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Protein coding circular RNAs in Human Diseases

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ABSTRACT

Circular RNAs (circRNAs) are a unique class of closed-loop RNA molecules formed through back-splicing of precursor mRNAs that exhibit diverse biological functions. Recent evidence highlights their emerging roles as protein-coding entities, particularly in cancer biology. This review explores the cap-independent translation mechanisms of circRNAs, focusing on internal ribosome entry sites and N6-methyladenosine modifications. We further summarize the biological roles of circRNA-encoded proteins and their relevance to human diseases. Additionally, the review addresses key challenges in circRNA research and provides perspectives on their promising potential in disease diagnosis and therapeutic applications.

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1. Introduction

Circular RNAs (circRNAs) are a unique class of closed-loop RNA molecules formed through back-splicing of precursor mRNAs, lacking 5' caps and 3' polyadenine tails, which confer enhanced stability and prolonged cellular retention [1, 2]. Initially dismissed as splicing errors, circRNAs are now recognized as key regulators of gene expression, interacting with microRNAs, proteins, and RNA polymerases to modulate transcription and signaling pathways [3-6]. Recent advancements, such as the discovery of circRNA translation into functional peptides and proteins [7-9], have expanded their biological significance, particularly in cancer, where circRNA-encoded proteins influence tumor progression and therapy response [10-12]. This review explores circRNA biogenesis, translation mechanisms, and their potential clinical applications.

The biogenesis of circRNAs involves a distinctive back-splicing mechanism of precursor mRNAs [13], producing a closed-loop structure regulated by intracellular factors and external signals. Unlike linear mRNAs, circRNAs are classified into three main types: exon circRNAs (entirely exons), circular intron RNAs (entirely introns), and exon-intron circRNAs (comprising both exons and introns) [2, 14, 15]. Inverted repeat sequences play a pivotal role in their formation by facilitating splice site proximity and enhancing splicing efficiency, often through interactions with splicing factors like SR proteins and hnRNPs [11, 16, 17]. Furthermore, the number and position of inverted repeat sequences significantly impact the abundance and stability of circRNAs and may play a role in the development of human diseases [13, 16]. Regulatory proteins such as QKI, RBM20, and CELF further modulate back-splicing under specific conditions, influencing circRNA diversity and abundance [18, 19]. This intricate regulation underscores the complexity of circRNA generation and its potential implications in health and disease.

2. Translation Mechanisms of circRNAs

CircRNAs were long considered none protein-coding due to their lack of canonical translation

features, including a 5' cap and 3' poly-A tail that are deemed essential for cap-dependent translation. They were initially misclassified as splicing errors or byproducts of mRNA processing and believed to lack open reading frames (ORFs), a deemed prerequisite for translation. However, with advancements in next-generation sequencing technology, bioinformatics, and experimental techniques, the ability of circRNAs to encode proteins via cap-independent mechanisms, such as internal ribosome entry sites (IRES) or m6A modifications, has come to light.

2.1 IRES-Mediated Translation Initiation

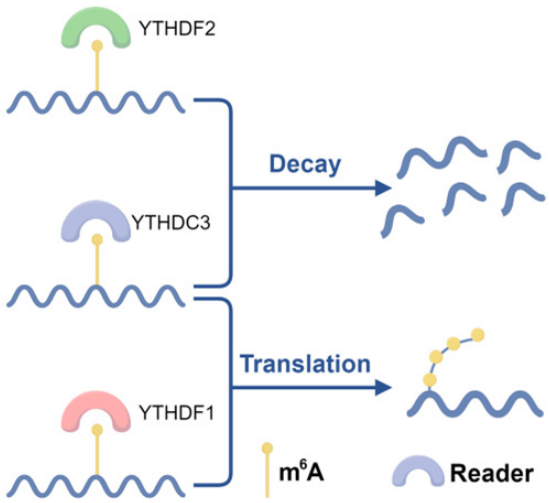
Ribosome recruitment to RNA is a crucial step for translation. In eukaryotes, while cap-dependent initiation is the primary pathway, translation can also proceed via cap-independent mechanisms involving IRESs. A systematic approach for identifying IRES elements had been proposed, which uncovers thousands of IRES-active sequences in human cells [20]. These functional sequences are broadly categorized into two types: one driven by short sequence motifs that confer localized sensitivity and the other by complex secondary structures that facilitate global sensitivity to ribosome binding [21]. For circRNAs, which inherently lack the canonical 5' cap structure, IRES-mediated initiation is the predominant mechanism for translation.

IRES elements regulate translation initiation through interactions with RNA-binding proteins (RBPs) and typically contain intricate secondary structures that serve as docking sites for ribosomes. These structures enable ribosome guidance via RNA-protein interactions, bypassing the need for the 5' cap [22, 23]. For instance, during viral infections the Sam68 protein undergoes methylation to translocate to the cytoplasm, where it enhances IRES-dependent translation [24]. Specifically, Sam68 recognizes stem-loops IV and V of the EV71 IRES and interacts with PCBP2 and PABP to increase translation efficacy [25-27]. Other RBPs such as FBP1, FBP2, G3BP1, hnRNPA1, CSDE1, and GARS, interact with IRES elements to form dynamic RNA-protein networks [28-33]. These protein-RNA networks respond to cellular stress and environmental changes to modulate translation initiation, thereby fine-tuning protein synthesis adapting to various cellular conditions.

2.2 N6-methyladenosine (m6A)-Mediated Translation

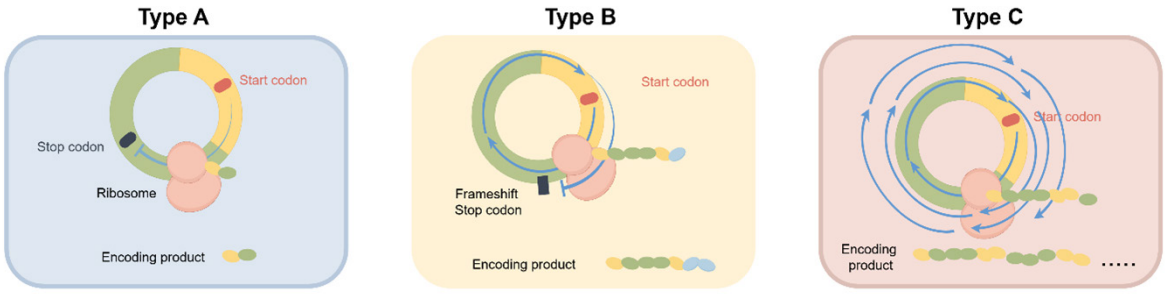
M6A is a prevalent RNA modification that plays a critical role in the regulation of gene expression,

Figure 1. Mechanisms of m6A Reader Proteins in circRNA Translation and Decay



particularly in translation [34]. This modification regulates translation efficacy by recruiting specific RBPs known as reader" proteins. Among these, YTH domain family proteins (YTHDFs) are key players. YTHDF1 enhances translation by binding to m6A-modified RNA and interacting with translation initiation factors such as eIF3[35-38]. YTHDF2 promotes mRNA degradation by directing m6A-modified mRNA to processing bodies[39, 40]. YTHDF3 cooperates with YTHDF1 and YTHDF2, sharing RNA targets to modulate both translation and decay of methylated RNA (Figure 1)[41-45]. M6A modifications present in the 5' untranslated region of mRNAs support cap-independent translation , and its levels dynamically change in response to cellular stress, causing significantly impacts to the translation of specific transcription factors [35-38]. For instance, during heat shock stress m6A modifications in the 5' untranslated region facilitate the translation of ATF4, which is a stress-responsive transcription factor to regulate a complex protein network associated with stress-induced translation [37, 46-48]. Moreover, m6A is also involved in the regulation of viral RNA replication and expression. Studies have shown that the replication of viruses, such as HIV and SV40, is positively regulated by m6A [49, 50]. Notably, m6A not only influences the translation of linear RNAs but also plays a role in the translation regulation of circRNAs [51-56]. It had been demonstrated by several studies that the translation of circRNAs, such as circMDK, is modulated by m6A modifications, thereby responsible for circRNA directed protein synthesis. In summary, these studies highlight the capability

Figure 2. Three types of protein translation mechanism mediated by circRNAs.



for m6A to mediated cap-independent translation of both mRNAs and circRNAs.

2.3 Alternative Mechanisms of circRNA Translation Initiation

Evidence indicates that circRNAs can initiate translation through splice-dependent mechanisms, notably mediated by the Exon Junction Complex (EJC)[7, 57-61]. The EJC bound to circRNAs acts as a molecular scaffold, recruiting eIF4A3, the eIF3 complex, and the 40S ribosome, thereby facilitating translation initiation. Furthermore, eIF4A3 exhibits an intrinsic ability to initiate internal translation in an eIF3-dependent manner.

Similar to mRNA, circRNAs can also initiate translation at Non-AUG Codons. For example, CircE7, identified in papillomavirus, translates efficiently despite lacking an ATG start codon. Similarly, Circ-PAPOLA, which lacks annotated AUG start codons, has been shown to support translation. These findings underscore the unique mechanisms underlying circRNA translation, expanding the current understanding of their regulatory roles and functional significance[62, 63].

2.4 Types of circRNA Translation

The translation enabled by the closed-loop architecture of circRNAs can be classified into two distinct types: conventional translation (Type A) and special translation (Types B and C), each with different outcomes depending on the presence of stop codons and nucleotide sequences. Figure 2 shows a graphic summary of these three types of circRNA translations.

2.5 Conventional Translation (Type A)

In the conventional translation of circRNAs, the ORFs follow a typical translation process similar to mRNA. When the ORFs in circRNAs[64-66]encounter a stop codon during the initial translation cycle, the translation process will terminate in a manner similar to the traditional translation process. It will result in the production of a peptide, and the translation process ceases once the stop codon is encountered. This type of translation follows the classical mRNA translation, producing a single peptide.

2.6 Special Translations

When the translation of circRNAs does not encounter a stop codon during the first cycle, special translation mechanisms become activated, which will result in the translation to proceed beyond the first cycle, leading to the possibility of two different types of special translation processes:

Non-Triplet Nucleotide Count (Type B): When the circRNA sequences do not consist of a nucleotide count that is a multiple of three, translation proceeds into the second cycle with a ribosomal frameshift. This will alter the reading frame, producing a peptide sequence that differs from the initial cycle's peptide[67, 68]. This ribosomal frameshift can continue for several cycles, usually not exceeding three, until a stop codon is eventually encountered. This mechanism introduces additional diversity of the peptide products that are not observed in proteins encoded by classically mRNA.

Multiple of Three Nucleotide Count (Type C): If the circRNA's nucleotide count is a multiple of three, translation theoretically continues indefinitely, producing the same peptide sequence repeatedly in a continuous loop. Under such conditions [69-71], translation will eventually be terminated by ribosomal programmed frameshifts and production of stop codons to pause translation.

3. Functional Diversity of circRNA-Encoded Proteins and Peptides

A thorough understanding of circRNAs in diseases, especially cancers, necessitates a functional classification. We categorize circRNAs encoding proteins based on their biological functions into tumor

Table 1.

circRNA	Cellular Localization	Initiation Mode	Length	Translation Type	Involved Diseases	Ref.
circFAM53B	Cytoplasm	IRES	219aa	Type A	Breast cancer	[64]
circDdb1	Cytoplasm	IRES	867aa	Type C	Muscle Atrophy	[69]
circSPECC1	Nucleus/ Cytoplasm	IRES	415aa	Type A	Glioblastoma	[65]
circFOXP1	Cytoplasm	IRES	231aa	Type B	Intrahepatic cholangiocarcinoma	[73]
circ-SLC9A6	Cytoplasm	m6A	126aa	Type B	Nonalcoholic fatty liver disease	[102]
circCOPA	Cytoplasm	IRES	99a	Type A	Glioblastoma	[72]
circCAPG	Cytoplasm	IRES	171aa	Type B	Breast cancer	[81]
circTmeff1	Cytoplasm	IRES	339aa	Type C	Muscle Atrophy	[70]
circMAP3K4	Cytoplasm	m6A	455aa	Type B	Hepatocarcinoma	[75]
circEIF6	Cytoplasm	IRES	224aa	Type A	Breast cancer	[82]
circGSPT1	Cytoplasm	IRES	238aa	Type A	Gastric carcinoma	[76]
circSEMA4B	Cytoplasm	IRES	211aa	Type A	Breast cancer	[83]
circATG4B	Cytoplasm	IRES	222aa	Type A	Colorectal cancer	[77]
circDIDO1	Nucleus	Unverified	529aa	Type A	Gastric carcinoma	[74]
circAXIN1	Cytoplasm	IRES	295aa	Type A	Gastric carcinoma	[103]
circE-Cad	Secreted/ Membrane	IRES	254aa	Type B	Glioblastoma	[67]
circEGFR	Secreted/ Membrane	Unverified	-	Type C	Glioblastoma	[71]
circMAPK14	Cytoplasm	IRES	175aa	Type A	Colorectal cancer	[104]
circSMO	Cytoplasm	IRES	193aa	Type A	Glioblastoma	[84]
circMAPK1	Cytoplasm	IRES	109aa	Type A	Gastric carcinoma	[105]
circPLCE1	Cytoplasm	IRES	411aa	Type A	Colorectal cancer	[66]
circCHEK1	Cytoplasm	IRES	246aa	Unverified	Myeloma	[85]
circFNDC3B	Cytoplasm	IRES	218aa	Type B	Colon cancer	[68]
circHER2	Cytoplasm	IRES	103aa	Type A	Breast cancer	[78]
circPPP1R12A	Cytoplasm	Unverified	73aa	Type A	Colon cancer	[80]
circβ-catenin	Cytoplasm	IRES	370aa	Type A	Liver cancer	[106]
circAKT3	Cytoplasm	IRES	174aa	Type A	Glioblastoma	[107]
circSHPRH	Cytoplasm	IRES	146aa	Type A	Glioblastoma	[87]
circGprc5a	Cytoplasm	Unverified	-	Unverified	Bladder cancer	[79]
circPINTexon2	Nucleus	IRES	87aa	Type A	Glioblastoma	[86]
circFBXW7	Nucleus	IRES	185	Type A	Glioblastoma	[108]

immunity, apoptosis regulation, autophagy, drug sensitivity modulation, epithelial-mesenchymal transition (EMT), tumor invasion, and specific signaling pathways. In each category, circRNA-encoded products have been demonstrated to exert key cellular activities that can be potentially utilized as biomarkers and therapeutic targets as summarized in Table 1.

3.1 Tumor Immunity

CircFAM53B was demonstrated to enhance anti-tumor immunity through encoding peptides that bind to MHC I and MHC II molecules, as well as T cell surface receptors [64]. It enables the encoded peptides to be presented to dendritic cells, leading to the activation of CD4+ and CD8+ T cells into effector T cells to exert effective cytotoxicity against tumor cells expressing these peptides. CircFAM53B-based vaccines have demonstrated strong tumor-suppressive effects in animal models, positioning circFAM53B as a promising candidate for cancer immunotherapy and the development of novel anti-tumor vaccines.

3.2 Regulation of Apoptosis

Protein coding circRNAs were reported to be pro-apoptotic to suppress tumor progression. CircCOPA encodes a protein that destabilizes the NONO-SFPQ complex, resulting in defects of RNA splicing and DNA repair in cancer cells. The peptide renders glioblastoma cells more vulnerable to chemotherapeutic agent such as temozolomide (TMZ) [72]. Similarly, circFOXP1 enhances ferroptosis in intrahepatic cholangiocarcinoma cells by modulating iron metabolism, leading to increased reactive oxygen species (ROS) accumulation and subsequent cell death [73]. CircDIDO1 encoding a protein DIDO1-529aa that can destabilize the antioxidant, PRDX2, promoting apoptosis in gastric cancer cells exposed to oxidative stress [74]. In contrast, other circRNAs were demonstrated to be anti-apoptotic to facilitate tumor cell survival and promoting tumor progression. For example, circMAP3K4 modulated by m6A encodes a peptide that inhibits the Bax/Bcl-2 ratio that led to reduced mitochondrial outer membrane permeability and suppression of apoptotic signaling in hepatocellular carcinoma [75]. Thus, circMAP3K4 helps tumor cells evade cell death mechanisms and survive under adverse conditions, contributing to liver cancer progression. The distinct roles of these circRNAs in apoptosis regulation provide important insights into the complex mechanisms of tumor progression and offer new directions for cancer treatment strategies targeting apoptosis. By further investigations on circRNAs, novel anticancer therapies may be developed to utilize these encoded proteins to regulate apoptosis to effectively inhibit tumor growth.

3.3 Regulation of Autophagy

Autophagy is a cellular degradation and recycling mechanism that plays dual pro- and anti-tumor roles in cancers. CircRNAs can regulate autophagy by encoding proteins, exemplified by circGSPT1, which encodes GSPT1-238aa to damage gastric cancer cells by inhibiting autophagy [76]. This protein reduces autophagy-related proteins such as LC3-II, prevents autophagosome maturation by binding to vimentin and blocks the fusion with lysosomes. In contrast, circATG4B encodes a protein that activates autophagic pathways by increasing LC3-II and Beclin-1 levels to enhance tumor cell resistance to chemotherapy in colorectal cancers [77]. Similarly, circCOPA in glioblastoma destabilizes the NONO-SFPQ complex, increasing the cancer cells' sensitivity to TMZ by compromising their DNA repair mechanisms [72]. These findings highlight that circRNAs play complex roles in cancer progression by either inhibiting or promoting autophagy.

3.4 Modulating Drug Sensitivity

In addition to circCOPA encoded protein that sensitizes therapy-induced DNA damage and circATG4B encoded protein that activates autophagy, the protein encoded by circSPECC1, SPECC1-415aa, enhances glioblastoma sensitivity to TMZ through inhibiting MGMT and DNA repair machinery [65]. Furthermore, HER2-103, encoded by circHER2, promotes tumor malignancy by sustaining AKT signaling through EGFR-HER3 dimerization [78]. HER2-103 shares high similarity with the CR1 domain of HER2, allowing pertuzumab to bind to HER2-103 and block its activity. These findings provide new avenues for developing more effective anticancer strategies and contribute significantly to precision medicine in cancer treatment.

3.5 Regulation of EMT and Tumor Invasion

CircRNA-encoded proteins were reported to regulate EMT, a critical process in which tumor cells acquire migratory and invasive properties, enabling them to metastasize. For instance, circFNDC3B encodes a protein that reduces Snail levels, inhibiting its transcriptional activity and suppressing EMT in colon cancer cells [68]. CircGprc5a encodes a pep-

tide that activates the ERK/MAPK and PI3K/AKT signaling pathways, enhancing bladder cancer cell invasion and metastasis [79]. Similarly, circPPP1R12A promotes YAP nuclear translocation, driving invasion and metastasis in colon cancer [80]. CAPG-171aa activates pathways like PI3K/AKT and Focal Adhesion, enhancing tumor growth and invasion in triple-negative breast cancer [81]. These findings emphasize the key roles of circRNAs in regulating EMT and tumor metastasis, providing insights into cancer progression and potential therapeutic targets.

3.6 Regulation of Specific Signaling Pathways

CircRNA encoded proteins also regulate several signaling pathways such as Wnt, AKT, Hippo-YAP, and Hedgehog, to influence cancer cell proliferation and invasion. CircEIF6 encodes EIF6-224aa to stabilize MYH9 and activate the Wnt/β-catenin signaling, which in turn promoting triple-negative breast tumors [82]. CircMAPK1 inhibits MAPK signaling to suppress gastric cancer cell growth [83], while circSEMA4B encodes SEMA4B-211aa to reduce AKT activation and limit breast cancer proliferation. In glioblastoma, circSMO enhances Smoothened activity in the Hedgehog pathway, driving tumor progression [84]. CircAKT3 inhibits the AKT pathway to reduce glioblastoma cell proliferation, and circPPP1R12A activates the Hippo-YAP pathway, promoting colon cancer metastasis [80]. Additionally, circPLCE1 regulates NF-kB to suppress colorectal cancer progression, and circGprc5a enhances bladder cancer metastasis by activating ERK/MAPK and PI3K/AKT pathways[79]. CircCAPG and circE-Cad promote tumor growth and invasion in triple-negative breast cancer and glioblastoma by activating multiple signaling pathways [67, 81]. Together, these finding offer new insights into cancer biology and potential targets for therapy.

3.7 Other Functions

Two other functions of circRNA encoding proteins are ferroptosis and chromosomal stability. CircFOXP1 promotes ferroptosis by enhancing iron accumulation and lipid peroxidation, increasing tumor sensitivity to oxidative stress, and reducing cholangiocarcinoma recurrence [73]. CircCHEK1 encodes CHEK1-246aa, which induces chromosomal instability by disrupting chromosome segregation, accelerating multiple myeloma progression [85]. CircLINC encodes a peptide that suppresses oncogenic transcriptional elongation in glioblastoma by interacting with RNA polymerase II, inhibiting tumor cell proliferation and invasion [86]. Additionally, circSHPRH encodes a protein that enhances DNA repair, reducing glioma formation by promoting homologous recombination and non-homologous end joining [87]. These circRNAs reveal the complexity of cancer biology and highlight their potential for therapeutic targeting.

4. Current Challenges and Future Perspectives

The investigation on circRNA-encoded proteins faces challenges due to their high diversity, tissue specificity, and complex roles in biological processes [1, 88]. Traditional CRISPR methods struggle with targeting circRNAs due to overlap with their linear mRNA counterparts, complicating functional validation [89]. Despite these hurdles, circRNA-encoded proteins hold significant promise for cancer therapy, offering new avenues for targeted treatments by influencing cellular signaling, autophagy, and metastasis [90]. Integrating advanced tools like long-read sequencing, high-resolution imaging, and CRISPR-Cas13 will help uncover the precise roles of these proteins in diseases [91]. Their stability and specificity make circRNAs potential targets for therapeutic interventions by using antisense oligonucleotides, small-molecule inhibitors, and cancer vaccines [92]. Moreover, the roles of circRNA-encoded proteins in non-cancer diseases like neurodegenerative and cardiovascular conditions warrant further exploration. These studies will facilitate the translation of circRNA research into clinical applications, advancing both diagnostic and therapeutic strategies [93].

Circular RNAs (circRNAs) are emerging as a promising platform for next-generation RNA vaccines due to their stability, ease of storage, low immunogenicity, and safety profile, as they do not integrate into the host genome. In 2022, circRNA vaccines made significant strides in COVID-19 prevention. Synthetic SARS-CoV-2 circRNA vaccines encoding the spike protein binding domain activated antigen-presenting cells, eliciting a strong immune response [94-96]. A circRNA vaccine designed with Zika virus envelope domain III (EDIII) and IgG1 Fc successfully triggered immune responses in mice, offering protection against Zika infection[97]. Beyond viral applications, circRNA vaccines have shown promise in cancer immunotherapy. A synthetic circRNA vaccine encoding chicken ovalbumin (OVA) demonstrated strong immune activation in melanoma [98-100]. Addition-

ally, in vitro-amplified endogenous circRNAs can induce potent anti-tumor responses. For example, circFAM53B-encoded peptides activate both CD4+ and CD8+ T cells, enhancing anti-tumor immunity[64]. Furthermore, circRNA vaccines derived from immunogenic peptides encoded by lncRNA-H19 have proven effective against gliomas [101]. Despite these advances, circRNA vaccine research is still in early stages, with challenges remaining in improving circRNA cyclization efficiency, antigen yield, vaccine purity, and delivery methods.

5. Conclusion

CircRNA-encoded proteins play crucial roles in cancer and other diseases by influencing key biological processes such as cellular signaling, autophagy, and apoptosis. Unlike traditional non-coding RNAs, circRNAs encoded proteins to impact cancer initiation, progression, and treatment response. As research progresses, circRNA-encoded proteins are emerging as potential therapeutic targets for diseases like cancer. Advancements in experimental techniques, gene editing, and bioinformatics will enhance our understanding of their mechanisms, offering new insights for diagnosis and treatment.

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