

# Epigenetic modifications in acute myeloid leukemia: The emerging role of circular RNAs (Review)

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**Abstract.** Canonical epigenetic modifications, which include histone modification, chromatin remodeling and DNA methylation, play key roles in numerous cellular processes. Epigenetics underlies how cells that possess DNA with similar sequences develop into different cell types with different functions in an organism. Earlier epigenetic research has primarily been focused at the chromatin level. However, the number of studies on epigenetic modifications of RNA, such as N<sup>1</sup>-methyladenosine, 2'-O-ribosemethylation, inosine, 5-methylcytidine, N<sup>6</sup>-methyladenosine (m<sup>6</sup>A) and pseudouridine, has seen an increase. Circular RNAs (circRNAs), a type of RNA species that lacks a 5' cap or 3' poly(A) tail, are abundantly expressed in acute myeloid leukemia (AML) and may regulate disease progression. circRNAs possess various functions, including microRNA sponging, gene transcription regulation and RNA-binding protein interaction. Furthermore, circRNAs are m<sup>6</sup>A methylated in other types of cancer, such as colorectal and hypopharyngeal squamous cell cancers. Therefore, the critical roles of circRNA epigenetic modifications, particularly m<sup>6</sup>A, and their possible involvement in AML are discussed in the present review. Epigenetic modification of circRNAs may become a diagnostic and therapeutic target for AML in the future.

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## 1. Introduction

Acute myeloid leukemia (AML) is the most common type of adult leukemia, with a wide range of biological and clinical characteristics (1). A total of 19,520 new cases of AML were reported in the US in 2018 (2), and 14,100 cases were reported in China in 2015, according to survey data (3). Genetic and epigenetic abnormalities have been identified to play key roles in the pathogenesis of AML (4,5).

Epigenomics, which refers to the epigenetic changes that modify the expression of a genotype into a particular phenotype without any alteration of the genetic material, play key roles in mammalian growth and maturation (6). Canonical epigenetics research had previously focused on the modifications and variations of DNA in chromatin, whereas epigenetic modifications of RNA, particularly those involving non-coding RNAs, have been attracting increasing attention recently. With the advancement of RNA deep sequencing technologies and bioinformatics approaches, circular (circ)RNAs have become increasingly significant among RNA species. Distinct from linear RNAs, circRNAs have loop structures that are covalently closed and lack 5' caps and 3' poly(A) tails due to back-splicing (7). Due to their stability (8), evolutionary conservatism (9) and abundance (10), circRNAs act as microRNA (miRNA/miR) sponges (4,11), RNA splicing factors (12) and parental gene expression modulators (13). In addition, circRNAs have been detected to serve as biomarkers for a wide range of diseases, including gastric and hepatocellular cancers (14). Furthermore, studies have shown that circRNAs are N<sup>6</sup>-methyladenosine (m<sup>6</sup>A) methylated (15,16), and methyltransferase-like (METTL)3/14 promotes their translation, whereas fat mass and obesity-associated (FTO) gene inhibits their translation (15). Both circRNAs and m<sup>6</sup>A participate in RNA processing, and both are associated with

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AML. Therefore, the aim of the present review is to report the role of canonical epigenetic effects in AML, summarize the progress of RNA epigenetics and circRNAs, and propose a possible link between AML and circRNA epigenetic modifications.

## 2. AML and canonical epigenetics

Epigenetic modifications are associated with numerous important biological processes and serve key roles in the development of an organism. Through epigenetic modifications, cells that bear a similar genome can differentiate into various cell types with different functions (17). The treatment of hematological malignancies, including AML, is challenging. Hence, studies on the association between AML and epigenetics may contribute to elucidating the pathogenesis of this disease. The conventional epigenetic processes include histone modification, chromatin remodeling and DNA methylation. In this section, the role of these epigenetic processes in AML pathogenesis is examined.

**AML and DNA methylation.** DNA methylation plays a key role in mammalian development (18). As a covalent alteration of genomic DNA, DNA methylation participates in gene expression modification and is involved in the transmission and perpetuation of epigenetic information via DNA replication and cell division (19). Two such functions that are linked to DNA methylation are regulation of genomic stability and gene expression control from the promoter region or another regulatory region containing CpG-rich regions, known as CpG islands (CGI) (20–22). Several studies involving knockout mouse models of DNA methylation enzymes have demonstrated the importance of DNA methylation in hematopoiesis. Hematopoietic stem cell (HSC) self-renewal, homing and apoptosis suppression have all been shown to require the maintenance of DNA-methyltransferase (DNMT)1 (18,23). Furthermore, DNMT1 plays a role in myeloid/lymphoid lineage commitment regulation (23), and multiple studies found that myeloid-specific loci were hypermethylated in lymphoid progenitors (24–26), substantiating this hypothesis. Conditional knockout HSC models confirmed that *de novo* DNMT3A and DNMT3B served a role in hematopoiesis (27).

Genetic and epigenetic changes are involved in the pathogenesis of AML (28,29), and aberrant DNA methylation patterns have been identified in various types of cancer (30). It was previously reported that dysregulation of DNA methylation is linked to hematological malignancies, suggesting that different subtypes of AML have different DNA methylation profiles (31). Furthermore, promyelocytic leukemia protein-retinoic acid receptor  $\alpha$  (PML-RAR $\alpha$ ) was shown to require DNMT3A to function as an oncogenic transcription factor in acute promyelocytic leukemia initiation, and DNMT3A DNA methyltransferase activity was confirmed to be essential for the enhanced self-renewal of PML-RAR $\alpha$ -transformed hematopoietic progenitors (32). Previously, DNMT3A mutations have been identified in ~20% of AML cases and are associated with poor clinical outcomes, including shorter overall survival (OS) and/or disease-free survival (33–35). Furthermore, Hájková *et al* (22) reported a possible association between DNA methylation and DNMT3A

mutations in patients with AML. DNA methylation levels were significantly lower in patients with mutated DNMT3A, and higher DNA methylation levels were associated with a lower incidence of relapse. The study indicated that patients with lower levels of DNA methylation had a worse OS compared to those with higher DNA methylation levels at multiple loci. Another previous study involving an analysis assay revealed a distinct significant hypomethylation profile in patients with AML with 11q23 abnormalities (31). Moreover, mixed lineage leukemia (MLL)-AF9 overexpression in human hematopoietic stem and progenitor cells (HSPCs) leads to a DNA methylation signature that was found to be similar to that of patients with MLL-AF9 AML (36), suggesting that the leukemic transformation could be due to a possible link between the MLL fusion protein and aberrant DNA methylation. Interestingly, patients with AML harboring various cytogenetic or genetic alterations have also been shown to possess distinct global patterns of DNA methylation, and PML-RAR $\alpha$  and AML1-eight-twenty one (ETO) exhibit highly distinct profiles of methylation (31,33,37). As a result, DNA methylation may be considered as an additional parameter in stratifying patients with AML.

**AML and TET2 mutations.** Another important group of epigenetic regulators involved in hematopoietic development is the ten-eleven translocation (TET) protein family. TET1 is commonly expressed in embryonic stem cells, whereas TET2 and TET3 are found in most adult tissues (38). TET2 is the most commonly expressed of the three TET family members in the hematopoietic lineage, and it is frequently mutated in hematological malignancies. Tet2 knockout mice developed splenomegaly, monocytosis and extramedullary hematopoiesis as a result of bone marrow defects with enlargement of the HSC compartment (39). HSCs with Tet2 deletion exhibited increased self-renewal capacity, allowing them to outcompete wild-type counterparts and predominate in the transplanted mice's peripheral blood (40). Furthermore, Tet2<sup>-/-</sup>-HSCs showed a transcriptional program similar to that of common myeloid progenitors, but with enhanced expression of self-renewal regulators Meis1 and Evi1, and decreased expression of myeloid-specific factors Cebpa, Mpo and Csf1 (40). These findings suggested that TET2 is vital for HSC self-renewal and differentiation into the myeloid lineage (39,40).

TET2 is commonly found to be aberrantly expressed in AML, myelodysplastic syndromes/myeloproliferative neoplasms and chronic myelomonocytic leukemia (41,42). Approximately 17% of patients with AML have loss-of-function mutations of TET2 (43). TET2 mutations can predispose HSCs to a pre-leukemic state, in which they retain the ability to differentiate to a wide range of mature blood cells. However, after acquiring additional genetic lesions, these pre-leukemic stem cells may transform into leukemia-initiating cells (44,45). This suggests that while TET2 mutations can promote leukemic transformation, they are insufficient for completing the process. TET2 mutations frequently co-occur with other mutations in KRAS, CCAAT enhancer-binding protein  $\alpha$ , AML1, nucleophosmin 1, FMS-like tyrosine kinase 3 (FLT3) and Janus kinase 2 in AML (46), suggesting that TET2 inactivation works in tandem with these other mutations to drive leukemogenesis. The findings that the synergistic action of

TET2 depletion and FLT3-internal tandem duplication (ITD) mutation dysregulates DNA methylation and interferes with normal hematopoietic cell differentiation, leading to HSPC and granulocyte-monocyte progenitor accumulation (47), further substantiates this hypothesis. Several hypermethylated regions of TET2 and FLT3-ITD mutations are located at gene regulatory elements, triggering the deregulation of self-renewal and differentiation genes (Gata1, Gata2, inhibitor of differentiation 1, myeloproliferative leukemia virus 1 and suppressor of cytokine signaling 2) (47). Furthermore, knocking out TET2 in pre-leukemic cells with AML1-ETO yielded genome-wide DNA hypermethylation, affecting ~25% of enhancer elements (48). As several hypermethylated enhancers are linked to tumor suppressor genes, this suggests that TET2 mutations play a role in leukemia development through an epigenetic mechanism.

**AML and histone modification.** The structural unit of chromatin is a nucleosome consisting of one H1, two H2A and H2B dimers, and one H3/H4 tetramer (49). Histone modification, which is a set of covalent post-translational modifications of histone proteins and modifications that commonly involve acetylation, methylation, phosphorylation, sumoylation, ubiquitination and ADP-ribosylation (49), has been shown to play a role in stem cell differentiation (50). For example, class I and II histone deacetylases (HDACs) that contain the two catalytic domains, function as the mammalian regulators of histone acetylation (50,51).

DNA methylation and histone modification are significant epigenetic mechanisms for gene expression. DNA hypermethylation in the promoter CGIs of tumor suppressor genes that trigger transcriptional silencing is considered to be essential in carcinogenesis (52-54). Histone proteins are assembled into nucleosomes that act as both transcriptional regulators and DNA packaging units. The histone amino-terminal tails protrude from the nucleosome and are subject to chemical modifications, such as acetylation, phosphorylation and methylation (55). Modifications to the post-translational histone tail, added or removed by histone-modifying proteins (HMPs), serve to control access to the underlying DNA and alter gene expression by affecting the structure of chromatin. It has been shown that altered HMP activity contributes to leukemogenesis in AML via gene transcription regulation and, since modifications of post-translational histones are reversible, they may be considered as possible therapeutic targets (56). In addition, removal of the H3K4 methyl group via lysine-specific histone demethylase 1A resulted in decreased expression of the tumor suppressor gene. Similarly, the aberrant recruitment of HDACs to promoters of hematopoietic genes was found in AML (56).

**AML and chromatin remodeling.** Chromatin remodeling is the chromatin architectural modification that controls transcription through nucleosome displacement and rearrangement. The chromatin remodeling mechanism is powered by ATP (57), and chromatin remodeling complexes comprise four main classes as follows: Imitation SWI, switch/sucrose non-fermentable, INO80 complex ATPase subunit and chromodomain-helicase-DNA-binding protein Mi-2 homolog (Mi2/CHDD) (58,59). Chromatin remodeling is fundamental

to transcription. Redner *et al* (60) outlined models of the normal control of chromatin remodeling during gene-specific transcription, and concluded that disruption of these mechanisms may lead to transcriptional disorders and leukemic transformation. They further suggested that chromatin therapy may emerge as a potential antileukemic strategy in the future. In addition, chromatin remodeler inhibition was reported to reduce the development of AML and sensitize AML cells to genotoxic drugs through increased DNA accessibility and impaired double-strand break repair (61).

The chromodomain-helicase-DNA-binding protein 4 (CHD4), an ATP-dependent chromatin remodeling factor, is part of the nucleosome remodeling and histone deacetylation nucleosome remodeling deacetylase complex and plays an important role in the regulation of epigenetic transcriptional genes (62). CHD4 has been associated with oncogenic processes, including cell cycle progression regulation (63-65), cancer metastasis, epithelial-to-mesenchymal transition, and epigenetic repression of tumor suppressor genes (66). Heshmati *et al* (67) indicated that CHD4 is important for the proliferation of different types of leukemic cells and AML development *in vivo*, but not for normal primary hematopoietic cell proliferation and survival. It was also confirmed that CHD4 was previously shown to be important for the proliferation of a broad range of cancer cells (67), as well as the capacity of AML cells to form colonies (61), suggesting that CHD4 may represent a cancer-specific dependency in a wider tumor repertoire. In another study, the activity of chromodomain-helicase DNA-binding protein-7 (CHD7), an ATP-dependent chromatin remodeling factor, was found to interact with the AML1/CBF $\beta$ -SMMHC complex and altering the expression of its target genes. Chd7 deficiency in Chd7<sup>f/f</sup>Mx1-CreCbf $\beta$ +56M mice expressing the Cbf $\beta$ -MYH11 fusion gene delayed Cbf $\beta$ -MYH11-induced leukemia in both primary and transplanted mice (68).

One mechanism via which miRNA dysregulation causes AML is epigenetic alterations by altered expression of transcription factors or oncogenic fusion proteins. Of note, the expression of AML1-ETO causes heterochromatic silencing of genomic regions that produce miR-223 by recruiting chromatin remodeling enzymes at the (Runt-related transcription factor 1) RUNX1-binding site of the pre-miR-223 gene (69). Furthermore, AML1-ETO induces heterochromatic silencing at the RUNX1-binding sites of miR-193a by recruiting chromatin remodeling enzymes and expanding the oncogenic function of the fusion protein (70). Taken together, these data demonstrated that chromatin remodeling may be crucial for leukemogenesis, including AML, and may influence its pathogenesis to a certain extent.

### 3. RNA modification

Epigenomics involves stable and inheritable gene expression variations without changes to the sequence of DNA (71). However, epigenetic changes occur in DNA as well as in RNA, termed the epitranscriptome; >100 forms of RNA modifications are involved in the epitranscriptome (72), and previous studies have identified RNA modifications mostly in transfer (t)RNAs, ribosomal (r)RNAs and small nuclear (sn)RNAs, whereas they are relatively infrequent in mRNAs (72,73).

However, technological advancements have been made in the last few years, increasing our ability to recognize alterations to the mRNA, and recent cellular transcriptome studies have focused attention on epitranscription (74). Numerous studies indicate that these modifications significantly enhance the role of RNA in promoting genetic diversity (71-73), and the common RNA modifications consist of N<sup>1</sup>-methyladenosine, pseudouridine, 5-methylcytosine (m<sup>5</sup>C), 7-methylguanosine, m<sup>6</sup>A and 2'-O-ribosemethylation (72,75). The most common types of RNA epigenetic modifications are summarized in this review.

#### 4. m<sup>6</sup>A modification

One of the most common mRNA modifications identified in all eukaryotes is the m<sup>6</sup>A modification, which is the methylation of position N6 of adenosine (76). To detect this alteration, earlier studies used mass spectrometry and showed that the relative content of m<sup>6</sup>A ranged from 0.1 to 0.4%, representing the modification of 3-5 sites in each mRNA (73,76). The m<sup>6</sup>A modification, which is decoded by m<sup>6</sup>A methyltransferase post-transcriptionally, is an abundant internal modification in eukaryotic mRNA (77) and often occurs in the RRACH (R=G or A; H=A, C or U) consensus sequence (78). The m<sup>6</sup>A-specific MeRIP-Seq method was previously used to detect and analyze the position of m<sup>6</sup>A, which was found to be localized predominantly in the 3' untranslated regions (UTRs) of mRNAs, long internal exons and stop codons (79). The distribution of m<sup>6</sup>A in tissue-specific sites was also analyzed, and this modification was found to be abundant in the heart, brain and kidney (79).

Another study used an m<sup>6</sup>A-Seq method and detected that the sites modified by m<sup>6</sup>A are highly conserved in humans and mice (80). To increase the resolution of m<sup>6</sup>A detection, researchers have developed antibody-based crosslinking methods (76-79). The terms 'writer', 'eraser' and 'readers' are used to accurately characterize the m<sup>6</sup>A activity, and these terms are commonly used for other types of modifications as well. METTL3, METTL14 and the regulatory subunit Wilms tumor 1 associated protein (WTAP) constitute the m<sup>6</sup>A methyltransferase (81-83). METTL14 exerts its enzymatic activity by interacting with METTL3 to methylate the conserved GGACU and GGAUU sequences (84). Although it does not have methyltransferase activity due to the lack of a catalytic center, by interacting with METTL3 and METTL14, WTAP may locate the methyltransferase complex into nuclear speckles (85). METTL3 knockdown was shown to induce alterations in splicing patterns and alternative polyadenylation that affected RNA stability, transcriptional silencing and translation (86-91). A study previously detected another mechanism of m<sup>6</sup>A modification: METTL16, a long unknown U6 small nucleolar (sn)RNA methyltransferase capable of controlling S-adenosylmethionine levels that affect m<sup>6</sup>A levels in most cells by controlling human MAT2A expression (92).

The identification of m<sup>6</sup>A demethylating enzymes, known as 'erasers', focused on the FTO (93) and AlkB homolog 5, RNA demethylase (ALKBH5) proteins, belonging to the Fe(II) and 2-oxoglutarate-dependent oxygenase superfamily (94,95), and they oxidize m<sup>6</sup>A via N6-hydroxymethyladenosine (hm<sup>6</sup>A) and N6-formyladenosine (f<sup>6</sup>A) intermediates (96). It was previously indicated that FTO is involved in several essential life

processes, including adipogenesis (97), the regulation of brain dopaminergic signaling (98), adipogenic regulatory factor mRNA splicing (99) and the enhancement of the transformation and leukemogenesis of leukemic oncogene-mediated cells (100). FTO and ALKBH5 are both essential for cells, and these demethylating enzymes also influence mRNA processing, nuclear export and metabolism in HeLa cells (94). Furthermore, it was previously reported that the development of cancer stem cells is driven by ALKBH5 and the depletion of m<sup>6</sup>A (101).

The m<sup>6</sup>A effector proteins known as 'readers' involve the YT521-B homology (YTH) family that encodes in mammals five proteins, namely the YTH domain family (YTHDF) proteins 1, 2 and 3, and the YTH domain-containing (YTHDC) proteins 1 and 2 (99,101). To date, four of these proteins have been shown *in vitro* and *in vivo* to display m<sup>6</sup>A selectivity (102,103,104). YTHDF2 and YTHDC1 have an m<sup>6</sup>A-specific conserved hydrophobic binding pocket and are involved in the mechanism controlling the methylation and transcript fate of mRNA (102,104,105). Furthermore, the high-resolution mapping of transcription-binding sites has shown that YTHDF1 and YTHDF2 tend to bind to the GGACU conserved mRNA sequence motif, which shows significant overlap with m<sup>6</sup>A methylation sites (103,106,107).

During the development of an organism, N6-methyladenosine plays a critical role, and changes in m<sup>6</sup>A levels affect several life processes, including tissue development, self-renewal (96,108) and differentiation of stem cells (99). m<sup>6</sup>A can also regulate the heat shock response (91), circadian clocks (98), as well as processes related to the fate and function of RNAs, such as RNA stability, splicing, transport, localization and translation (89,90,96,102,107,108), primary processing of miRNAs (109,110) and RNA-protein interactions (80,81,111). A substantial body of research however, suggests a link between m<sup>6</sup>A and certain diseases, including AML. m<sup>6</sup>A has been associated with obesity, diabetes and cancer (112). m<sup>6</sup>A modifications may be used in combination with tumor therapy. A study analyzed The Cancer Genome Atlas (TCGA) datasets and discovered that changes in m<sup>6</sup>A regulatory genes were linked to TP53 mutations in patients with AML. Moreover, alterations in the m<sup>6</sup>A regulatory genes were found to lower the survival rates of patients with AML. Therefore, m<sup>6</sup>A regulatory genes may serve as potential new molecular targets for AML therapy (113). In addition, Su *et al* (114) reported the antitumor activity of R-2-hydroxyglutarate in patients with AML harboring an isocitrate dehydrogenase (IDH) mutation by blocking FTO to induce MYC degradation. In tissue cells with an IDH mutation, TCGA data showed high MYC and low FTO levels. Numerous studies have recently investigated the regulation of mRNA metabolism by m<sup>6</sup>A modifications, revealing m<sup>6</sup>A modification characteristics and associated regulatory mechanisms in AML (Table I) (100,115,116).

#### 5. Other RNA modifications

DNA has been the subject of the majority of studies on m<sup>5</sup>C, and m<sup>5</sup>C is not frequently found in RNA (84). Researchers have found however, that m<sup>5</sup>C is enriched in 3'-UTRs (117). 3-Methylcytidine (m<sup>3</sup>C) was first detected in *Saccharomyces cerevisiae* total RNA (118). Previous findings demonstrated

Table I. Roles of some m<sup>6</sup>A key members in AML.

First author, year	Protein	Role	Functional classification	Mechanism	(Refs.)
Vu <i>et al</i> , 2017	METTL3	Oncogene	Inhibiting differentiation along with promoting cell growth <i>in vitro</i> Inducing differentiation and apoptosis, and preventing leukemia <i>in vivo</i>	Promotes c-MYC, BCL2 and PTEN translation	(115)
Weng <i>et al</i> , 2018	METTL14	Oncogene	Inhibiting differentiation of AML. Promoting leukemia stem cell self-renewal	Regulates the stability of mRNA as well as MYB and MYC translation, and was inhibited by SPI1	(116)
Li <i>et al</i> , 2017	Fat mass and obesity-associated	Oncogene	Promotes cell transformation together with leukemogenesis, enhancing the inhibition of cell differentiation in AML	Regulates the expression of targets like ASB2 and RAR $\alpha$ by decreasing m <sup>6</sup> A levels in these mRNA transcripts	(100)

AML, acute myeloid leukemia; METTL, methyltransferase-like; SPI1, transcription factor PU.1; ASB2, Ankyrin repeat and SOCS box protein 2; RAR $\alpha$ , retinoic acid receptor  $\alpha$ ; PTEN, phosphatase and tensin homolog.

that METTL2 and METTL6 participate in m<sup>3</sup>C modifications, in particular in tRNAs, and that METTL8 only causes m<sup>3</sup>C changes in mRNA in humans and mice (119). Another study identified RNA methylation in mixtures of either RNA isomers or non-isomeric RNA types and detected modifications in RNA methylation, such as 3-methyluridine, m<sup>5</sup>C, m<sup>6</sup>A and 5-methyluridine, by top-down mass spectrometry (120). A relatively abundant form of RNA modification is also pseudouridylation, and the relative amount of pseudouridine in RNA is 0.2-0.6% (121). Two mechanisms are involved in the formation of pseudouridine: One is dependent on tRNA-pseudouridine synthase (PUS)I, whereas the other relies on a type of H/ACA box small nucleolar RNA (122,123). In rRNA, pseudouridine is mainly found in peptidyl transferase centers, decoding centers and the A-site finger region (124). This modification may therefore be involved in rRNA processing, ribosome assembly, as well as advanced structure maintenance (125). It has been shown that pseudouridine is highly conserved in snRNA (U1, U2, U3, U4, U5 and U6) in various species (126). In 2011, a study showed that, through pseudouridylation, stop codons may be transformed into sense codons (127). HSPCs are also particularly sensitive to changes in pseudouridine and protein synthesis. In this regard, silencing PUS7 causes a decrease in a specific type of tRNA-derived small fragment containing 5' terminal oligoguanine (mTOG), resulting in increased protein synthesis and severe HSPC differentiation blockage (128). Protein synthesis is disrupted in patients with myelodysplastic syndrome due to PUS7 and mTOGs dysfunction, which is characterized by a high rate of transformation to aggressive leukemia (128). The irreversible deamination of adenosine to inosine, known as A-to-I editing, is another commonly studied RNA modification. Inosine is a normal and necessary post-transcriptional modification of the RNA introduced by specific deaminases (129) and this process is catalyzed by adenosine deaminase acting on tRNA, while adenosine deaminase acting on RNA catalyzes the process in mRNAs and non-coding RNAs (130). Hematopoiesis

involves A-to-I RNA editing. During myeloid differentiation, adenosine deaminases that act on RNA (ADAR)1 and ADAR2 are modulated. ADAR1 expression was shown to be upregulated in AML and was linked to the proliferation of leukemia cells. Silencing ADAR1 promoted AML cell cycle arrest and reduced Wnt effector expression (128). The alteration in the splicing pattern of protein tyrosine phosphatase non-receptor type 6 and its association with leukemogenesis is another example of the effect of RNA editing in AML (128).

## 6. Epigenetic modifications of circRNAs

circRNAs are an abundant class of RNA species formed from the ligation of a downstream splice donor to an upstream acceptor. They have a cyclically ordered structure, and are involved in a variety of physiological and pathological processes (4,131), have structural stability, sequence conservation and tissue-specific expression. circRNAs have more recently become one of the most frequently studied RNA species. Due to the aforementioned unique characteristics, circRNAs are known to act as miRNA sponges (4,11), and they are capable of being translated into proteins through an internal ribosome entry site (IRES)-driven process (132). Furthermore, several circRNAs have been suggested to serve as potential biomarkers for several diseases, including several types of cancer (14). Although numerous biological functions of circRNAs remain unclear, there is a continuous exploration of this research field. In 2017, circRNAs were identified to be widely methylated by m<sup>6</sup>A, and this was determined by m<sup>6</sup>A immunoprecipitation of RNase R exoribonuclease-treated RNA samples, and they were effectively translated as IRESs in human cells via short sequences consisting of the m<sup>6</sup>A site (15). Initiation of this m<sup>6</sup>A-mediated translation involves the eukaryotic translation initiation factor 4G2 and a YTH m<sup>6</sup>A RNA-binding protein (YTHDF)3 reader, and their mechanism of translation involves METTL3/14 and is inhibited by FTO (Fig. 1). In addition, that study detected that when



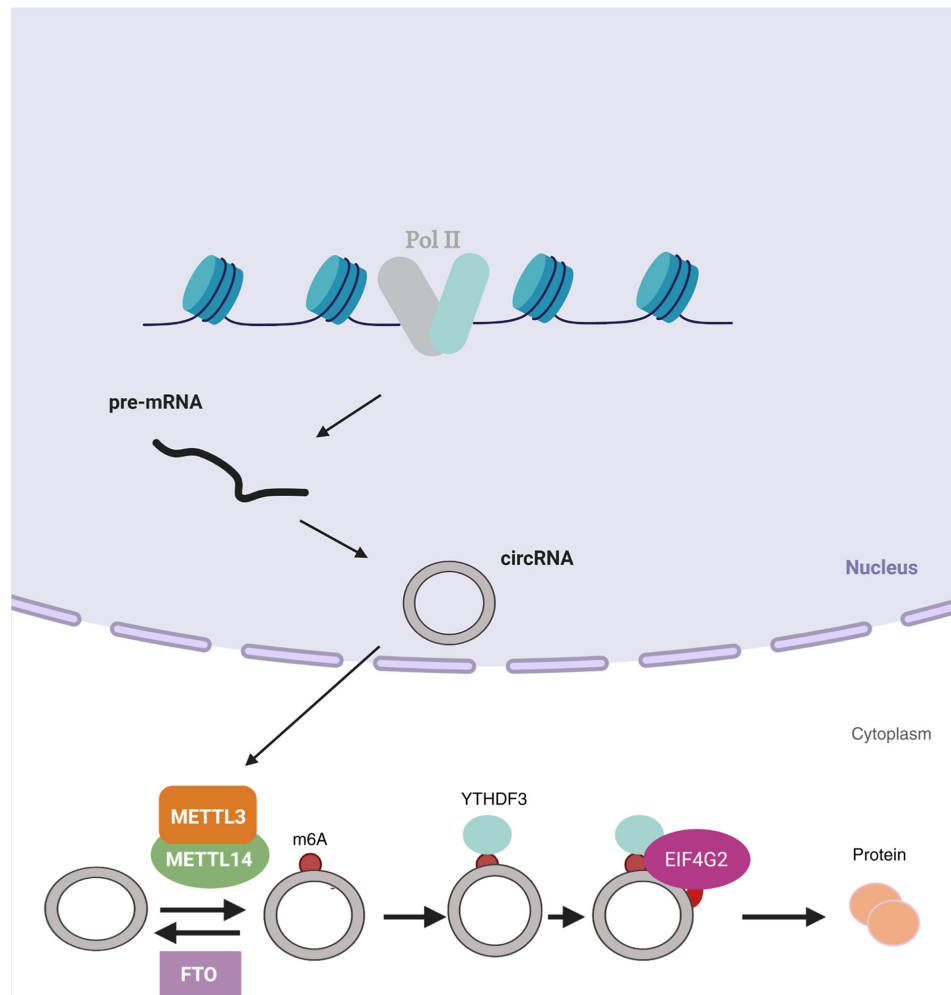


Figure 1. Regulation of circRNAs by m<sup>6</sup>A modifications. The cytoplasm is where m<sup>6</sup>A modifications affect circRNA via a regulatory mechanism. The demethylase FTO and methyltransferase complex METTL3/14 regulate circRNA. The METTL3/14 methyltransferase complex induces circRNA m<sup>6</sup>A methylation modifications, whereas the FTO demethylase removes circRNA m<sup>6</sup>A methylation. The m<sup>6</sup>A methylation site is recognized by YTHDF3, which then recruits eIF4G2 to the circRNA, resulting in circRNA translation. As a result, m<sup>6</sup>A can modify circRNAs, and the methylated circRNAs can encode proteins. circRNA, circular RNA; FTO, fat mass and obesity-associated; METTL, methyl transferase-like; YTHDF3, YTH domain family protein 3; eIF4G2, Eukaryotic translation initiation factor 4  $\gamma$ 2.

circRNAs were subjected to heat, their translational function improved, suggesting that circRNA-encoded proteins may be essential under conditions of stress (15). Other researchers also built an AutoCirc computational pipeline to analyze RNA and m<sup>6</sup>A immunoprecipitation results, and further confirmed that m<sup>6</sup>A modifications are largely observed in circRNAs (16). m<sup>6</sup>A circRNAs were shown to possess highly cell-specific expression, and found that circRNAs with m<sup>6</sup>A modifications also had long single exons (16). Moreover, m<sup>6</sup>A circRNAs and m<sup>6</sup>A mRNAs were compared by the researchers, and it was validated that the methylated exons in mRNAs were distinct from the exons that form m<sup>6</sup>A circRNAs. In addition, they indicated that m<sup>6</sup>A circRNAs were correlated with mRNA stability via the interaction with YTHDF1/YTHDF2 (16).

**Role of m<sup>6</sup>A methylation in the regulation of circRNAs.** Current RNA research indicates that the dysregulation of m<sup>6</sup>A modification is linked to various diseases, including cancer. Aberrant m<sup>6</sup>A modification contributes to tumorigenesis and tumor progression in the majority of cases. Researchers have recently focused their attention on m<sup>6</sup>A-modified mRNA, as

m<sup>6</sup>A functions primarily by influencing RNA metabolism. Currently, m<sup>6</sup>A-modified ncRNAs as well as m<sup>6</sup>A-modified circRNAs, need to be further explored. The role of m<sup>6</sup>A modification in the regulation and function of circRNA is summarized here.

Studies have revealed that certain circRNAs can encode proteins (132,133) and that m<sup>6</sup>A can drive the translation process (15). The transcription initiation elements are located on the 5' end cap structure of mRNA, and the translation mechanism is associated with the transcription initiation elements-cap structure or mechanism (134). In the absence of a dissociative 5' end, this traditional cap-dependent mechanism does not function in a closed circular transcript. As a result, some cap-independent translation initiation mechanisms, such as the IRES-dependent and m<sup>6</sup>A-dependent mechanisms, were proposed to explain how some circRNAs can code for proteins. IRESs are sequences that mediate ribosome-RNA binding and, thus, initiate translation. circZNF609 in myogenesis (132), circMbL in fly head extracts (133), circSHPRH and circFBXW7 in glioma tumorigenesis (135,136), and circ $\beta$ -catenin in liver cancer growth (137) are examples

of protein-coding circRNAs driven by IRESs. A study by Yang *et al* (15) however, broadened our understanding of the coding landscape of the m<sup>6</sup>A-human transcriptome. In cellular responses to environmental stress, an m<sup>6</sup>A-driven translation pathway was proposed and validated. circRNA m<sup>6</sup>A containing motifs were found to be translated, and translation efficiency was found to be modulated by the m<sup>6</sup>A level. It is worth noting that these two cap-independent translation pathways may not function independently. Legnini *et al* (132) reanalyzed m<sup>6</sup>A-Seq and immunoprecipitation (IP) data and combined it with other m<sup>6</sup>A IP results in myoblasts alone (132). The results revealed that the IRES-activated protein-coding circRNA, circZNF609, exhibited high m<sup>6</sup>A methylation levels, suggesting a possible link between these two cap-independent pathways.

Circular RNAs are naturally more stable than their parental linear RNAs due to their closed circular structure, as they are not the primary targets of foreign chemicals or exonucleases. This was confirmed in several studies associated with the characterization of circRNAs (138,139). In Actinomycin D and RNase R treatment, circRNAs are rarely degraded before their corresponding parental linear RNAs (140). However, little is known about how circRNAs are degraded and what factors contribute to circRNA degradation. One of the pathways by which m<sup>6</sup>A-modified RNAs are degraded is the endoribonucleolytic cleavage pathway. As emerging research in the field of RNA research, m<sup>6</sup>A-modified circRNAs were also discovered to be endoribonuclease-cleaved via a YTHDF2-HRSP12-RNase P/MRP axis (141). HRSP12 is an adaptor protein that connects YTHDF2 (m<sup>6</sup>A reader protein) and RNase P/MRP (endoribonucleases) to form the YTHDF2-HRSP12-RNase P/MRP complex, with YTHDF2 serving as the guide. When an m<sup>6</sup>A-modified circRNA is recognized by YTHDF2, regardless of whether it occupies an HRSP12-binding site, RNase P/MRP always performs its endonuclease function. The only difference is that the presence of the HRSP12 binding site improves endoribonucleolytic cleavage efficiency significantly. The m<sup>6</sup>A-modified circRNA is then selectively downregulated. The biological function of circRNAs is altered as a result (142). Thus, it can be deduced that one of the means by which m<sup>6</sup>A modification regulates circRNA biological function is by affecting their degradation.

Interesting emerging studies suggest a possible link between m<sup>6</sup>A modification of circRNAs and certain diseases, including cancer. A recent study suggested m<sup>6</sup>A modification of human endogenous circRNAs played a key role in the inhibition of innate immunity. This study also indicated that exogenous circRNAs were found to induce antigen-specific T- and B-cell activation, antibody production and antitumor immunity *in vivo*, while m<sup>6</sup>A modifications of these exogenous circRNAs inhibited activation of immunity. Furthermore, YTHDF2 was also suggested to be required for inhibiting innate immunity by recognizing m<sup>6</sup>A (143). m<sup>6</sup>A modification was shown to play a key role in stabilizing circCUX1 expression, inhibiting caspase-1 expression and conferring radiotherapy resistance to hypopharyngeal squamous cell carcinoma (140). Moreover, it was observed that m<sup>6</sup>A modification facilitated the cytoplasmic export of circNSUN, which promoted colorectal carcinoma metastasis (139). Taken together, these findings suggest that circRNAs may

regulate the progression of cancer, possibly including AML, via m<sup>6</sup>A modification. However, further evidence is required to determine the regulatory mechanisms involved. These findings indicate that the regulatory mechanisms involved in circRNA interaction with m<sup>6</sup>A members could be essential for cancer progression, which may provide new insights into tumorigenesis.

## 7. circRNAs and AML

The accumulation of abnormal and immature hematopoietic progenitor cells (HPCs) in the bone marrow and peripheral blood is caused by a variety of genetic and epigenetic abnormalities that arrest hematopoietic cell differentiation and maturation. Lethal infection, organ infiltration and cytopenias are frequently associated with these abnormalities (4,9). The progression and pathogenesis of hematopoietic malignancies and solid tumors including AML have been linked to aberrant circRNA expression (Table II). This was further validated in a recent study in which hundreds of circRNAs were found to be differentially expressed in AML, and several of these circRNAs were transcribed from genes implicated in leukemia biology (144). miRNAs are short stretches of RNA (~23 nt in length) that are linked to a variety of biological processes (2), and circRNAs have also been associated with tumorigenesis, metastasis and drug resistance (145). Interestingly, the most well-known mechanism of action of circRNAs is their 'sponge' function, which involves binding to miRNAs (15,146), proteins (139-141) or DNA (147,148). circRNAs modulate mRNA stability and translation by sequestering the mRNA and protein transcripts, and this is the most well-known role of circRNAs in AML (149,150). A brief review of several AML studies suggests that circRNAs could become possible biomarkers in AML (Table II) (150-156). Although the roles of circRNAs in AML requires further exploration, it is evident that circRNA levels are dynamically modulated in AML. Thus, these findings suggest that circRNAs may play an important role in AML.

*Sponging interaction with miRNAs and RNA-binding proteins (RBPs).* The first function of circRNAs, which was discovered in 2013, was that of miRNA sponging, and the most well-established function of circRNAs is to sponge miRNAs and proteins. ciRS-7 has >70 conserved miR-7 binding sites, and it can bind to the Argonaute (AGO) protein (11,147). The sequestration of miRNAs by circRNAs supports the translational machinery to bind to the specific mRNA, resulting in gene derepression in the case of circRNA-miRNA sponge formation (Fig. 2A). Increased expression of genes that are involved in cell proliferation, differentiation and migration may support the development of leukemia (147). Both *cis*- and *trans*-acting factors (157), the latter also termed as RBPs, regulate circRNA biogenesis (147). Since RBPs are also involved in cell cycle progression as well as the biogenesis of circRNAs, circRNA-RBP interactions (Fig. 2B) or indirect circRNA-miRNA-RBP interactions by circRNAs may also induce the development of leukemia (158).

With regard to the role of circRNA-miRNA interaction in AML, a study by Wu *et al* (150) revealed that circDLEU2 suppressed miRNA-496 expression, which has protein kinase

Table II. List of circRNAs and related miRNA sponges reported in acute myeloid leukemia.

First author, year	Circ base ID (name of circRNA)	Change in expression	miRNA target sponge	Functions	Target gene(s)	(Refs.)
Ping <i>et al.</i> , 2019	hsa_circRNA_100053 (circ_0009910)	Up	miR-20a-5p	Biomarker	RUNX3, Rab27B, Smad	(151)
Fan <i>et al.</i> , 2018	hsa_circ_100290	Up	miR-203	Oncogene	Rab10	(152)
Chen <i>et al.</i> , 2018	hsa_circRNA_101141 (circ-ANAPC7)	Up	miR-181 family	Oncogene, biomarker	Numerous	(153)
Wu <i>et al.</i> , 2018	hsa_circ_0000488 (circ-DLEU2 )	Up	miR-496	Biomarker, therapeutic target	PRKACB	(150)
Li <i>et al.</i> , 2017	hsa_circ_0004277	Down	miR-138-5p miR-30c-1-3p miR-892b	Biomarker, therapeutic target	SH3GL2, PPARGC1A	(154)
Shang <i>et al.</i> , 2019	hsa_circ_0100181 (circ-PAN3)	Up	miR-153-5p miR-183-5p	Drug resistance	XIAP	(155)
Hirsch <i>et al.</i> , 2017	hsa_circ_0075001 (circNPM1)	Up	miR-181	Biomarker	TLR signaling pathway genes	(156)

circRNA, circular RNA; miRNA/miR, microRNA.

cAMP-activated catalytic subunit  $\beta$  (PRKACB) as a downstream target gene (Fig. 3A). PRKACB encodes the catalytic subunit of the cyclic AMP-dependent protein kinase, which uses cAMP to regulate various signaling processes, such as proliferation and differentiation. miR-496 inhibited PRKACB expression, whereas circ-DLEU2 sponging miR-496 increased PRKACB expression. As a result, increased circ-DLEU2 expression promoted leukemic cell proliferation and inhibited apoptosis *in vitro*, and promoted the formation of AML tumors *in vivo*. These findings suggested that circ-DLEU2 may be essential for the development of AML (150).

The interaction between circRNAs and RBPs, as well as the associated potential functional aspects, are becoming increasingly clear (159). AGO (4,11), RNA polymerase II (9), Muscleblind protein (12), Quaking I (147) and elongation initiation factor 4A3 (160) are some of the RBPs that have been identified. These RBPs play a role in cellular processes by regulating gene expression. Some upregulated interacting RBPs serve key roles in RNA splicing and maintaining the leukemic condition, according to CRISPR-Cas9-based RBP screening in AML. When RBM39, the network's main regulator, is knocked out, the splicing of essential mRNAs for AML is disrupted, resulting in AML cell apoptosis (161). Furthermore, as comprehensively reviewed previously (162), mutational profiling of leukemic patients has revealed somatic genetic mutations in RBPs that are linked to splicing. In addition, in patients with AML with ITD mutations in the FLT3 gene, high expression of circMyb-related protein B (MYBL2), a product of the MYBL2 gene, was reported. The circMYBL2 and FLT3-ITD mutant kinase were found to have a positive regulatory relationship. circMYBL2 was identified to improve mutant FLT3 kinase protein expression, as a result, FLT3-ITD-dependent signaling pathways were activated.

circMYBL2 enhanced FLT3 kinase translational efficiency by promoting the binding of polypyrimidine tract-binding protein 1 (PTBP1) to mutant FLT3 kinase mRNA. In addition to inhibiting AML cell proliferation and supporting differentiation *in vitro* and *in vivo*, circMYBL2 knockdown compromised the cytoactivity of cells with the FLT3-ITD mutation against quizartinib (Fig. 3B) (163).

*Regulation of gene transcription.* circRNAs are primarily located in the cytoplasm due to their stable structure, nonetheless, some circular isoforms (EicircRNA) can also be found in the nucleus. These circular isoforms bind to chromatin modifiers, causing the gene to be repressed or activated (164,165). RNA polymerase II interacts with certain EicircRNAs, such as circEIF3J and circPAIP2, to recruit U1 small nuclear ribonucleoprotein to promote gene transcription (13). Furthermore, some circRNAs positively regulate the expression of their parent gene, as seen in the case of circRNA, ci-ankrd52, which reduces the expression of ankrd52 without affecting the expression of the surrounding genes (9). By binding to its cognate DNA, circRNA derived from the SEP3 gene controls expression of the linear transcript. circRNA-SEP3 has a linear counterpart with the same sequence that binds to DNA with a low affinity. Hence, transcriptional repression together with the generation of a SEP3 linear transcript with exon skipping are likely outcomes of circRNA-DNA formation (166). Furthermore, promoter-associated RNA suppresses rRNA gene expression by recruiting DNMT3b to the TTF-I (transcription factor) target site via complementarity with the rDNA promoter. By binding to genomic DNA and forming a DNA-RNA triplex, the circRNA, like other RNA species, may affect DNA replication (167). These findings suggest that circRNAs may bind to DNA to regulate gene expression and DNA replication.



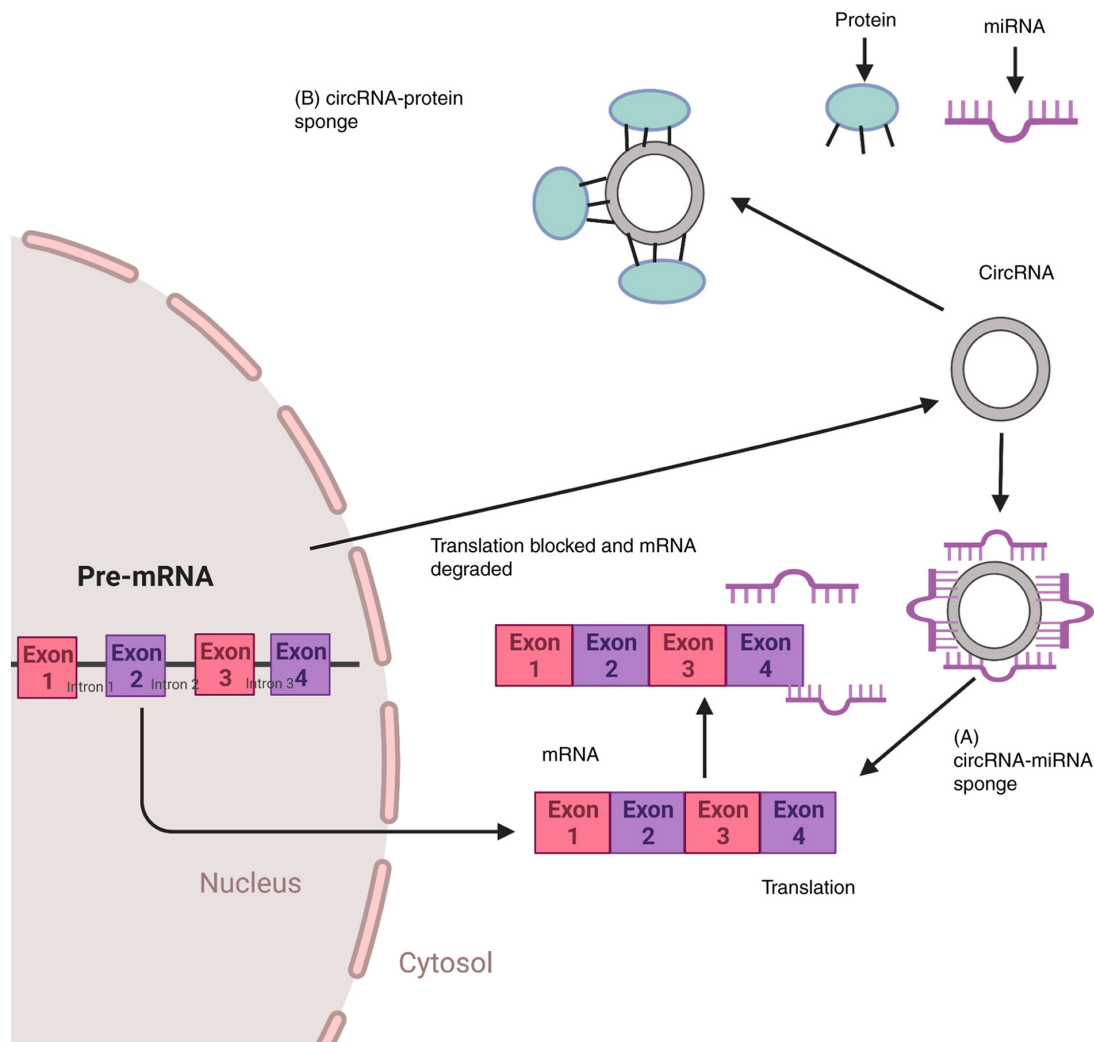


Figure 2. circRNAs sponging interactions with miRNAs and RBPs. (A) circRNAs act as miRNA sponges to regulate mRNA translation. circRNAs may act as miRNA sponges to compete for miRNA binding sites and thus reducing the impact of miRNA-mediated regulatory functions. (B) circRNA may sponge with proteins (RBP) that regulate gene expression and thus play a role in a variety of biological processes. circular RNA; miRNA, microRNA; RBP, RNA binding protein.

**Translation.** Even though circRNAs have an open reading frame, they often lack essential translational components, such as a poly(A) tail and a 7-methylguanosine cap (133). Nonetheless, mounting evidence suggests that circRNAs are capable of translation (133). For example, the RNA modification motif m<sup>6</sup>A, which is abundantly present in circRNAs, aids circRNA translation in human cells (15). Other mechanisms exist for circRNA translation. circRNAs containing an IRES which drives translation, such as circ-ZNF609 and circMbl3, have been found to translate proteins (132,133). Furthermore, Sun *et al* (163) suggested that circMYBL2 regulated FLT3 translation by recruiting PTBP1 to enhance FLT3-ITD AML progression. Generally, circRNA translational mechanisms in AML are not well understood and require further investigation.

## 8. circRNA epigenetic modifications and their possible roles in AML

m<sup>6</sup>A is one of the most abundant patterns of methylation in mRNAs, and was also previously detected in circRNAs, as

described above (15,16). It was further demonstrated that m<sup>6</sup>A is important for the regulation of the fate and function of RNA, which are essential for differentiation and development (99). In addition, FTO, as an m<sup>6</sup>A demethylating enzyme, was found to be overexpressed and to play a critical oncogenic role in AML by promoting cell transformation and leukemogenesis, and inhibiting cell differentiation (100).

By acting as miRNA sponges, circRNAs are involved in regulating RNA processing, such as alternative splicing, pre-RNA splicing and RNA editing (11,168,169). Furthermore, aberrant circRNA expression (mainly upregulation), has been identified as a potential biomarker in AML (Table II). The mechanisms by which circRNAs regulate AML remains unclear. Previous findings suggest that circRNAs may regulate tumorigenesis, at least partly via m<sup>6</sup>A modification (139,140,143).

m<sup>6</sup>A regulators have been identified to be responsible for the dysregulation of m<sup>6</sup>A epigenetic modifications in circRNAs. One such regulator, METTL3, was found to induce circ1662 expression by introducing m<sup>6</sup>A modifications in circ1662 flanking reverse complementary sequences.

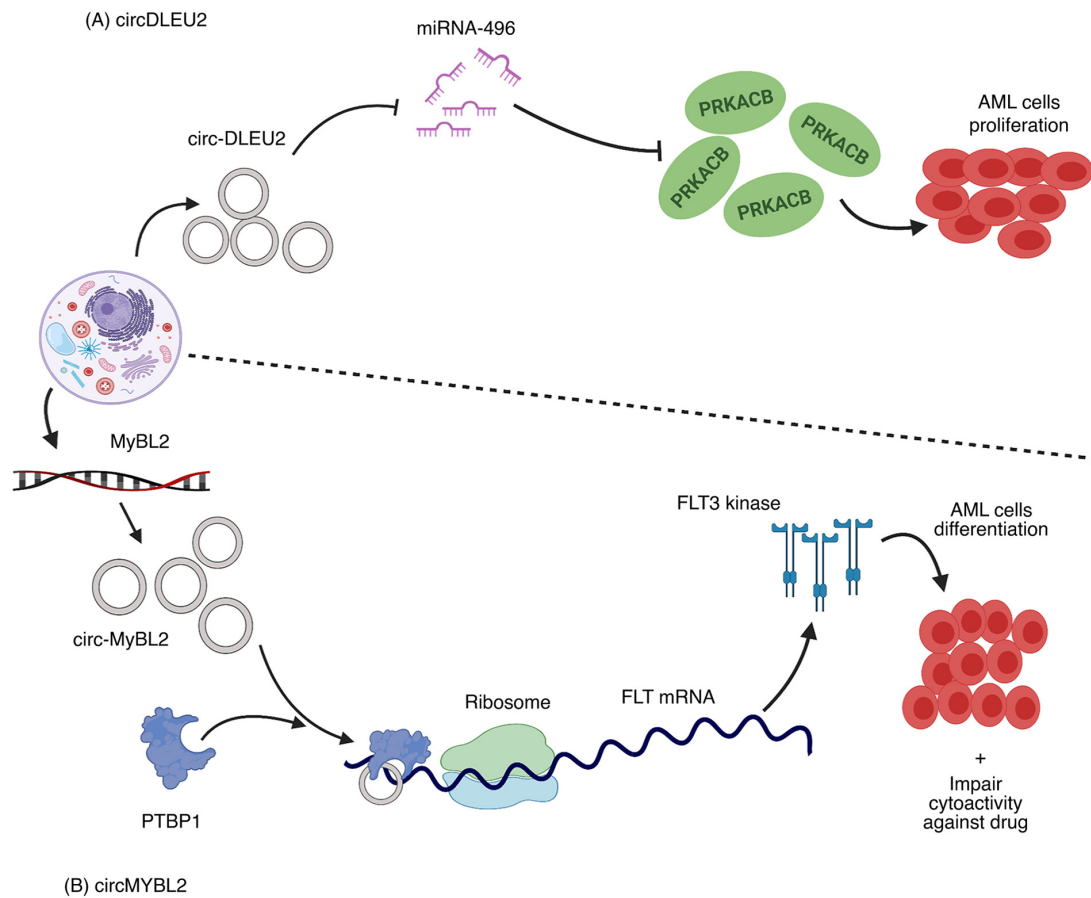


Figure 3. Potential mechanisms of circRNAs in AML. (A) miRNA-496 binds to the related mRNA sites, leading to PRKACB repression; however, circDLEU2-miRNA-496 sponge formation supports the translational machinery to bind to the mRNA, resulting in gene derepression. AML cell proliferation is linked to increased PRKACB expression. (B) The recruitment of PTBP1 to the FLT3-ITD in an mRNA transcript, circMYBL2, which is derived from the MYBL2 gene, positively regulates the FLT3-ITD mutant gene's expression. AML, acute myeloid leukemia; miRNA, microRNA; PRKACB, cAMP-dependent protein kinase catalytic subunit  $\beta$ ; PTBP1, polypyrimidine tract-binding protein 1; FLT3-ITD, FMS-like tyrosine kinase 3- internal tandem duplication; MYBL2, Myb-related protein B.

This study suggested that METTL3 facilitated colorectal cancer (CRC) cell invasion and migration through the circ1662-YAP1-SMAD3 axis, and further analysis confirmed METTL3-induced circ1662 promoted EMT, accelerating CRC metastasis via the YAP1-SMAD3 signaling pathway (170). In another study, METTL3 mediated the m<sup>6</sup>A methylation of circCUX1 and stabilized its expression in hypopharyngeal squamous cell carcinoma (HPSCC), which lead to radio-resistance of HPSCC through the caspase-1 pathway (140). Chen *et al* (139) also revealed that circNSUN2 was exported by another m<sup>6</sup>A regulator, YTHDC1, from the nucleus to the cytoplasm in an m<sup>6</sup>A methylation-dependent manner and this was essential for CRC cells' invasive ability.

At present, the mechanism of m<sup>6</sup>A modification of circRNA in AML is unclear and related studies are yet to be reported. As a result, several hypotheses on how epigenetic modification of circRNAs may influence AML disease are proposed in this present study. It is speculated that the epigenetic modification of circRNAs might prevent miRNA-mRNA binding in AML by occupying the miRNA binding sites. Studies have indicated that circRNAs participate in AML pathogenesis by sponging miRNAs to inhibit their function and promote the expression of the miRNA target genes (Table II). In addition, it has been demonstrated that m<sup>6</sup>A modification was found to promote

miRNA degradation as well as the translational inhibition of downstream target genes. However, m<sup>6</sup>A modification was suggested to protect mRNA degradation mediated by miRNA (171). Taken together, m<sup>6</sup>A modification of circRNA may facilitate circRNA sponging miRNA interaction, which is found in the pathogenesis of several diseases including AML. Second, during AML pathogenesis, epigenetically modified circRNAs may transmit information to the microenvironment via exosomes. Exosomes, which were first identified in 1983, are 50-nm vesicles that play an important role in intracellular and extracellular communication (172). Pre-mRNAs containing Dicer, AGO2 and *trans*-acting regulatory RBP were found in the exosomes of breast cancer cells according to a previous study (173). Furthermore, an AML study revealed that exosomes, emerging as key modulators of hematopoiesis, were found to suppress hematopoiesis in AML (174). That study found that exosomes released from leukemia blasts were able to suppress HPC function in two ways: i) Through stromal reprogramming of niche retention factors and ii) through AML exosome-directed miRNA delivery to HPCs. These could transform the bone marrow niche into a leukemia growth-permissive microenvironment. Third, certain circRNAs can be translated into proteins, and these proteins are suggested to be involved in RNA processing. As a result,

it is speculated that epigenetic modifications of circRNAs, such as m<sup>6</sup>A, may play a key role in AML by influencing RNA splicing and processing. Furthermore, the fact that circRNAs are potential therapeutic targets, or diagnostic or prognostic markers in AML, means their epigenetic modification may affect RNA stability and promote AML pathogenesis.

## 9. Conclusion

In conclusion, the role of circRNAs in carcinogenesis, including AML, is currently a major focus of cancer research. Although alterations in circRNA epigenetic modifications may have an impact on hematopoiesis and AML development, further studies are required to confirm this hypothesis. Therefore, it may be necessary to identify alterations in circRNA epigenetic modifications in AML, as well as the regulatory mechanisms behind these modifications, which could further elucidate the specific roles of circRNAs in this disease. These studies may provide new insights into AML pathogenesis and therapy.

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## Availability of data and materials

Not applicable.

## Authors' contributions

MAI, DW and JS contributed to study conceptualization. MAI was the primary contributor with support from FZ, WZ, HF and HZ in writing the original draft, and it was reviewed and edited by MAI, YL and RC. All the authors have read and approved the final manuscript. Data authentication is not applicable.

## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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