

# CIRCULAR RNA: AN EMERGING LANDSCAPE IN RNA BIOLOGY AND BEYOND

## Abstract

Circular RNAs (circRNAs) have recently emerged as a fascinating and abundant class of non-coding RNAs, capturing the attention of researchers in the field of biotechnology. These covalently closed RNA molecules were once considered as by-products of splicing errors. However, advances in high-throughput sequencing and bioinformatics have revealed their pervasive presence across various species and tissues. Our chapter intends to summarise the main implication of circRNAs in their biogenesis and degradation mechanisms, their role in altering gene expression, translation into several proteins, as biomarkers for human diseases, their therapeutic potential, and their application in stem cell biology and various computational approaches and tools for their analysis. This chapter aims to encourage the research of circular RNA in the molecular biology field.

**Keywords:** Circular RNA, Biogenesis, Biomarker, RNA therapeutics, mi-RNA sponge.

## Authors

### **Dalini D**

Fish Genetics and Biotechnology Division

ICAR-CIFE

Mumbai, India.

[dalinidavid99@gmail.com](mailto:dalinidavid99@gmail.com)

### **Subashini V**

Fish Genetics and Biotechnology Division

ICAR-CIFE

Mumbai, India.

## I. INTRODUCTION

Circular RNAs are the emerging class of RNA transcripts with numerous applications in various fields such as medicine, agriculture and other allied sectors. They are distinguished from mRNA and other long non-coding RNA molecules by the absence of free 5' and 3' ends and polyadenylated tails. They have a covalently closed loop structure arising from back splicing. They are the component of non-coding RNA molecules; still, some are translated into proteins. They play a key role in regulating gene expression at the post-transcriptional level by acting as miRNA and protein decoys. Diener [1], in 1969 discovered the first RNA molecule with a circular structure in potatoes affected with spindle tuber disease. The single stranded covalently closed circRNA molecules were found in viroids of hepatitis delta virus in 1976 [2]. Later circular transcription of the SRY gene in the testis of mice, circular RNA working as competitive endogenous RNA and miRNA sponge were discovered [3]. Circular RNA is still a research hotspot due to their multiple cellular processes. The simpler circular RNA found in potato spindle tuber is single-stranded RNA about 250-400 nucleotides. In contrast it is about 1.7 kb in case of hepatitis delta virus, a human pathogen. Circular RNAs are more stable and highly resistant to exonuclease degradation due to the absence of free 5' and 3' ends. Circular RNAs have a high half-life of about 48h, whereas mRNA is only about 10h. Exons and introns subjected to circularization are three times longer than normal exons and introns, suggesting that length plays a significant role in deciding which exon and intron to circularize [4]. Compared to the mRNA of its host gene, circular RNA has a higher tissue selectivity, suggesting that it has high analytical specificity, robustness, reproducibility and repeatability. They exhibit different temporal and spatial expression patterns, and due to their stable circular shape, many of them are evolutionarily conserved [5]. Circular RNA conservation in mice and humans is 92.6%, 3.9%, 2.3%, 1%, and 0.1% in the brain, heart, liver, skin, and lung, respectively [6]. Circular RNA's highly conserved nature has made them a biomarker for a variety of human disorders, including cancer. Because of their localization at synapses, the high expression of over 10,000 circular RNA in the brain implies that they have a role in healthy brain function, particularly learning and memory [7]. It has been discovered that the timely expression of circular RNA in muscle plays an important function in mammalian growth control and may act as a growth marker. The onset and progression of many diseases alter the homeostatic expression of circular RNA. Understanding the mechanism of circular RNA will lead to the development of novel diagnostic and prognostic tools, as well as targeted therapeutics for a variety of disorders.

## II. BIOGENESIS OF CIRCULAR RNA

The mechanism of formation of various types of circular RNA is highly diverse. pre-mRNA, pre-tRNA, and pre-rRNA are the primary sources for forming circular RNA [8]. Lariat formation and direct back splicing are the two main mechanisms through which pre-mRNA can be spliced into circular RNA [9]. Lariat-derived circRNA formation can be achieved through exon skipping and direct circularisation of the intronic lariat. A hetero-lariat structure formation is there in case of exon skipping resulting in the formation of exon-intronic circular RNA (EircRNA), which sometimes continue with the removal of all introns and leads to the origination of exonic circular RNA (ecircRNA) [10]. Direct circularisation of intronic lariat is by pre-mRNA cleavage at the 5' end with the help of small nuclear RNA (U1snRNA) and ligated between 5' guanine and 2' adenosine. The formed intronic circular RNA is retained inside the nucleus [11]. Back splicing can generate all three

types of circular RNA; exonic circular RNA, exon intronic circular RNA, and intronic circular RNA [12]. Back splicing follows RNA binding protein-driven and intron-pairing-driven circularisation. Two flanking introns can be bridged through RNA binding proteins, which form a circularised structure and a linear product [13]. The direct base pairing of Alu elements happens in intron-pairing driven circularisation, which facilitates the ligation of the 5' donor and 3' splice acceptor site and forms a circularised structure and a linear product. The introns are removed or retained to create an ecircRNA or an EIciRNA (exon-intron circRNA) [14]. A circRNA called tRNA-driven intronic circular RNA [15] and rRNA-derived circular RNA [16], follows the same process which is reported in bacteria and to some extent in eukaryotes [17]. A detailed explanation of biogenesis is given in Figure 1.

### III. REGULATORY MECHANISMS OF CIRCULAR RNA

Circular RNAs can regulate various cell functions due to their high stability and prolonged half-life. Different regulatory mechanisms of circular RNAs are listed below.

- 1. Circular RNA- Gene Expression Regulators:** CircRNAs can control the expression of parental genes in various ways, including microRNA (miRNA) sponges, mRNA traps and control of transcription and splicing. Circular RNAs are primarily formed in the nucleus and exported to the cytoplasm by various exportin proteins such as UAP56 and URH49, but the export mechanism is still unclear. Nevertheless, several circular RNAs remain in the nucleus and regulate the pol II-mediated transcription. They usually regulate the transcription at initiation, elongation and post-transcription all levels. The pre-initiation complex formed in the promoter of eukaryotic transcript is further stabilised by various transcription factors, which stimulate the transcription rate. To find whether other factors or non-coding RNAs are involved in promoting transcription, Li et al. in 2015 [18] performed an immune precipitation assay using RNA polymerase II specific antibody, where they found about 111 circular RNAs are involved in promoting pol II transcription. Of these 111 circular RNAs, 15 of them are EIcircRNA. Fluorescent *In situ* Hybridization (FISH) proved that two among them are circEIF3J and circPAIP2, which are exclusively localised in the nucleus. Knockdown of these two resulted in decreased transcripts produced, proving that binding circular RNA in the initiation complex promotes the transcription initiation level. It is almost believed that introns are an unused part of transcription, but it has been found that several intron-derived circular RNAs are involved in regulating transcription elongation [19]. ci-ankrd52 is formed from the second intron of the ankrd52 gene, which is engaged in promoting transcription elongation, synthetic antisense oligonucleotides, which is complementary to the ci-ankrd52 which, when used, will reduce the number of mRNA produced, which proves that intronic circular RNAs are engaged in transcription elongation [20]. The primary function of circular RNA found to date is its ability to act as a mi-RNA sponge. Almost about 18,000 microRNA have been found to date. These mi-RNAs will down regulate the gene expression by binding to the mRNA. Circular RNA promotes the expression of mRNA by binding to those inhibitory mi-RNAs. ciRS- 7, generated from the antisense transcript of CDR1as, is the first reported circRNA. It acts as a mi-RNA sponge and negatively regulates miR-7 by expressing in brain tissue, neuroblastoma and astrocytoma. More than 60 binding sites for miR-7 are found in CDR1as, and its resistance to miRNA-mediated degradation makes it the perfect ceRNA for miR-7 [21]. Apart from CDR1as, several other circular RNA such as cir-SRY, cir-ITCH, circ-HIPK3, circ-PVT1, circRNA-CER, circRNA-MYLK etc. are

involved in miRNA sponging function [22]. The circRNA may act as an mRNA trap by securing the start site, producing a non coding linear transcript, and reducing the expression level of the beneficial protein when the host gene's translation start site is present. Duchenne muscular dystrophy (DMD) sufferers have also been discovered to have the mRNA trap phenomena. According to a study by Gualandi *et al.* 2003 [23], in people with specific deletion mutations, greater dystrophin EcircRNA production may result in inactive dystrophin transcripts and lower quantities of functional proteins [24].

2. **Circular RNA in Protein Translation:** Circular RNA, which falls under the long non-coding RNA, sometimes gets translated into proteins. The presence of open reading frames, internal ribosome entry sites that acts as RNA scaffold interacting with translational machinery, and N6-methyladenosine modifications that interact with YTH domain family protein 3 (YTHD3) and recruit eIF4G2 to start the translation indicate that the circular RNA undergoes cap-independent translation [25] [26]. Proteins produced from circRNA are involved in myoblast proliferation (circZNF609 - 250 AA) [27], inhibit transcription elongation (circPINTexon2 in glioblastoma cells – 87aa) [28], inhibits ubiquitination of  $\beta$ -catenin and activates Wnt/ $\beta$ -catenin signalling (circ $\beta$ -catenin - 370 AA) [29].
3. **Circular RNA – Protein Interactions:** Even though most circRNAs lack numerous protein-binding sites, many circRNAs can function well as protein sponges. Circ-Amotl1 binds to PDK1 and AKT1 through binding sites to mediate wound healing [30]. CircRNAs operate as protein decoys, collaborating with the target protein in the appropriate cellular site to modify its normal physiological action. Circ-Amotl1, for example, may bind and retain c-Myc in the nucleus, where it stabilises c-Myc and up-regulates its target genes, resulting in greater cell proliferation and reduced apoptosis [31]. CircRNAs can also function as protein scaffolds, enabling two or more proteins to interact and co-localize [30]. CircRNAs may also recruit certain proteins to specific cellular sites, leading to gene expression. e.g. FECR1, a circRNA, recruits and controls the host gene FLI1's promoter region via recruiting Ten-Eleven Translocation methylcytosine dioxygenase 1 (TET1) [32]. To examine circRNA-protein interactions, RNA pull-down experiments or RNA immune precipitation (RIP) are utilised
4. **Circular RNA as Biomarkers for Human Disease:** Due to its high stability and vast utility, Circular RNA can be used as biomarkers for various diseases, particularly cancer. Circular RNA is the primary key player in almost all types of cancer as a biomarker. Apart from cancer, it can be used as a diagnostic biomarker in other pathologies such as hypertension, cardiac diseases, diabetes, brain disorders etc. also it can be used as a prognostic biomarker for several ageing and age-related disorders. In addition to these disorders, circular RNAs are involved in several gynaecological and reproduction-related disorders regulating follicular maturity. Table 1 discusses the circRNAs that serve as biomarkers in various diseases and their targets.

#### IV. DEGRADATION PATHWAYS OF CIRCULAR RNA

Due to the absence of free 5' and 3' ends, endo-nucleolytic cleavage is the only possible way of circular RNA degradation. RNase L, which is circRNA endonuclease involved in globally degrading circular RNA [51]. Kim et al.'s [52] discovery that HRSP12,

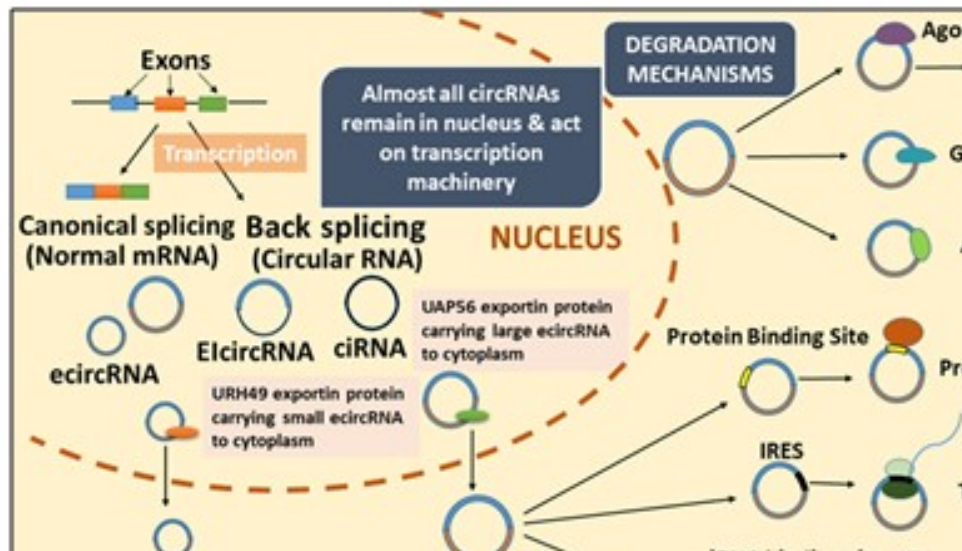
which RNase P/MRP particularly downregulates, is required for the relationship between a subset of circRNAs containing m6A and YTHDF2 is supported by this. Finding that m6A can mediate both circRNAs and mRNA degradation appears to have raised awareness of this circRNA degradation mechanism among researchers [53]. In several situations, binding of mi-RNA to circular RNA leads to

**Table 1: Circular RNAs as Disease Biomarkers and its Respective Targets.**

Disease	circRNA	Target	Effect on the Disease	References
Ovarian cancer	circ_0072995 (Upregulated)	miR-147a	Promotes cell proliferation and invasion and suppresses apoptosis	[34]
	circ_MTO1 (Downregulated)	miR-10a	Suppresses cell proliferation and promotes apoptosis	
Gastric cancer	circ_0000936 (Upregulated)	miR-582-3p/HUR/VEGF	Promotes cell proliferation, migration, invasion and angiogenesis	[35]
Breast cancer	circRNA_002178 (Upregulated)	miR-328-3p/COL1A1	Promotes cell proliferation, invasion, angiogenesis, and inflammatory response and decreases overall survival.	[36]
	circ_0000515 (Upregulated)	miR-269-5p/CXCL10		
Hepatocellular carcinoma	circRNA-100338 (Upregulated)	MMP9/MVD/ VE-Cadherin /ZO-1	Promotes invasion, metastasis and angiogenesis	[37]
Renal Cell Cancer	circPRRC2A (Upregulated)	TRPM-3	Promotes angiogenesis and metastasis	[38]
Bladder cancer	circSEMA5A (Upregulated)	miR-330-5p/ ENO1/ SEMA5A	Promotes cell proliferation, suppresses apoptosis, facilitates migration, accelerated invasion, enhanced angiogenesis, and promotes glycolysis	[39]
Prostate Cancer	circRNA_001587 (Downregulated)	miR-223/SLC4A4	Inhibits migration, invasion and angiogenesis	[40]
Osteosarcoma	circTADA2A (Upregulated)	miR-203a-3p/ CREB3	Promotes metastasis and inhibits apoptosis	[41]
Hypertension	Hsa_circ_0005870 (Upregulated)	hsa-miR-6807-3p, hsa-miR-5095, hsa-miR-1273g-3p, hsa-miR-5096, hsa-miR-619-5p	-	[42]
Cardiovascular disease	circHRCR (Downregulated)	miR-223	Inhibits cardiac hypertrophy and heart failure	[43]

	circHECTD1 (Upregulated)	miR-142- TIPAR	Astrocyte activation and cerebral infarction in stroke	[44]
	circMFACR (Upregulated)	miR-MTP18	Cardio myocyte death in myocardial infarction	[45]
	CircFOXO3 (Upregulated)	ID-1, E2F1, FAK, HIF1 $\alpha$ (Transcription factors)	Promotes cardiac senescence by arresting ID- 1, E2F1, FAK, and HIF1 $\alpha$ in the cytoplasm	[46]
	circANRIL (Downregulated)	PES1	Controls ribosome biogenesis in atherosclerosis	[47]
Alzheimer's disease	ciRS-7 (Downregulated)	miR-7- UBE2A	Modulates the $\alpha$ -synuclein aggregation pattern	[48]
Parkinson's disease	ciRS-7 (Upregulated)	miR-7-PD		
Diabetic retinopathy	circ_0005015 (Upregulated)	miR-519d-3p	Facilitates retinal endothelial angiogenic function	[49]
	circHIPK3 (Upregulated)	miR-30a	Regulates retinal endothelial cell function and vascular dysfunction	
Osteoporosis	circRUNX2 and circ_0002060 (Downregulated)	miR-203	Promotes the expression of osteogenic differentiation- related proteins	[50]

endonucleolytic cleavage of circular RNA and finally subjects to complete degradation. When miR-671 binds to CDR1as, the endonuclease Argonaute-2 (Ago2) directly cleaves the RNA, producing a 5' and 3' free end. The broken-down RNA is destroyed by various exonucleases on both ends [54]. Another study found that the depletion of GW-182 in DL1/S2 cells in *Drosophila* increased the amount of circular RNA transcripts, proving that the gawky 182 protein is responsible for circular RNA degradation [55]. Further research confirmed that RNA binding proteins UPF-1 and G3BP1 are involved in the structure-mediated degradation of circular RNAs. Similar to the gawky-mediated degradation, the depletion of UPF-1 and G3BP1 promotes the upregulation of circular RNA, evidencing that those RBPs are responsible for the degradation of circular RNAs [56].



**Figure 1:** Biogenesis and Degradation Mechanisms of Circular RNA. IRES: Internal Ribosome Entry Site; Ecircrna: Exonic Circular RNA; Eicircrna: Exon-Intron Circular RNA; Cirna: Circular Intronic RNA.

## V. CIRCARNAS IN DEVELOPMENT AND PHYSIOLOGY

Circular RNAs are expressed in several organs following a spatio-temporal pattern, implying their potential bio functions [57]. CircRNAs have been associated in the development of mammalian tissues such as brain development [58], osteogenic differentiation [59], skeletal muscle development [60], and haematopoiesis [61]. CircRNAs promote myogenesis in skeletal muscle by functioning as miRNA sponges that hinder myoblast differentiation. Circ-zfp-609, for example, stimulates myogenesis by sponging miR-194-5p and upregulating BCLAF1 [62]. CircRNAs, specifically circBIRC6, promote pluripotency in human embryonic stem cells by serving as a miRNA (miR-34a and miR-145) sponge, alleviating the inhibition of NANOG, OCT4 and SOX2 [63]. Circular RNAs are important in the osteogenic differentiation of Bone Marrow Stem Cells (BMSCs), collagen production, and FGF or TGF- signalling pathways for chondrogenic differentiation [64].

## VI. THERAPEUTIC POTENTIAL OF CIRCARNAS

CircRNA dysregulation impacts cancer cell proliferation, migration, angiogenesis, apoptosis, or therapy resistance, and so regulates the progression of numerous cancer types. Targeted circRNA therapy has less off-target effects than microRNAs and siRNAs, which have considerable off-target effects due to their short length and half-life [65]. Due to their closed-loop structure, protein-coding capabilities, and greater stability compared to their linear mRNA counterparts, circular RNAs provide a novel, viable technique for delivering vaccines and therapeutics. Artificial circular RNA (circRNA) sponges (circmiRs) have therapeutic miRNA antagonistic potential [66]. The successful development of COVID-19 mRNA vaccines has prompted researchers to investigate the use of circRNAs in the development of higher-stability nucleic acid-based COVID-19 vaccines. CircRNA vaccines at low doses may elicit strong immune responses [67]. Researchers have also attempted to

leverage circRNA stability to guide the generation of chimeric antigen receptor (CAR) T cells by introducing CAR-expressing circRNA into T cells to avoid the time-consuming approach of producing CAR-T cells via T cell separation followed by engineering [68]. The use of nanoparticles as a delivery vehicle has considerably increased the *in vivo* efficiency of circRNA-based therapies [69].

## VII. CIRCULAR RNA AND RNA EDITING

Circular RNAs are cleaved *in vivo* by delivering siRNA/shRNA lipid-based polymers to their back-splice junction. This RNAi-mediated knockdown of circular RNA has several drawbacks, including fast nuclease degradation, limited intracellular transport efficiency, lack of cell-specificity, immunological response, and other off-target effects [70]. Conditional circRNA knockout/knockdown uses a cre-dependent short hairpin RNA (shRNA) to activate circRNA cleavage in certain cells *in vivo*, which is then processed into short interfering RNA (siRNA) [68]. Using endogenous or exogenous ligands, a siRNA delivery method mediated by lipid nanoparticles (LNPs) targets circRNAs in the cytoplasm of particular cells. Because nanoparticles cannot penetrate the nucleus, this method can only target circRNAs in the cytoplasm [69]. Using lentiviral and adenoviral circRNA expression plasmids, circRNA vectors are overexpressed *in vivo* [71]. CRISPR/Cas9-mediated circular RNA knockout removes the intronic complementary region flanking the circularised exon, which is necessary for circRNA synthesis. This method has also been used to knock off and knockdown genes, respectively, targeting the entire gene locus and a transcription factor. Because of the complexity of circRNA synthesis, determining the intron to be targeted is difficult. This barrier can be overcome by CRISPR/Cas13-mediated circRNA knockdown, which targets circRNA back-splice junctions rather than introns to induce circRNA cleavage with fewer off-target consequences [72].

## VIII. COMPUTATIONAL APPROACHES FOR CIRCULAR RNA ANALYSIS

With the advent of RNA sequencing, researchers can now profile the transcriptome for circRNAs. Several bioinformatics methods have been critical in determining the functional importance of circular RNA utilising RNA sequencing data or even transcript sequence characteristics. PolyA selection, polyA depletion, rRNA depletion, RNase R treatment, RNase R treatment, followed by polyadenylation and polyA (+) RNA depletion are some of the procedures used to prepare circRNA libraries. CircRNA identification methods are classified into three types according to their implementation: back splicing junction (BSJ)-based, integration-based, and machine learning-based. BSJ's circRNA identification techniques are further characterised as split-alignment-based (Segmented based) and pseudo-reference-based (Candidate based). Segmented-based techniques, which are considered *de novo* tools, identify BSJ by aligning reads to the reference genome, whereas pseudo-reference tools depend on gene annotation files to identify junction reads [73]. Some tools follow integration pipelines for identifying circular RNA with high reliability. Machine-learning algorithms for circRNA identification leverage standard circRNA properties such as ALU repetitions, structural motifs, and sequence motifs to develop reliable classification models. Some circRNA identification tools also allow you to measure circRNA expression levels.

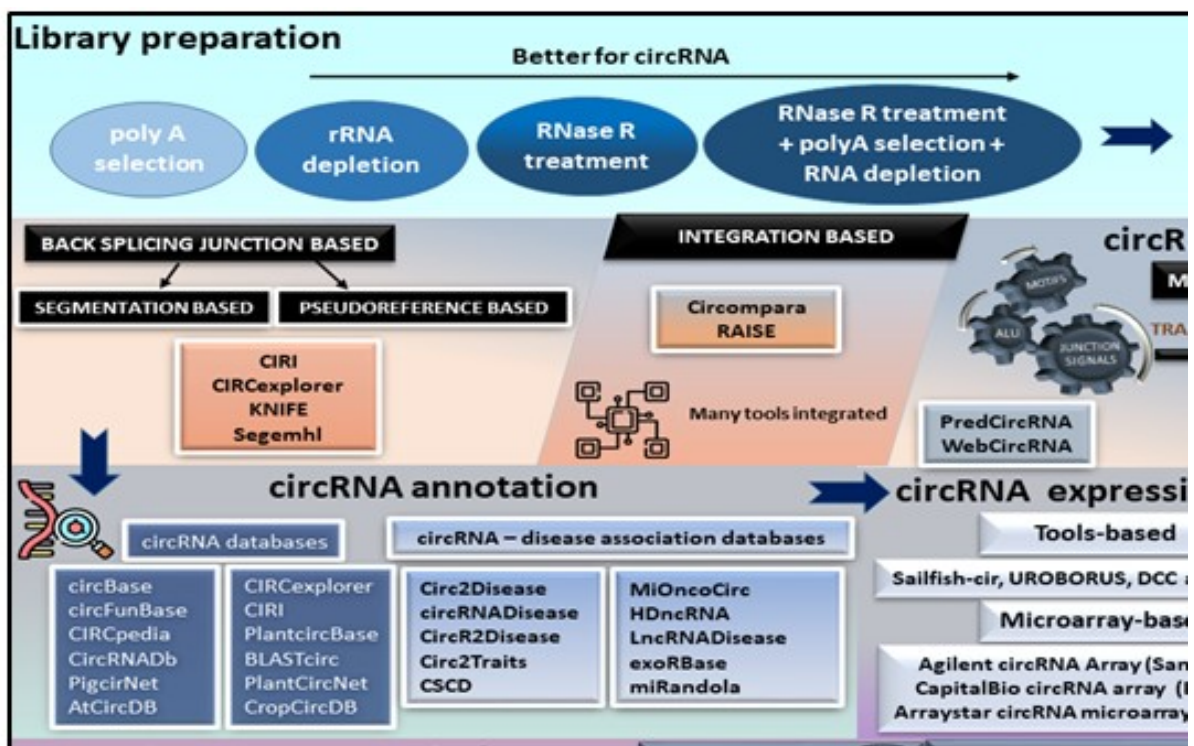
Many circRNA databases mine the literature for putative circRNAs using various identification techniques and particular NGS datasets. Certain databases gather all ncRNA



information that includes circRNA, circRNAs linked to illnesses, their miRNA interactions, RNA binding proteins, and Transcription factors (TFs). Various techniques for downstream circRNA analysis can identify alternative splicing, circRNA assembly, primer design, structure prediction, and visualisation. Figure 2 depicts the computational tools for circRNA and their applications in a bioinformatics process. Despite the existence of multiple extensively managed circRNA databases, there is a lack of common nomenclature for circRNA annotation, which results in non-universal IDs being used throughout different databases. This is a huge barrier in circRNA research.

## IX. CONCLUSION

Researchers were already beginning to uncover various functional roles of circRNAs, including their involvement in gene regulation, RNA-binding protein interactions, and potential roles in disease development. As circular RNA (circRNA) research advances, several challenges must be addressed. Accurate detection and quantification of circRNAs can be challenging due to their low abundance compared to linear RNAs, which require more tools that are reliable. Determining the specific functions of circRNAs remains a complex task. Comprehensive studies of circRNA across different cell types and tissues remain unexplored as they exhibit tissue-specific expression patterns.



**Figure 2:** Bioinformatics Pipeline for Circrna Identification and Analysis with Thelist of Computational Tools Used in each Step

In the future, we require a deeper understanding of the specific functions of circRNAs and how they contribute to cellular processes. Identifying and linking circRNAs to specific diseases could open new avenues for diagnostics, targeted therapies and biomarker development for prognostics and disease monitoring. CircRNAs' tissue-specific expression

patterns and disease associations could contribute to personalised medicine approaches, enabling tailored treatments based on individual patient profiles. Integration of circRNA data with omics data is necessary to provide a holistic view of cellular processes and biological mechanisms. Considering the stability and cytoplasmic localisation of circRNAs, engineered circRNAs could be used for various molecular tools or therapies. As circRNA research matures, it will likely intersect with other areas of biology, genomics, and medicine, leading to exciting discoveries and applications. However, researchers must address the challenges associated with circRNA research to fully unlock their potential and shed light on their roles in health and disease.

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