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Critical Reviews and Perspectives

Translation of circular RNAs

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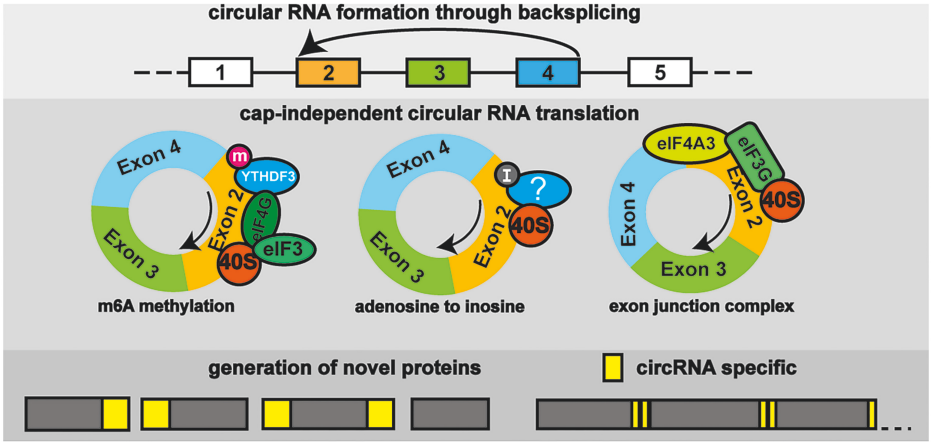
Abstract

Circular RNAs (circRNAs) are covalently closed RNAs that are present in all eukaryotes tested. Recent RNA sequencing (RNA-seq) analyses indicate that although generally less abundant than messenger RNAs (mRNAs), over 1.8 million circRNA isoforms exist in humans, much more than the number of currently known mRNA isoforms. Most circRNAs are generated through backsplicing that depends on pre-mRNA structures, which are influenced by intronic elements, for example, primate-specific Alu elements, leading to species-specific circRNAs. CircRNAs are mostly cytosolic, stable and some were shown to influence cells by sequestering miRNAs and RNA-binding proteins. We review the increasing evidence that circRNAs are translated into proteins using several cap-independent translational mechanisms, that include internal ribosomal entry sites, N6-methyladenosine RNA modification, adenosine to inosine RNA editing and interaction with the eIF4A3 component of the exon junction complex. CircRNAs are translated under conditions that favor cap-independent translation, notably in cancer and generate proteins that are shorter than mRNA-encoded proteins, which can acquire new functions relevant in diseases.

Graphical abstract



Introduction

Circular RNAs contribute considerably to transcriptome diversity

Thirty-three years ago, reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of the tumor suppressor gene DCC (Deleted in Colorectal Carcinoma) revealed the order of exons to be reversed in a small portion of messenger RNAs (mRNA). For example, for an RNA corresponding to a genomic clone with the order exon B – exon C – exon D, the orientation exon D to exon B was observed (1). It was later found that eukaryotic genes form circular RNAs (circRNA) using canonical splice sites (2,3), which explained the scrambled arrangement. Early reports indicated that a circRNA made from an archaea pre-23S rRNA is translated into an endonuclease of 194 amino acids (4). Technical progress in RNA sequencing showed then a widespread expression of circRNAs that are present in all eukaryotes tested (5,6).

CircRNAs are covalently closed RNAs generated from pre-mRNA. With some exceptions, for example circHIPK3 (6), circRNAs are less abundant than linear mRNAs, making it necessary to enrich most circRNAs for detection using rRNA removal and digestion of linear mRNA with RNase R, which is followed by next generation sequencing. However, highly abundant circRNAs are present in standard RNA

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Table 1. Resources and databases for circRNAs

FL-circAS	Full-length circRNA sequences, shows internal sequences and alternative splicing	(10)
circVis	Visualization of circRNA, including open reading frames	(69)
circNET 2.0	Regulatory network in cancer	(139)
circMine	Disease related circRNAs	(140)
riboCirc	Translatable circRNA	(68)
transCirc	Translatable circRNA	(62)
Circ2Disease	CircRNAs in human disease	(141)
CircInteractome	Database of circRNA interaction with miRNAs and proteins	(59)

List of web-based resources useful to study circRNAs.

sequencing (RNA-seq) libraries. This approach identifies the backsplice junction, which allows prediction of the RNA structure based on the annotated exons. However, with this approach the sequence information outside the reads of the backsplice site remains unknown. Recently, rolling circle amplification of circRNAs, followed by adaptor addition and long range nanopore sequencing allowed identification of full-length circRNAs (7–9). These circRNAs were collected in databases (10) (Table 1). Collectively, sequencing efforts identified >880 000 backsplicing events in humans that potentially generate >1.8 million human circRNA isoforms. Depending on the library preparation, between 6 and 19% of these circRNAs were detected by more than five reads (10). This diversity compares to about 320 000 human mRNA isoforms from annotated genes and over 30 million transcripts from other human genomic loci (11), suggesting that circRNAs play a so far overlooked role in the eukaryotic transcriptome.

Formation of circRNAs through backsplicing that depends on intronic regions

CircRNAs can be generated by several mechanisms, including circularization of lariat introns, circularization during transfer RNA (tRNA) and ribosomal RNA (rRNA) splicing [reviewed in (12)] and self-splicing of group I introns (13). In humans, most circRNAs are generated from pre-mRNA through backsplicing, where a downstream 5′ splice site is connected to an upstream 3′ splice site. Backsplicing contrasts standard pre-mRNA splicing where this order is reversed and an upstream 5′ splicing site is linked to a downstream 3′ splice site, resulting in removal of an intron (Figure 1A and B).

The backsplice splice sites can be thousands of base pairs apart in the pre-mRNA, indicating that additional mechanisms are necessary to bring the backsplice sites into apposition. Pre-mRNAs secondary structures formed by complementary intronic sequences are likely a major contributor to circRNA formation (6,14,15), reviewed in (16). The complementary sequences are often provided by repetitive elements, for example Alu elements in primates or B1 elements in rodents.

Alu elements are about 300 nt long repetitive elements and comprise 11% of the human genome (17). RNA *in situ* conformation sequencing showed that Alu elements promoted >37% of all RNA–RNA interactions across enhancers (18). It is likely that Alu elements play a similar prominent role in forming pre-mRNA structures, a model which is supported by the findings that in humans circRNA backsplice sites are often surrounded by Alu elements (6) and these likely ac-

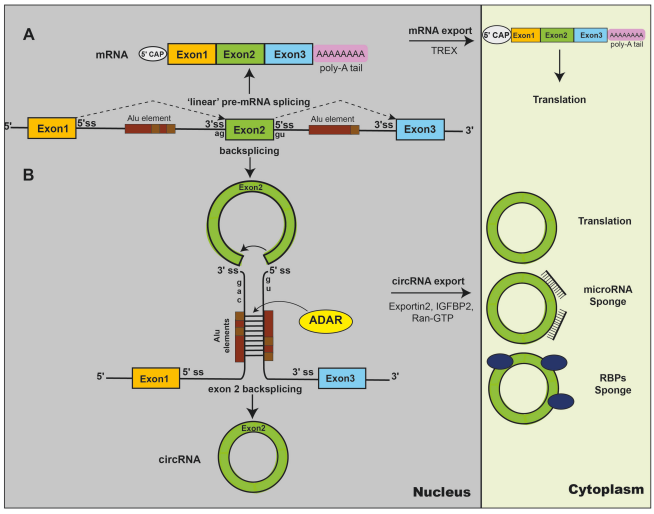

Figure 1. Generation and overall functions of mRNAs and circRNAs. (**A**) A pre-mRNA containing three exons and two introns flanking the central exon is processed into a mRNA containing a 5′ cap and a poly adenosine tail using the 'linear' pre-mRNA pathway, dashed lines. The invariant AG-GT nucleotides of the 3′ and 5′ splice sites are indicated. mRNAs are predominantly exported by the TREX (transcription and RNA export) complex from the nucleus to the cytosol where they are translated. (**B**) A small fraction of the pre-mRNA can undergo backsplicing when the 5′ and 3′ splice site are brought into close proximity, which is promoted by RNA secondary structures or mediated by proteins. In humans, pre-mRNA structures are often introduced by Alu elements that form long complementary sequences, which are the major substrate for ADAR enzymes. CircRNAs are exported via an exportin-2-IGFBP2-RanGTP complex. In the cytosol they can sequester miRNAs, RNA binding proteins or act as translational templates.

count for the numerous human-specific circRNAs (10). Alu elements are similar to the 5′ and 3′ end of the 7SL RNA from which they originated but lack a 155 nt long 7SL-specific part. Alu elements consist of a left arm composed of a head to tail dimer of two similar sequences, each of which is about 130 nt long. Following a stretch of adenosines, the right Alu-arm contains an additional insert (19). Reflecting the highly structured 7SL RNA, Alu elements exhibit complementarity through direct RNA interactions, especially when present in a sense to antisense orientation in the pre-mRNA. Double-stranded RNAs are the substrate of ADAR enzymes (adenosine deaminase acting on RNA) that change adenosines to inosines. In humans >99% of all edited RNAs are derived from Alu elements (20,21), demonstrating that intramolecular double-strand RNA (dsRNA) regions formed by Alu elements are common in pre-mRNAs.

As Alu elements are species-specific (17), they could contribute to the species-specificity reported in databases for some circRNAs (10). The survival of motoneuron (SMN) gene is an example for an Alu-rich gene, as it contains >40 Alu elements, occupying about 40% of the SMN gene sequence. A systematic PCR-based study showed that the human SMN gene generates at least 53 circRNAs, that include four novel exons, which are not expressed in mouse (22,23).

Mouse B1 elements are related to human Alu elements and are also derived from 7SL-RNA. However, B1 elements are more diverse and shorter (about 140 nt) than Alu elements, consisting of only one monomer, which likely decreases the preponderance of self-complementarity and overall RNA editing (24).

Back splicing can also be promoted by proteins. For example, the interaction between Alu element RNA can be increased by SLU7 Homolog-Splicing Factor (SLU7) binding to Alu sequences flanking circCAPG, which promotes circRNA generation (25). Protein-dependent promotion of circRNA formation can occur without Alu elements, and >15 RNA-binding proteins were shown to promote circRNA formation, among them QKI (26), RBM20 (27), hnRNP L (28) and FUS (29) (reviewed in 30). Conversely, the nuclear RNA helicase DHX9 reduces the number of circRNAs, likely by resolving Alu element-dependent secondary structures (31,32).

CircRNAs are cytosolic, more stable than mRNAs and use a nuclear-cytoplasmic export pathway different from mRNAs

Due to their generation through backsplicing, circRNAs lack a 7-methyl guanosine cap at their 5′ end (5′ cap) and also do not have a poly A tail at their 3′ end. CircRNAs collected in recent databases have an average (median) length of around 750 nt and about 20% of circRNAs are longer than 500 nt, the largest up to 81 000 nt in length. CircRNAs are mostly composed of exons that are already found in linear RNAs, i.e. contain exons annotated in the database (10). Nanopore sequencing of full-length brain circRNAs showed that 3.2% of exons in circRNAs are completely new, and 5.3% show a new splicing pattern of known exons (33). circRNAs are generated in the nucleus and can be exported into the cytosol. A prominent export mechanism depends on insulin-like growth factor 2 mRNA-binding protein (IGF2BP1). The circRNA-IGF2BP1 complex is exported into the cytosol through interaction with exportin 2 and Ran-GTP (34). It is likely that other pathways exist that depend on the length of the circRNA. For example, exportin 4 (35) and UAP56 (DDX39B) are involved in export of long circRNAs, whereas URH49 (DDX39A) (36) promotes export of shorter circRNAs.

Thus, the circRNA export mechanisms are distinct from mRNAs. Cytosolic export of the vast majority of mRNAs depends on pre-mRNA splicing, where the exon junction complex is recognized by the TREX (transcription export) complex (37,38). The transport of mRNAs into the cytosol is further promoted by numerous other factors, notably SR-proteins (39,40) and poly (A) binding protein (PABP) (41).

The nuclear-cytosolic distribution of circRNAs can be influenced by external stimuli, such as hypoxia that leads to a translocation of circLRP6 from the nucleus to the cytosol. This transport is possibly mediated by binding of the circRNA to hnRNP M that accumulates in the cytosol under hypoxic conditions (42). The export of circRNAs can be cell-type specific. For example, in pluripotent cell, but not differentiated cells, adenosine-rich circRNAs are retained in the nucleus through interaction with PABPC1 (poly A binding protein C1) (43).

The high stability of circRNAs is another striking difference between circular and mRNAs. Half-lives have been measured for several protein coding circRNAs, for example, for circYAP1 (44), circTRCP (45), circARHGAP35 (46) and circHER2 (47). In cell-based assays, circRNA half-lives were found to be longer than 24 h, which is the upper limit of detection, indicating that the true half-lives may be longer. The data reflect the half-lives (18.8–23.7 h) previously measured for 61 circRNA (48). In comparison, mRNAs have a half-life of 4.3 h (49) making circRNA considerably more stable.

CircRNAs can function by binding to nucleic acids and proteins

Interaction with nucleic acids

Despite the widespread expression of circRNAs, their biological functions are poorly understood. CircRNAs were found to bind to nucleic acids and proteins and could thus act by sequestering these molecules. Most circRNAs are expressed at low levels, which needs to be taken into account when interpreting their interaction with proteins and RNAs. Binding to miRNAs was the first function identified for circRNAs where the unusually abundant circRNA ciRS-7/CDR1as binds to miR-7 and exerts biological effects through miR-7 sequestration ('sponging') (50,51). Later studies showed that ciRS-7/CDR1as is part of a network of non-coding RNAs that likely controls spatial and temporal miR-7 availability in neurons (52) and ciRS-7/CDR1as knock-out impacts synaptic transmission (53). Sequestration of miRNAs by circRNAs has been reported for other circRNAs (reviewed in 54,55). Binding to DNA via base-pair interaction is also possible. For example, in arabidopsis thaliana the circRNA made from exon 6 of the SEPALLATA3 gene binds to its own DNA locus, forming an R-loop of circRNA and DNA (56). Expression of circRNAs from the SMN gene changed expression of 15% of all genes in HEK293 cells, underlining the possible influence that circRNAs can have on gene expression, although the molecular mechanisms remain unclear (57).

Binding to proteins

CircRNAs were shown to bind to numerous proteins, mainly RNA binding proteins, but some circRNAs, like circFOXO3 were reported to sequester p21 and CSK2, which are proteins without known RNA binding activity (58). Computational predicted binding sites were collected in databases (59). Sequestration of proteins by circRNAs can effect transcription, cell cycle progression, apoptosis and cell migration (reviewed in 60).

Evidence for translation of circRNAs

Most circRNAs contain open reading frames and initially over 4000 circRNAs were predicted to encode proteins (61). That number has increased to >300 000 in the recent transCirc database. The updated prediction for translation of circRNAs is based on available mass-spectrometry data, the association with polysomes, the presence of internal ribosomal entry sites (IRESes) as well as known and predicted m6A sites (62). There is now an increasing number of studies showing translation of endogenous circRNAs.

Early evidence that circRNAs can be translated came from studies in 1988 using the archaebacterium *Desulfurococcus mobilis*, where an intron from the pre-23S rRNA is excised as a stable circRNA that encodes an endonuclease of 194 amino acids that can spread through other strains (4). In mammalian systems, exon 2 of circSLC8A1 (previously named NCX1) was shown in 1999 to encode a protein that transports calcium across a membrane (63). Proof of principle studies in 1995 showed that circRNAs can be translated *in vitro* if they contain IRESes (64), which has been confirmed for different IRESes (65). Rolling circle translation has been observed even in chemically modified circRNAs containing phosphoramidate linkages (66). The translational potential of circRNAs

was controversial, as initial screens of circRNAs with open reading frames did not detect translation (67). However, further studies validated the translation of a growing number of circRNAs in physiological systems (Table 2). Evidence that circRNAs are translated comes from their association with polysomes, indicating active translation; validation of backsplice site-encoded peptides using mass-spectrometry and detection of circRNA-encoded proteins using specific antisera.

Association with polysomes

Over 3000 publicly available Ribosome profiling (Ribo-seq) databases were analyzed for the presence of circRNAs, identifying 1969 polysome associated circRNAs in six different species, which were collected in the riboCirc database (68). Similarly, the circVIS database reported 9859 polysome associated circRNAs (69), which expanded the previous transCirc database (62). Polysome association was confirmed for numerous transcripts using RT-PCR of independent polysome preparations (70). Similarly, numerous studies focusing on a defined circRNA showed polysome association, indicated in Table 2.

Ribosome foot printing experiments showed a significant increase in circRNAs when the polysomes were prepared in the presence of detergent (0.1% Triton), indicating that circRNA translating ribosomes are associated with membranes (71). Since detergent is omitted in most polysome preparation protocols, the actual number of circRNAs associated with polysomes could be underestimated. Translation and polysome association of circRNAs could be specific for the developmental stages of a given cell. For example, erythrocytes and platelets contain high levels of circRNAs, but only 32 circRNAs were polysome associated in the progenitor erythroblasts (72).

Validation by mass-spectrometry

The translation of several circRNAs was also confirmed by mass-spectrometry that detected a peptide corresponding to the backsplice junction. One of the first of these proteins was *Drosophila* muscleblind (71) for which backsplice site peptides of numerous human proteins were identified and similar mass-spectrometry evidence has been found for other circRNA-encoded proteins (Table 2). During sperm development, mRNAs are degraded in pachytene spermatocytes, leading to an enrichment of circRNAs. Mass-spectrometry analysis using mouse testes identified over 1600 peptides corresponding to backsplice junctions, indicating widespread circRNA translation (73). Analysis of human heart tissue showed 8878 circRNAs, of which 40 were associated with polysomes and mass-spectrometry evidence for translation was found for six of these proteins (74), showing translation of human circRNAs in a differentiated tissue.

Detection of circRNA-encoded proteins using specific antisera

Proteins encoded by circRNAs often have amino and carboxy termini that are specific for the circRNA and are not found in the mRNA (Figure 3A). These peptides encoded only by circRNAs make it possible to generate circRNA-encoded protein specific antisera (Table 2). A common problem is that these circRNA-specific sequences (shown in Supplementary Table S1) are usually short, which can make it difficult to generate specific antisera.

Detection of circRNA-encoded proteins using reporter genes

Transfection studies with reporter genes were first used to prove the possibility of circRNA translation. Usually, these reporter genes contain a protein tag like a 3× Flag tag that is located upstream of the start codon and thus depends on circRNA formation for translation. It is possible that translation initiates from the linear RNA, using intronic start codons upstream of the flag tag (75). These putative start codons need to be removed from the reporter constructs. The circRNA translational reporters often use heterologous flanking intronic sequences, provided for example by the ZKSCAN1 gene that strongly expresses circZKSCAN1. However, heterologous and authentic flanking introns can give different amounts of circRNAs and circRNA-encoded protein expression (76).

Precautions in interpreting circRNA translation experiments

As circRNAs usually have low expression levels, investigating their translation needs to be carefully controlled (77). The ectopic expression from reporter gene assays can produce artefacts, such as *trans*-splicing (78), and protein expression from linear byproducts (75). These problems can be addressed by controls, including comparison with mutated backsplice sites where the invariable GU is changed to UU, removal of the introns necessary for circularization, introduction of in frame stop codons, and splitting the epitope tag in the backsplice site. Splice site mutations will affect both *trans*-splicing and circRNA-generating backsplicing. However, only circRNAs will be resistant to RNaseR treatment, which can be used to discriminate between *trans*-spliced mRNA and circRNA. Additional controls are vectors that use group I self-splicing as a different mechanism for circularization (79), which can be used to show translation of the circRNA (79,80).

The association of circRNAs with ribosomes does not prove that these circRNAs are translated. Ribo-seq analysis of translated mRNA shows a triplet periodicity that has not been observed with most circRNAs (67,77), suggesting that most ribosome-associated circRNAs are not actively translated and modulate ribosomal function, similar to proteins like SMA (81) and other ribosome associated proteins (82). Furthermore, it is possible that the triplet periodicity cannot be detected due to the low number of circRNA reads.

Several reports indicate that the interaction between circRNAs and ribosomes inhibits translation. For example, circHIPK2 binds directly to the pre-ribosomal initiation factor RPL7, which inhibits myogenesis (83). Similarly, circTRPS1 binds to several ribosomal proteins, which reduces translation (84). Testing translation of specific mRNAs, it was found that circMALAT1 inhibits PAX5 mRNA translation (85), circSMAD2 sequesters eIF4A3 which inhibits translation of linear SMAD2 (86). However, as detailed below, circRNA translation depends on RNA modifications (79,87,88), which were not considered in the Ribo-seq analysis. Finally, interpreting mass-spectrometry results of short, circRNA-specific peptides is challenging, as the mass-spectrometric false discovery rate

Table 2. Validated proteins made from circRNAs

#	Name	nb_aa	Identification	Function	Summary function	CircRNA length [n]	Alu elements	Number of exons	References
1	LINC-PINT	87	MS, AS	Suppresses glioblastoma cell proliferation	LINC-PINT (Long Intergenic Non-Protein Coding RNA [also p53 Induced Transcript]) forms a circRNA from exon 2, There is no protein translation from the LINC-PINT transcript; however, there is translation from the circRNA. The 87 amino acid long protein binds to PAF1 (polymerase associated factor (PAF1) complex. The protein could arrest the PAF1 complex on promoters of oncogenes, leading to a suppression of cell proliferations in glioblastoma.	1084	no	1	(124)
2	circSHPRH	146	MS, AS	Inhibits tumor cell proliferation	SHPRH (Histone Linker PHD RING Helicase [SNF: sucrose non fermenting]), forms a 440 nt long circRNA through backsplicing from exons 29–26. The 440 nt long circRNA is translated into circSHPRH-146aa. The protein could act by sequestering the ubiquitin E3 ligase DTL that then no longer acts and destabilizes the linear SHPRH protein. The full length SHPRH causes PCNA degradation, and thus circSHPRH-146aa indirectly stops proliferation of glioblastoma cells. circSHPRH-146 is downregulated in glioblastoma. The protein starts and stops within the same 4 nucleotides: TGATG. It starts at ATG, and due to frameshift uses TGA as stop.	440	left	4	(142,143)
3	circFBXW7	185	MS, AS	Represses glioma tumorigenesis	FBXW7 (F-Box And WD Repeat Domain Containing 7, E3) is part of an ubiquitin ligase complex acting as a E3 ligase. The circFBXW7 protein corresponds to the N-terminus and lacks the WXD40 domain necessary for substrate recognition. circFBXW7 competes with the linear FBPXW7 for binding to the deubiquitinase USP28. Thus, an increase in amount of circFBXW7 frees linear FBPXW7 protein for substrate degradation, which was shown for c-Myc. circFBXW7 interacts with catenin, influences Wnt signal, leading to cancer cell resistance.	620	no	2	(144,145)
4	circCDH1	254	MS, AS	Activates EGF receptor	circCDH1 (Cadherin 1) is generated through backsplicing from exons 10–7 in the E-cadherin gene. Due to a frameshift after one round of translation a unique C-terminus is created. The circRNA was detected in 84% of glioma, but not in controls. The circCDH1 activated STAT3, PI3K-AKT and MAPK-ERK signaling in glioblastoma. In contrast to linear E-cadherin that is localized in the plasma membrane, circCDH1 is secreted out of cells and binds and activates EGFR using its circRNA-specific region. The circRNA-encoded protein contain cadherin domains but lacks a transmembrane domain and the signal peptide. The activation of EGFR promotes tumor formation and prevents the therapeutic effect of an anti EGFR-antibody (nimotuzumab).	733	both	4	(127)
5	circINSIG1	131	MS, AS	Induces cholesterol biosynthesis and colorectal cancer progression.	INSIG1 (Insulin Induced Gene 1) is an endoplasmic reticulum membrane domain protein with six transmembrane regions. Backsplicing from exons 4–3 generates circINSIG1 that has a specific C-terminus. circINSIG1 recruits a ubiquitination adaptor complex made from the proteins CUL5 and ASB6. This complex promotes ubiquitination and degradation of the linear INSIG1 protein, which promotes cholesterol biosynthesis and colorectal cancer proliferation and metastasis.	292	no	2	(113)
6	circNFIB	RC	PS	Inhibits breast tumor growth, decreases arachidonic acid	NFIB (Nuclear Factor I B) is a transcriptional activator. Backsplicing of exons 6–3 generates a 361 circRNA that undergoes rolling circle translation. The circNFIB is downregulated in breast cancer. Its overexpression prevents cancer cell proliferation, whereas knock down has the opposite effect.	361	no	4	(146)
7	circZNF609	250	PS, RP	Controls myoblast proliferation	ZNF609 (zinc finger protein 609) forms a circRNA through backsplicing of exon 2.	874	both	1	(147)
8	circYAP	220	MS, AS, PS	Binds to LATS1	CircYAP is generated through backsplicing of exons 7–2 of YAP1. circYAP competes with linear YAP for binding to the kinase LATS1, causing loss of phosphorylation of linear YAP that translocated into the nucleus and turns on oncogenic transcription program. The translation occurs via METT3/14, YTHDF3 eIF4G2.	842	both	5	(44)
9	circFAM53B	219	MS, AS	Inhibits tumor growth	circFAM53B (Family with Sequence Similarity 53 Member B) regulates the Wnt signaling pathway by regulating beta-catenin (CTNNB1) nuclear localization.	659	no	1	(134)
10	circ Beta TRCP (HUGO name: BTRC)	343	MS, AS	Protein mediates tratuzumab resistance by binding to NRF2 transcription factor	Beta-Transducin Repeat Containing E3 Ubiquitin (BTRC) generates circBeta TRCP through backsplicing of exons 13–7. circBeta TRCP contains WD40 repeats that bind to NRF2 and promotes trastuzumab resistance in breast cancer. In contrast to the linear proteins, circ Beta TRCP lacks an F box needed to bind to the ubiquitination complex SKP1-Cul1-Rbx1. Thus, circ Beta TRCP prevents NRF2 from being ubiquitinated, leading to an increase in NRF2, which promotes HER2-positive breast cancer.	913	both	7	(45)

Table 2. Continued

#	Name	nb_aa	Identification	Function	Summary function	CircRNA length [n]	Alu elements	Number of exons	References
11	circMET	404	PS, AS, MS	Promotes glioblastoma	MET (MET Proto-Oncogene, Receptor Tyrosine Kinase) generates circMET through backsplicing its exon 2, which is the first coding exon in the pre-mRNAs. The circRNA is translated after undergoing m6A modification, mediated by YTHDF2. The circMET protein contains the signal peptide and the protein is secreted and binds to the extracellular domain of the linear MET protein, which promotes dimerization without the physiological HGF (hepatocyte growth factor) ligand. circMET promotes glioblastoma tumorgenicity through MET activation.	1214	no	1	(148)
12	circCAPG	171	MS	Promotes breast cancer by binding of serine/threonine kinase 38 (STK38) to SMAD-specific E3 ubiquitin protein ligase 1 (SMURF1)	CAPG (Capping Actin Protein, Gelsolin Like) binds to the barbed ends of F-actin filaments regulating the filament's length. Backsplicing of exons 8–6 generates circCAPG. The circCAPG levels are elevated in triple negative breast cancer and promote tumor growth. It sequesters serine threonine kinase 38, which ultimately prevents MEKK2 proteasomal degradation. The formation of the circRNA is repressed by the splicing factor SLU7, possibly acting on the flanking Alu elements.	376	both	3	(25)
13	circRSRC1	161	PS, MS	Regulates assembly of mitochondrial ribosomes. Loss of the protein decreases male fertility.	RSRC1 (Arginine and Serine Rich Coiled-Coil 1), acts in alternative splicing. Backsplicing from exons 3–2 creates a circRSRC1, that is highly conserved between mouse and human. Knock out in mice reduced spermatogenesis. circRSRC1 binds to C1qbp (Complement C1q Binding Protein), a multifunctional protein. Through interaction with C1qbp, circ RSRC1-161aa could influence mitochondrial ribosome assembly.	322	right	2	(149)
14	circTMEFF1	RC 3×	PS, RP	Promotes muscle atrophy through binding to TDP-43	TMEFF1 (Transmembrane Protein with EGF Like And Two Follistatin Like Domains 1) in involved in receptor signaling. It forms a circRNA by backsplicing of exons 7–5 that is upregulated in muscular atrophy. circTMEFF1 promotes atrophy in cell and mouse models, which can be antagonized by siRNAs.	339	right	3	(150)
15	circPPP1R12A	72	MS, RG	Activates YAP, promotes metastasis	PPP1R12A (Protein Phosphatase 1 Regulatory Subunit 12A), also known as Myosin phosphatase target subunit 1, regulates myosin-actin interaction. Backsplicing of exons 25–24 generates circPPP1R12A. The encoded protein is in a different reading frame than the linear protein and has no orthologs in the protein database. The protein promotes metastasis by indirectly affecting the phosphorylation of YAP, which activates oncogenes.	1138	right	2	(125,151)
16	circGSPT1	238	MS	Binds to vimentin tumor suppressor	GSPT1 (G1 To S Phase Transition 1) is acting in translational termination, also known as eukaryotic release factor 3A (eRF3A). Backsplicing from exons 11–4 generates circ GSPT1that promotes autophagy and apoptosis in cancer cell models by binding to vimentin/beclin/14–3-3. CircGSPT1is downregulated in gastric cancer, and act like a tumor suppressor.	826	left	6	(152)
17	circMAPK14	175	MS	Binds to MKK6	MAPK14 (Mitogen-Activated Protein Kinase 14) is a serine/threonine kinase activated by environmental stress or cytokines. It generates circMAPK14 through backsplicing of exons 10–4. circMAPK14 is downregulated in colorectal colon cancer, which reduces proliferation. Both linear and circular MAPK14 bind to *MAP2K6 (aka* MKK6, Mitogen-Activated Protein Kinase Kinase 6) and circMAPK14 antagonizes the linear MAPK14 MAP2K6 interaction, leading to a change in a transcriptional program.	506	left	6	(153)
18	circPLCE1	411	MS, RP	Influenced NFKb through ubiquitination	PLCE1 (Phospholipase C Epsilon 1) hydrolyzes phosphatidylinositol-4,5-bisphosphate generating inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). Backsplicing of its exon 2 generates circPLCE1 that is downregulated in colorectal carcinoma. CircPLCE1 promotes cancer cell proliferation and metastasis. By sequestering HSP90alpha in the HSP90alpha/RPS3 complex, leading to RPS3 ubiquitination and degradation. This pathway is disturbed in cancer cells due to the reduced circPLCE1 expression, which ultimately leads to NF-κB activation in the nucleus and a tumor program.	1570	no	1	(154)
19	circCTNNB1	370	MS, RP	Promotes migration and proliferation of cancer cells.	CTNNB1 (Catenin Beta 1) is part of adherents junctions that regulate cell–cell interactions and forms circCTNNB1 by backsplicing of exons 7–2. circCTNNB1 is upregulated in liver cancer and non-small lung cell carcinoma tissue and competes with the full length CTNNB1 for phosphorylation by GSK3. As the phosphorylation leads to degradation of the protein, circCTNNB1 'protects' full length CTNNB1 from degradation, allowing the full-length protein to activate the Wnt/beta catenin pathway.	1068	no	5	(155,156)
20	circARHGAP35	1289	MS, RP, PS	Encoded protein promotes cancer cell progression	ARHGAP35 (Rho GTPase Activating Protein 35) is a cytosolic GTPase activating protein. It creates circARHGAP35 by backsplicing exons 2–3, The circRNA is upregulated in hepatocellular carcinoma and promotes proliferation and metastasis. In contrast to the cytosolic linear protein, the circular protein is nuclear and still contains FF domains, necessary for binding to TFII-I, which likely starts a cancerogenic expression program.	4014	left	2	(46)
21	circEGFR	RC	MS, RP	Prevents receptor endocytosis	EGFR (Epidermal Growth Factor Receptor) is a transmembrane protein acting as protein kinase upon acidification. Backsplicing of exons 15–14 creates circEGFR encode an infinite open reading frame (iORF). The circprotein remains associated with the cell membrane and prevents endocytosis and inactivation of the receptor. The circRNA is upregulated in glioblastoma and expression levels correlate with survival.	249	both	2	(123)

Table 2. Continued

#	Name	nb_aa	Identification	Function	Summary function	CircRNA length [n]	Alu elements	Number of exons	References
22	CircAPP	175	MS, RP, Brain tissue	circAPP was identified in sporadic AD	APP (Amyloid Beta Precursor Protein) is a cell surface receptor that is cleaved by secretases into a number of peptides, some of them form protein aggregates involved in Alzheimer's disease. The gene creates a circRNA by backsplicing exons 17–14.	524	both	4	(157)
23	circFNDC3B	218	MS	Inhibits proliferation of cancer cells	FNDC3B (Fibronectin Type III Domain Containing 3B) is a single pass membrane protein present in the endoplasmic reticulum and the plasma membrane. It generates circFNDC3B through backsplicing from exons 6–5. circFNDC3B expression is downregulated in colorectal cancer cells. circFNDC3B inhibits the proliferation of colorectal cancer cells, possibly by inhibiting the expression of SNAIL, a transcriptional regulator that inhibits FBP1 (Fructose-Bisphosphatase 1) expression.	526	left	2	(158)
24	circRTN4 (mouse)	RC	MS, RP	To be determined	Mouse Reticulon 4 (RTN4) generates a circRTN4 by backsplicing exons 3–2.	2457	both	2	(159)
25	circNLGN (mouse)	173	AS, MS	Promoter activation	Neuroligin 1 is a neuronal cell surface protein. It generates circNlgn through circularization of its first coding exon that includes the signal peptide. The encoded protein has nine circRNA specific amino acids at its C-terminus that interact with Lamin B1, leading to the nuclear localization of the circRNA protein where it activates promoters of ING4 and C8orf44-SGK3 genes that impacts on cardiac fibroblast proliferation. Transgenic mice with circNlgn-173 have a heart phenotype.	813	both	1	(160)
26	circAXIN1	295	AS, MS	Highly expressed and associated with lymph node metastasis in gastric cancer	AXIN1 (Axis Inhibition Protein 1) encodes a protein phosphatase. Circularization of exon 2 generates circAXIN1 that contains the start codon of the linear protein but has a unique C-terminus consisting of two amino acids TD. CircAXIN1 binds competitively with APC, i.e. removes linear AXIN1. This releases beta catenin from a larger cytosolic complex. Beta catenin translocate into the nucleus and activates the wnt pathway, promoting gastric cell cancer growth.	959	right	1	(161)
27	circEIF6	224	PS, MS, AS.	proliferation, and invasion of triple negative breast cancer cells	eIF6 (Eukaryotic Translation Initiation Factor 6) prevents the association of the 40S and 60S rRNA subunits during translation. It also known as Integrin beta 4 binding protein (ITGB4BP) and is part of hemidesmosomes that link the basal membrane to the cytoskeleton. Backsplicing from exons 7–3 generates circEIF6 promotes proliferation, and invasion of triple negative breast cancer cells. It binds to MYH9, which prevents MYH9 ubiquitination and degradation, leading to an activation of the Wnt/beta-catenin pathway.	906	right	5	(162)
28	circHER2 (circERBB2)	103	PS, MS, AS	Promotes heterodimerization between EGFR and HER3 receptor	HER2 (Erb-B2 Receptor Tyrosine Kinase 2) is part of the epidermal growth factor receptor family. Through heterodimerization it enhances ligand binding and activation of other receptor kinases. Through backsplicing from exons 7–3, it creates circHER2, that is 100% identical to the linear one. CircHER2 expression correlates with poor survival in breast cancer.	750	no	5	(47)
29	circMAPT 12 to7	RC	RP	Tau aggregation	MAPT (microtuble associated protein tau) generates circTau RNA through backsplicing of exons 12–7. The encoded protein promotes aggregation of linear tau protein in reporter cells.	752	both	5	(76)
30	circMAPT 12 to 10	RC	RP,MS	Tau aggregation	MAPT (microtuble associated protein tau) generates circTau RNA through backsplicing of exons 10–7. This circTau RNA lacks a start codon, but is translated after undergoing A > I editing, likely creating a AUI start codon.	288	both	3	(76)
31	circMAN2A1-400	186	RP	circMAN2A1 expressoin correlated with AD	circMAN2A1 RNA expression correlates with Alzheimer's disease progression. The encoded protein lacks a catalytic domain.	400	both	2	(118)
32	circSDHAF2	146	RP	Tumor growth	SDHAF2 (Succinate Dehydrogenase Complex Assembly Factor 2) is necessary for the flavination of a succinate dehydrogenase complex subunit A. SDHAF2 creates circSDHAF2 by backsplicing exons 3–2. Overexpression of circSDHAF2 promotes tumor growth.	334	both	2	(114)
33	circSLC8A1	605	WB	Ca^{++} exchanger	SLC8A1 (previously called NCX1) generates circSLC8A1 through backsplicing of exon 2. The protein lacks the hydrophobic domain of the linear protein but can still transport Ca^{++} across a membrane.	1832	no	1	(63)
34	circFGFR1	328	MS, AS	Dominant negative to FGFR1 receptor, suppresses cell proliferation	FGFR1 (Fibroblast growth factor receptor 1) creates a circRNA of 1093 nt by backsplicing exons 7–2. The circFGFR1 protein contains the signal peptide, but lacks the receptor kinase domain and acts in a dominant negative way to suppress cell proliferating under heat shock conditions.	1093	both	7	(104)

Summary of validated proteins encoded by circRNAs. Alu elements indicate the presence of Alu elements in introns directly flanking the first and last exon on the circRNA, left: Alu element present in upstream (left) intron; right: Alu element present in the downstream (right) intron; both: Alu element present in both introns flanking the circRNA. For iORFs generated by rolling circle, the length of one round is indicated. RC, rolling circle; aa_nb, number of amino acids; validation: MS, mass-spectrometry; AS, antisera; PS, polysome, RP, reporter; AD, Alzheimer's disease.

of peptides from the whole proteome does not necessarily apply to a small subset of backsplice-junction-encoded peptides from circRNAs (77).

Mechanisms of circRNA translation

Cap-dependent translation initiation of mRNAs

Cellular circRNAs were initially considered non-protein-coding RNAs, because they lack a 5′ 7-methyl guanosine (7mG) cap or known ribosomal entry sites necessary for translational initiation. Thus, circRNAs cannot be translated similar to the vast majority of mRNAs in a cap-dependent manner. For mRNAs, the 40S ribosomal subunit binds to a ternary complex of eukaryotic initiation factors eIF2 bound to Met-tRNA(i) and GTP, which binds to the 40S ribosomal subunit with the help of eIF1, eIF1A, eIF3 and eIF5, generating the 43S ribosomal pre-initiation complex. This 43S complex is recruited to the mRNA via the eIF4F complex composed of eIF4E, eIF4G and eIF4A and interacts with the cytosolic poly A binding protein (PABPC) (89). The 43S preinitiation complex then scans the mRNA for the AUG start codon. Thus, cap-dependent translation occurs on a mRNA structure that is closed through protein interactions bringing the 7mG cap in proximity to the poly adenosine tail (90,91) (Figure 2A). Similarly, translation of circRNAs occurs on a circular structure.

Cap-independent translation initiation of mRNAs

Under cellular stress conditions, caused by hypoxia, nutrient starvation, oxidative or endoplasmic reticulum stress, mRNAs can be translated without a 7mG cap (91). Cap-independent translation has been first discovered in viruses, where an IRES is present (92). The IRES forms tertiary RNA structures that interact with the 40S rRNA allowing to recruit the 43S pre-initiation complex (reviewed in 93). IRESes act through various mechanisms, including direct rRNA-IRES contacts and *trans*-acting factors mediated contacts. After their discovery in viruses, IRESes have been identified in many cellular mRNAs (93) and cap-independent translation using IRES sequences is potentially used by up to 15% of human mRNAs (94,95).

A second mechanism of cap-independent translation is methylation of adenosine on the N6 position, called m6A. M6A modifications are one of the most common RNA modifications likely present in all classes of RNAs (reviewed in 96). The m6A methylation is catalyzed by METTL3 (methyltransferase like 3), which needs cofactors for positioning that is provided by METTL14 for circRNAs. M6A modification are reversible and can be removed by fat mass and obesity associated protein (FTO) and AlkB homolog 5 (ALKBH5). M6A modifications are recognized by the YTH domain [yeast two hybrid, after YTH521-B, now YTHDC1 (97)]. M6A modifications influence RNA stability, tertiary structure, subcellular localization and pre-mRNA processing. M6A modification promotes circRNA formation, likely mediated by its nuclear reader YTHDC1 (88). YTHDF1 promotes cap-dependent translation of m6A modified mRNAs by interacting with eIF3 (98), which can make mRNA translation independent of the 7mG cap under cellular stress conditions (99) (Figure 2B). Thus, using various mechanisms, numerous mRNAs undergo cap-independent translation that usually occurs under cellular stress conditions. These conditions include hypoxia which is frequently observed in cancer (91).

Translation of circRNAs in prokaryotes

Bacterial RNA lacks a 5′ cap and translational initiation is mediated by mRNA–ribosome interaction. This explains why circRNAs can also be translated in bacteria, which has been observed *in vitro*, using the first translated circRNAs from archaea (4) and has been suggested as a protein expression platform. In this approach circRNAs are generated as a group I intron and are translated by *Escherichia coli* if the Shine–Dalgarno Sequence and the downstream sequence element surrounding the AUG start codon are present (100). This mechanism could be physiologically important, as viroid-like circRNAs were discovered in the microbiome of the human gut (101,102).

Four major models have been proposed for cap-independent circRNA translation (103): IRES, m6A methylation, adenosine to inosine RNA editing and 40S recruitment via eIF4A3 that is deposited on exon junctions.

IRESes of circRNAs

Early proof of principle studies showed that the introduction of viral IRESes into circRNA strongly increased translation through eIF4G recruitment, leading to translational initiation (64). Screens using circGFP-reporter that took circRNA structure and complementarity to 18S rRNA into account identified over 17 000 possible circRNA IRES sequences. One of these endogenous IRESes regulated translation of the circFGFR RNA (104). A systematic screen for shorter ribosomal entry sites using GFP reporters identified 97 purine rich hexamer sequences in circRNAs that promote translation (105). The identified hexamers partially overlap with m6A sites but given the presence of adenosines these sequences could also be substrates for A > I editing. The potential m6A sites are annotated in databases (10) and computational analysis using known translated circRNAs identified short, A-U rich putative ribosomal entry sites (106). The presence of these distinct classes of IRESes indicate that circRNAs use several molecular mechanisms for translational initiation.

m6A methylation of circRNAs promotes their translation

A second mechanism of cap-independent translation of circRNAs is due to the methylation of adenosine on the N6 position, called m6A. Using an siRNA screen, it was found that the m6A reader YTHDF3 is responsible for the translation of circRNAs, but YTHDF3 knock out did not affect the translation of linear mRNAs and could thus be specific for circRNAs. YTHDF3 binds directly to eIF4G2, leading to cap-independent circRNA translation (87,88) (Figure 2C). At least 13% of circRNAs show m6A modification, indicating that m6A could contribute to the translation of numerous circRNAs. Analysis of pachytene spermatocytes showed that m6A enrichment correlated with both an increase of back-splicing and circRNA translation, resulting in the detection of 1600 translated circRNAs (73). These data demonstrate a general role of m6A modification in circRNA translation and expression levels.

Splicing-promoted translation of circRNAs through the exon junction complex

During the splicing reaction, an exon junction protein complex is deposited on the nascent mRNA. The exon junction complex is necessary for RNA export into the cytosol and

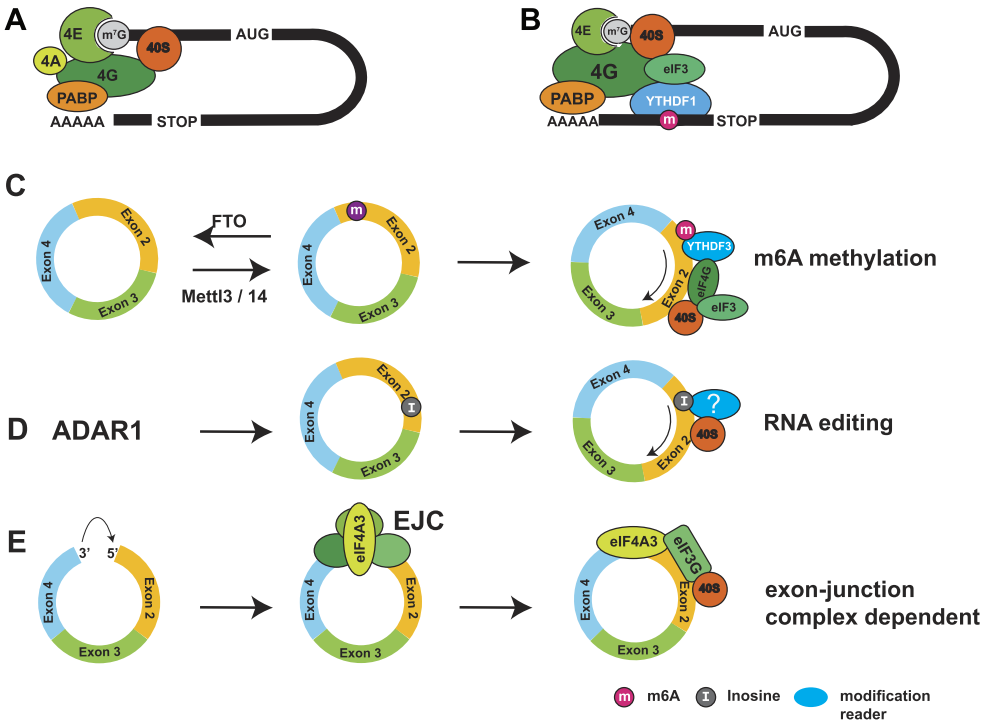

Figure 2. Translation initiation mechanisms used by mRNAs and circRNAs. (**A**) Cap-dependent translational initiation: Schematic illustration of the cap-dependent translational initiation complex. Eukaryotic initiation factors and RNA modification readers are indicated by color. (**B**) Cap-dependent translational initiation of m6A-methylated mRNA: M6A methylation promotes formation of the cap-dependent translational initiation complex by stabilizing the interaction of the 40S subunit through interaction of the m6A reader YTHDF1 with eIF3. (**C**) m6A-methylation-dependent translation of circRNAs: The complex of methyltransferases 3 and 14 catalyzes m6A methylation of adenosines. M6A binds to the YTHDF3, which binds to eIF3G that recruits the 40S rRNA subunit. (**D**) RNA editing-dependent translation of circRNAs: ADAR activity (ADAR1, ADAR2, adenosine deaminase acting on RNA) modifies adenosines to inosines that bind to an unknown reader, which binds to the 40S rRNA subunit, initiating translation at the AUG indicated (arrow). (**E**) Exon-junction-dependent translation of circRNAs: eIF4A3 is part of the exon junction complex, which binds to the border of former exons spliced together (arrow). eIF4A3 interacts with eIF3G that initiates translation. For clarity, eIF4A3 is shown only on the backsplice junction, but is likely present in all exon junctions.

is composed of eIF4A3, MAGOH, Y14/RBM8A and CASC3 (107,108).

Every circRNA generated by backsplicing contains at least one exon–exon junction on which an exon-junction complex is likely deposited. CircRNAs made from one exon contain the backsplice junction and addition of other exons to the circRNAs adds exon junctions also found in linear RNAs. A key component present in the exon junction complex is eIF4A3, which could be bound to backsplice sites or other exon-junctions present in the circRNAs. EIF4A3 likely plays a role in circRNA biogenesis as it promotes formation of some circRNAs (109–111). eIF4A3 initiates translation by recruiting eIF3 through direct interaction with its subunit eIF3G. eIF3 recruits the small 40S ribosomal subunit that scans for a start codon, which initiates translation. This mechanism has been found for luciferase-reporter genes and translation of the circCTNNB1 (beta catenin) RNA (112), circINSIG1 (113) and for the translation of succinate dehydrogenase assembly factor 2 (circSDHAF2) (114). CircISIG1 translation is likely promoted by hypoxia acting on eIF4A3. Notably, circSDHAF2 contains no known m6A-enhancers or m6A modifications. Its translation is further enhanced by the presence of introns, suggesting that translation of this circRNA mainly depends on the exon junction complex (114). Reporter gene studies indicate that generation of exon junction complexes also promote circRNA expression levels. For example, addition of internal introns within circSMN reporter constructs increased circSMN expression levels, either by recruitment of splicing factors during circRNA formation or an increase in circRNA stability (115).

Inosine-dependent translational initiation

CircRNAs form intramolecular double-stranded regions (76,116) making them substrates for ADAR enzymes (adenine deaminase acting on RNA) that recognize double-stranded RNA structures and convert adenosines into inosines (117). A screen of *trans*-acting factors found that ADAR1 and ADAR2 strongly increase translation of the circTau RNAs (76). In humans, there are three different ADAR genes: ADAR1-ADAR3. ADAR1 is expressed in all tissues with an interferon-induced cytosolic (p150) and a constitutive nuclear (p110) isoform; ADAR2 is weakly expressed in brain and the catalytic inactive ADAR3 gene is highly expressed in brain. ADAR1-p150 has the strongest effect on circRNA translation (118), possibly due to its cytosolic localization. ADAR activity results in decoding of RNAs, as an inosine is read as a guanosine and thus an AUA changed into an AUI could serve as a start codon. The decoding was shown for circTau 12→10 RNA, generated by backsplicing of exons 12–10. This 288 nt long circRNA does contain an infinite open reading frame (iORF), but lacks a start or stop codon. ADAR activity changes an AUA to AUI, which initiates translation in reporter gene assays, showing that in principle ADAR activity can change the amino acid usage of circRNAs (76) (Figure 3B). RNA-seq of reporter genes

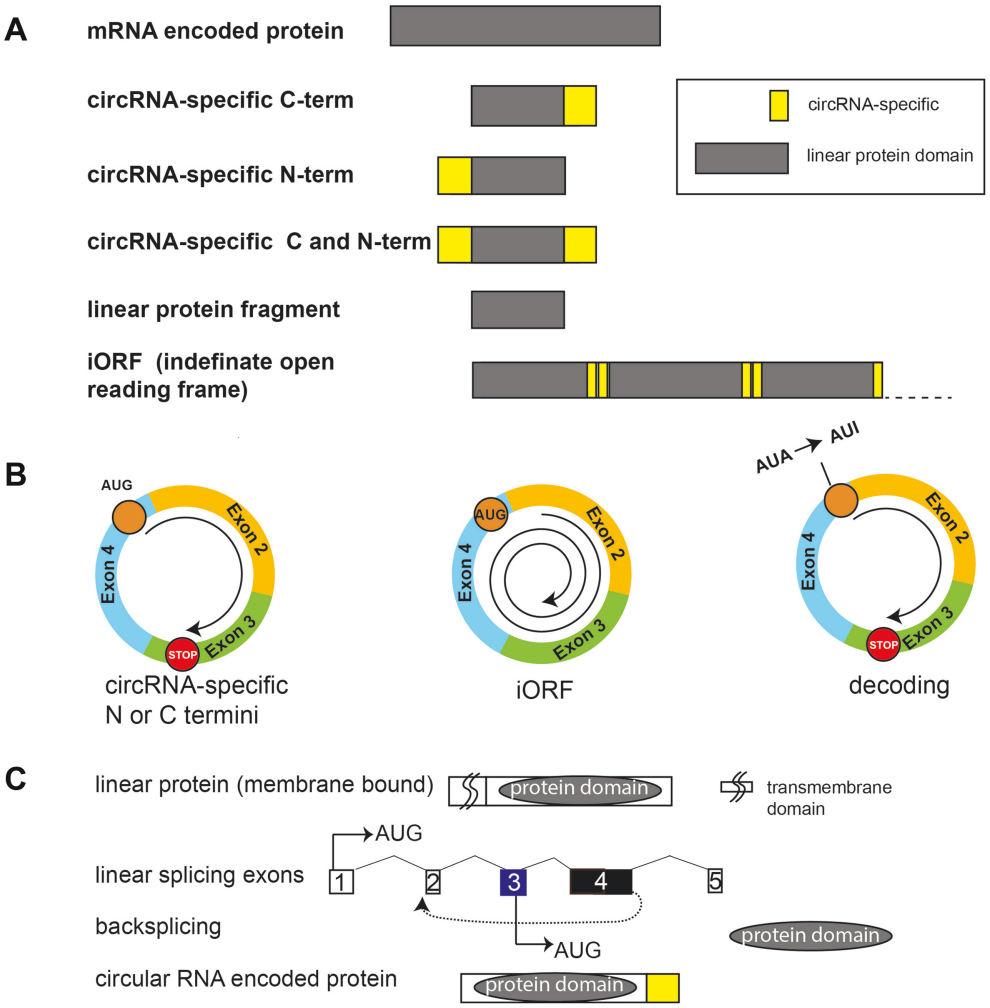

Figure 3. Protein variants generated from circRNAs. (**A**) Schematic structures of proteins made from circRNAs, shown in Table 2. Sequences are in Supplementary Table S1. (**B**) Rolling circle translation. CircRNA-encoded proteins are translated from a methionine usually, but not aways, in the same reading frame as in the linear mRNA until a stop codon is encountered. If there is a change in reading frame between exon 4 and exon 2, a circRNA-specific N- and C-terminus is generated. Adenosine to inosine RNA editing can generate novel start codons by changing a AUA (Ile) into an AUI start codon. In the absence of a stop codon, rolling circle translation generates a infinite ORF, where protein production stops after several rounds of translation via an unknown mechanism. (**C**) CircRNAs can encode proteins lacking a transmembrane domain. Most transmembrane domains are encoded by the first exon that lacks a 3′ splice site and is not included in the circRNAs. Thus, the resulting circRNA-encoded protein lacks the transmembrane domain and is likely cytosolic.

in the presence of co-transfected ADAR enzymes showed a widespread change of adenosines to inosines. In most positions <10% of a given adenosine site is changed. The editing profile of the circRNA depends on the type of Alu element in the flanking introns, suggesting that at least some of the editing occurs in the nucleus, before intron removal. Thus, Alu elements surrounding circRNAs could promote circRNA translation by influencing A > I editing. In addition, Alu elements could promote circRNA biogenesis.

Using circTau reporter genes the interaction between RNA modifications was tested. There was no strong increase in translation when m6A-dependent circRNA translation was initiated. However, when both pathways were activated, an additive effect was seen (119). The mechanism leading to inosine-dependent translation of circRNAs remains to be determined, as the *trans*-acting factors connecting circRNA translation and inosines are unknown. Several proteins, NOGO, p54nrb and vigilin/HDLBP were shown to predominantly bind to inosines (120) and vigilin/HDLBP directly interacts with the 40S rRNA subunit (121), suggesting that vigilin/HDLBP might promote translational initiation of edited circRNAs, i.e. it could act as a reader for inosines, which needs further experimental validation.

General features of proteins made from circRNAs

In their capacity to encode proteins, circRNAs are distinct from linear RNAs as they can potentially undergo rolling circle translation, where the ribosome moves around the circRNA. Due to the backsplicing mechanism, circRNAs lack the first and last exons of mRNAs that cannot provide a 3′ or 5′ splice site, respectively.

CircRNAs can increase proteome diversity through frameshifts during rolling circle translation

The potential of circRNA rolling circle translation is illustrated by a 220 nt long circRNA that represent the viroid-

like satellite RNA of the rice yellow mottle virus. Viroids are single-stranded circRNAs that infect flowering plants (116) and that potentially are also present in prokaryotes (101). The 220 nt long viroid-like satellite RNA can undergo three rounds of rolling circle translation where the reading frame shifts during each round. The three frameshifts generate three distinct proteins of 16, 18 and 23 kDa from only 220 nt genetic information (122).

The circRNA translation allows the generation of novel proteins that contain parts of the full-length linear proteins. If the number of nucleotides of a circRNA cannot be divided by three (is not an integer multiple of three) frameshifts will occur during translation, which generates proteins with a specific amino-terminus, carboxy terminus or both a specific amino and carboxy terminus (Figure 3A). If the circRNA lacks stop codons and the number of nucleotides can be divided by three, multimers of proteins can be generated using rolling circle translation of an iORF (Figure 3A and B). Translation of iORFs have been observed in transfection assays, and the number of rounds of translation can be from 1.5 (76) to >5 (123) and depends on the circRNA and cell type. In some cases, the circRNA encodes a completely new protein using a circRNA-specific reading frame (124,125).

Backsplicing removes membrane-translocation signals if they are located in the first exon

Proteins can be inserted into the membrane or enter the endoplasmic reticulum when they express a signal peptide at their amino-terminus. This 16–30 amino acid long hydrophobic sequence binds to the signal recognition particle (SRP) and enters the endoplasmic reticulum through the SRP receptor. The signal peptide is usually located in the first exon (126) that lacks a 3′ splice site and can thus not participate in backsplicing. Thus genes encoding proteins localized in the endoplasmic reticulum, Golgi or endosomes as well as membrane proteins can generate cytosolic variants using backsplicing (Figure 3C), which has been confirmed for circCadherin (127) and circINSIG1 (113). The presence of non-coding exons in the 5′ UTR are exceptions to this mechanism. For example, the first exon of the Fibroblast growth factor receptor 1 is non coding and circFGFR1 generated by exon 7 to exon 2 backsplicing contains the signal sequence, making the translated circFGFR1 protein membrane bound (104). Thus, changing the intracellular localization by removal of the transmembrane signal could be a common feature of circRNA-encoded proteins.

Currently known translated circRNAs

Experimentally validated circRNA-encoded proteins are summarized in Table 2. As the nomenclature of circRNAs is currently refined (128), we include their protein sequences in Supplementary Table S1, where circRNA-specific protein parts are indicated. Most of the circRNA-encoded proteins play a role in cancer, which reflects the current research focus and possibly the hypoxia conditions in tumors that promote circRNA translation. Reflecting the backsplicing mechanism, the circRNA-encoded proteins are smaller than mRNA-encoded proteins, with an average size of 268 aa [human mean 456 aa (129)]. 38% (13/34) of the experimentally studied proteins show a high propensity (>0.8) for phase separation predicted by MolPhase (130), indicating that they could form aggregates. The mean molecular recognition features (131) for circRNA-encoded proteins is 12.08%, compared to 1% to eukaryotic proteins, indicating that circRNA-encoded proteins have a high likelihood to interaction with other proteins and adopt a structure upon binding (Supplemental Table S2). Thus, circRNA-encoded proteins likely have different biophysical characteristics than mRNA-encoded proteins and could change cellular properties once translated.

Outlook

CircRNAs generate an astonishing number of isoforms that are less abundant, but more stable than mRNAs. The number of known circRNA isoforms far exceeds the number of mRNA isoforms suggesting that circRNAs could provide so far unknown functions to an organism, especially the nervous system, which has the highest expression of circRNAs.

Evidence reviewed here shows that one of these functions is to serve as templates for translation. CircRNAs must use cap-independent translational mechanisms, that are likely more employed when cap-dependent translation is repressed, frequently under pathophysiological conditions. Due to their cytosolic localization, presence of internal secondary structures and high stability, circRNAs can accumulate base modifications, some of which allow translation of circRNAs. Since circRNA translation is often increased under pathophysiological conditions, circRNA-encoded proteins could be novel biomarkers, as well as new therapeutic targets (132,133), including cancer vaccines (134–136).

As >170 RNA modifications are known, it is possible that other RNA modifications could lead to circRNA translation, and there is some evidence N1-methyladenosine could contribute to circRNA translation (137). These modifications could potentially change translation itself, like N1-methyl pseudouridine that promotes translational frameshifts (138). The interplay between RNA modification and translation makes circRNAs a fascinating research area to understand transcriptome and proteome diversity.

Data availability

No new data were generated analysed in support of this research.

Supplementary data

Supplementary Data are available at NAR Online.

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Conflict of interest statement

None declared.

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