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REVIEW

Guidance of circular RNAs to proteins' behavior as binding partners

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Abstract

Circular RNAs (circRNAs) are single-stranded and covalently closed back-splicing products of pre-mRNAs. They can be derived from exons, introns, or exons with intron retained between exons of transcripts, as well as antisense transcripts. CircRNAs have been reported to function as microRNA sponges, regulate gene transcription mediated by RNA polymerase II, and modulate the splicing or stability of mRNA. However, emerging studies demonstrate that they affect the behavior of proteins via direct interactions with them. Here, we summarize that by binding directly with proteins; circRNAs can facilitate their nuclear or cytoplasmic localizations, regulate their functions or stability, promote or inhibit the interactions between them, or influence the interactions between them and DNA. Furthermore, these circRNA-binding proteins contain transcription factors, RNA processing proteins, proteases, and some other RNA-binding proteins. As a consequence, circRNAs are involved in the regulation of multiple physiological or pathological processes, including tumorigenesis, atherosclerosis, wound repair, cardiac senescence, myocardial ischemia/reperfusion injury, and so forth. Nonetheless, it is worthwhile to further explore more types of proteins that interact with circRNAs, which would be helpful in revealing other unknown biological functions of circRNAs that guide the variation in behavior of cellular proteins.

Keywords Circular RNA · CircRNA–protein interaction · Protein localization · Protein function

Introduction

Circular RNAs (circRNAs), which are products generated from back-splicing of pre-mRNA transcripts, are single-stranded and covalently closed where the 3′ and 5′ RNA ends are joined together. Although circRNAs were discovered more than two decades ago, they were formerly regarded as byproducts of aberrant splicing, and only a few circRNAs have been found and characterized [1–4]. Benefiting from the advancement of high-throughput sequencing technology and computational algorithms, circular RNAs were recently identified as a large class of RNAs in animals [5, 6]. CircRNAs can be circularized natural antisense transcripts (e.g., antisense to the cerebellar degeneration-related protein 1 transcript (CDR1as)) [7] or products from back-spliced exons [e.g., sex-determining region Y (*Sry*)] that have been defined as exonic circRNAs [4, 8]. However, a class of circular intronic RNAs that are derived from intronic sequences were identified and characterized in human cells and termed as ciRNAs [9]. Furthermore, another kind of circRNAs, which are composed of circularized exons with intron retained between exons, were named Exon–Intron circular RNAs (EIciRNAs) [10]. Recently, a class of fusion circRNAs were found to be generated from transcribed exons of distinct genes during aberrant chromosomal translocations in cancer cells [11]. Possibly due to back-splicing being far less efficient than canonical splicing in cells, circRNAs are prevalent on average only about 1% of their linear cognate RNAs [12, 13]. CircRNAs have been reported to play key roles in various physiological or pathological processes, such as myogenesis [14], brain function [15], epithelial–mesenchymal transition [16], ischemic myocardial injury [17], vascular diseases [18], tumorigenesis and tumor progression [19–22], and so forth.

Although many circRNAs are considered to be non-coding RNAs [23–25], emerging studies have demonstrated that a number of endogenous circRNAs possess translation potential [14, 26–30]. A well-defined function of circRNAs is binding with microRNAs (miRNAs) and sequestering them away from their target mRNAs as competitive

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endogenous RNAs. In 2013, a human cytoplasmic circRNA CDR1as was found to harbor 63 conserved binding sites for miR-7, preventing their function as a sponge, and leading to expression release of miR-7-inhibited mRNA [5]. Additionally, another circRNA, *Sry*, serves as a sponge of miR-138 [6]. Lately, an increasing number of studies confirmed that circRNAs act as binding platforms for the miRNA–AGO complex and function as a class of post-transcriptional regulators [31–33]. However, circRNAs have also been reported to be involved in the transcription regulation of their parent coding genes, a process that is mediated by RNA polymerase II. Ci-ankrd52, as an example of ciRNA, largely accumulates to the transcription sites of its parental gene *ankrd52*, associates with elongation Pol II machinery, and acts as a positive regulator of Pol II-mediated *ankrd52* transcription [9]. Besides this, EIciRNAs also promote Pol II-mediated parental gene transcription by interacting with U1 snRNA [10], a small nuclear RNA that promotes early transcriptional events by enhancing preinitiation complex assembly [34, 35]. Furthermore, circRNA can regulate the splicing of its cognate mRNA [36] or bind with mRNA and enhance its stability [37].

Except for the association with RNAs, increasing numbers of studies have shown that circRNAs function via direct interaction with cellular proteins. Although the biological implications and functions of circRNA–protein interactions have been reviewed earlier elsewhere [38], we focus here on the behavior of cellular proteins affected by direct binding of circRNAs, including subcellular localization of protein, protein–protein interaction, protein function or stability, and protein–DNA interaction. In addition, the working models of how circRNAs regulate the behavior and function of proteins, as well as the prediction and validation methods of circRNA–protein interactions, are also introduced in this article. Moreover, we categorize the proteins directly bound by circRNAs, together with the corresponding biological or physiological processes they are involved in.

CircRNAs regulate the subcellular localization of proteins

CircRNAs promote the nuclear localization of proteins

Emerging studies have demonstrated that through direct interaction, circRNAs can modulate the nuclear localization of proteins as well as their corresponding biological functions [39–42] (Fig. 1a). Circ-Amotl1, a circular RNA originating from exon 3 of the angiomotin like 1 (AMOTL1)

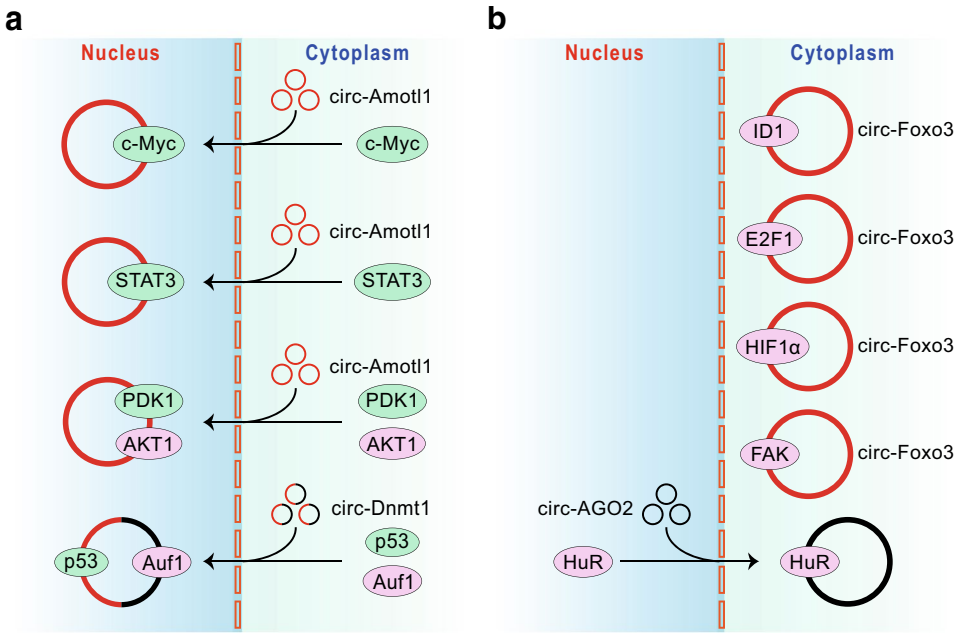

Fig. 1 CircRNAs regulate the subcellular localization of proteins. **a** Circ-Amotl1 can promote the nuclear localization of c-Myc, STAT3, PDK1, and AKT1 by direct interaction with them. Through its different regions, circ-Dnmt1 can bind to p53 and Auf1, and facilitate their nuclear localization. **b** Circ-Foxo3 is predominantly localized in the cytoplasm and interacts with ID1, E2F1, HIF1α, and FAK, leading to their cytoplasm retention. Through the interaction with HuR protein, circAGO2 facilitates its translocation from the nucleus to the cytoplasm. Please see more detail in the text. *STAT3* signal transducer and activator of transcription 3, *PDK1* phosphoinositide-dependent kinase 1, *AKT1* AKT serine/threonine kinase 1, *ID1* inhibitor of DNA binding 1, *E2F1* E2F transcription factor 1, *HIF1α* hypoxia inducible factor 1 subunit alpha, *FAK* Focal adhesion kinase, *HuR* human antigen R

gene, is expressed at high levels in breast carcinoma and breast cancer cell lines [39]. It not only enhances cell proliferation and colony formation, but also inhibits cell apoptosis. Moreover, tumor growth assay by nude mouse xenograft models showed that circ-Amotl1 promotes tumorigenesis. Mechanistically, circ-Amotl1 interacts with c-Myc protein, a well-known oncogenic transcription factor [43], and facilitates its nuclear translocalization. Consequently, expression of c-Myc-regulated target genes is promoted, resulting in enhanced cancer growth.

By RNA immunoprecipitation and pull-down assays, it has been demonstrated that specific physical association also exists between circ-Amotl1 and transcription factor STAT3 [40]. As a consequence, circ-Amotl1 increases the nuclear translocation of STAT3. Subsequently, STAT3 binds to the *Dnmt3a* promoter and activates its transcription. As a DNA methyltransferase, Dnmt3a can methylate the promoter of miR-17 and suppress the expression of miR-17-5p. Furthermore, the inhibitory effect of miR-17-5p on its target genes *Stat3*, *Dnmt3*, and extracellular matrix gene *fibronectin* are eliminated. By promoting the expression of these genes and enhancing cell proliferation, survival, adhesion, and migration, circ-Amotl1 accelerates wound repair, which was demonstrated in a mouse wound healing model [40].

Additionally, circ-Amotl1 is highly expressed in neonatal human cardiac tissue, and its ectopic expression decreased doxorubicin-induced cardiomyopathy and cardiomyocytes death in mice [41]. AKT1, one kind of serine/threonine kinase, has been reported to be involved in the transduction of the anti-apoptotic signaling cascade and inhibit cell death in cardiomyocytes [44]. Nonetheless, phosphoinositide-dependent kinase 1 (PDK1) is an upstream kinase that can phosphorylate AKT1 in cardiomyocytes [45]. Circ-Amotl1 can physically interact with both PDK1 and AKT1, facilitate their nuclear translocation, and promote phosphorylation of AKT1. Subsequently, more phosphorylated AKT1 are localized in the nucleus, where they directly phosphorylate downstream intracellular proteins that facilitate cell proliferation and survival [41]. Therefore, circ-Amotl1 may reinforce the cardio-protective role mediated by AKT1.

Another circRNA circ-Dnmt1, which is derived from back-splicing between exon 7 and exon 6 of *Dnmt1*, is highly expressed in breast cancer cell lines and breast carcinoma tissues [42]. Circ-Dnmt1 can bind to both p53 and Auf1, although through different regions, and promote their nuclear translocation. Consequently, on one hand, nuclear p53 facilitates autophagy [46, 47]. On the other hand, Auf1 nuclear translocation results in a decrease of its cytosol accumulation, which causes the release of its suppression to Dnmt1 mRNA's stability in the cytoplasm [48]. Subsequently, upregulated Dnmt1 inhibits p53 transcription in the nucleus by binding to its regulatory region [49]. Therefore, circ-Dnmt1 plays a central role in increasing cell autophagy and enhancing cell proliferation, survival, and tumor growth, possibly by regulating the localization and expression of tumor suppressor p53.

CircRNAs promote the cytoplasmic localization of proteins

In addition to nuclear localization, cytoplasm localization of proteins is also regulated by circRNAs [50, 51] (Fig. 1b). Circ-Foxo3, a highly expressed circular RNA in the heart tissues of elderly patients and mice, is derived from exon 2 of *Foxo3* [50]. Integrated in vitro and in vivo assays have revealed the role of circ-Foxo3 in senescence. Ectopic expression of circ-Foxo3 not only promoted the cellular senescence of mouse embryonic fibroblasts, but also exacerbated doxorubicin-induced cardiomyopathy in mice. Besides, circ-Foxo3 is predominantly localized in the cytoplasm, where it interacts with the anti-senescence proteins ID1 and E2F1 and anti-stress proteins FAK and HIF1α, leading to cytoplasm retention of these proteins. Consequently, their function as transcription factors (ID1, E2F1, and HIF1α) in the nucleus and anti-stress protein (FAK) in the mitochondria was repressed, which facilitates cellular senescence. Therefore, circ-Foxo3 enhances cardiac senescence by regulating the subcellular localization of these protein factors.

Another circRNA, circAGO2 derived from the partial first intron of *AGO2*, enhances the cytoplasm localization of HuR protein [51]. CircAGO2 is upregulated in several cancer types, such as gastric cancer, colorectal cancer, prostate cancer, and neuroblastoma, and exhibits an oncogenic role in tumorigenesis in vitro and in vivo. As an RNA-binding protein, HuR can stabilize mRNAs by specifically binding to the AU-rich elements located in their 3′ untranslated regions (UTRs), a major target region in miRNA-mediated post-transcriptional regulation [52]. CircAGO2 can interact with HuR and facilitate its translocation from the nucleus to the cytoplasm, thereby increasing its binding to the 3′-UTR regions of target mRNAs. As a consequence, the binding of AGO2 protein to the mRNAs is restrained and the gene silencing effect mediated by AGO2–miRNA complexes is suppressed. Therefore, through HuR-mediated inhibition of the function of AGO2–miRNA complexes and release of the expression of their downstream target genes, circAGO2 promotes cancer progression [51].

CircRNAs regulate the function or stability of proteins

Besides subcellular localization, the function of proteins can also be regulated by circRNAs via their direct interaction. An autophagy-related circular RNA (ACR), also named

mmu_circRNA_006636, was implicated in the regulation of cardiomyocyte autophagy and myocardial infarction [53]. It suppressed autophagy and cell death not only in cultured cardiomyocytes but also in the hearts of mice upon ischemia/reperfusion injury. Mechanistic studies demonstrated that ACR physically binds to DNA methyltransferase Dnmt3B and represses Dnmt3B-mediated methylation of the Pink1 promoter, leading to increased Pink1 expression (Fig. 2a). Furthermore, Pink1 phosphorylates FAM65B at serine 46, which then restrains autophagy and cell death in cardiomyocytes. Therefore, ACR exerts its resistance effect on autophagy via the Pink1/FAM65B axis and reduces myocardial ischemia/reperfusion injury [53].

Muscleblind like splicing regulator 1 (MBNL1) is another protein whose function is affected by direct binding of circRNA [54]. MBNL1 not only modulates the alternative splicing of pre-mRNAs as a splicing factor, but also regulates circRNA biogenesis [54]. It strongly and specifically binds to the highly conserved binding sites in the flanking introns of the second exon of *MBNL1* in flies and humans. As a consequence, it promotes circularization of the exon and production of circular RNA circMbl. Nevertheless, circRNA biogenesis and pre-mRNA splicing compete with each other, probably because both circularization of exons and linear splicing depend on canonical splicing machinery [54, 55]. Additionally, a direct and strong interaction exists between circMbl and MBNL1, which serves to explain a possible feedback regulation of MBNL1 by circMbl. Once cellular MBNL1 protein is excessive, it enhances the biogenesis of circMbl, which results in the reduction of MBNL1 mRNA for the competition. Moreover, circMbl attenuates the cellular function of MBNL1 by binding and sponging out the excess MBNL1 protein (Fig. 2b).

In addition to cytoplasm localization [51], one recent study reported that circular RNA also influences the function of HuR directly [56], which has been demonstrated to regulate the stability and translation of target mRNAs [57, 58]. Through RNA immunoprecipitation (RIP) followed by microarray analysis, a mass of HuR-binding circular RNAs were identified in HeLa cells [56]. Hsa_circ_0031288, which is also named circPABPN1 because it originates from the exon 6 of *PABPN1*, is one of the most enriched circRNAs by HuR. RIP and polysome distribution analysis revealed that HuR can bind to the PABPN1 mRNA and promote its translation, respectively. However, circPABPN1, maybe functioning as a sponge of HuR (Fig. 2c), sequesters HuR away from the PABPN1 mRNA, therefore leading to attenuation of PABPN1 translation and protein expression [56].

Furthermore, the stability of proteins can also be modulated by circRNA. CircNOL10, a circRNA formed via circularization of exons 6–12 of NOL10 pre-mRNA, has low expression in lung cancer tissues and inhibits lung cancer progression in vitro and in vivo [59]. Mass spectrometer detection after an RNA pull-down assay of circNOL10-binding proteins revealed that circNOL10 interacts with the transcription factor Scm polycomb group protein like

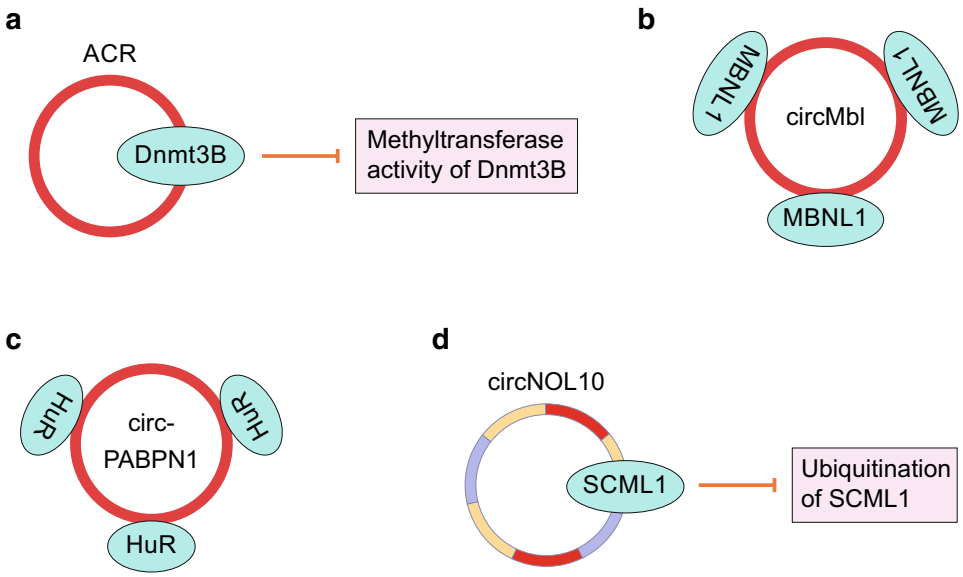

Fig. 2 CircRNAs regulate the function or stability of proteins. **a** ACR physically binds to Dnmt3B and suppresses its function as a DNA methyltransferase. **b** CircMbl attenuates the cellular function of MBNL1 as a sponge of MBNL1 proteins. **c** CircPABPN1 functions as a sponge of HuR proteins and sequesters them away from the PABPN1 mRNA, which results in attenuated translation of PABPN1. **d** CircNOL10 interacts with SCML1 and increases its stability by inhibiting its ubiquitination. Please see more detail in the text. "perpendicular sign" indicates an inhibition effect. *ACR* autophagy-related circular RNA, *MBNL1* Muscleblind like splicing regulator 1, *SCML1* Scm polycomb group protein like 1

1 (SCML1) (Fig. 2d). Moreover, circNOL10 enhances the expression of SCML1 in lung cancer cells by inhibiting its ubiquitination. Subsequently, several members of the HN polypeptide family, which are regulated by SCML1 at the transcriptional level, affect mitochondrial function and multiple tumor signaling pathways, eventually contributing to decreased cell cycle progression and cell proliferation, and increased apoptosis. Consequently, circNOL10 restrains lung cancer development via the SCML1/HN polypeptide family axis.

CircRNAs affect the interaction between proteins

CircRNAs promote the interaction between proteins

Recently, it was discovered that the interaction between proteins can be reinforced by circRNAs [60, 61]. One study demonstrated that circ-Foxo3 is conserved in human and mouse and suppresses cell cycle progression and cell proliferation by strengthening the interaction between p21 and CDK2 [60]. p21, a known cyclin-dependent kinase inhibitor, can interact with CDK2 and restrain the formation of the CDK2–cyclin E complex, therefore blocking the transition of cells from the G1 to the S phase [62]. However, circ-Foxo3 can bind to both p21 and CDK2 with high affinity, reinforce the interaction between them (Fig. 3a), and enhance the inhibitory effect of p21 on CDK2 activity. As a downstream consequence, the formation of CDK2–cyclin A complexes are also reduced, resulting in failure of the cells to pass through the S phase. Therefore, circ-Foxo3 controls cell cycle entry of cells by forming ternary complexes with p21 and CDK2 [60].

Another study revealed that circ-Foxo3 is down-regulated in specimens of patients with breast carcinoma, and its ectopic expression in breast cancer cells inhibited xenograft tumor growth and prolonged mouse lifespan [61]. MDM2, a nuclear-localized E3 ubiquitin ligase, was identified as a protein that interacts with circ-Foxo3 and regulates its intracellular effect. MDM2 has been reported to promote the protein degradation of p53 [63] and Foxo3 [64] via the ubiquitin–proteasome pathway. Circ-Foxo3 can interact with both MDM2 and p53, leading to enhanced interaction between them [61] (Fig. 3b). Subsequently, MDM2 mediates the poly-ubiquitination of p53 and promotes its degradation. However, circ-Foxo3 has only a low binding affinity to Foxo3, another target protein of MDM2, therefore failing to promote the binding of MDM2 to it. As a consequence of the circ-Foxo3–MDM2–p53 interaction, Foxo3 is relieved from the poly-ubiquitination and degradation modulated by MDM2. Upregulated Foxo3 then activates the expression of its downstream target PUMA, which contributes to increased cell apoptosis [65, 66].

CircRNAs suppress the interaction between proteins

However, one recent study reported that circRNA suppresses the interaction of proteins by competitive binding [67]. CircANRIL is derived from an *ANRIL* transcript on

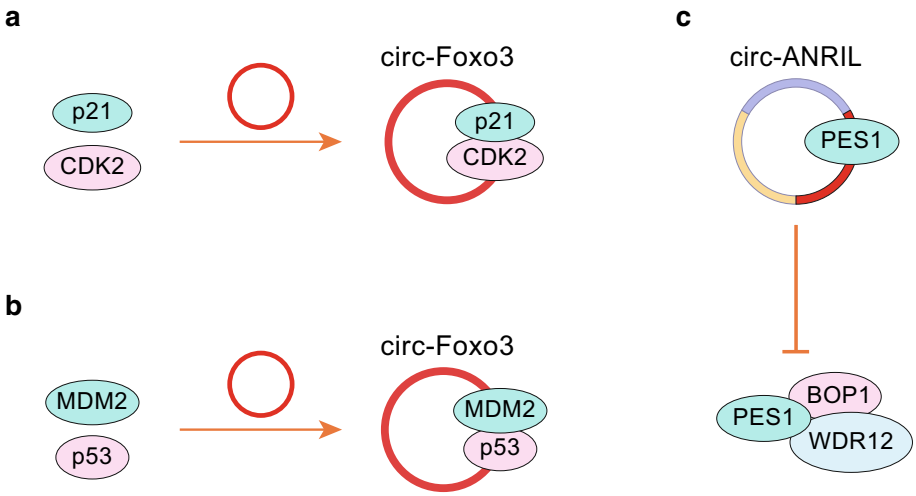

Fig. 3 CircRNAs affect the interaction between proteins. **a** Circ-Foxo3 can interact with both p21 and CDK2, strengthen their interaction, and heighten the inhibitory effect of p21 on CDK2 activity. **b** Circ-Foxo3 can bind to both MDM2 and p53, reinforce their interaction, and facilitate MDM2-mediated poly-ubiquitination of p53. **c** CircANRIL competitively binds to PES1 and prevents the assembly of the PeBoW protein complex that is consistsed of PES1, BOP1, and WDR12. Please see more detail in the text. "down arrow" indicates a promotion effect, but "perpendicular sign" indicates an inhibition effect. *CDK2* cyclin dependent kinase 2, *MDM2* MDM2 proto-oncogene, *PES1* Pescadillo ribosomal biogenesis factor 1, *BOP1* BOP1 ribosomal biogenesis factor, *WDR12* WD repeat domain 12

chromosome 9p21 and contains exon 5, 6, and 7, where exon 7 is covalently joined to exon 5. It was found to bind Pescadillo ribosomal biogenesis factor 1 (PES1) and affect ribosome biogenesis [67]. A causal correlation of circANRIL expression with 9p21 haplotypes in peripheral blood mononuclear cells from coronary artery disease patients indicates an atheroprotection role of circANRIL. PES1, which constitutes the nucleolar PeBoW complex with Bop1 and WDR12 [68], was identified as a protein with the strongest binding to circANRIL. As a result of circANRIL's competitive binding to the C-terminal domain of PES1 (Fig. 3c), the assembly and function of the PeBoW protein complex in pre-rRNA processing and the maturation of the 60S ribosomal subunit are arrested. Subsequently, nucleolar stress and p53 accumulation are induced, leading to increased apoptosis and decreased proliferation that restrain the development of atherosclerosis [69, 70]. Therefore, by controlling the pathway associated with atherogenesis, circANRIL contributes to atheroprotection [67].

CircRNAs influence the interaction between protein and DNA

In addition to the interaction between proteins, interaction between protein and DNA was also found to be promoted [71] or inhibited [72] by circRNA. FLI1 exonic circular RNA (FECR1) is a circRNA generated by the circularization of exons 4, 3, and 2 of *FLI1*, which functions potentially as an oncogenic driver in multiple types of cancer [71]. In accordance with the high abundance of FLI1 in advanced and metastatic breast cancer tissues, FECR1 is also expressed in breast cancer tissues and promotes the invasion of breast cancer cells. The investigations of molecular mechanisms revealed that on one hand, FECR1 binds to the promoter of DNMT1, a kind of methyltransferase responsible for maintaining the methylation of DNA, and downregulates its transcription. On the other hand, FECR1 interacts with the GC-rich region of the FLI1 promoter and further recruits demethylase TET1 to it, resulting in the demethylation of DNA [73] (Fig. 4a). Together, FECR1 serves to induce widespread DNA demethylation of the FLI1 promoter and epigenetically activates FLI1's expression. In turn, the oncoprotein FLI1 contributes to metastasis in breast cancer [71].

Nevertheless, another study reported that circRNA suppresses the interaction between protein and DNA through competitive binding [72]. Cyclic GMP–AMP synthase (cGAS) is a cytosolic DNA sensor, whose binding with DNA allows it to catalyze the synthesis of a second messenger cGAMP and stimulate downstream STING signaling [74]. The cGAMP/STING axis then induces the production of type I interferons (IFNs), which mediate the exhaustion of quiescent hematopoietic stem cells (HSCs) [75, 76]. A circular RNA antagonist for cGAS named cia-cGAS is composed of exons 4, 5, and 6 of mouse *D430042O09Rik* gene transcripts, with exon 6 linked to exon 4 [72]. Cia-cGAS is highly expressed in the nucleus of long-term hematopoietic stem cells (LT-HSCs) and contributes to the maintenance of quiescent HSCs. Its deficiency in mice results in the increase of type I IFNs in LT-HSCs and significant reduction of dormant LT-HSCs in bone marrow. The functional mechanism studies revealed that cia-cGAS harbors a stronger affinity to

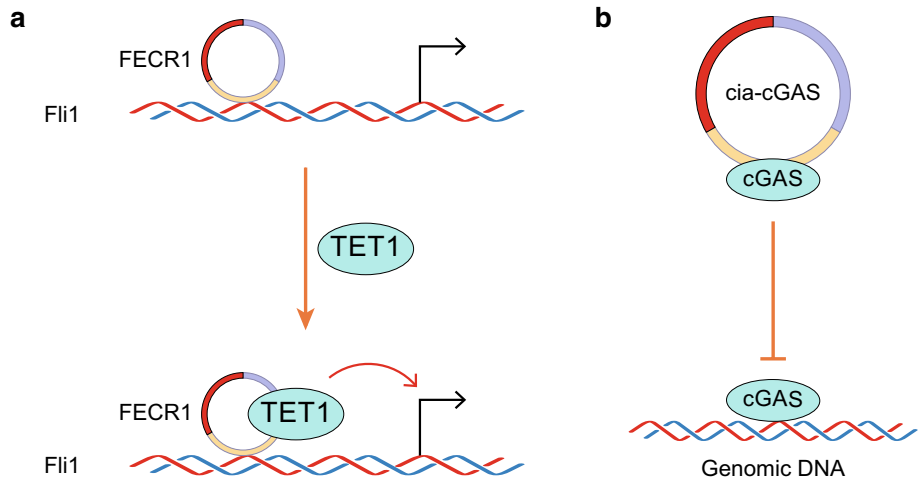

Fig. 4 CircRNAs influence the interaction between protein and DNA. **a** FECR1 binds to the FLI1 promoter and further recruits the demethylase TET1 to it, which mediates the DNA demethylation and activates the transcription of FLI1. **b** Cia-cGAS competitively binds to cGAS and restrains the interaction between genomic DNA and cGAS that is pivotal to the enzymatic activity of cGAS. Please see more detail in the text. "down arrow" indicates a promotion effect, but "perpendicular sign" indicates an inhibition effect. *FECR1* FLI1 exonic circular RNA, *TET1* tet methylcytosine dioxygenase 1, *cGAS* cyclic GMP-AMP synthase, *cia-cGAS* an circular RNA antagonist for cGAS

cGAS than that of its self-genomic DNA (Fig. 4b). Through the competitive binding to cGAS with genomic DNA, cia-cGAS blocks the enzymatic activity of cGAS in the nucleus of LT-HSCs and restrains cGAS-catalyzed production of type I IFNs. As a consequence, cia-cGAS defends dormant HSCs from cGAS/Type I IFNs-modulated exhaustion [72].

Prediction, screening, and validation of proteins directly bound by circRNAs

Physical association between protein and circular RNA can be predicted on the basis of bioinformatics analysis, to which Burton Yang's group has devoted a lot of work [39, 41, 42, 61]. The first step in their prediction was to determine the secondary structure of circRNA with lowest free energy using biological software such as Mfold. The best putative secondary structure defined with dot-bracket notation was then analyzed for tertiary structure prediction by software such as RNAComposer. Subsequently, NPDock was utilized to perform in silico molecular docking analysis of circRNA and protein, which is based on the tertiary structure of the circRNA and the 3D structure of the protein. Additionally, the secondary structure of circRNAs can also be predicted using the RNAstructure web server [59, 77] or the Vienna RNA package [67, 72, 78], and the prediction of protein–circRNA binding has also been performed with the catRAPID [59, 67, 79] or the RNA–Protein Interaction Prediction (RPISeq) software [67, 80].

The experimental method of cross-linking and immunoprecipitation followed by RNA sequencing (CLIP-Seq) is commonly used to identify cellular circRNAs that bind to one known protein [56]. However, researchers also searched for potential binding proteins of one known circular RNA by means of a proteomic screen. Holdt et al. constructed an expression vector of circANRIL with BoxB RNA hairpin sequences inserted into the coding sequence of exon 6, without impairing the circularization of exon 5 and 7 [67, 81]. Due to the high affinity of bacteriophage l transcriptional anti-terminator protein N (λN-peptide) to the BoxB RNA hairpin, λN-peptide coupled to *d*-desthiobiotin can mediate the pull-down of circANRIL–protein complexes in cellular lysates by streptavidin beads [82]. The captured proteins bound to circANRIL were then identified by mass spectrometric analyses [67]. Additionally, to identify the proteins interacting with one specific circRNA, Chen et al. performed an RNA pull-down assay by incubating the biotin-labeled circRNA probe with cell lysates and enriching the circRNA–protein complexes with streptavidin beads, and then detected the proteins by mass spectrometry assays [51].

One regular method to validate the interaction between circRNA and protein is the RNA immunoprecipitation (RIP) assay, in which antibodies against protein are utilized to immunoprecipitate the protein factor associated with the circRNA, followed by quantitative PCR analysis for the circRNA [39–42, 50, 53, 54, 56, 60, 61, 67, 71, 72]. Another regular method is the RNA pull-down assay, in which a complementary and biotinylated probe to circRNA is used, and the circRNA in cell lysates is pulled down by streptavidin beads; the protein bound to the circRNA is then co-precipitated and detected by western blot assay [39–42, 50, 51, 53, 56, 59–61, 67]. Expression of circRNA within cells or tissues can also be detected by fluorescent in situ hybridization (FISH) with a mixture of dye-labeled DNA oligo probes paired with circRNA [39, 50, 51, 59, 61, 72]. Moreover, a combination of FISH to circRNA and immunofluorescence staining with an antibody against protein makes it feasible to observe the colocalization of circRNA and protein with confocal laser scanning microscopy [39, 50, 51, 61, 72]. Additionally, the blocking oligo, which is complementary to the binding sites of protein on circRNA and aims to impair protein–circRNA binding, can be delivered into cells for checking the downstream variation of cellular function, further confirming the protein–circRNA interaction [40–42]. Besides, the electrophoretic mobility shift assay (EMSA) after the incubation of circRNA and protein was considered as another technique to prove the direct circRNA–protein interaction [51, 59, 72].

Conclusions and perspectives

As a large class of long non-coding RNAs in mammalians, circRNAs are known for their function as miRNA sponges. However, it is worth noting that unlike CDR1as and *Sry*, which have 67 and 16 putative binding sites for miR-7 [5] and miR-138 [6], respectively, a single predicted miRNA binding site within a circRNA sequence is not adequate to justify its sponge effect on the miRNA. Furthermore, the relative expression levels of circRNA and miRNA in the cells should be taken into account when the sponge function of the circRNA is claimed. Moreover, experimental verification, such as luciferase reporter assays for the test of circRNA's suppression to miRNA's knockdown potential, as well as demonstration of direct binding between circRNA and miRNA [6], would be essential for the evaluation of circRNA's sponge efficiency to miRNA. Additionally, circRNAs have been revealed to regulate the stability of mRNA via direct interaction with it [37], or to affect transcription by binding with DNA promoters of genes [10].

However, in this article, we have comprehensively summarized four working models on how circRNAs modulate the action of proteins by direct interaction. One is to promote the nuclear localization [39–42] or cytoplasmic localization of proteins [50, 51] (Fig. 1); the second is to regulate the function [53, 54, 56] or stability [59] of proteins (Fig. 2);

Table 1 The proteins directly bound by circRNAs and the biological processes they regulated

Type	Protein	CircRNA	Type of circRNA	Parent gene of circRNA	Function of circRNA	Experimental validation of methods for circRNA–protein interaction	Reference
Transcription factor	cMyc	circ-Amotl1	Exonic	*AMOTL1*	Promote tumorigenesis	RIP assay, RNA pull-down assay, FISH & IF assay	[39]
	Stat3	circ-Amotl1	Exonic	*AMOTL1*	Accelerate wound repair	RIP assay, RNA pull-down assay, oligo blocking assay	[40]
	p53 and AUF1	circ-DNMT1	Exonic	*DNMT1*	Facilitate breast cancer progression	RIP assay, RNA pull-down assay, oligo blocking assay	[42]
	Id1, E2F1, HIF1α, and FAK	circ-Foxo3	Exonic	*FOXO3*	Promote cardiac senescence	RIP assay, RNA pull-down assay, FISH & IF assay	[50]
	SCML1	circNOL10	Exonic	*NOL10*	Inhibit lung cancer progression	EMSA, RNA pull-down assay	[59]
RNA processing protein	MBL	circMBL	Exonic	*MBL*	Sponge out excess MBL proteins	RIP assay	[54]
	PES1	circANRIL	Exonic	*ANRIL*	Regulate atherosclerosis	RIP assay, RNA pull-down assay	[67]
Protease	PDK1 and AKT1	circ-Amotl1	Exonic	*AMOTL1*	Reinforce the cardio-protective role mediated by AKT	RIP assay, RNA pull-down assay, oligo blocking assay	[41]
	Dnmt3B	ACR	Intergenic	*N/A*	Reduce myocardial ischemia/reperfusion injury	RIP assay, RNA pull-down assay	[53]
	CDK2	circ-Foxo3	Exonic	*FOXO3*	Retard cell cycle progression	RIP assay, RNA pull down assay	[60]
	MDM2	circ-Foxo3	Exonic	*FOXO3*	Induce tumor apoptosis	RIP assay, RNA pull down assay, FISH & IF assay	[61]
	TET1	FECR1	Exonic	*Fli1*	Promote metastasis in breast cancer	RIP assay	[71]
	cGAS	cia-cGAS	Exonic	*D430042O09Rik*	Protect long-term hematopoietic stem cells from exhaustion	RIP assay, EMSA assay, FISH & IF assay	[72]
RNA-binding protein	HuR	circAGO2	Intronic	*AGO2*	Promote cancer progression	RNA pull-down assay, EMSA assay, FISH & IF assay	[51]
	HuR	circPABPN1	Exonic	*PABPN1*	Suppress translation of PABPN1	RIP assay, RNA pull-down assay	[56]

RIP assay RNA immunoprecipitation assay, *FISH* fluorescent in situ hybridization, *IF* immunofluorescence, *EMSA* electrophoretic mobility shift assay, *FISH & IF assay* an combination of FISH assay to circRNA and IF assay to protein to observe the colocalization of circRNA and protein with confocal microscopy

the third is to facilitate [60, 61] or restrain [67] the interaction between proteins (Fig. 3); and the fourth is to affect the interaction between protein and DNA [71, 72] (Fig. 4). The circRNAs and the proteins they interact with, together with the biological processes they are implicated in, are summarized in Table 1. The proteins bound by circRNAs can be classified as transcription factors [39, 40, 42, 50, 59], RNA processing proteins [54, 67], proteases [41, 53, 60, 61, 71, 72], and RNA-binding proteins [51, 56]. Accordingly, circRNAs are implicated in the regulation of multiple physiologic and pathologic processes, including tumorigenesis, atherosclerosis, wound repair, cardiac senescence, myocardial ischemia/reperfusion injury, and so forth (Table 1).

In view of distinct circRNA–protein complexes that exist in mammalian cells [24], interactions between circRNAs and protein factors, as well as the caused variation of cellular biological functions, would be far beyond what we described in this article. For instance, the regulation of circRNAs to proteins′ action in other organelles except for the cell nucleus and cytosol, including the endoplasmic reticulum, Golgi apparatus, and mitochondria, has not been reported. In addition, it would be worthwhile to investigate whether circRNAs can affect protein modification unreported, such as protein acetylation or phosphorylation, except for the protein localization, activity or stability reviewed in this article. Besides protein–protein or protein–DNA interactions, studies focused on whether circRNAs affect the interactions between proteins and mRNAs or other non-coding RNAs should also be given importance. Furthermore, the identification of other types of unexplored proteins interacting with circRNAs, such as the chromatin regulatory proteins that control chromatin architecture, will further unveil more unknown functions of circRNAs. Finally, the improvement in methods or techniques for identifying unexplored proteins directly interacting with circRNAs would propel the discovery of circRNAs′ guidance to protein behaviors.

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