CellPress

Trends in Biochemical Sciences

Review

Circular RNAs in physiology and non-immunological diseases

Liang Chen,[1] Chuan Huang [2,3,*] and Ge Shan [1,4,*]

Circular RNAs (circRNAs) are covalently closed single-stranded RNAs. Four subclasses of circRNAs have been identified in animal cells, and they have unique features in their biogenesis, degradation, and transport. CircRNAs have diverse molecular functions in sponging miRNAs, regulating transcription, modulating RNA-binding proteins, and even encoding proteins. Some circRNAs are important regulators of various physiological processes to maintain homeostasis. Dysregulation of circRNAs is associated with human disorders, and individual circRNAs are crucial factors that contribute to major diseases including non-immunological diseases such as cancers, neurological disorders, cardiovascular disease, and metabolic disease. Debates on circRNAs have also been raised in recent years, and further studies would help to resolve these disputes and potentially lead to biomedical applications of circRNAs.

Highlights

All circRNAs are covalently closed single-stranded RNAs, but they are diverse in their sequence composition, metabolism, roles, and functional mechanisms.

The dynamic expression and functional roles of circRNAs are closely associated with different physiological states, and various circRNAs are known to serve as crucial regulators of normal homeostasis.

Perturbations in circRNA expression are linked to an array of complex diseases. In particular, numerous individual circRNAs repress or induce the initiation and/or progression of various non-immunological diseases.

CircRNAs hold great potential as clinical diagnostic and prognostic markers, therapeutic targets, and even treatment reagents.

Overview of animal and human circRNAs

CircRNAs are a large class of covalently closed single-stranded RNAs [1–8]. They are common outputs of eukaryotic transcription and RNA processing, and some have been found to play crucial roles in diverse molecular and cellular events [2–6]. The development of research methods (Box 1) has substantially improved our understanding of the basic properties of circRNAs and their roles in normal physiology and major human diseases. Although circRNAs have been characterized in the context of immunological diseases and viral infections [9–11], we focus here on the newly described functional relevance of individual circRNAs to normal physiology and major non-immunological human diseases. We first provide a brief introduction to the molecular functions and mechanisms of circRNAs, and then summarize their physiological and pathological roles and discuss ongoing debates.

Major molecular mechanisms of circRNA function

Four subclasses of circRNAs have been identified in animal cells, including exonic circRNAs (EcircRNAs), exon–intron circRNAs (ElciRNAs), and intronic circRNAs (ciRNAs) that are encoded by the nuclear genome (Figure 1A) [2–6,12]. Another subclass has recently been identified as mitochondria-encoded circRNAs (mecciRNAs) (Figure 1A) [13]. Features of the biogenesis (Figure 1A), cellular export (Figure 1B), and degradation (Figure 1C) of these circRNAs are described in Box 2.

Inhibiting miRNA activities

Multiple EcircRNAs are presumed to serve as **competing endogenous RNAs** (**ceRNAs**; see Glossary) or microRNA (miRNA) sponges [2–6,14,15] (Figure 2A). A functional ceRNA would possess multiple copies of binding sites for the same miRNA. For example, *Cdr1as* is a conserved mammalian circRNA, and human *Cdr1as* has 74 predicted binding sites for *miR-7*, many of which are functional [14,15]. A circRNA in mice, *circSry*, contains 16 putative binding sites for *miR-138*, and experimental data also support a role for *circSry* as a *miR-138* sponge

[1]Department of Clinical Laboratory, First Affiliated Hospital of the USTC, Hefei National Laboratory for Physical Sciences at Microscale, Chinese Academy of Sciences (CAS) Key Laboratory of Innate Immunity and Chronic Disease, School of Basic Medical Sciences, Division of Life Science and Medicine, University of Science and Technology of China (UTSC), Hefei, 230027, China
[2]School of Life Sciences, Chongqing University, Chongqing 401331, China
[3]Center of Plant Functional Genomics, Institute of Advanced Interdisciplinary Studies, Chongqing University, Chongqing 401331, China
[4]Sir Run-Run Shaw Hospital, Zhejiang University School of Medicine, Hangzhou 310016, China

*Correspondence:
chuanhuang@cqu.edu.cn (C. Huang) and shange@ustc.edu.cn (G. Shan).

 https://doi.org/10.1016/j.tibs.2021.11.004

Box 1. Methods and biotechnologies used in circRNA studies

For global circRNA profiling, two main methods are used to prepare RNA fractions for next-generation sequencing (NGS). RNAs depleted for ribosomal RNAs are fractionated, which allows both mRNA and circRNA characterization in the same sample. CircRNAs can be enriched by RNase R treatment to degrade linear RNAs, and this method has been improved by replacing K^+ in the reaction buffer by Li^+ to facilitate the complete degradation of linear RNAs [82]. Third-generation sequencing (TGS) has recently been utilized to profile circRNAs [83,84]. One of the advantages of TGS is the much longer reads than NGS, and the full-length circRNAs can be deduced more reliably [83,94].

Molecular or biochemical methods such as real-time RT-PCR, northern blotting, and fluorescent *in situ* hybridization (FISH) have been adapted for the detection of individual circRNAs based on their unique circular junction sequences. Overexpression of circRNAs is achieved by plasmid- or virus-based systems that utilize reverse complementary sequences or simply the cognate flanking sequences bracketing the circularization region. The expression of circRNAs can be knocked down using RNA interference or RNase H-based antisense oligonucleotides (ASOs) that target the circular junction, without adverse effects on transcription of the host gene [85].

CRISPR-based genome editing is used to generate circRNA knockout animals by removing intronic sequences that facilitate circularization [44,67]. In these knockouts, the targeted circRNA is successfully eliminated, and the expression of mRNAs and the levels of the corresponding proteins are not substantially affected. Other CRISPR-based technologies that primarily directly target circRNAs have also been developed to identify their protein partners, for imaging, or to screen circRNAs in functional studies [3–5,55].

A proof-of-concept example of a circRNA drug has been provided by brain-specific delivery of *circSCMH1* encapsulated in engineered rabies virus glycoprotein extracellular vesicles to alleviate symptoms in mouse and nonhuman primate stroke models [73]. In this case, *circSCMH1* was 'prepared' via overexpression in cultured human HEK293 cells. CircRNAs are synthesized from the circularization of *in vitro* transcribed linear RNAs, and circularization is achieved through chemical ligation, DNA or RNA ligases, or ribozymes [86,87]. These *in vitro* synthesized circRNAs function as artificial ceRNAs or RNA aptamers in cells; when an IRES is included, circRNAs can efficiently express proteins in cultured cells [86,88,89]. Optimistically, engineered circRNAs have the potential to be developed into vaccines or drugs.

[11]. Notably, only a few human circRNAs have over 10 computationally predicted miRNA binding sites [16].

Crosslinking immunoprecipitation sequencing (CLIP-seq) data for Argonaute (AGO) proteins from human HEK293 cells revealed that ~12% of circRNAs contain functional miRNA binding sites as AGO footprint regions, whereas most of these circRNAs have only one such region [17]. Even for EcircRNAs that bind to miRNAs, most of them may not function individually, and instead groups of circRNAs may collectively sponge specific miRNAs. However, in many studies the role of a circRNA as a ceRNA role is based largely on circRNA or miRNA overexpression at high levels, and caution is needed when interpreting these results.

Regulating transcription

ElciRNAs are mostly retained in the nucleus (Figure 1A), and at least two of these, *circEIF3J* and *circPAIP2* in human cells, recruit U1 small nuclear ribonucleoprotein (snRNP) to promote transcription initiation by RNA polymerase II (Pol II) at the promoter of host genes [12]. In addition, several EcircRNAs regulate transcription by interacting with chromatin (Figure 2A). *circFECR1*, encoded by Friend leukemia virus integration 1 (*FLI1*), activates *FLI1* transcription in *cis* by recruiting the DNA demethylase TET1 to induce extensive promoter demethylation [18], and *circSMARCA5*, derived from exons 15–16 of *SMARCA5*, forms an RNA:DNA hybrid (**R-loop**) with the coding DNA, resulting in Pol II stalling at exon 15 [19]. Finally, a ciRNA which is also retained in the nucleus (Figure 1A), *ciankrd52*, forms an R-loop *in cis* and promotes transcription elongation by Pol II [20].

Modulating the functions of RBPs

CircRNAs can act as scaffolds or decoys of **RNA-binding proteins (RBPs)** (Figure 2A). For example, the *Drosophila* gene *muscleblind* (*mbl*) and its human homolog muscleblind-like protein 1 (*MBNL1*) encode a circRNA, *circMbl*, which binds to the Mbl or MBNL1 protein [21].

Glossary

Asthenozoospermia: an infertility condition in male mammals that is characterized by reduced sperm motility.
Backsplicing: an alternative form of splicing in which a splice donor site is joined to a splice acceptor site further upstream in the primary linear transcript, leading to the formation of a circular RNA.
Canonical splicing: the typical splicing reaction that joins the pre-mRNA splice sites in genomic order, and thus introns are removed and exons are connected together in the 5′ to 3′ direction.
Circular junction: a unique RNA region that is only generated through backsplicing or other circularization mechanisms. It consists of sequences that are 5′ and 3′ to each other and non-continuous a linear RNA, but become a contiguous sequences following circularization that links the 5′ and 3′ sites together.
Competing endogenous RNAs (ceRNAs): RNAs that impede other RNA transcripts from miRNA-mediated inhibition or degradation by competing for shared miRNAs.
Internal ribosome entry site (IRES): a class of *cis*-acting RNA elements that recruit ribosomes and initiate translation in a cap-independent manner.
Lariat RNAs: also known as intron lariats, this class of RNA is derived from spliced introns during canonical splicing. Once generated, they are generally subject to rapid debranching enzyme-mediated degradation.
MicroRNA (miRNA) pathway: an interconnected and dynamic process that induces the degradation and/or translational repression of targeted RNAs guided by miRNAs loaded onto Argonaute proteins.
N^6-methyladenosine (m^6A) modification: a class of RNA internal modification involving adenosine methylation at the nitrogen-6 position. It is the most abundant internal RNA modification on mRNAs and long noncoding RNAs (lncRNAs) in higher eukaryotic organisms, and is crucial for the transcription, translocation, translation, and degradation of some RNAs.
RNA-binding proteins (RBPs): proteins that recognize and bind to RNAs. These proteins modulate the transcription, translocation, translation, and degradation of RNAs, thereby playing important roles in RNA biology and in regulating gene expression.

Mbl and MBNL1 facilitate the biogenesis of *circMbl* by binding to flanking introns, thus decreasing the production of the *mbl* or *MBNL1* mRNA [21]. As a result, *circMbl* acts as a protein sponge (or decoy) for Mbl (or MBNL1) to autoinhibit circRNA generation and favor mRNA biogenesis.

circ-Foxo3 in mice functions as a scaffold by binding to the cell-cycle proteins cyclin-dependent kinase 2 (CDK2) and cyclin-dependent kinase inhibitor 1 (or p21) [22]. The formation of a *circ-Foxo3*–p21–CDK2 ternary complex results in inhibition of CDK2 activity [22]. As a scaffold, *circ-Foxo3* also facilitates the degradation of mutant p53 and at the same time inhibits Foxo3 degradation by modulating double-minute 2 (MDM2)-mediated ubiquitination in murine cells [23]. CDK2, p21, and MDM2 are so-called **non-canonical RBPs**.

R-loop: an RNA–DNA hybrid formed by base-pairing between an RNA and one strand of the genomic DNA duplex.
Non-alcoholic steatohepatitis (NASH): a common class of liver diseases characterized by liver inflammation and damage. NASH occurs in people who rarely drink alcohol, and is mainly induced by an excess of fat in the liver.
Non-canonical RBP: RNA-interacting proteins that lack classical RNA-binding domains.

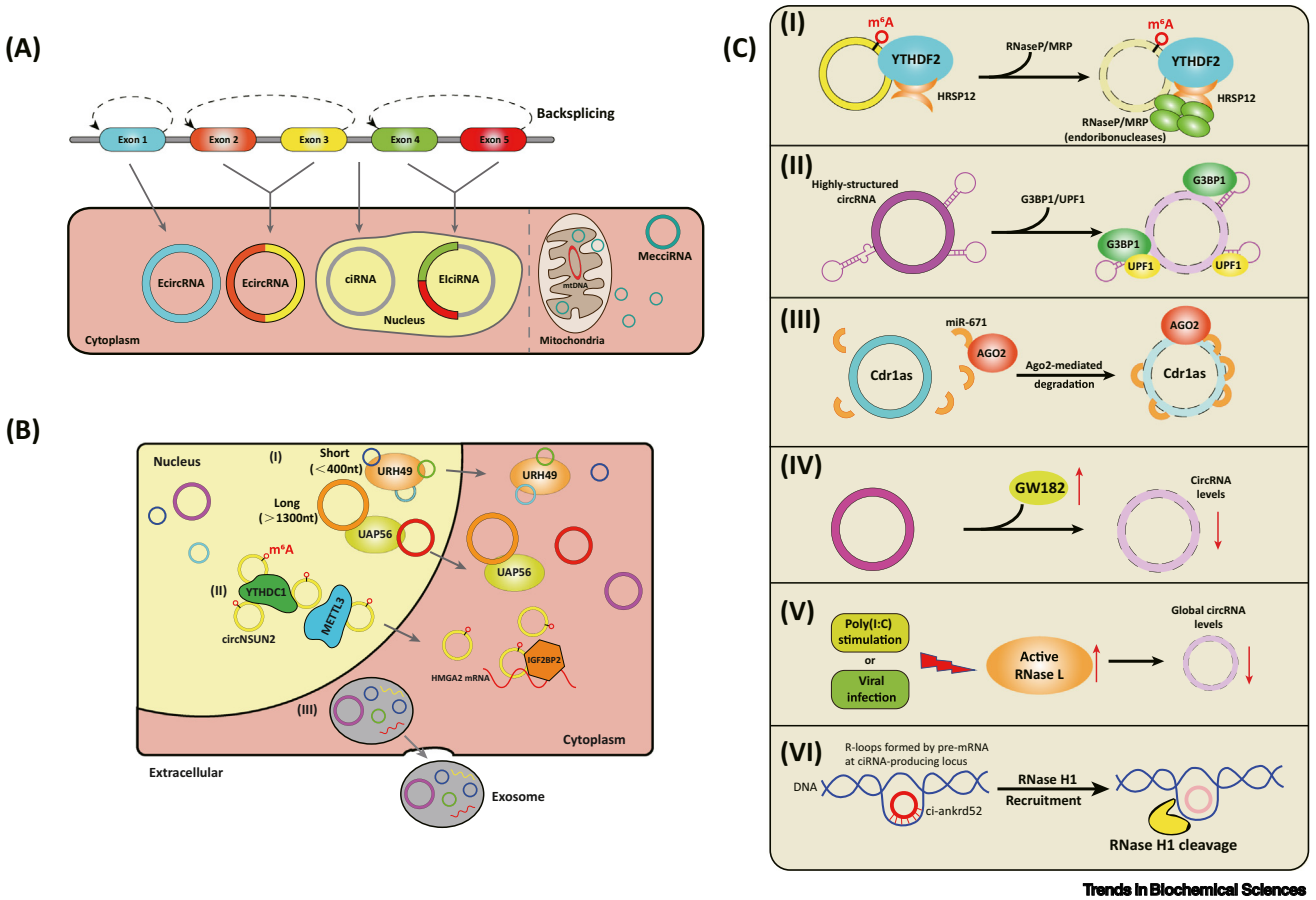


Figure 1. Subclasses and RNA homeostasis of circular RNAs (circRNAs). (A) The biogenesis and subclasses of circRNAs. Nuclear genome-derived circRNAs comprise three groups: exonic circRNAs (EcircRNAs) that are generated by backsplicing and localize predominantly in the cytoplasm, exon–intron circRNAs (ElciRNAs) that are circularized with intronic sequences retained between the backsplicing exons that are localized predominantly in the nucleus, and intronic circRNAs (ciRNAs) from intronic **lariat RNA** precursors that localize in the nucleus. MecciRNAs are mitochondria-encoded circRNAs that are distributed in the mitochondria and the cytosol. (B) Transport of circRNAs. (I) Length-related nuclear export of circRNAs: *Drosophila* Hel25E and its human homolog UAP56 regulate the nuclear export of long circRNAs (>1300 nt), whereas Hel25E and another human homolog URH49 modulate the export of short circRNAs (<400 nt). (II) The m^6A-mediated nuclear export of circRNAs: export is facilitated by YTHDC1 in a METTL3-mediated m^6A methylation-dependent manner, and *circNSUN2* is shown as an example. (III) Intercellular transport of circRNAs via extracellular exosomes: many circRNAs are packaged into exosomes and transported to other cells or various bodily fluids. (C) Degradation of circRNAs. (I) Degradation of m^6A-modified circRNA: m^6A-modified circRNAs are recognized by YTHDF2 and HRSP12, which then recruit the RNase P/MRP complex to mediate circRNA endonucleolytic cleavage. (II) Degradation of highly structured circRNAs regulated by the RNA helicase UPF1 and its associated endonuclease G3BP1. (III) The **microRNA (miRNA) pathway** mediates circRNA degradation, as exemplified by *miR-671:Cdr1as*. (IV) GW182 regulates circRNA stability in an Argonaute-independent manner. (V) Activated RNase L decreases global circRNA levels. (VI) R-loop-dependent *ciankrd52* degradation mediated by RNase H1 cleavage.

Box 2. The basic properties of circRNAs

EcircRNAs and ElciRNAs are generated by backsplicing (see Figure 1A in main text), and their junction sites are 5′–3′ linkages. The flanking introns of circularizing exons usually consist of complementary repeat elements which facilitate circularization [2–6]. Several RBPs, such as the alternative splicing factor QKI, act analogously to complementary sequences in backsplicing [3–6,90]. ciRNAs are derived from lariat RNAs that escape debranching during **canonical splicing** (Figure 1A), and thus their junction sites are 5′–2′ linkages [2–6]. The subclass of mecciRNAs has recently been identified [13,91], and mecciRNAs in the murine brain have been verified by TGS [84] (Figure 1A). The biogenesis of mecciRNAs requires further study.

Most EcircRNAs are cytoplasmic, whereas ElciRNAs and ciRNAs are predominantly nuclear. The *Drosophila* RNA helicase Hel25E and its human homologs UAP56 and URH49 modulate the nuclear export of EcircRNAs (Figure 1B) [2–4,92]. The m^6A modification regulates the nuclear export of a small portion of EcircRNAs (Figure 1B) [63,93]. In addition, mecciRNAs are distributed in both the mitochondria and the cytosol but the mechanism is not well understood [13]. In addition to intracellular transport, EcircRNAs are packaged into exosomes and transported to other cells or through various bodily fluids (Figure 1B) [94,95].

CircRNAs are generally much less abundant than their linear RNA counterparts (with some exceptions), although circRNAs can accumulate, especially in non-dividing cells [2–4]. An explanation for the accumulation is that circRNAs are highly stable and are not susceptible to exonuclease degradation [2–4]. However, emerging studies have investigated their degradation (Figure 1C). The m^6A-modified circRNAs are recognized by the reader YTHDF2 and its adaptor HRSP12, which then recruits the RNase P/MRP complex to mediate circRNA endonucleolytic cleavage [96]. Some highly structured circRNAs are degraded by the RNA helicase UPF1 and the associated endonuclease G3BP1 [97]. The **microRNA (miRNA) pathway** contributes to the degradation of some circRNAs [74,98]. For example, *miR-671* has perfectly matched sites on *Cdr1as*, and *miR-671* invokes AGO2-mediated slicing of *Cdr1as* [98]. GW182, a miRNA pathway component, appears to regulate circRNA stability in an AGO2-independent manner [99]. In response to encephalomyocarditis virus infection, RNase L is rapidly activated and decreased circRNA levels are observed [100]. One ciRNA, *ciankrd52*, forms an R-loop with its cognate DNA, and then RNase H1, an RNase specific for the R-loop, degrades *ciankrd52* [20]. MecciRNAs do not appear to be stable as nucleus-encoded circRNAs, but the mechanism responsible for their degradation remains unknown [13].

The translation of circRNAs

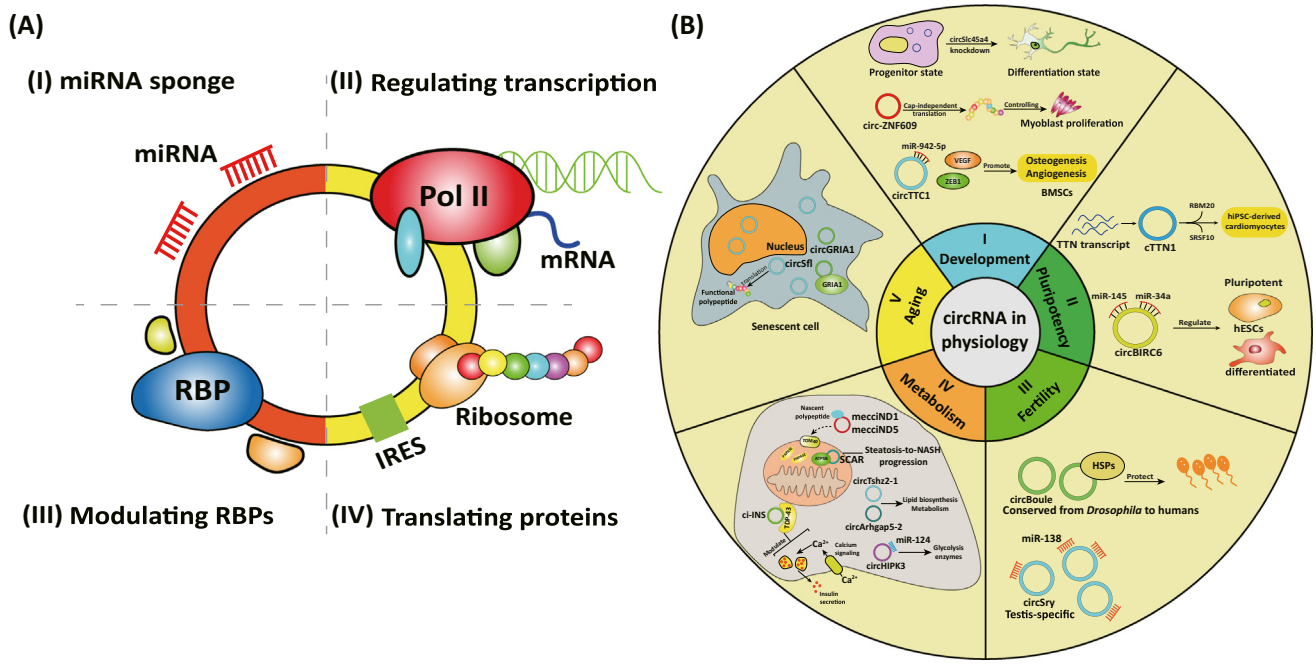
Some EcircRNAs may be translated, and their polypeptide products have been shown to play roles in physiology and disease [24–31] (Figure 2A). For example, *Drosophila circSf1*, which uses the same start codon as the *Sfl* mRNA and contains an in-frame stop codon after the **circular junction**, can be translated into a shorter protein (~25 kDa), and its function in aging is discussed in the following text [24]. The presence of an **internal ribosome entry site (IRES)** and/or ***N^6*-methyladenosine (m^6A) modification** in circRNAs may contribute to ribosome recruitment and translation initiation [29–31]. Caution is needed when interpreting the coding ability of circRNAs from overexpression constructs. In the example of *circZNF609* overexpression, aberrant linear transcripts are generated from the overexpression constructs, and translation of these linear transcripts rather than from the overexpressed circRNAs is pervasive [32,33]. Although circRNAs may encode proteins, most circRNAs are unlikely to serve as translation templates because the majority of cytoplasm-localized circRNAs are not associated with polyribosomes [32,34,35].

CircRNAs in normal physiology

Multiple circRNAs are known to function as crucial regulators of animal and human physiology (Figure 2B). The functions of individual circRNAs in development, pluripotency, fertility, metabolism, and aging have been documented, and strong genetic, molecular, and cellular evidence is available for some circRNAs (Table 1).

Development

Specific circRNAs have been shown to function in neurogenesis, myogenesis, and osteogenesis (Figure 2B). *circSLC45A4*, a circRNA that is conserved from *Xenopus* to humans, is expressed at high levels in the embryonic frontal cortex of humans [36]. Knockdown of *circSlc45a4* causes spontaneous neuronal differentiation of SH-SY5Y human neuroblastoma cells, and disrupts the cell fate switch from apical to basal progenitors in developing mouse cortex [36]. Decreased levels



Trends in Biochemical Sciences

Figure 2. Molecular and physiological roles of circular RNAs (circRNAs). (A) Major molecular mechanisms of circRNA functionality. CircRNAs exert their functions by (I) inhibiting miRNAs as ceRNAs, (II) modulating transcription, (III) modulating the functions of RBPs, and (IV) being translated into proteins. (B) Physiological functions of circRNAs and the corresponding mechanisms. (I) In development: knockdown of the vertebrate circRNA *circSLC45A4* disrupts the cell fate switch from apical to basal progenitor in the developing mouse cortex; *circZNF609* may be translated to control myoblast proliferation; *circTTC1* functions as *miR-942-5p* sponge to regulate the expression of ZEB1 and VEGF and to promote the osteogenesis-angiogenesis coupling in BMSCs. (II) Pluripotency: *cTTN1* interacts with RBM20 and SRSF10 and subsequently modulates the splicing of key muscle genes, and *circBIRC6* functions as a ceRNA for *miR-34a* and *miR-145* to regulate pluripotency genes. (III) Fertility: *circSry* plays roles in the mouse testis as a *miR-138* sponge; *circBoule*, conserved from *Drosophila* to humans, binds to and promotes the ubiquitination of HSPs. (IV) Metabolism: mecciRNAs (e.g., *mecciND1* and *mecciND5*) facilitate the mitochondrial entry of nucleus-encoded proteins; the mecciRNA *SCAR* interacts with ATP5B to regulate the permeability of mitochondrial ROS; *ci-INS* in humans (*ci-Ins2* in rodents) interacts with TDP-43 to modulate Ca^{2+} signaling and insulin secretion; *circTshz2-1* and *circArhgap5-2* regulate genes involved in lipid biosynthesis and metabolism; *circHIPK3* suppresses the expression of glycolysis-related enzymes by sponging *miR-124*. (V) In aging: *circSfl*-derived protein is sufficient to extend the lifespan of *Drosophila*; the mammalian *circGRIA1* negatively regulates GRIA1 transcription and inhibits synaptic plasticity and synaptogenesis during brain aging. Abbreviations: BMSCs, bone marrow stem cells; hESC, human embryonic stem cell; hiPSC, human induced pluripotent stem cell; IRES, internal ribosome entry site; NASH, non-alcoholic steatohepatitis; Pol II, RNA polymerase II; RBP, RNA-binding protein; ROS, reactive oxygen species.

of *circSlc45a4* promote the expression of particular signaling, adhesion, and development-regulating genes, and suppress the expression of genes in multiple biosynthetic pathways through a so far unknown mechanism [36].

Global changes in circRNA expression are observed during myogenesis in cultured human and mouse myoblasts [27]. Based on features such as conservation and expression dynamics, 25 of these circRNAs have been selected for further functional screens. *circ-ZNF609* (or *circZNF609*) is the most effective [30]; it is also is downregulated during myogenesis, and knockdown of *circ-ZNF609* in undifferentiated myoblasts leads to reduced proliferation [30]. Genetic and biochemical data indicate that *circ-ZNF609* encodes a protein that is responsible for its function, although the authors have some reservations about a direct link between *circ-ZNF609* function and its protein-coding capacity [30].

The circRNA *hsa_circ_0074834* (or *circTTC1*) encoded by *TTC1* was identified by comparing the circRNA profiles between bone marrow mesenchymal stem cells (BMSCs) isolated from patients with bone nonunion and normal controls [33]. Nonunion is an arrest in the fracture repair process

Table 1. circRNAs in physiology and non-immunological diseases

circRNA	Physiology or disease	Physiological functions or pathology	Molecular mechanism	Refs
mecciND1 and *mecciND5*	Metabolism	Protects mitochondria from UV irradiation or oxidative stress	Interacts with nucleus-encoded proteins and facilitates their mitochondrial import	[13]
circFECR1	Breast cancer	Promotes breast cancer metastasis	Modulates transcription in *cis*	[18]
circSMARCA5	Breast cancer	Inhibits DNA damage repair and improves drug sensitivity of cancer cells	Modulates transcription via R-loop formation	[19]
circ-Foxo3	Breast cancer	Decreases MDM2-mediated polyubiquitination of Foxo3	Interacts with MDM2 and p53	[23]
circSfl	Aging	Extends the lifespan of fruit flies	Is translated into a short version of Sfl	[24]
circSMO	Glioblastoma	Maintains the self-renewal and tumorigenicity of cancer cells	Is translated into SMO-193a.a	[25]
circZNF609	Myogenesis and Duchenne muscular dystrophy	Sustains human myoblast growth	Is translated into a short version of ZNF609	[30]
circSLC45A4	Development: neurogenesis	Promotes spontaneous neuronal differentiation and disrupts the cell-fate switch from apical to basal progenitor	N.D.[a]	[36]
circTTC1	Development and bone nonunion	Promotes bone tissue regeneration during fracture healing	Sponging of *miR-942-5p*	[37]
circFOXP1	Pluripotency	Maintains mesenchymal stem cell identity in the process of bone repair	Sponging of *miR-127-5p*, *miR-17-3p* and *miR-370-3p*	[38]
circBIRC6	Pluripotency	Determines the pluripotent state of hESCs	Sponging of *miR-34a* and *miR-145*	[40]
cTTN1	Pluripotency and dilated cardiomyopathy (DCM)	Maintains the homeostasis of hiPSC-derived cardiomyocytes	Regulates the nuclear localization and splicing activity of RBM20	[41]
circSry	Fertility	Testis-specific, may regulate mice spermatogenesis	Sponging of *miR-138*	[14,15,43]
circBoule	Fertility and asthenozoospermia	Protects male reproductive function from heat stress	Interacts with non-canonical RBPs (heat-shock proteins)	[44]
ci-INS	Metabolism and type 2 diabetes	Controls insulin secretion	Interacts with the RBP TDP-43	[46]
circTshz2-1 and *circArhgap5-2*	Metabolism and obesity	Promotes adipogenesis	N.D.	[47]
SCAR	Metabolism and NASH	Inhibits mROS output and fibroblast activation	Interacts with the non-canonical RBP ATP5B	[48]
circGRIA1	Aging	Regulates synaptic plasticity and synaptogenesis	Modulates transcription in *cis*	[52]
circTP63	Lung squamous cell carcinoma	Promotes cell proliferation and cell cycle progression	Sponging of *miR-873-3p*	[56]
circCCDC66	Colorectal carcinoma	Regulates proliferation and promotes tumor growth	Sequesters *miR-185*, *miR-33b*, and *miR-93*	[59]
circ2082	Glioblastoma	Enhances tumorigenicity of glioblastoma cells	Binds to the RBP RBM3, leading to nuclear accumulation of DICER	[60]
circAKT3	Gastric cancer (GC)	Promotes DNA damage repair and inhibits apoptosis of GC cells	Sponging of *miR-198*	[61]
circ-E-Cad	Glioblastoma	Enhances stemness, proliferation, invasion, anti-apoptosis and senescence resistance of glioblastoma cells	Is translated into a E-cadherin variant	[62]
circNSUN2	Colorectal carcinoma	Promotes liver metastasis	Interacts with the RBP IGF2BP2	[63]
circPABPC1	Hepatocellular carcinoma (HCC)	Suppresses HCC metastasis and destabilizes cancer cell adhesion to extracellular matrix	Interacts with the non-canonical RBP ITGB1	[64]
circURI1	Gastric cancer	Suppresses GC metastasis	Interacts with the splicing factor hnRNPM	[65]
circACC1	Metabolism and colorectal cancer	Modulates both the β-oxidation of fatty acids and glycolysis	Enhances the enzymatic activity of the non-canonical RBP AMPK	66

(continued on next page)

Table 1. (continued)

circRNA	Physiology or disease	Physiological functions or pathology	Molecular mechanism	Refs
Cdr1as	Neuropsychiatric disorders	Regulates synapse transmission	Sponging of *miR-7* and *miR-671*	[67]
circTLK1	Ischemic stroke	Aggravates neuronal injury and neurological deficits	Sponging of *miR-335-3p*	[71]
circDLGAP4	Ischemic stroke	Attenuates neurological deficits and decreases infarct areas and blood–brain barrier damage	Sponging of *miR-143*	[72]
circSCMH1	Development and Ischemic stroke	Improves brain plasticity, facilitates motor function recovery, and reduces the activation of astrocytes and microglia	Interacts with MeCP2	[73]
circAnks1a	Neuropathic pain	Induces neuropathic pain induced by nerve injury	Sponging of *miR-324-3p* and modulates transcription the *Vegfb* gene	[75]
circSLC8A1	Aging and Parkinson's disease (PD)	Increases the main PD-inducing risks	Sponging of *miR-128*	[76]
circFndc3b	Myocardial infarction	Improves neovascularization and left ventricular functions	Interacts with the RBP FUS	[78]
cZNF292	Atherosclerosis	Promotes angiogenic sprouting and tube formation	N.D.	[79]
circNlgn	Fibrosis and heart failure	Aggravates cardiac fibrosis and leads to heart failure	Is translated into Nlgn173	[80]

[a]Abbreviation: N.D., not determined.

which requires the migration and differentiation of BMSCs. Notably, *hsa_circ_0074834* is significantly downregulated in BMSCs from nonunion patients [37]. *circTTC1* promotes osteogenesis by cultured BMSCs, and the migration and angiogenesis of cultured HUVECs; these effects of *circTTC1* have been subsequently confirmed *in vivo* with transplanted BMSCs in nude mice [37]. Mechanistically, *circTTC1* functions as a *miR-942-5p* sponge to modulate the expression of key genes involved in bone repair, such as *ZEB1* and vascular endothelial growth factor (*VEGF*) [37]. *circFOXP1*, a specific marker of mesenchymal stem cells (MSCs), maintains MSC identity during bone repair [34]. *circFOXP1* may function as a ceRNA against miR-127-5p/miR-17-3p/miR-370-3p [38]. The ceRNA roles of *circTTC1* and *circFOXP1* may need more extensive examination because the published studies mainly relied on overexpression methods [37,38].

Pluripotency

Thousands of circRNAs are differentially expressed during the generation or differentiation of human induced pluripotent stem cells (hiPSCs) [39]. *circBIRC6* and *circCORO1C* were subsequently found to be expressed at high levels in hiPSCs and embryonic stem cells (ESCs); both play positive roles in maintaining the pluripotent state [40] (Figure 2B). *circBIRC6* (but not *circCORO1C*) is associated with AGO2, and functions as a ceRNA with four binding sites for *miR-34a* and *miR-145*, two sites for each miRNA [41]. Recently, the titin gene (*TTN)* has been found to produce heart-specific circRNAs whose biogenesis relies on the splicing factor RMB20 [41]. Among them, *cTTN1* regulates the function of the splicing factor SRSF10 in cardiac homeostasis through an SRSF10 binding motif at its circular junction [41]. *cTTN1* also regulates the nuclear localization and splicing activity of RMB20, and *cTTN1* knockdown disrupts sarcomere structure and induces cell death in hiPSC-derived cardiomyocytes [41] (Figure 2B). Hopefully, circRNAs will be used to modulate reprogramming efficiency and hiPSC differentiation to facilitate stem cell applications.

Fertility

Many circRNAs are specifically expressed in the reproductive organs of animals and humans [42]. In particular, a mouse testis-specific circRNA, *circSry*, is one of the earliest identified mammalian circRNAs [43] (Figure 2B). Surprisingly, the human *SRY* gene generates only linear transcripts but

no circRNA [44]. By contrast, conserved *circBoule* RNAs generated from the testis-specific gene *Boule* have been identified to play a role in preserving male fertility [44]. Genetic knockout of *circBoule* in both flies and mice results in reduced male fertility, especially under heat-stress conditions. Mechanistically, *circBoule* functions as a scaffold to directly bind to heat-shock proteins (HSPs; Hsp60C and Hsc4 in flies, and HSPA2 in mammals) via conserved RNA motifs and then promote the ubiquitination and degradation of these HSPs [44] (Figure 2B). Patients with **asthenozoospermia** have lower *circBoule* levels in sperm, and the protein level of HSPA2 is negatively correlated with *circBoule* levels in normozoospermia but not in asthenozoospermia [44]. Most studies examining the roles of circRNAs in reproduction have been conducted in males, and studies of circRNAs in females are needed [45].

Metabolism

Nucleus-localized *ci-Ins2* or *ci-INS*, which is derived from the intron of the insulin 2 (*Ins2*) gene in rats and mice or the insulin (*INS*) gene of humans, modulates the expression of genes involved in calcium signaling and insulin secretion at least in part by interacting with the RBP TDP-43 [46] (Figure 2B). Extensive changes in circRNA expression have been observed during adipogenesis; among these, *circTshz2-1* and *circArhgap5-2* modulate the expression of genes involved in lipid biosynthesis and metabolism through an unidentified mechanism [47].

MecciRNAs such as *mecciND1* and *mecciND5* promote the mitochondrial import of specific nuclear-encoded proteins (Box 2) [13]. *mecciND1* and *mecciND5* are closely associated with mitochondrial DNA levels and cellular physiology. Cells exposed to UV irradiation or oxidative stress and liver cancers show upregulated *mecciND1* and *mecciND5* expression, indicating that they may play roles in the adaptation of mitochondria to stress [13]. A mammalian conserved mecciRNA, *SCAR* (steatohepatitis-associated circRNA ATP5B regulator), suppresses mitochondrial reactive oxygen species (ROS) output and fibroblast activation by interacting with ATP5B, a subunit of mitochondrial ATP synthase, and then closing the mitochondrial permeability transition pore [48].

Aging

The expression of circRNAs in neuronal tissues increases substantially relative to their linear counterparts in an age-dependent manner in both flies and mice [49–51]. In long-lived insulin mutant flies, circRNA accumulation with age slows in a brain-specific manner [24]. *circSfl* is one of the most highly upregulated circRNAs in most tissues of insulin mutant flies, and extensive investigations using genetic and molecular cell biological methods have shown that *circSfl* encodes a shorter protein that corresponds to the N-terminal part of the Sfl protein [24] (Figure 2B). Overexpression of *circSfl* or the shorter protein from a linear transcript is sufficient to prolong lifespan [24].

In the prefrontal cortex and hippocampus of rhesus macaques, a circRNA conserved in mammals, *circGRIA1*, shows age-related and male-specific increased levels [52]. *circGRIA1* localizes predominately in the nucleus and exhibits an increased association with the promoter of *GRIA1* during brain aging (Figure 2B). This circRNA negatively regulates the transcription of its host gene in hippocampal slices and cultures [52]. Furthermore, *in vivo* knockdown of hippocampal *circGRIA1* through microinjection of adeno-associated virus (AAV) viral particles expressing small interfering RNAs (siRNAs) leads to increased levels of *GRIA1* mRNA and protein in rhesus macaques [52]. More strikingly, *circGRIA1* knockdown increases hippocampal synaptogenesis and synaptic plasticity as well as Ca^{2+} homeostasis in aged males [52]. Based on the examples of *circSfl* and *circGRIA1*, at least some circRNAs appear to play active roles in aging rather than only exhibiting passive associations.

CircRNAs in non-immunological diseases

Various circRNAs are involved in the initiation and progression of human diseases. We focus here on non-immunological diseases such as cancers, neurological disorders, cardiovascular diseases (CVDs), and metabolic diseases (Figure 3 and Table 1). The majority of human diseases are related to immunology, and here non-immunological diseases refer to disorders that are not directly caused by infections or by severe abnormalities or over-reaction of the immune system. For immunology-related roles of circRNAs, the interested reader is referred to additional reviews [9–11].

Cancer

CircRNAs in cancers have been extensively investigated, and numerous studies have revealed that the global expression of circRNAs is disturbed in almost all cancer types. Cancer cells generally express circRNAs at reduced levels [53–55]. Comprehensive databases of circRNAs detected in tumor tissues, such as MiOncoCirc, are available for studies in this field [55]. Many circRNAs are also upregulated in cancer; for example, *circTP63* is upregulated in lung squamous cell carcinoma [56]. In addition, some circRNAs exhibit inconsistent changes in different cancers. One example is *circHIPK3*, which is upregulated in colorectal cancer (CRC) but downregulated in bladder cancer [57,58]. Many of these dysregulated circRNAs function as either oncogenes or tumor suppressors in key cellular events of cancer [54] (Figure 3A).

Regulating cell proliferation

CircRNAs have been observed to regulate cancer cell proliferation by acting as miRNA sponges and by modulating RBPs (Figure 3AI). The circRNA *circCCDC66*, which is expressed at high levels in CRC, is reported to regulate proliferation and promote tumor growth by sequestering *miR-185*, *miR-33b*, and *miR-93* from *c-Myc* mRNA [59]. In glioblastoma (GBM) cancer stem-like cells (GSCs), *circ2082*, derived from the genomic region encoding the long noncoding RNA (lncRNA) *MALAT1*, increases cell proliferation and tumorigenesis by binding to the RBP RBM3, and then the *circ2082*–RBM3 complex facilitates the nuclear accumulation of DICER, which eventually leads to suppression of miRNA biogenesis [60]. Pathologically, the size of tumors from mouse brains implanted with *circ2082*-depleted GSCs is significantly smaller than that in the non-depletion group [60].

Regulation of apoptosis

Gastric cancer (GC)-specific *circAKT3* has been found to activate PI3K signaling by inhibiting *miR-198*-mediated suppression of PIK3R1, a regulatory subunit of PI3K, thereby slowing the apoptosis of cancer cells [61]. Cisplatin (CDDP) is a common chemotherapeutic drug that induces apoptosis in cancers, including GC. *circAKT3* enhances the cisplatin resistance of GC cells in mice, and, clinically, *circAKT3* is expressed at high levels in CDDP-resistant GC tissues [61]. In another case, *circ-Foxo3* increased the levels of PUMA, a proapoptotic protein, through the MDM2-mediated ubiquitination and degradation pathway [23] (Figure 3AII). It seems that circRNAs are harnessed in cancer cells as antiapoptotic factors.

Regulating the self-renewal of cancer stem cells

At least two circRNAs regulate the self-renewal of cancer stem cells (CSCs) by being translated into small proteins (Figure 3AIII). *circSMO* encoded by the smoothened (*SMO*) gene produces the short protein SMO-193a.a. [25]. Higher levels of SMO-193a.a. in GBM have been verified with specific antibodies, and this short protein regulates Hedgehog signaling by activating full-length SMO, thereby promoting the self-renewal of CSCs [25]. Another GBM-specific circRNA, *circ-E-Cad*, is translated to generate a secretory E-cadherin protein variant (C-E-Cad), and this protein and its production from the *circ-E-Cad* template have been extensively authenticated with specific monoclonal antibodies and protein mass spectrometry in conjunction with other

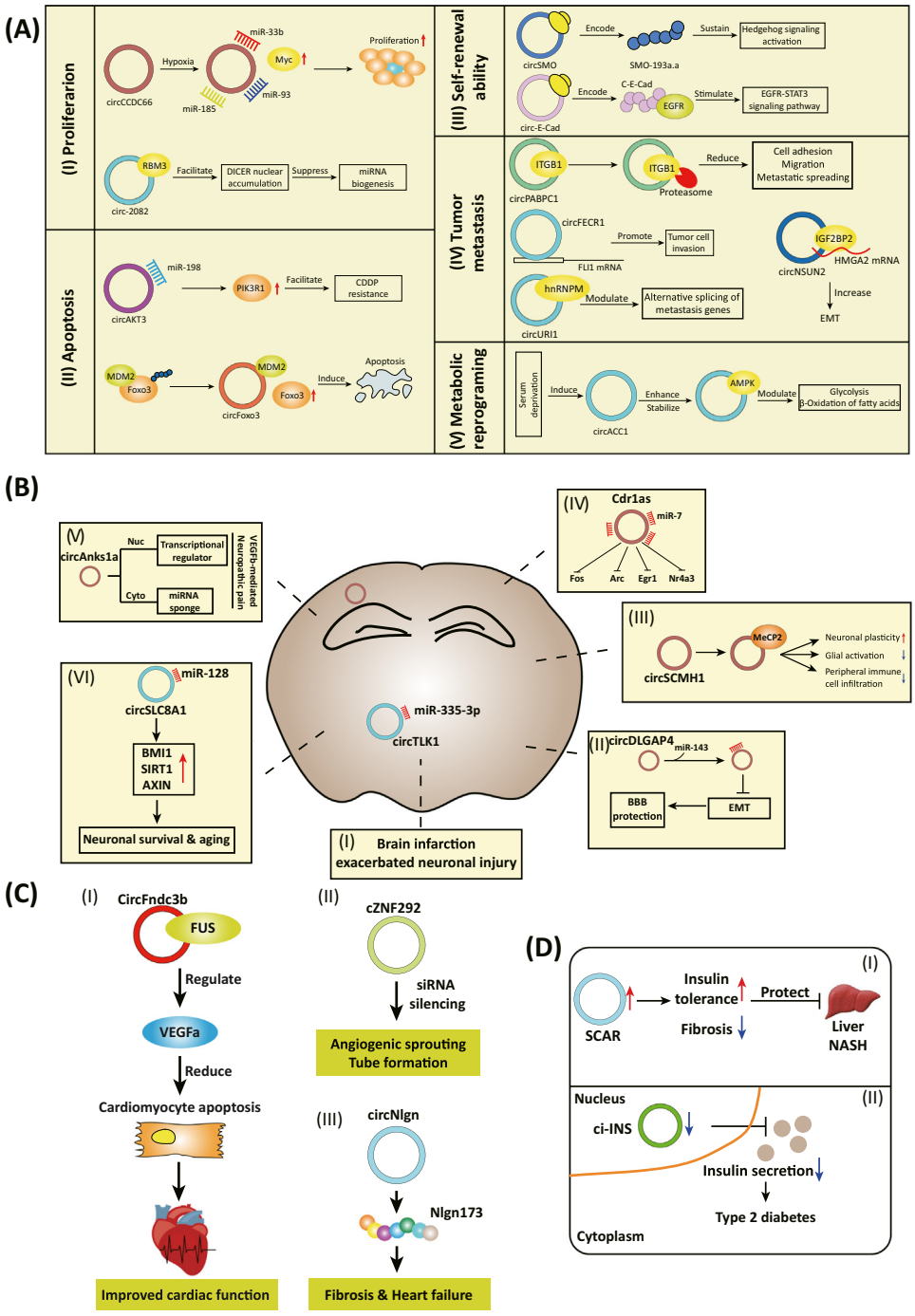


Figure 3. Roles of circular RNAs (circRNAs) in non-immunological diseases. (A) Roles of circRNAs in key events of cancer. (I) Proliferation: *circCCDC66* sponges *miR-185*, *miR-33b*, and *miR-93* to regulate oncogenes; *circ2082*-RBM3 facilitates the nuclear accumulation of DICER. (II) Apoptosis: *circAKT3* facilitates CDDP resistance by sponging *miR-198*; *circ-Foxo3* decreases the MDM2-mediated polyubiquitination of Foxo3. (III) Self-renewal of CSCs: *circSMO* encodes SMO193a.a. to sustain Hedgehog signaling; *circ-E-Cad* encodes C-E-Cad to activate EGFR signaling. (IV) Tumor

(Figure legend continued at the bottom of the next page.)

methods [62]. C-E-Cad directly interacts with EGFR to stimulate EGFR–STAT3 signaling in CSCs of GBM, and then promotes the expression of stemness-related genes and attenuates the expression of differentiation-related genes [62].

Regulating cancer metastasis

Metastasis is a complex process, and circRNAs are involved in multiple ways (Figure 3AIV). The expression of *circNSUN2* is significantly upregulated in the serum of CRC patients with liver metastasis [63]. Silencing of *circNSUN2* robustly attenuates the migration and invasion of cultured CRC cells and inhibits tumor metastasis in a mouse model [63]. Mechanistically, by forming a complex with the RBP IGF2BP2 and *HMGA2* mRNA, *circNSUN2* stabilizes the *HMGA2* mRNA, which leads to increased levels of HMGA2 protein that promotes the epithelial-to-mesenchymal transition (EMT) during metastasis [63]. In hepatocellular carcinoma (HCC), *circPABPC1* physically connects the non-canonical RBP ITGB1 to the proteasome and facilitates ITGB1 degradation [64]. ITGB1 is a positive regulator of intrahepatic metastasis and HCC migration is inhibited [64]. The regulatory effect of *circPABPC1* on the proteasome is evidenced by negative staining in electron microscopy as well as by other lines of evidence [64]. FLI1 is closely associated with hematological malignancies and some solid tumors, and *circFECR1* promotes the invasiveness of breast cancer cells by activating *FLI1* transcription [18]. In GC, *circURI1* is identified as a factor that inhibits metastasis by functioning as a decoy of the splicing factor hnRNPM to modulate the alternative splicing of key genes involved in cell migration, such as *VEGFA* [65].

Regulation of metabolic reprogramming

A hallmark of cancer is metabolic reprogramming, and some circRNAs regulate this process [54]. A good example is *circACC1* which is upregulated in cells in response to serum deprivation [66] (Figure 3AV). By functioning as a scaffold, *circACC1* stabilizes and enhances the enzymatic activity of AMPK, and then modulates both glycolysis and fatty acid β-oxidation in CRC [66].

Neurological disorders

One of the first- characterized circRNAs, *Cdr1as*, is brain-specific, and *Cdr1as* knockout mice are also the first mammalian circRNA knockout model [14,15,67]. CircRNAs are generally more abundant in neuronal tissues, and some brain-accumulated circRNAs are evolutionarily conserved in their expression and sequences. Moreover, the expression patterns of circRNAs, compared to their corresponding mRNAs, change more dramatically following the induction of neuronal activity and plasticity [68,69]. ciRNAs were recently found to be generated from disease-causing DNA sequences with hexanucleotide (GGGGCC) repeat expansions – the leading genetic cause of diseases such as amyotrophic lateral sclerosis and frontotemporal dementia [70]. In addition, individual circRNAs have been shown to play crucial roles in multiple neurological diseases (Figure 3B and Table 1).

metastasis: *circNSUN2* forms a *circNSUN2*–IGF2BP2–*HMGA2* mRNA complex to increase the EMT; *circPABPC1* facilitates ITGB1 degradation by the proteasome; *circFECR1* promotes invasiveness by activating FLI1 transcription; *circURI1* interacts with hnRNPM to modulate alternative splicing of metastasis genes. (V) Metabolic reprogramming: *circACC1* enhances and stabilizes the AMPK complex. (B) Neurological disorders. (I) *circTLK1* sponges *miR-335-3p*, resulting in exacerbated neuronal injury; (II) *circDLGAP4* sponges *miR-143* to attenuate BBB damage; (III) *circSCMH1* releases MeCP2-mediated repression to exert anti-stroke effects; (IV) knockout of *Cdr1as* in mice leads to synaptic transmission deficiency and aberrant behavior; (V) *circAnks1* functions in VEGFb-mediated neuropathic pain; (VI) upregulated *circSLC8A1* in PD patients sponges *miR-128* to promote neurodegeneration. (C) Cardiovascular diseases. (I) *circFndc3b* interacts with FUS to regulate VEGFa and reduce cardiomyocyte apoptosis; (II) silencing of *cZNF292* inhibits angiogenic sprouting and tube formation; (III) *circNlgn* encodes Nlgn173 to transcriptionally regulate gene expression in fibrosis and heart failure. (D) Metabolic diseases. (I) *SCAR* exerts protective effects during the progression of steatosis to NASH; (II) *ci-INS* levels are reduced in individuals with type 2 diabetes, and *ci-INS* silencing decreases insulin secretion. Abbreviations: BBB, blood-brain barrier; Cyto, cytoplasm; EMT, epithelial–mesenchymal transition; NASH, non-alcoholic steatohepatitis; Nuc, nucleus; PD, Parkinson's disease.

In animal models and perhaps in ischemic stroke patients, *circTLK1* acts as a *miR-335-3p* sponge to increase TIPARP expression, resulting in increased brain infarction and exacerbated neuronal injury [71]. Similarly, *circDLGAP4* functions as a ceRNA of *miR-143* to increase the expression of key genes related to the EMT during blood–brain barrier (BBB) disruption, and thus has a neuroprotective function to reduce BBB damage in mouse stroke models [72] (Figure 3BII). The expression of *circSCMH1*, one of the most abundant circRNAs in neurons, exhibits a continuous decrease during the acute phase of stroke, and is an example of an RBP–circRNA interaction [73]. *circSCMH1* binds to MeCP2 (methyl-CpG binding protein 2) and subsequently relieves the repressive effect of MeCP2 on target gene transcription. Impressively, brain-specific delivery of *circSCMH1* after stroke can facilitate motor function recovery and reduce the activation of astrocytes and microglia in mouse and nonhuman primate models [73] (Figure 3BIII). Loss of *Cdr1as* in mice leads to *miR-7* degradation, deficits in excitatory synaptic transmission, and aberrant memory and exploratory behaviors [67] (Figure 3BIV). In fact, the lncRNA Cyrano, that is conserved in vertebrates, contains a perfect match for *miR-7*; Cyrano, *Cdr1as*, *miR-7*, *miR-671*, and mRNA targets of the two miRNAs form a complex RNA regulatory network in mouse brain [74]. As shown in a recent study, *circAnks1* functions in VEGFb-mediated neuropathic pain [75]. In the nucleus, *circAnks1* recruits YBX1 to the *VEGFB* promoter to activate transcription, whereas *circAnks1* in the cytoplasm inhibits *miR-324-3p* to increase the translation of *VEGFB* mRNA [75] (Figure 3BV).

Neurological diseases are major threats to human health, and multiple circRNAs have established roles in these disorders. Thousands of circRNAs are dysregulated in patients with Alzheimer's disease (AD) and Parkinson's disease (PD) [76,77]. For example, *circSLC8A1* is upregulated in the substantia nigra of patients with PD, and this circRNA increases the expression of many neurodegenerative and aging-related proteins, possibly through a circRNA–miRNA–mRNA circuit [77]; *circSLC8A1* possesses seven functional *miR-128* binding sites [77] (Figure 3BVI).

Cardiovascular diseases

CVDs are another class of major health problems, and individual circRNAs have been implicated. After myocardial infarction, *circFndc3b*, a significantly downregulated circRNA, directly interacts with the RBP FUS to regulate VEGFa expression and signaling in the heart [78] (Figure 3CI). Overexpression of *circFndc3b* not only reduces oxidative stress-induced apoptosis and promotes tube formation at the cellular level, but also increases capillary density and reduces infarct size in the hearts of mouse models [78]. The endothelial circRNA *cZNF292*, which is substantially upregulated by hypoxia, may be associated with the risk of developing atherosclerosis [79]. Silencing of *cZNF292* robustly inhibits the sprouting of spheroids and the proliferation of endothelial cells through an unknown mechanism [79] (Figure 3CII). *circNlgn* is significantly upregulated in myocardial tissues from patients with specific congenital heart defects under cardiac overload [80]. *circNlgn* is translated into a polypeptide of 173 aa (Nlgn173) which functions as a transcription factor to regulate the expression of genes such as *ING4* and *SGK3,* and also contributes to fibrosis and heart failure in *circNlgn* transgenic mice and very possibly in patients [80] (Figure 3CIII).

Metabolic diseases

CircRNAs that regulate normal metabolism could also play crucial roles in metabolic diseases. For example, expression of the mecciRNA *SCAR*, which is more abundant than its linear counterpart, shows continuous reduction during the progression of steatosis to **non-alcoholic steatohepatitis (NASH)** in patients [48]. Overexpression of *SCAR* robustly improves tolerance to insulin and reduces fibrosis in liver tissues of mice fed a high-fat diet by regulating the mitochondrial permeability of ROS [48] (Figure 3DI). The aforementioned *ci-Ins2* and *ci-INS* are specifically expressed in β cells and are present at reduced levels in the pancreatic islets of rodent diabetes models and patients with type 2

diabetes [42]. Silencing of *ci-Ins2* or *ci-INS* specifically decreases insulin secretion from cultured rat or human β cells treated with glucose or potassium [46] (Figure 3DII).

Concluding remarks

Early studies associated circRNAs with 'scrambled exons' or 'mis-splicing' [1–4,7,8]. To date researchers have identified at least some circRNAs that are not noise from gene expression and are instead regulatory molecules. Significant progress has been achieved in the characterization of the molecular and cellular features and functions of circRNAs. In particular, the roles of circRNAs in physiology and disease have been the focus of studies in recent years, but numerous issues remain to be resolved (see Outstanding questions).

The vast numbers and the considerable diversity of circRNAs may enable many more regulatory complexities. Some circRNAs, including *circBoule* and *circSLC45A4*, have conserved roles in animal physiology and human disorders, whereas other circRNAs are specifically expressed in humans or animal species (e.g., *circSry* in rodents). Many investigations on circRNAs in normal physiology and diseases are either association studies or are purely descriptive, and more molecular and cellular details will be necessary to pinpoint the precise significance of circRNAs.

A recent publication estimated that >97% of circRNAs generated by **backsplicing** are nonfunctional products of splicing errors, based mainly on their low abundance and lack of conservation across humans, macaques, and mice [81]. We have summarized the importance of an array of circRNAs in physiology and disease, and future studies will help to resolve the debate regarding whether circRNAs are erroneous noise or specific regulators. As further progress is achieved, circRNAs have the potential to be developed into clinical diagnostic and prognostic markers, therapeutic targets, or even RNA drugs.

Acknowledgments

This work was supported by funding from the National Key R&D Program of China (2019YFA0802600 and 2018YFC1004500), the National Natural Science Foundation of China (31725016, 31930019, 91940303, 32070633), the Strategic Priority Research Program 'Biological Basis of Aging and Therapeutic Strategies' of the Chinese Academy of Sciences (XDB39010400), the Fundamental Research Funds for the Central Universities of China (2021CDJZYJH-002, 2020CDJQY-A076), the Natural Science Foundation of Chongqing China (cstc2019jcyjmsxmX0085), and the Innovation Support Program for Overseas Returned Scholars of Chongqing China (cx2019142).

Declaration of interests

The authors declare no conflicts of interest.

References

1. Salzman, J. *et al.* (2012) Circular RNAs are the predominant transcript isoform from hundreds of human genes in diverse cell types. *PLoS One* 7, e30733
2. Chen, L. *et al.* (2015) Circular RNAs in eukaryotic cells. *Curr. Genomics* 16, 312–318
3. Kristensen, L.S. *et al.* (2019) The biogenesis, biology and characterization of circular RNAs. *Nat. Rev. Genet.* 20, 675–691
4. Chen, L.L. (2020) The expanding regulatory mechanisms and cellular functions of circular RNAs. *Nat. Rev. Mol. Cell Biol.* 21, 475–490
5. Wilusz, J.E. (2018) A 360 degrees view of circular RNAs: from biogenesis to functions. *Wiley Interdiscip. Rev. RNA* 9, e1478
6. Xiao, M.S. *et al.* (2020) Biogenesis and functions of circular RNAs come into focus. *Trends Cell Biol.* 30, 226–240
7. Nigro, J.M. *et al.* (1991) Scrambled exons. *Cell* 64, 607–613
8. Cocquerelle, C. *et al.* (1993) Mis-splicing yields circular RNA molecules. *FASEB J.* 7, 155–160
9. Yan, L. and Chen, Y.G. (2020) Circular RNAs in immune response and viral infection. *Trends Biochem. Sci.* 45, 1022–1034
10. Choudhary, A. *et al.* (2021) Circular RNA as an additional player in the conflicts between the host and the virus. *Front. Immunol.* 12, 602006
11. Zhou, Z. *et al.* (2019) Roles of circular RNAs in immune regulation and autoimmune diseases. *Cell Death Dis.* 10, 503
12. Li, Z. *et al.* (2015) Exon–intron circular RNAs regulate transcription in the nucleus. *Nat. Struct. Mol. Biol.* 22, 256–264
13. Liu, X. *et al.* (2020) Identification of mecciRNAs and their roles in the mitochondrial entry of proteins. *Sci. China Life Sci.* 63, 1429–1449
14. Hansen, T.B. *et al.* (2013) Natural RNA circles function as efficient microRNA sponges. *Nature* 495, 384–388
15. Memczak, S. *et al.* (2013) Circular RNAs are a large class of animal RNAs with regulatory potency. *Nature* 495, 333–338
16. Guo, J.U. *et al.* (2014) Expanded identification and characterization of mammalian circular RNAs. *Genome Biol.* 15, 409
17. Ragan, C. *et al.* (2019) Insights into the biogenesis and potential functions of exonic circular RNA. *Sci. Rep.* 9, 2048

Outstanding questions

How many circRNAs are truly regulatory molecules? The functional circRNAs included in this review may indeed be outliers, and future in-depth functional studies on circRNAs using multiple approaches may be needed. Even circRNAs that are 'real' gene expression noise can still be functional – or at least must be handled or controlled properly.

Does RNA homeostasis of circRNAs play a crucial role in normal physiology; furthermore, do abnormalities in circRNA homeostasis contribute substantially to the pathology of specific diseases? The biogenesis, intracellular export, and degradation of circRNAs may be tightly regulated, and these regulatory mechanism may be crucial to their physiological activities at the cellular and organismal levels. In addition, aberrant circRNA biogenesis, intracellular export, or degradation may be associated with or even contribute significantly to the etiology of particular diseases.

Are there any other fundamental physiological or pathological mechanisms by which circRNAs exert their functions? One possible scenario is that one specific circRNA or a group of circRNAs might serve as an 'information' carrier for intercellular communication (e.g., circRNAs loaded into extracellular exosomes). Alternatively, circRNAs may participate in epigenetic 'memory' at the cellular level or even as intergenerational and transgenerational messages to offspring.

What other information will be necessary to enable real-world clinical applications of circRNAs? The development of clinical applications of small RNAs, mRNAs, and lncRNAs has achieved some successes, although the path has been challenging. Past achievements and lessons from other types of RNAs will surely be beneficial.

18. Chen, N.F. *et al.* (2018) A novel FLI1 exonic circular RNA promotes metastasis in breast cancer by coordinately regulating TET1 and DNMT1. *Genome Biol.* 19, 218
19. Xu, X. *et al.* (2020) CircRNA inhibits DNA damage repair by interacting with host gene. *Mol. Cancer* 19, 128
20. Li, X. *et al.* (2021) Linking circular intronic RNA degradation and function in transcription by RNase H1. *Sci. China Life Sci.* 64, 1795–1809
21. Ashwal-Fluss, R. *et al.* (2014) circRNA biogenesis competes with pre-mRNA splicing. *Mol. Cell* 56, 55–66
22. Du, W.W. *et al.* (2016) Foxo3 circular RNA retards cell cycle progression via forming ternary complexes with p21 and CDK2. *Nucleic Acids Res.* 44, 2846–2858
23. Du, W.W. *et al.* (2017) Induction of tumor apoptosis through a circular RNA enhancing Foxo3 activity. *Cell Death Differ.* 24, 357–370
24. Weigelt, C.M. *et al.* (2020) An insulin-sensitive circular RNA that regulates lifespan in *Drosophila*. *Mol. Cell* 79, 268–279
25. Wu, X.J. *et al.* (2021) A novel protein encoded by circular SMO RNA is essential for Hedgehog signaling activation and glioblastoma tumorigenicity. *Genome Biol.* 22, 33
26. Zhang, M.L. *et al.* (2018) A peptide encoded by circular form of LINC-PINT suppresses oncogenic transcriptional elongation in glioblastoma. *Nat. Commun.* 9, 4475
27. Zhao, J.W. *et al.* (2019) Transforming activity of an oncoprotein-encoding circular RNA from human papillomavirus. *Nat. Commun.* 10, 2300
28. Li, J. *et al.* (2020) Circular HER2 RNA positive triple negative breast cancer is sensitive to pertuzumab. *Mol. Cancer* 19, 142
29. Pamudurti, N.R. *et al.* (2017) Translation of circRNAs. *Mol. Cell* 66, 9–21
30. Legnini, I. *et al.* (2017) Circ-ZNF609 is a circular RNA that can be translated and functions in myogenesis. *Mol. Cell* 66, 22–37
31. Yang, Y. *et al.* (2017) Extensive translation of circular RNAs driven by N6-methyladenosine. *Cell Res.* 27, 626–641
32. Hung, H.X. *et al.* (2020) Comprehensive analysis of translation from overexpressed circular RNAs reveals pervasive translation from linear transcripts. *Nucleic Acids Res.* 48, 10368–10382
33. Jiang, Y. *et al.* (2021) Overexpression-based detection of translatable circular RNAs is vulnerable to coexistent linear RNA byproducts. *Biochem. Biophys. Res. Commun.* 558, 189–195
34. Hansen, T.B. (2021) Signal and noise in circRNA translation. *Methods* 196, 68–73
35. Schneider, T. *et al.* (2016) CircRNA–protein complexes: IMP3 protein component defines subfamily of circRNPs. *Sci. Rep.* 6, 31313
36. Suenkel, C. *et al.* (2020) A highly conserved circular RNA is required to keep neural cells in a progenitor state in the mammalian brain. *Cell Rep.* 30, 2170–2179
37. Ouyang, Z. *et al.* (2019) CircRNA hsa_circ_0074834 promotes the osteogenesis–angiogenesis coupling process in bone mesenchymal stem cells (BMSCs) by acting as a ceRNA for miR-942-5p. *Cell Death Dis.* 10, 932
38. Cherubini, A. *et al.* (2019) FOXP1 circular RNA sustains mesenchymal stem cell identity via microRNA inhibition. *Nucleic Acids Res.* 47, 5325–5340
39. Lei, W. *et al.* (2018) Signature of circular RNAs in human induced pluripotent stem cells and derived cardiomyocytes. *Stem Cell Res Ther* 9, 56
40. Yu, C.Y. *et al.* (2017) The circular RNA circBIRC6 participates in the molecular circuitry controlling human pluripotency. *Nat. Commun.* 8, 1149
41. Tijsen, A.J. *et al.* (2021) Titin circular RNAs create a back-splice motif essential for SRSF10 splicing. *Circulation* 143, 1502–1512
42. Quan, G.B. and Li, J.L. (2018) Circular RNAs: biogenesis, expression and their potential roles in reproduction. *J. Ovarian Res.* 11, 9
43. Capel, B. *et al.* (1993) Circular transcripts of the testis-determining gene Sry in adult-mouse testis. *Cell* 73, 1019–1030
44. Gao, L. *et al.* (2020) Circular RNAs from BOULE play conserved roles in protection against stress-induced fertility decline. *Sci. Adv.* 6, eabb7426
45. Dang, Y. *et al.* (2016) Tracing the expression of circular RNAs in human pre-implantation embryos. *Genome Biol.* 17, 130
46. Stoll, L. *et al.* (2020) A circular RNA generated from an intron of the insulin gene controls insulin secretion. *Nat. Commun.* 11, 5611
47. Arcinas, C. *et al.* (2019) Adipose circular RNAs exhibit dynamic regulation in obesity and functional role in adipogenesis. *Nat. Metab.* 1, 688–703
48. Zhao, Q. *et al.* (2020) Targeting mitochondria-located circRNA SCAR alleviates NASH via reducing mROS output. *Cell* 183, 76–93
49. Westholm, J.O. *et al.* (2014) Genome-wide analysis of drosophila circular RNAs reveals their structural and sequence properties and age-dependent neural accumulation. *Cell Rep.* 9, 1966–1980
50. Gruner, H. *et al.* (2016) CircRNA accumulation in the aging mouse brain. *Sci. Rep.* 6, 38907
51. Rodriguez, S.A. *et al.* (2016) Global genome splicing analysis reveals an increased number of alternatively spliced genes with aging. *Aging Cell* 15, 267–278
52. Xu, K.Y. *et al.* (2020) CircGRIA1 shows an age-related increase in male macaque brain and regulates synaptic plasticity and synaptogenesis. *Nat. Commun.* 11, 3594
53. Chen, S. *et al.* (2019) Widespread and functional RNA circularization in localized prostate cancer. *Cell* 176, 831–843
54. Chen, L. and Shan, G. (2021) CircRNA in cancer: fundamental mechanism and clinical potential. *Cancer Lett.* 505, 49–57
55. Vo, J.N. *et al.* (2019) The landscape of circular RNA in cancer. *Cell* 176, 869–881
56. Cheng, Z.A. *et al.* (2019) circTP63 functions as a ceRNA to promote lung squamous cell carcinoma progression by upregulating FOXM1. *Nat. Commun.* 10, 3200
57. Zeng, K.X. *et al.* (2018) CircHIPK3 promotes colorectal cancer growth and metastasis by sponging miR-7. *Cell Death Dis.* 9, 417
58. Li, Y.W. *et al.* (2017) CircHIPK3 sponges miR-558 to suppress heparanase expression in bladder cancer cells. *EMBO Rep.* 18, 1646–1659
59. Hsiao, K.Y. *et al.* (2017) Noncoding effects of circular RNA CCDC66 promote colon cancer growth and metastasis. *Cancer Res.* 77, 2339–2350
60. Bronisz, A. *et al.* (2020) The nuclear DICER-circular RNA complex drives the deregulation of the glioblastoma cell microRNAome. *Sci. Adv.* 6, eabc0221
61. Huang, X.X. *et al.* (2019) Circular RNA AKT3 upregulates PIK3R1 to enhance cisplatin resistance in gastric cancer via miR-198 suppression. *Mol. Cancer* 18, 71
62. Gao, X.Y. *et al.* (2021) Circular RNA-encoded oncogenic E-cadherin variant promotes glioblastoma tumorigenicity through activation of EGFR–STAT3 signaling. *Nat. Cell Biol.* 23, 278–291
63. Chen, R.X. *et al.* (2019) N6-methyladenosine modification of circNSUN2 facilitates cytoplasmic export and stabilizes HMGA2 to promote colorectal liver metastasis. *Nat. Commun.* 10, 4695
64. Shi, L. *et al.* (2021) A tumor-suppressive circular RNA mediates uncanonical integrin degradation by the proteasome in liver cancer. *Sci. Adv.* 7, eabe5043
65. Wang, X. *et al.* (2021) CircURI1 interacts with hnRNPM to inhibit metastasis by modulating alternative splicing in gastric cancer. *Proc. Natl. Acad. Sci. U. S. A.* 118, e2012881118
66. Li, Q.D. *et al.* (2019) CircACC1 regulates assembly and activation of AMPK complex under metabolic stress. *Cell Metab.* 30, 157–173
67. Piwecka, M. *et al.* (2017) Loss of a mammalian circular RNA locus causes miRNA deregulation and affects brain function. *Science* 357, eaam8526
68. Rybak-Wolf, A. *et al.* (2015) Circular RNAs in the mammalian brain are highly abundant, conserved, and dynamically expressed. *Mol. Cell* 58, 870–885
69. You, X. *et al.* (2015) Neural circular RNAs are derived from synaptic genes and regulated by development and plasticity. *Nat. Neurosci.* 18, 603–610
70. Wang, S. *et al.* (2021) Nuclear export and translation of circular repeat-containing intronic RNA in C9ORF72-ALS/FTD. *Nat. Commun.* 12, 4908

71. Wu, F.F. *et al.* (2019) Circular RNA TLK1 aggravates neuronal injury and neurological deficits after ischemic stroke via miR-335-3p/TIPARP. *J. Neurosci.* 39, 7369–7393
72. Bai, Y. *et al.* (2018) Circular RNA DLGAP4 ameliorates ischemic stroke outcomes by targeting miR-143 to regulate endothelial–mesenchymal transition associated with blood–brain barrier integrity. *J. Neurosci.* 38, 32–50
73. Yang, L. *et al.* (2020) Extracellular vesicle-mediated delivery of circular RNA SCMH1 promotes functional recovery in rodent and nonhuman primate ischemic stroke models. *Circulation* 142, 556–574
74. Kleaveland, B. *et al.* (2018) A network of noncoding regulatory RNAs acts in the mammalian brain. *Cell* 174, 350–362
75. Zhang, S.B. *et al.* (2019) CircAnks1a in the spinal cord regulates hypersensitivity in a rodent model of neuropathic pain. *Nat. Commun.* 10, 4119
76. Dube, U. *et al.* (2019) An atlas of cortical circular RNA expression in Alzheimer disease brains demonstrates clinical and pathological associations. *Nat. Neurosci.* 22, 1903–1912
77. Hanan, M. *et al.* (2020) A Parkinson's disease CircRNAs Resource reveals a link between circSLC8A1 and oxidative stress. *EMBO Mol. Med.* 12, e11942
78. Garikipati, V.N. *et al.* (2018) Circular RNA circFNDC3b modulates cardiac repair after myocardial infarction via FUS-1/VEGF-A axis. *Nat. Commun.* 10, 4317
79. Boeckel, J.N. *et al.* (2015) Identification and characterization of hypoxia-regulated endothelial circular RNA. *Circ. Res.* 117, 884–890
80. Du, W.W. *et al.* (2021) A neuroligin isoform translated by circNlgn contributes to cardiac remodeling. *Circ. Res.* 129, 568–582
81. Xu, C.A. and Zhang, J.Z. (2021) Mammalian circular RNAs result largely from splicing errors. *Cell Rep.* 36, 109439
82. Xiao, M.S. and Wilusz, J.E. (2019) An improved method for circular RNA purification using RNase R that efficiently removes linear RNAs containing G-quadruplexes or structured 3' ends. *Nucleic Acids Res.* 47, 8755–8769
83. Xin, R. *et al.* (2021) isoCirc catalogs full-length circular RNA isoforms in human transcriptomes. *Nat. Commun.* 12, 266
84. Zhang, J. *et al.* (2021) Comprehensive profiling of circular RNAs with nanopore sequencing and CIRI-long. *Nat. Biotechnol.* 39, 836–845
85. Song, Z.X. *et al.* (2021) Antisense oligonucleotide technology can be used to investigate a circular but not linear RNA-mediated function for its encoded gene locus. *Sci. China Life Sci.* 64, 784–794
86. Breuer, J. and Rossbach, O. (2020) Production and purification of artificial circular RNA sponges for application in molecular biology and medicine. *Methods Protoc.* 3, 42
87. Obi, P. and Chen, Y.G. (2021) The design and synthesis of circular RNAs. *Methods* 196, 85–103
88. Wesselhoeft, R.A. *et al.* (2019) RNA circularization diminishes immunogenicity and can extend translation duration in vivo. *Mol. Cell* 74, 508–520
89. Litke, J.L. and Jaffrey, S.R. (2019) Highly efficient expression of circular RNA aptamers in cells using autocatalytic transcripts. *Nat. Biotechnol.* 37, 667–675
90. Conn, S.J. *et al.* (2015) The RNA binding protein quaking regulates formation of circRNAs. *Cell* 160, 1125–1134
91. Liu, X. and Shan, G. (2021) Mitochondria encoded non-coding RNAs in cell physiology. *Front. Cell. Dev. Biol.* 9, 713729
92. Huang, C. *et al.* (2018) A length-dependent evolutionarily conserved pathway controls nuclear export of circular RNAs. *Genes Dev.* 32, 639–644
93. Zhou, C. *et al.* (2017) Genome-wide maps of m6A circRNAs identify widespread and cell-type-specific methylation patterns that are distinct from mRNAs. *Cell Rep.* 20, 2262–2276
94. Li, Y. *et al.* (2015) Circular RNA is enriched and stable in exosomes: a promising biomarker for cancer diagnosis. *Cell Res.* 25, 981–984
95. Hulstaert, E. *et al.* (2020) Charting extracellular transcriptomes in the human biofluid RNA atlas. *Cell Rep.* 33, 108552
96. Park, O.H. *et al.* (2019) Endoribonucleolytic cleavage of m6A-containing RNAs by RNase P/MRP complex. *Mol. Cell* 74, 494–507
97. Fischer, J.W. *et al.* (2020) Structure-mediated RNA decay by UPF1 and G3BP1. *Mol. Cell* 78, 70–84
98. Hansen, T.B. *et al.* (2011) miRNA-dependent gene silencing involving Ago2-mediated cleavage of a circular antisense RNA. *EMBO J.* 30, 4414–4422
99. Jia, R.R. *et al.* (2019) Defining an evolutionarily conserved role of GW182 in circular RNA degradation. *Cell Discov.* 5, 45
100. Liu, C.X. *et al.* (2019) Structure and degradation of circular RNAs regulate PKR activation in innate immunity. *Cell* 177, 865–880