RNAs might be circular in 1980 [7]. The decade between late 1970s and 1980s with research about RNA splicing had demonstrated that introns were spliced out as another nonlinear form of "lariat" [8]. An important discovery coming out later was that an RNA exonuclease RNase R could digest all linear RNAs in isolated total RNA and leave only the loop portion of the intronic lariat and circRNAs [9, 10]. Mammalian cells might also have endogenous intronic circRNAs presumably coming from the

loop of intronic lariat [10, 11]. The resistance to RNase R digestion has now become one of the golden markers of circRNA.

It has taken more than two decades before the realization that a large number of circRNAs actually exist in mammalian cells. In 1991, Nigro *et al.* identified several "abnormally" spliced transcripts corresponding to a tumor suppressor gene DCC, in which exons were joined accurately at consensus splice sites, but in an order different from that present in the primary transcript [12]. The authors called these transcripts "scrambled exons". One year later, another example of scrambled exons was reported in which a human c-ets-1 transcript demonstrated a scrambled order of exons different from that in genomic DNA [13]. The authors also showed that the scrambled transcript was non-polyadenylated, and was not a product either from genomic rearrangement or from any ets-1 pseudogene. Finally in 1993, two reports came out about the formation of circRNAs from exons. In the first report, the previously identified scrambled transcripts of human ets-1 were demonstrated to be circRNA molecules [14]. In the second report, the important testis-determining gene sex determining region Y (Sry) was shown to express high abundant circular transcripts in testis of adult mice [15]. In the following years, examples of circRNAs formed by exonic sequences were discovered for genes such as rat P450 2C24, rat androgen binding protein gene, mouse Formin, human P450 2C18, human MLL, human dystrophin, mammalian sodium-calcium exchanger, and *Drosophila* muscleblind [16-24]. Examples of circRNAs corresponding to linear noncoding RNA (ncRNA) as well as antisense RNA were seen in 2010 and 2011 [25, 26]. It was in 2012 and 2013, with the development of high-throughput RNA sequencing (RNA-seq) and bioinformatic analysis, that circRNAs have begun to be recognized as large species of

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RNAs with thousands of members in mammalian cells [27-29].

Salzman J *et al.* reported in the 2012 paper, that a significant portion of the spliced transcripts from hundreds of human genes were circRNAs based on deep sequencing data from multiple normal and abnormal cell types [27]. In a 2013 paper, Jeck WR *et al.* demonstrated that a number of circRNA species derived from exons of coding genes were present in human fibroblast cells through RNA-seq of libraries from ribosome-depleted RNA with or without the RNase R digestion [28]. In another 2013 paper, Memczak S *et al.* reported that circRNAs were present with thousands in number in human, mouse, and *C. elegans* cells [29]. What was more important, they showed indications and lines of evidence that at least certain circRNAs might possess regulatory functions [29]. These findings have immediately inspired enormous interests to uncover circRNA biology in recent years.

The circular form of RNAs in eukaryotic cells may include plant viroids, genome of some single-stranded RNA viruses, circular intermediate of some tRNAs (permutated tRNAs and some excised tRNA introns) [30], loop part of intronic lariats, circRNAs from noncoding genes or antisense transcripts, as well as exonic circRNAs (Fig. **1**). Currently exonic circRNAs consist of the majority of circRNAs identified in animal cells, and for this review, the term of circRNA refers to exonic circRNAs from back-splicing exons of coding genes unless specified. We would like to summarize and discuss here knowns and unknowns for circRNAs, and hopefully to promote future explorations in this field.

circRNAs IN DIVERSE ORGANISMS

Human and Mammals

circRNAs in human and mice have been extensively profiled [27-29, 31]. In human fibroblasts, it was estimated that 14.4% of actively transcribed genes gave rise to circRNAs, and for some genes the abundance of circRNAs exceeded the corresponding linear mRNA [27]. Analyzing RNA-seq data from the ENCODE project identified ~7,000 human circRNAs with abundance of at least 10% of the transcripts from the corresponding genomic loci [31]. Most circRNAs are expressed with cell specificity [27-29, 31]. Some circRNAs are orthologs between human and mice. For example, Jeck and coauthors identified 69 circRNAs expressed in murine testis that were coded in precisely orthologous genomic sequences to human circRNAs [28]. In another study, it was shown that of about one fifth of the 635 mouse circRNAs annotated were orthologous to human circRNAs, although the sequence conservation of these circRNAs was not higher comparing to that of their neighboring exons [31]. Even most circRNAs are cytoplasmic and some of them possess start codon (e.g. exon 1 is included in the circRNA), the majority of circRNAs should be noncoding as they are not found to associate with polyribosomes [31, 32]. A recent report demonstrated relative higher abundance of circRNAs in murine brain as compared to heart, liver, lung and testis [32].

Drosophila and C. elegans

The first indication about the existence of an exonic circRNA was reported for the *Drosophila* muscleblind gene in

2006 [24]. A large number of circRNAs have now been identified in *Drosophila* and also in another important metazoan model organism *C. elegans*. A survey with over 100 RNA libraries from diverse developmental stages and cell types of *Drosophila* identified more than 2,500 exonic circRNAs [33]. Across multiple *Drosophila* species, the generation of circRNAs from hundreds of genes is conserved. RNA-seq with several life stages of *C. elegans* has recently identified ~1,100 exonic circRNAs [29, 34].
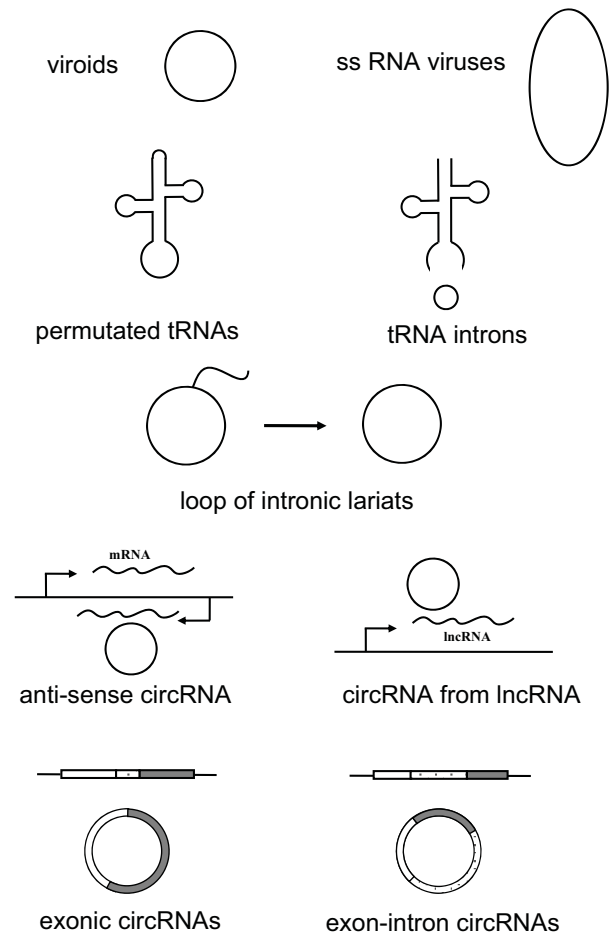
Fig. (1). A variety of circular RNAs in eukaryotic cells. The circular RNAs in eukaryotic cells include plant viroids, some single-stranded RNA viruses, permutated tRNAs, some excised tRNA introns, loop of intronic lariats, circRNAs from noncoding genes, circRNAs corresponding to antisense transcripts, exonic circRNAs from coding genes, and exon-intron circRNAs from coding genes.

The Other Eukaryotic Organisms

A survey for circRNAs in yeasts (*Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*), protists (*Plasmodium falciparum* and *Dictyostelium discoideum*) and a plant (*Arabidopsis thaliana*) has been conducted [35]. A substantial number of exonic circRNAs are identified in all these species. Two points of particular interest are the relative small introns in *S. cerevisiae*, *S. pombe*, *Dictyostelium*, *P. falciparum* and *Arabidopsis* and the very rare alternative splicing in *S. pombe and P. falciparum*. These two features are related to mechanisms of circRNA biogenesis in the following discussion.

The Prokaryotes

circRNAs may have a deep root in the evolution as they are also found in Archaea. It was reported that the archaeon *Sulfolobus solfataricus P2* has circRNAs as excised tRNA introns, rRNA processing intermediates, and a large portion of circular molecules corresponding to ncRNAs (e.g. C/D box RNAs and RNase P RNA), although it seems that no circRNA is generated from coding genes in this archaeon [36, 37]. Many of these circRNAs were conserved in another archaeon *Sulfolobus acidocaldarius* [36]. There is essentially no circRNA in eubacteria with only several of interesting candidates corresponding to rRNAs and tRNAs [37].

circRNA BIOINFORMATICS

Genome-wide identification of circRNAs relies on an efficient bioinformatic analysis. There are multiple pipelines developed to focus on reads from the back-spliced junctions for the identification of circRNAs from RNA-seq data. These include find_circ [29], Segemehl [38], CIRCexplorer [39], CIRC [40] and PFOR [41, 42]. All of them except PFOR depend on the reference genome. In brief, reads that aligned contiguously to the reference genome are filtered out, then the unmapped reads are analyzed further for possible dual alignments as two segments mapped to two genomic sites in the reverse (head-to-tail) order. The resulting reads are considered as circRNA junctions. PFOR is independent on the genome, suitable for *de novo* assembly of circRNAs based on the overlapped reads to pinpoint the full sequences of circRNAs from small RNA-seq data [41, 42].

circRNA BIOGENESIS AND STABILITY

Biogenesis of circRNAs has been recent research hotspot. It had been noticed that relatively long introns and the complementary repeat sequences flanking the region of circularization were associated respectively with the circularization of circEts-1 and circSry [13, 43]. Several genome-wide bioinformatic analyses along with some experimental data now confirmed the association of flanking long introns and the presence of bracketing intronic repeats such as Alu elements to the biogenesis of mammalian circRNA [28, 39, 44, 45]. The association of flanking long introns and complementary sequences with most backsplicing exons has two important indications for mammalian circRNA biogenesis. First, the regions of circularization are marked with hard-to-splice long introns. Secondly, the two splicing sites of backsplicing are brought together by the flanking complementary sequences or some other interactions (Fig. **2**). There are also many exceptions in which circRNAs are formed without association with long boarding introns and intronic repeats [44-46]. Besides flanking intronic sequences, the exonic sequences involved in backsplicing may be crucial also for some circRNAs [44-46]. It seems that the exonic sequences and intronic repeats must collaborate with one another for circRNA biogenesis [44].

Is there any protein specific for circRNA biogenesis? Recently, the RNA-binding protein quaking I 5 (QKI5) was shown to participate in the circularization of some exons [47]. Another interesting example is the "self-regulation" of circRNA biogenesis by muscleblind (MBL) protein on circMbl. circMbl and its flanking introns contain MBL binding sites, and thus MBL can modulate the generation of circMbl [48]. As both QKI5 and MBL are known factors of alternative splicing for selective mRNAs, there is a concern about involuntary involvement of these proteins in backsplicing [49].
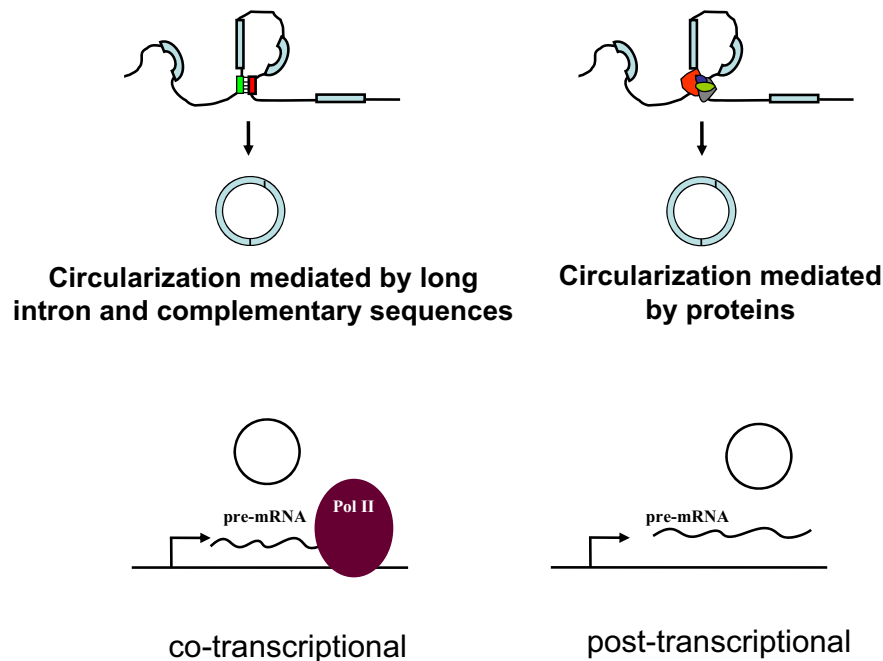
Fig. (2). circRNA Biogenesis. Biogenesis of circRNAs may be mediated by flanking long introns and intronic complementary sequences. RNA binding proteins such as QKI, MBL and ADAR are shown to associate with the biogenesis of certain circRNAs. circRNAs may be generated co-transcriptionally or post-transcriptionally.

circRNAs are ubiquitously present in essentially all mammalian cell types, and for this reason, any essential factor for circRNA biogenesis should be present in all cells. On the other hand, there is no doubt that circRNAs are expressed with cell-type and gene specificity and are not piggybacking byproducts of alternative splicing [27-29, 50]. The molecular ratio of circRNAs to mRNAs for individual genes has also to be managed for different cells under different conditions [45, 50]. Molecular mechanisms of these regulations are waiting for further investigations.

As splicing is generally co-transcriptional, the backsplicing for the generation of circRNAs may also be co-transcriptional. Ashwal-Fluss R *et al.* provided data to show the co-transcriptional produce of circRNAs; backsplicing of circRNA and linear splicing of mRNA may compete against each other [48]. On the other hand, Liang and Wilusz found with overexpressing plasmids that the pre-mRNA requires a stable 3′ end for the favor of circRNA biogenesis, arguing strongly that circRNA biogenesis may be post-transcriptional at least in their experimental system [44] (Fig. **2**).

Researches on circRNAs in organisms other than mammals provide additional insights to circRNA biogenesis. It was found that *C. elegans* circRNAs were significantly enriched with flanking reverse complementary sequences in introns, and the double-strand RNA-editing enzyme ADAR1 mitigated circRNA expression presumably via melting double-stranded structures formed by these complementary sequences [34]. The facts that yeasts, protists, and *Arabidopsis* having exonic circRNAs but without long intron and the lack of pervasive alternative splicing in *S. pombe and P. falciparum* indicate strongly that circRNAs may have a splicing independent biogenesis in these species.

The amount of each circRNA and the expression profile of circRNAs are decided presumably together by circRNA biogenesis and degradation. How circRNAs are degraded is largely an unexplored subject. circRNAs are more stable due to their inaccessibility to exonucleases such as RNase R [9, 10]. Questions such as how circRNAs are degraded intracellularly (and/or intercellularly), whether and how this process is regulated, and could circRNA degradation be regulated for individual circRNAs are waiting for future investigations.
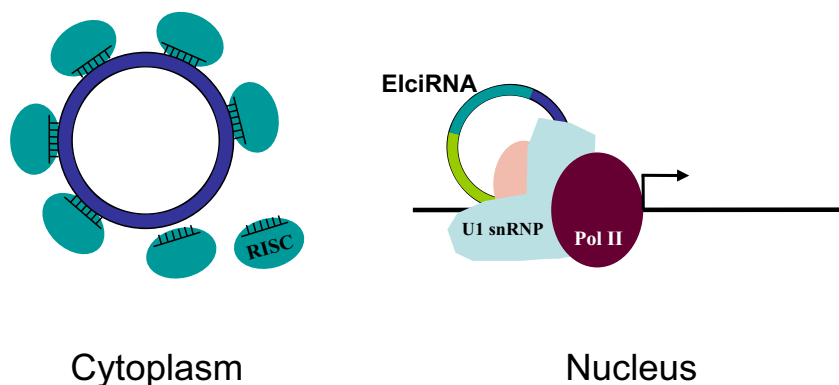
circRNA FUNCTIONS AND FUNCTIONAL MECHANISMS

Multiple lines of evidence suggest strongly that circRNAs may play crucial regulatory roles in eukaryotic cells. From yeast to human cells, circRNAs are shown to demonstrate specific expression profiles corresponding to specific cellular conditions, developmental stages, and cell types [27-29, 33, 34, 45, 50].

The first serious circRNA functional studies demonstrated that two circRNAs function as microRNA sponge [29, 51]. The mammalian circRNA CDR1as harbors over 70 conserved binding sites for miR-7, and indeed overexpression CDR1as suppressed miR-7 function [29, 51]. The murine circRNA circSry might be a microRNA sponge with more than ten miR-138 binding sites, although these sites are missing in human circSry [31, 51]. Both CDR1as and circSry are cytoplasmic, which is in consistence with their functional role as microRNA sponge. Most circRNAs are found in the cytoplasm, and findings about these two circRNAs indicated immediately a regulatory mechanism as microRNA sponge as a common way for circRNA function. Although later analysis showed that only several out of all the thousands human circRNAs harbor multiple computational binding sites for individual microRNAs, it is unlikely for human circRNAs to play a general role as microRNA sponge [31]. Furthermore, *P. falciparum* and *S. cerevisiae* lack microRNA pathway but both have circRNAs [35]. Interestingly, *Drosophila* circRNAs do harbor over a thousand predicted miRNA sites conserved across the genus [33].

A specific subtype of circRNAs called exon-intron circRNA (EIciRNA) is found to localize predominantly in the nucleus [45]. EIcircRNAs are also distinct in their sequences; unlike the other exonic circRNAs with all introns spliced out, EIciRNAs are intron-retained or composed with both exonic and intronic sequences. EIciRNA promotes the transcription of RNA polymerase II (pol II) *in cis* via RNA-RNA interaction between EIciRNA and U1 snRNA. The interacting site of EIciRNA and U1 snRNA is the 5' splicing site of the EIciRNA retained intron [45].

Cellular localization would be linked directly with physiological function and functional mechanism of circRNAs. The pervasive distribution in the cytoplasm for

Cytoplasm   Nucleus

Fig. (3). circRNA Function & Functional mechanism. Two circRNAs (CDR1as and circSry) play roles as microRNA sponge in the cytoplasm, and EIciRNAs regulate transcription in the nucleus.

circRNAs may be passive due to the leaky and breakdown of nuclear membrane in mitosis. This simple assumption can not explain the nuclear localization of EIciRNA and the cytoplasmic localization of circRNAs in non-dividing cells (e.g. neurons) [32]. Interestingly, punctae enriched with circRNAs are noticed recently in the neuronal dendrites [32]. All these suggest strongly that active transporting or localization mechanisms for circRNAs must exist, correlated with physiological functions of specific circRNAs and maybe with some cell specificity.

DIVERSITY OF circRNAs

The way to view exonic circRNAs as a family with common features in their sequences, biogenesis, cellular localization, functions and functional mechanisms may need to be abandoned. The discovery of EIciRNA with its function and functional mechanism provides a paradigm of circRNA diversity [45]. The composition of both exonic and intronic sequences makes EIciRNA distinct from the other circRNAs. The inclusion of intron against intron exclusion may be subjected to regulation in EIciRNA biogenesis. The nuclear localization of EIciRNAs may be due to their association with nuclear proteins (e.g. proteins of U1 snRNP), or some other mechanism may be involved. Finally the function and functional mechanism as transcriptional regulator may be unique to EIciRNAs but not to the other circRNAs. We see several discrepancies or even disputes in circRNA biology such as roles of flanking long introns and reverse complementary sequences in circRNA biogenesis, cotranscriptional or posttranscriptional generation of circRNAs, and being microRNA sponge or not. It is possible that these are not really discrepancies but rather indications of great diversity within circRNAs. The only common feature of all circRNAs may just be that they are circular.

circRNAs AND HUMAN DISEASES

There are indications about the association between some human diseases and circRNAs. Upon the finding of circRNA corresponding to the INK4/ARF-associated ncRNA, it was noticed that this circRNA might have some correlation with risk of atherosclerosis [25]. The discovery of CDR1as an miR-7 sponge promoted thoughts about the link between circRNAs and neurodegenerative diseases (NDs) such as Alzheimer's disease (AD), as miR-7 and some other microRNAs are directly related to NDs [52-55]. Several studies showed association of circRNAs with cancers [56-59]. The overall circRNA abundance is lower in tumor cells and correlates negatively with cell proliferation [59]. Interestingly, levels of circRNAs increase relative to linear mRNAs during aging [33, 34]. There is a database for SNP (single nucleotide polymorphism) associated with diseases for genomic sites with corresponding circRNAs [60]. Exactly to how much degree that circRNAs are involved in human diseases and the actual roles of circRNAs underneath the etiology of particular disease remain for further studies.

CONCLUSION

A large number of circRNAs have been identified in eukaryotes. With multiple lines of evidence, these molecules are believed to be crucial regulatory elements and are not splicing noise as previously thought. The field of circRNA has bloomed in recent years with researches related to their expressing profiles, biogenesis, physiological relevance and functional mechanisms. However, we are still at the beginning of this field with a lot of unknowns waiting for further investigations.

We may need to learn from the development in the research field of linear lncRNA to answer the fundamental questions such as how circRNAs are generated, degraded, localized, and what are their functions and functional mechanisms. The first examples of lncRNAs such as XIST had been identified as potent epigenetic regulator [61]. Researches within the past two decades have now demonstrated that lncRNAs are a diverse family with thousands of members functioning with a plethora of molecular mechanisms in an array of physiological relevancies [62-66]. The circRNA field may need to realize the potentially great diversity among circRNAs, and have to start from detailed studies of circRNA subtypes (e.g. EIciRNAs). The full picture of circRNA biology may not be able to assemble until specific subtypes of circRNAs are explored.

circRNAs may be directly linked to future biomedical research for disease etiology, diagnosis and treatment, and more understanding in circRNAs would be essential for the development of RNA biology and biomedicine.

BIOINFORMATIC RESOURCE

find_circ, http://circbase.org/cgi-bin/downloads.cgi

Segemehl, http://www.bioinf.uni-leipzig.de/Software/segemehl/

CIRCexplorer, https://github.com/YangLab/CIRCexplorer

CIRC, https://sourceforge.net/projects/ciri/

PFOR2, http://journals.plos.org/plospathogens/article?id=10.1371/journal.ppat.1004553

Circ2Traits, http://gyanxet-beta.com/circdb/

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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