CellPress

Antiviral Immunity and Circular RNA: No End in Sight

Cristhian Cadena[1,3] and Sun Hur[2,3,*]
[1]Program in Virology, Division of Medical Sciences, Harvard University, Cambridge, MA 02138, USA
[2]Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115, USA
[3]Program in Cellular & Molecular Medicine, Boston Children's Hospital, Boston, MA 02115, USA
*Correspondence: sun.hur@childrens.harvard.edu
http://dx.doi.org/10.1016/j.molcel.2017.07.005

In this issue of *Molecular Cell*, two papers by Chen et al. (2017) and Li et al. (2017) describe new insights into circRNA biogenesis and function, connecting circRNAs to innate immune pathways.

Double-stranded RNA (dsRNA)-binding proteins constitute a large part of the host antiviral innate immune system. These proteins include pattern recognition receptors, such as RIG-I, MDA5, and TLR3, which couple viral dsRNA binding to antiviral cytokine production, and effector molecules, such as PKR, ADAR1, and NF90/110, which upon dsRNA binding induce a variety of changes in cellular and viral RNA processes to suppress viral replication. The two reports by Chen et al. (2017) and Li et al. (2017) in this issue of *Molecular Cell* suggest circular RNAs (circRNAs), derived from both host and viruses, as a new class of RNA molecules that interact with these dsRNA binding antiviral proteins and interface the host-virus interaction.

CircRNAs are RNAs in which the 5′ and 3′ ends are covalently connected to form a closed-loop structure. One way that circRNA is generated is through "back-splicing," wherein a splice donor is joined with an upstream splice acceptor instead of a downstream acceptor as in a normal splicing reaction (Figure 1A). This leads to an exon skipping and generation of a circRNA containing the skipped exon. Back-splicing occurs at low frequency but can be promoted when the splice donor and acceptor are brought together through either an RNA secondary structure or a protein binding (Figure 1A). Due to the lack RNA ends, circRNAs are resistant to cellular exoribonucleases and thus are extremely stable.

CircRNAs were first described more than 20 years ago and have long been thought to be byproducts of rare mis-splicing reactions with no specific functions (Capel et al., 1993; Cocquerelle et al., 1993). However, accumulating evidence suggests that circRNAs are generated from a broad range of transcripts, and some circRNAs have regulatory functions in gene expression (Lasda and Parker, 2014). For example, two abundant circRNAs, ciRS-7/CDR1 and Sry circRNA, were shown to soak up and suppress miRNA-7 and miRNA-138, respectively, by having multiple repeats of cognate miRNA-binding sites (Hansen et al., 2013; Memczak et al., 2013). CircRNAs were also shown to sequester RNA-binding proteins, such as MBL, thereby functioning as protein decoys (Ashwal-Fluss et al., 2014). Finally, circRNAs were thought to be more prone to adopt secondary or tertiary structures due to the constrained circular geometry, although whether this is true and whether such structures confer functions remain to be tested.

In the article by Chen et al. (2017), the authors showed an unexpected function of circRNAs in eliciting antiviral immune response. They found that transfection of purified circRNA, independent of sequence or the synthesis mechanism, leads to activation of RIG-I (Figure 1B). This recognition cannot be explained by the known preference of RIG-I for 5′-triphosphate-containing dsRNA end. Intriguingly, Chen et al. (2017) found that, when circRNAs were synthesized in cells (from plasmids) instead of being synthesized in vitro and transfected into cells, the immune stimulatory activity was dependent on the splicing mechanism. CircRNAs generated with endogenous introns (for example, from the human ZKSCAN1 gene) and spliced by cellular spliceosomes did not stimulate RIG-I. In contrast, circRNAs made with self-splicing introns (for example, from phage td intron) stimulated RIG-I. Interestingly, mass spectrometry analysis showed that "self" circRNAs generated by the host splicing machineries co-purify with a series of RNA-binding proteins, as well as the splicing proteins, while self-spliced "non-self" circRNAs have few proteins bound. This led to the model that host spliceosomes mark endogenous circRNAs as "self" using the protein-binding partners, which in turn protect host circRNAs from RIG-I detection. In other words, circRNAs intrinsically harbor an unknown RIG-I-stimulatory element, and cellular circRNA, unlike viral circRNA, evades RIG-I recognition by protecting itself using native protein partners (Figure 1B).

The observations by Chen et al. (2017) raise several intriguing questions. Do the specific identities of the "self" circRNA-bound proteins matter in evading RIG-I detection? Or is it the total level of bound proteins, independent of their identities, that matters? How does protein binding prevent circRNA from stimulating RIG-I? Is it by altering the RNA structure, masking the RIG-I recognition element? Or do proteins affect nuclear export efficiency, thereby indirectly changing the RIG-I accessibility? What is the relative importance of circRNAs versus 5′ ppp-containing canonical RNA ligands for RIG-I during viral infection? Lastly, and perhaps most urgently, precisely how are circRNAs recognized by RIG-I despite lacking a 5′ end? Chemical structural probing analysis (SHAPE analysis) by Chen et al. (2017) showed little difference in secondary structures between linear and circular RNAs. But, it is possible that these RNAs, once introduced in cells, can adopt more

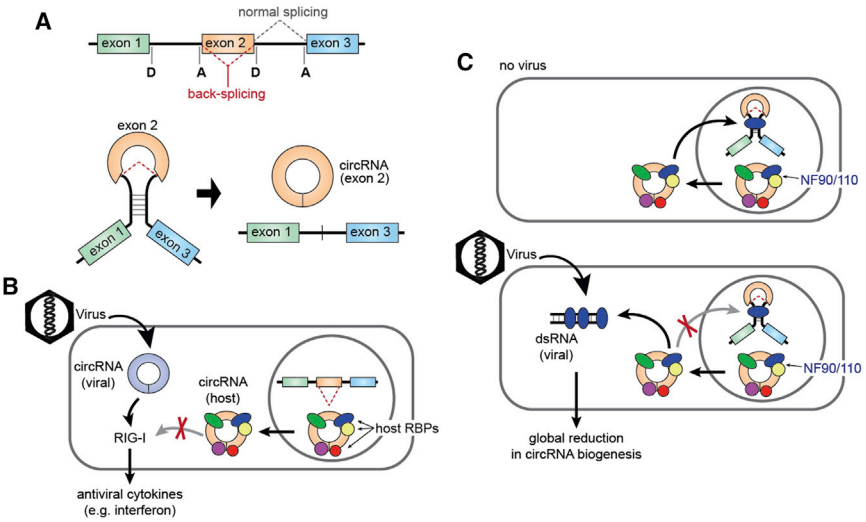

Figure 1. CircRNAs and Antiviral Immunity

(A) Schematic of circRNA biogenesis.

(B) CircRNAs from virus, but not host, are recognized by the host antiviral immune receptor RIG-I. This is because circRNA has an intrinsic ability to stimulate RIG-I, and the host circRNAs evade the recognition using the protein-binding partners.

(C) Antiviral dsRNA-binding proteins, NF90/110, stabilize the intronic RNA secondary structure that promotes circRNA biogenesis. NF90/110 also serve as global regulators of circRNA biogenesis by lowering its nuclear level during viral infection.

complex structures that in turn stimulate RIG-I.

The report by Li et al. (2017) provides a related, but somewhat different, perspective of how the antiviral innate immune system interacts with circRNAs. Unlike in Chen et al. (2017)'s paper, which focuses on how viral circRNAs affect the host immune function, Li et al. (2017) report how the host immune system and viral infection affect host circRNA biogenesis. Li et al. (2017) used a genome-wide short hairpin RNA (shRNA) library screening to identify genes involved in host circRNA biogenesis. Intriguingly, many of the viral RNA receptor genes, including RIG-I and NF90/110, were identified as important factors for circRNA generation. NF90 and NF110, both dsRNA-binding proteins, are splice variants from the common gene ILF3 and are known to be involved in a broad range of RNA biology, from transcription to host-virus interactions. Li et al. (2017) found that both NF90 and NF110 bind RNA duplexes that were formed by inverted repeat elements in the introns flanking the two splice sites. This stabilizes the RNA hairpin structure that promotes back-splicing. Accordingly, depletion of NF90/110 leads to a global decrease in the level of circRNAs.

Can the level of NF90/110, and thus circRNAs, be regulated in normal physiological settings? Li et al. (2017) found that nuclear NF90/110 is depleted upon vesicular stomatitis virus infection or transfection of long dsRNA mimic, polyIC, into the cells. The observed nuclear depletion was caused by cytosolic sequestration of NF90/110, and Li et al. (2017) proposed that this sequestration is mediated by PKR-dependent block of nuclear import and/or sequestration of NF90/110 by viral dsRNA in the cytoplasm. Curiously, Li et al. (2017) noted that NF90/110 binds not only intronic dsRNA during circRNA biogenesis, but also mature circRNA devoid of introns. Additionally, binding assays showed that NF90/110 have higher affinities for circRNAs than for the linear counterpart. These observations intriguingly align with those made by Chen et al. (2017) that circRNA, but not linear RNA, stimulate another dsRNA binding protein, RIG-I. Whether there is a common structural element within circRNA that can explain the preferential recruitment of RIG-I and NF90/110 is yet unknown. Additionally, whether circRNAs, either individually or as a group, can influence the functions of cytosolic antiviral dsRNA-binding proteins also remains to be investigated.

Another intriguing finding by Li et al. (2017) is that circRNA biogenesis is affected not only by the nuclear population of NF90/110, but also by non-nuclear proteins, such as RIG-I and TLR3, which are known to be functional in the cytoplasm and endosomal lumen, respectively. This raises the question of how these proteins affect circRNA biogenesis and whether RIG-I and TLR3 can influence biogenesis through their common downstream antiviral signaling pathways. This finding thus suggests another potential link between circRNAs and antiviral immune molecules beyond their dsRNA-binding activities.

CircRNAs, which were previously considered byproducts of erroneous splicing reaction, are emerging as a new class of non-coding RNAs with biological functions. The two studies in this issue of *Molecular Cell* offer the latest addition to the growing list of the functions of circRNAs. They do so by providing evidence for a complex layer of interactions between circRNAs and dsRNA-binding proteins in antiviral immunity. It is certain that more detailed investigation of how circRNAs interact with these antiviral proteins will shape our understanding of circRNA biology.

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