Review article

Understanding the roles of N^6-methyladenosine writers, readers and erasers in breast cancer ☆, ☆☆

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Abstract

Breast cancer is believed to be driven by epigenetic regulation of genes implicated in cell proliferation, survival, and differentiation. Recently, aberrant N^6-methyladenosine (m6A) decorations turned up as crucial epigenetic regulator for malignant breast cancer, which may serve as new targets for breast cancer treatment. Here we briefly outline the functions of m6A and its regulatory proteins, including m6A "writers," "readers," and "erasers" on RNA life fate, recapitulate the latest breakthroughs in understanding m6A modification and its regulatory proteins, and the underlying molecular mechanisms that contribute to the carcinogenesis and the progression of breast cancer, so as to provide potential epigenetic targets for diagnosis, treatment and prognosis in breast cancer.

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Introduction

Breast cancer is the leading cancer and the main cause of cancer-related mortality for women all around the world according to the research in 2021 [1]. Although progress has been made in both understanding and treating breast cancer, nearly 30 percent of patients suffer relapse or metastasis which is the major cause of breast cancer-related mortality due to the shortage of effective treatment or preventive strategy [2]. Despite progressive genetic abnormalities, accumulating studies revealed that breast cancer can be also driven by epigenetic alterations [3]. However, the molecular mechanisms by which epigenetic alterations are regulated and drive breast cancer remain elusive.

N^6-methyladenosine (m6A) modification was discovered and partially characterized in a great variety of cellular mRNAs in the 70s decade [4,5]. It is the most abundant internal methylation in mRNA [6]. Establishment of methylated RNA immunoprecipitation sequencing (MeRIP-seq/m6A-seq) enables the investigation of the m6A RNA methylomes and the mapping over 18,000 m6A sites in the transcripts of more than 7,000 human genes [7,8]. Approximately 90% of all m6A sites are conserved with G-A-C and A-A-C motifs [9]. m6A modification is a post-transcriptional methylation which formed by transferring the methyl group to the nitrogen atoms at 6th position of adenosine from *S*-adenosylmethionine [10]. m6A modification is regulated by the methyltransferase ("writer"), demethyltransferase ("eraser") and m6A recognized RNA binding protein ("reader"), and determines RNA life fate, including RNA splicing, translocation, stability and translation [11,12]. Accumulating evidence indicates that dysregulation of m6A modification and its corresponding proteins contribute to the tumorigenesis and the progression of cancer [13].

In the current review, we briefly outline the roles of m6A modification and its regulatory proteins on RNA life fate, recapitulate the recent advances in understanding m6A modification and its regulatory proteins, and the underlying molecular mechanisms that assist breast cancer carcinogenesis and progression, so as to provide potential epigenetic targets for diagnosis, treatment and prognosis in breast cancer.

m^{6}A modification determines RNA life fate

The reversible modification of m^{6}A is regulated by the cooperation of methyltransferase ("writer") and demethyltransferase ("eraser") [11]. The m^{6}A "writer" complex methyltransferase complex is composed of core methylation subunits, including methyltransferase-like 3 (METTL3) and methyltransferase-like 14 (METTL14), and methylation cofactors, including Wilms tumor 1-associated protein (WTAP), RNA binding motif protein 15/15B (RBM15/15B), Vir like m^{6}A methyltransferase associated protein (VIRMA, synonym: KIAA1429), zinc finger CCCH-type containing 13 (ZC3H13) and Cbl proto-oncogene like 1 (CBLL1, synonym: HAKAI) [14]. Among them, METTL3 plays a methyltransferase catalytic role, METTL14 structurally serves as an upholder for METTL3 [15]. Methylation cofactors, such as WTAP, promotes m^{6}A modification through guiding METTL3 and METTL14 to the nuclear speckles, while RBM15/RBM15B could interact with METTL3 in a WTAP-dependent manner and participate in the modulation of m^{6}A modification of certain RNAs [12]. VIRMA associates with alternative polyadenylation and preferentially mediates m^{6}A methylation of mRNA close to the stop codon and 3′ UTR [16]. ZC3H13 cooperates with other cofactors, like WTAP, to affect 3' UTR methylation [17]. CBLL1 interacts with WTAP/ZC3H13/VIRMA complex and affects some extents of m^{6}A [18]. In addition, METTL16 was currently discovered as an m^{6}A "writer" that primarily targets ncRNAs, lncRNAs, and pre-mRNAs [19]. Two reported demethyltransferases those erase m^{6}A modification are AlkB homolog 5 (ALKBH5) and fat mass and obesity-associated (FTO) protein, both belonging to the AlkB dioxygenase family which demethylate N-methylated nucleic acids [12]. Notably, FTO has moderate effect on m^{6}A or m^{6}A$_m$ levels in mRNA but preferred to demethylate N^6, 2'-O-dimethyladenosine (m^{6}A$_m$) in snRNA [20]. m^{6}A$_m$ is abundant in both mRNA and snRNA, and is involved in the RNA's stability and splicing [21,22]. m^{6}A modification and m^{6}A "readers" have an important role in the metabolism and the translation of mRNA [6]. Different sub-location of "readers" may exert different functions on RNA life fate [23]. The "readers" located in the nucleus, such as heterogeneous nuclear ribonucleoproteins (hnRNPs), including HNRNPG, HNRNPC, and HNRNPA2B1 and YTH domain containing 1 (YTHDC1) are involved in RNA stabilization, RNA splicing, RNA export, RNA structure switching, X chromosome inactivation and microRNA maturation [23,24]. The "readers" located in the cytoplasm, such as YTH domain-containing family protein 1-3 (YTHDF1-3), YTH domain containing 2 (YTHDC2), Fragile X mental retardation 1 (FMR1), proline-rich coiled-coil 2A (PRRC2A), insulin-like growth factor 2 mRNA-binding proteins 1-3 (IGF2BP1-3) and eukaryotic initiation factor 3 (eIF3), mainly contribute to the translation and degradation of mRNA [6,24,25] (Fig. 1).

The expression of m^{6}A modification and regulatory proteins in breast cancer

Epigenetic marks, such as DNA methylation, histone modifications, can be prognostic and predictive biomarkers in oncology. As the most abundant internal methylation in mRNA, m^{6}A modification along with its regulatory proteins can serve as promising biomarkers for breast cancer.

The expression of m^{6}A writers in breast cancer

Although, several studies reported that the expressions of m^{6}A methylation, METTL3 and METTL14 were increased in breast tumor and promoted breast cancer progression [26−29], another studies showed opposite perspective that METTL3 and METTL14 may be low expressed in breast cancer [30]. Wu et al explored the mRNA expression of m^{6}A regulators in both breast cancer samples and public datasets (TCGA and ONCOMINE), revealing that METTL3 and METTL14 were down-regulated in breast cancer [30]. Additionally, Gong et al also analyzed the expression of METTL14 and ZC3H13 in multiple bioinformatics databases, and found that these 2 genes negatively associated with the overall survival and may play a tumor-suppressing role in breast cancer [31]. While the inconsistency between serval previous studies and Wu's or Gong's study may due to the heterogeneity of cancer cell and the unequal expression of proteins and mRNAs which is frequently occurred in cancer. It is probably that METTL3 and METTL14 are indeed not prognostic markers in breast cancer, but involved in specific process and stage of pathogenesis which contribute to the regulation of breast cancer. TCGA data show that the expression of WTAP is reduced 1.9-fold in breast cancer samples as compared with normal tissue. Moreover, low mRNA level of WTAP is associated with ER (+) or PR (+) status, and high mRNA level is found to be associated with basal-like and normal breast-like breast cancer [30]. The expression of VIRMA mRNA is high and negatively related to patients' survival time in breast cancer [32].

The expression of m^{6}A erasers in breast cancer

The m^{6}A "erasers", FTO and ALKBH5, were reported to be tumor promotor in breast cancer [33,34]. The increase of FTO was observed in human HER2(+) breast cancer and highly correlated with poor prognosis of patients [33]. TCGA data also reveal that the mRNA expression of ALKBH5 is elevated 1.5-fold in breast cancer [30]. However, analysis of data from ONCOMINE revealed that FTO and ALKBH5, were decreased in breast cancer [30].

The expression of m^{6}A readers in breast cancer

YTHDF1/2/3 and YTHDC1/2 have recently been identified as "reader" of m^{6}A modification on mRNA, they display a preferential reorganization for m^{6}A-methylated mRNA [35]. A recent study reported that YTHDF1 and YTHDF3 were most frequently amplified and associated with poor survival time of breast cancer patients through analyzing TCGA data [36]. IGF2BP family proteins (IGF2BP1/2/3) have been recognized as important regulators in breast cancer progression for a long time [37,38]. Although the expression level of IGF2BP1 is low in breast cancer, it is necessary for clonogenic growth of breast cancer cells [39]. IGF2BP2 and IGF2BP3 are aberrantly overexpressed in triple-negative breast cancer (TNBC) and associated with poor patients' survival [38]. eIF3 is a huge complex comprised of 13 non-identical subunits, from eIF3a to eIF3m, in human cells. It has been reported that the expressions of eIF3d and eIF3e are increased in breast cancer cell lines and promote carcinogenesis of breast cancer [40,41]. It was reported that eIF3f was significantly down-regulated in ER (+) breast cancer cells [42], while the expressions of eIF3m and eIF3h are obviously higher in TNBC than those in non-TNBC or in normal breast tissues [43,44]. The expression levels of HNRNPA2B1 and HNRNPC are also higher in breast cancer cells [45,46]. However, Liu, et al reported that in their study, higher expression of hnRNPA2B1 correlated with longer median survival time of breast cancer patients [47].

In summary, m^{6}A modification is probably not a prognostic marker for breast cancer, and the expression of m^{6}A regulatory proteins is more complex in breast cancer or cell lines than m^{6}A modification. The targets regulated by m^{6}A modification and m^{6}A regulatory proteins probably exert the function in breast cancer, which raise an open question and need to be understood.

m^{6}A modification regulates breast cancer progression

Recently, accumulating evidence indicates that m^{6}A modification plays important roles in breast cancer progression. m^{6}A modification and m^{6}A regulatory proteins regulate the expressions of proto-oncogenes or tumor
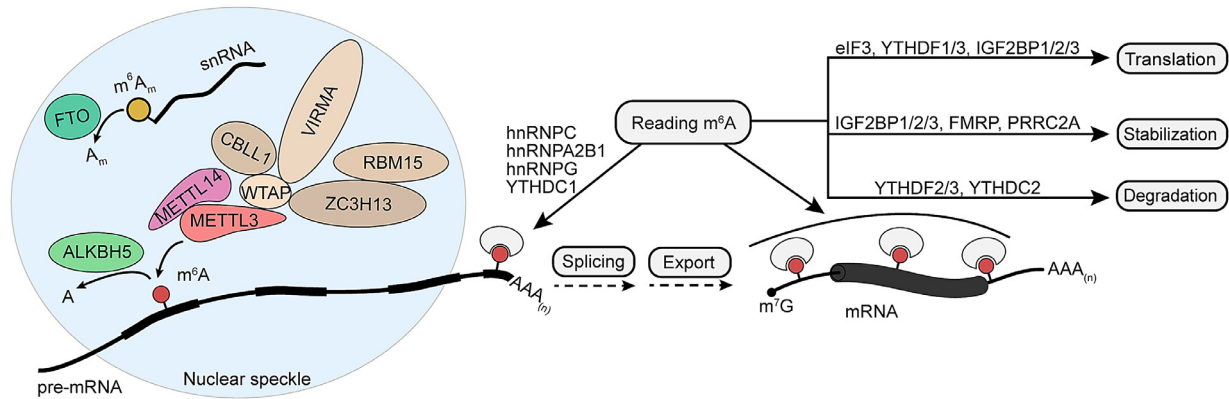
Fig. 1. m^{6}A modification determines RNA life fate. Shown here is the working model of methyltransferase complex, demethylases, and m^{6}A binding proteins. A, adenosine; m^{6}A, N^6-methyladenosine; m^{6}A$_m$, N^6, 2'-O-dimethyladenosine; A$_m$, 2'-O-methyladenosine; snRNA, small nuclear RNA; m^{7}G, N^7-methylguanosine; AAA$_{(n)}$, polyadenylation.

suppressor genes through modulating the RNA life fate during breast cancer progression [27,33]. Alternatively, m^{6}A regulatory proteins also participate in breast cancer progression via m^{6}A independent manner [32,48]. m^{6}A regulatory proteins are highly connected with breast cancer cell proliferation, metastasis, invasion, drug-resistance, and so on [30]. Thus, m^{6}A regulatory proteins are promising targets for breast cancer treatment.

m^{6}A modification modulates the growth of breast tumor

m^{6}A "writer" complex components exhibit either promoting or inhibiting effects on the growth of breast tumor. The interfering of METTL3 expression could decrease cell proliferation and promote apoptosis in breast cancer. Using gene-specific m^{6}A-qPCR, Wang et al identified Bcl-2 as the downstream effector of METTL3 [26]. Another study revealed that METTL3 showed high co-expression with hepatitis B X-interacting protein (HBXIP) which is reported as an oncogene for breast cancer[27]. HBXIP is able to increase the expression METTL3 through inhibiting miRNA let-7g. In turn, increased METTL3 upregulates the expression of HBXIP through enhancing m^{6}A modification in HBXIP's mRNA which results in tumor malignant growth [27]. A recent study revealed that METTL3 regulated m^{6}A-induced expression of LINC00958. m^{6}A-modified LINC00958 is up-regulated in breast cancer cells and facilitates the tumor progression via miR-378a-3p/YY1 axis [49]. During METTL14-mediated m^{6}A methylation in breast cancer, long non-coding RNA LNC942 exerts carcinogenic function as the upstream regulator for METTL14, whereas, CXCR4 and CYP1B1 are identified as direct targets of METTL14 which is also regulated by LNC942. LNC942 is able to promote the mRNA stability of CXCR4 and CYP1B1 for breast cancer proliferation and progression [29]. However, another study demonstrated opposite results that overexpression of METTL14 significantly suppressed cell viability and colony formation ability of MDA-MB-231 cells [30]. Due to the conflict results, we queried breast cancer cell lines data from DepMap portal (https://depmap.org/portal/), which is a cancer dependency database generated by cell depletion assay using either CRISPR or RNAi method, to help evaluate the role of METTL14. Using CRISPR method, METTL14 was essential in most of breast cancer cell lines. However, using RNAi method, METTL14 was non-essential in most of breast cancer cell lines (Table 1), which suggests low expression of METTL14 is enough to exert its function on the viability of breast cancer cell. A recent study showed that VIRMA was able to promote breast cancer proliferation. Through RIP-seq analysis, Qian et al found that the mRNA of CDK1 in the cell cycle was mostly associated with the oncogenic activities of VIRMA. Interestingly, VIRMA increased the stability of CDK1 mRNA in

an m^{6}A-independent manner [32]. CBLL1 negatively contributes to the carcinogenesis and malignant of breast cancer. CBLL1 functions as a co-repressor of ERα through interfering with the recruitment of SRC-1 and SRC-2 which are coactivators of ERα. Functionally, Gong et al revealed that CBLL1 overexpression could hindered the proliferation and migration of breast cancer cells [50].

Like the "writer" complex, m^{6}A "eraser" also exert essential roles during breast cancer cell proliferation [51]. FTO enhances cell proliferation and colony formation of breast cancer cells [33]. Mechanistically, FTO induces the mRNA degradation of BNIP3, an apoptosis-inducing protein, through modulating m^{6}A in the 3'UTR [33]. FTO also play an oncogenic role in breast cancer through modulating energy metabolism, which involves the promotion of glycolysis and lactic acid production through PI3K/AKT signaling pathway [52]. Knockdown of ALKBH5 inhibits breast cancer cell proliferation and colony formation [34]. The expression of ALKBH5 can be induced by hypoxia. Induced ALKBH5 promotes the mRNA stability and the expression of NANOG, which in turn enhances breast cancer stem cells (BCSCs) [34]. According to these independent studies, m^{6}A "erasers" play an oncogenic role in breast cancer. Thus, the m^{6}A "erasers" may be promising targets for breast cancer treatment. m^{6}A "readers" are major players in breast cancer due to their RNA binding property. The expression of IGF2BP1 is low in breast cancer cells, but it is necessary for the clonogenic growth of breast cancer cells [39]. Furthermore, IGF2BP2 interacts with pseudogene-transcribed RPSAP52 to stimulate the proliferative pathways in breast cancer [53]. Consistently, IGF2BP3 contributes to breast cancer cell proliferation via binding the mRNA of CD44 and enhancing the expression of CD44, which increases IGF2 levels in fibroblasts [54]. Additionally, IGF2BP3 promotes cell proliferation by blocking the maturation of miR-3614, which protects TRIM25 mRNA from degradation mediated by miR-3614 [55]. Additionally, IGF2BP3 serves as an RNA sponge for long non-coding RNA CERS6-AS1. Due to the binding of CERS6-AS1 to IGF2BP3, the mRNA stability of CERS6 is increased, which results in the promotion of cell proliferation and the suppression of cell apoptosis [56]. Although the authors didn't investigate whether these functions of IGF2BPs relied on m^{6}A modification of the RNA, the functions of IGF2BPs was exerted through their RNA binding capacity, indicating that m^{6}A modification might contribute to the recognition of RNAs. Knockdown of eIF3d suppresses breast cancer cell proliferation through inhibiting Wnt/β-catenin signaling [40]. eIF3h serves as a prognostic marker, of which amplification or overexpression regulates the proliferation, survival, and transformation of breast cancer cells [57]. Inhibition of HNRNPC hindered the proliferation of MCF-7 and T47D cells [46]. In this process, dsRNA

Table 1

List of reported functions of m^{6}A regulatory proteins in breast cancer.

Gene name	Role in RNA modification	Role in cancer	Mechanism	m^{6}A regulation	References	DepMap[a]
METTL3	writer	Oncogene	Promoting the expression of Bcl-2 through m^{6}A modification	m^{6}A dependent	[26]	Essential
		Oncogene	Promoting the expression of HBXIP through enhancing m^{6}A modification and be suppressed by let-7g	m^{6}A dependent	[27]	
		Oncogene	Promoting the expression of LINC00958 through enhancing m^{6}A	m6A dependent	[49]	
		Tumor suppressor	Inhibiting the expression of COL3A1 by m^{6}A modification	m^{6}A dependent	[48]	
		Oncogene	Promoting the expression of miR-221-3p by increasing pri-miR-221-3p m^{6}A mRNA modification	m^{6}A dependent	[65]	
METTL14	writer	Oncogene	Promoting the expression of hsa-miR-146a-5p	Unkown	[28]	Essential[b]
		Oncogene	Promoting the expression of CXCR4 and CYP1B1 through m^{6}A modification which are the targets of LNC942	m^{6}A dependent	[29]	
VIRMA	cofactor	Oncogene	Increasing the stability of CDK1 mRNA	m^{6}A independent	[32]	Very essential[b]
CBLL1	cofactor	Tumor suppressor	Interfering with the recruitment of coactivators of ERα: SRC-1 and SRC-2	m^{6}A independent	[50]	Essential
FTO	eraser	Oncogene	Inducing BNIP3 mRNA degradation through modulating m^{6}A	m^{6}A dependent	[33]	Partial-essential[c]
		Oncogene	Promoting glycolysis and lactic acid production through PI3K/AKT signaling pathway	Unkown	[52]	
		Oncogene	Promoting miR-181b-3p/ARL5B signaling pathway	Unkown	[58]	
ALKBH5	eraser	Oncogene	Inducing by hypoxia and promoting the mRNA stability and the expression of NANOG	m^{6}A dependent	[34]	Partial-essential
YTHDF3	reader	Oncogene	Promoting the translation of ST6GALNAC5, GJA1 and EGFR through m^{6}A modification	m^{6}A dependent	[62]	No data
IGF2BP1	reader	Tumor suppressor	Promoting the degradation of UCA1 through recruiting the CCR4-NOT1 deadenylase complex	Unkown	[63]	Partial-essential[c]
		Oncogene	Interacting with lncRNA KB-1980E6.3 and promoting c-Myc mRNA stability through modulating m^{6}A	m^{6}A dependent	[66]	
IGF2BP2	reader	Oncogene	Interacting with pseudogene-transcribed RPSAP52	Unkown	[53]	Partial-essential
		Oncogene	Suppressing the transcription of miR-200a by destabilizing the mRNA of the progesterone receptor (PR)	Unkown	[38]	
IGF2BP3	reader	Oncogene	Promoting the expression of CD44 via binding the mRNA of CD44	Unkown	[54]	Partial-essential[c]
		Oncogene	Inhibiting TRIM25 mRNA degradation mediated by miR-3614 through blocking the maturation of miR-3614	Unkown	[55]	
		Oncogene	Promoting the mRNA stability of CERS6 as an RNA sponge for long non-coding RNA CERS6-AS1	Unkown	[56]	
		Oncogene	Suppressing the transcription of miR-200a by destabilizing the mRNA of the progesterone receptor (PR)	Unkown	[38]	
		Oncogene	Promoting the expression of SOX2 by binding to the mRNA of SLUG	Unkown	[64]	
		Oncogene	Promoting the expression of BCRP via binding to BCRP mRNA	Unkown	[70]	
eIF3d	reader	Oncogene	Promoting Wnt/β-catenin signaling	Unkown	[40]	Very essential[c]
eIF3h	reader	Oncogene	Catalyzing the deubiquitylation of YAP and resulting in the stabilization of YAP	m^{6}A independent	[44]	Essential[c]
HNRNPC	reader	Oncogene	Regulating Alu-enriched dsRNA and the down-stream interferon response	Unkown	[46]	Very essential
	reader	Oncogene	Promoting ERK1/2 and STAT3 pathway	Unkown	[58]	Partial-essential[c]
HNRNPA2B1		Tumor suppressor	Regulating ERK-MAPK/Twist, GR-β/TCF4, STAT3 and WNT/TCF4 signaling pathways	Unkown	[47]	

[a] Data were retrieved from breast cancer cell lines dataset from DepMap portal (https://depmap.org/portal/). Very essential, dependency score $\leq$ -1.0; Essential, -0.1 < dependency score $\leq$ -0.5; Partial essential, -0.5 < dependency score $\leq$ 0; Non-essential, dependency score > 0. For RNAi data, Combined RNAi (Broad, Novartis, Marcotte) dataset was queried. For CRISPR data, CRISPR (Avana) Public 21Q1 dataset was queried. Lower dependency score from either CRISPR or RNAi data was chosen for default.
[b] Conflict with RNAi data.
[c] Supported by both RNAi data and CRISPR data.

sensor RIG-I induced interferon response, resulted in the suppression of cell proliferation. Moreover, suppression of HNRNPC also induces Alu-enriched dsRNA which eventually resulted in nonsense-mediated RNA decay [46]. HNRNPA2B1 increases the carcinogenesis of breast cancer which is attributable to ERK1/2 and STAT3 pathway stimulation [58].

m6A modification modulates the metastasis of breast tumor

Breast cancer metastasis is an enormous challenge for treatment. m6A modification and regulatory proteins also participate in the process of metastasis. COL3A1 promotes the metastatic ability of TNBC cells. METTL3 can methylate the mRNA of COL3A1 resulting in decreased expression of COL3A1 and inhibited metastasis [48]. Additionally, increased expression of METTL14 and METTL14-mediated m6A modifications enhance the metastasis capacity of breast cancer cells through reshaping the miRNA/mRNA network which is most enriched in cancer [28]. hsa–miR–146a–5p is identified as one of the miRNA targets of METTL14 in this remodeled miRNA/mRNA network [28]. VIRMA is able to promote breast cancer metastasis via increasing the mRNA stability of CDK1 [32]. CBLL1 inhibits breast cancer cell migration through suppressing ERα activation [50]. RBM15/15B is reported positively correlated with invasive breast carcinoma [59,60]. m6A "writer" most likely modulates a complicated group of targets, which form a network and feed multiple cancer pathways. Further researches need to consider to extend their downstream investigation to a larger scope, which might figure out the discordance presented in the current independent studies. m6A "erasers", FTO and ALKBH5, were also supposed to contribute to breast cancer metastasis [33,34]. FTO is able to promote the migration and the invasion of HER2(+) breast cancer cells through miR-181b-3p/ARL5B axis [61]. m6A "readers" are also major players in breast cancer metastasis. Recently, an important study reported that YTHDF3 was correlated with the prognosis of breast cancer and promoted breast cancer brain metastasis [62]. Chang et al found that YTHDF3 knockdown inhibited transmigration across the blood-brain barrier in a mouse model [62]. Mechanistically, YTHDF3 increased the translation of the key brain metastasis genes such as ST6GALNAC5, GJA1 and EGFR in an m6A-dependent manner [62]. Another study showed that IGF2BP1 inhibited lncRNA UCA1-mediated breast cancer cell invasion. IGF2BP1 interacts with lncRNA UCA1 and triggers UCA1 degradation via recruiting the CCR4-NOT1 deadenylase complex [63]. Similarly, IGF2BP2 and IGF2BP3 were reported to promote the metastasis of TNBC collaboratively through recruiting CCR4-NOT1 deadenylase complex [38]. They can suppress the transcription of miR-200a by destabilizing the mRNA of the progesterone receptor (PR). In turn, miR-200a could directly target the mRNAs of IGF2BP2 and IGF2BP3 [38]. Interestingly, Zhou, et al have confirmed that elevated eIF3h in breast cancer cells exerts an oncogenic role as deubiquitylating enzyme which catalyzes the deubiquitylation of YAP, resulting in the stabilization of YAP and breast tumor invasion and metastasis [44]. It is worth noting that eIF3h is known as a subunit of translation initiation factor eIF3, which is also known as m6A reader. eIF3h functioned neither as translation initiation factor nor m6A reader in Zhou's research. eIF3d can also contribute to breast cancer invasion through Wnt/β-catenin pathway [40]. Liu, et al revealed that HNRNPA2B1 expression was not only negatively associated with breast cancer metastasis. Through multiple in vitro and in vivo experiments, Liu, et al also confirmed that HNRNPA2B1 could inhibit breast cancer metastasis, which involved complicated regulation of ERK-MAPK/Twist, GR-β/TCF4, STAT3 and WNT/TCF4 signaling pathways [47]. However, whether HNRNPA2B1 worked depending on its m6A reader activity remains unclear (Fig. 2).

m6A modification modulates the clinical outcomes of breast cancer

Drug tolerance and stemness of cancer cells are 2 important things that affect clinical outcomes of breast cancer [64]. METTL3/miR-221-

3p/HIPK2/Che-1 axis is found to be involved in Adriamycin resistance in MCF-7 breast cancer cells. METTL3 is able to methylate pri-miR-221-3p resulting in elevated expression of mature miR-221-3p. Consequently, miR-221-3p down-regulates HIPK2 through targeting the 3'UTR of HIPK2 resulting in increased level of Che-1 which is a target of HIPK2. Thus, the METTL3/miR-221-3p/HIPK2/Che-1 axis enhances drug resistance in adriamycin-resistant MCF-7 cells [65]. A recent study revealed that a hypoxia-induced lncRNA KB-1980E6.3 could promote the stem-like properties of breast cancer stem cells (BCSCs) through the recruitment of IGF2BP1. The interaction between IGF2BP1 and lncRNA KB-1980E6.3 is important for m6A-induced mRNA stability of c-Myc [66]. IGF2BP3 is also reported to associate with TNBC [67] and facilitates the initiation of tumor and the stemness of BCSCs in TNBC through regulating SOX2 expression. In this process, SLUG, as an important functional downstream of IGF2BP3, mediates the regulation of SOX2 by IGF2BP3 [68]. Besides, IGF2BP3 was shown to be essential for the stemness of breast cancer cells, which could be regulated by miR-34a [69]. Preferential expression of IGF2BP3 in TNBC contributes to the resistance to many chemotherapeutics [70]. Suppression of IGF2BP3 in TNBC cells significantly increased the sensitivity to doxorubicin and mitoxantrone of cancer cells. The mechanism underlines that IGF2BP3 regulates breast cancer resistance protein (BCRP), which is a major effector of drug resistance in breast cancer [70].

The expression of HNRNPA2B1 is higher in tamoxifen-resistant LCC9 cells than that in MCF-7 cells which are tamoxifen-sensitive cells. In MCF-7 cells, ectopic expression of HNRNPA2B1 could alter miRNA transcriptome [45]. However, the involved pathways, the roles of altered miRNA transcriptome and downstream effectors involving in endocrine-resistance necessitates further elucidation of detailed mechanisms [45].

Since some conflict results were observed in multiple independent studies. We also checked the roles of m6A regulatory proteins in DepMap portal. Through the results we can conclude that most of m6A regulatory proteins are at least partial essential in breast cancer cell lines (Table 1).

Experimental methods to identify m6A specific regulation of individual RNA

It has been difficult for researchers to pick out individual RNAs of interest that bear m6A marks and this has required the development of a number of new techniques. Researchers also could not exclude the interferences from contaminating RNA species which was co-purified with target mRNA. A lot of studies didn't distinguish whether their purposed model worked in m6A dependent manner. m6A-seq (or MeRIP-seq) engaging immunoprecipitation and high-throughput sequencing technologies opens up the era of "Epitranscriptome" [71]. Dedicated and detailed reviews on technologies investigating epitranscriptome were previously published [23]. To facilitate the following studies focus on m6A modification or m6A regulatory proteins in breast cancer, we summarized necessary experiments those identifying individual RNAs that contained m6A, and those exclude the interferences from contaminating RNA species which was co-purified with target mRNA.

MeRIP-qPCR

Methylated RNA immunoprecipitation (MeRIP) is widely used in m6A related studies. Immunoprecipitated and purified with m6A antibody, m6A marked RNAs could be subjected to reverse transcription and qPCR to confirm the m6A enrichment of RNA of interest (ROI) or to compare m6A enrichment of ROI between different samples/treatments [7] (Fig. 3A).
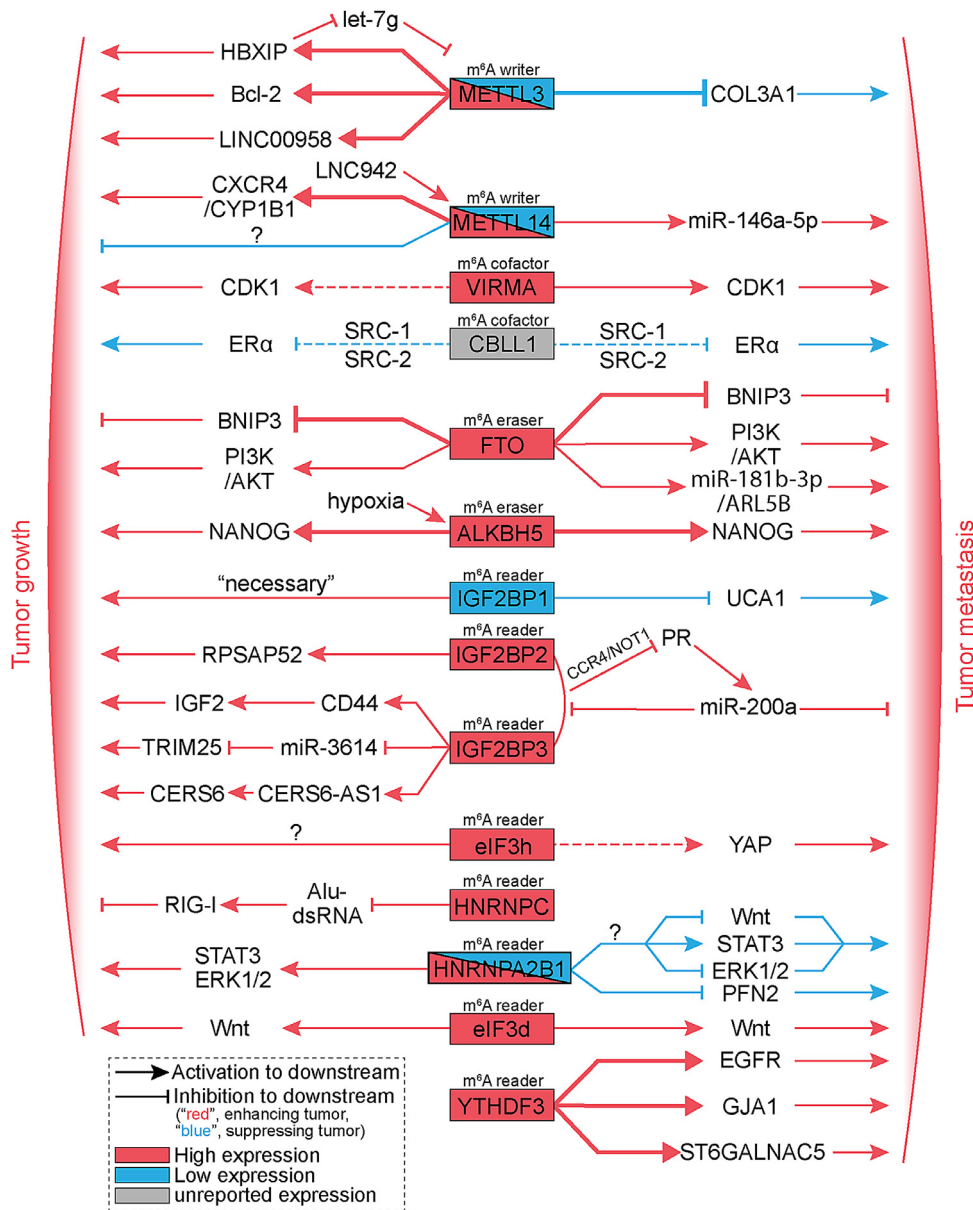
Fig. 2. m^{6}A modification modulates the growth and the metastasis of breast tumor. Shown here is molecules and pathways regulated by m^{6}A regulatory proteins resulting in enhanced or suppressed breast tumor growth and metastasis. Arrows indicate activation to downstream molecules or pathways, and flat lines indicate inhibition to downstream molecules or pathways. Bold arrows indicate m^{6}A dependent manner, dashed lines indicate m^{6}A independent manner, and solid arrows or lines indicate unknown manner. Red arrows or flat lines indicate to enhance breast tumor growth and metastasis, blue arrows or flat lines indicate to suppress breast tumor growth and metastasis. The red box means high expression, the blue box means low expression, and the gray box means unreported expression in breast cancer. "?", unknown mechanism. (Color version of figure is available online.)

RIP-qPCR or CLIP-qPCR

RNA immunoprecipitation (RIP)-qPCR or cross-linking immunoprecipitation (CLIP)-qPCR is useful for the determination of interaction between the protein of interest and the RNA of interest. Both techniques depend on antibody to immunoprecipitate RNA-protein complex, and include reverse transcription, and qPCR analysis of RNA of interest. The differences are CLIP-qPCR uses ultraviolet (UV) light to crosslink proteins to RNAs that are in close proximity, and the following isolation is done stringently [72] (Fig. 3A).

Dual luciferase reporter assay

Dual luciferase reporter assay uses a reporter DNA conjugated with luciferase to quantify the regulation to the reporter. Weng, et al inserted wild type or m^{6}A sites-mutated (A to T) 3' coding region of MYB or MYC right before the stop codon of luciferase to show that METTL14 regulated the mRNA stability of MYB or MYC through m^{6}A sites [73]. This method is an alternative easy way to confirm an m^{6}A specific regulation of target RNA, when the m^{6}A sites in RNA of interest are known (Fig. 3B).
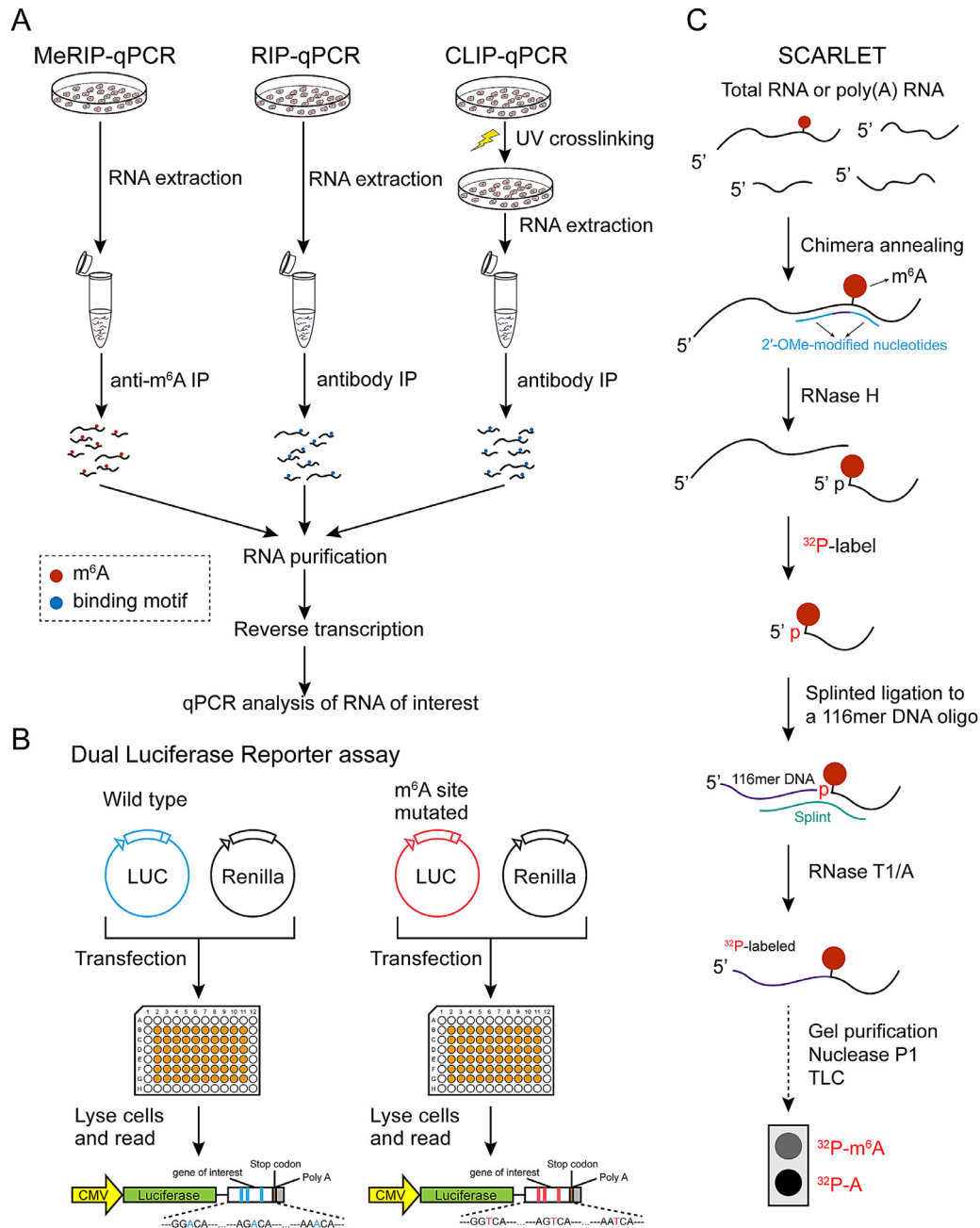
Fig. 3. Experiments identify m^{6}A specific regulation of individual RNA. Shown here is schematic diagrams of (A) methylated RNA immunoprecipitation (MeRIP)-qPCR, RNA immunoprecipitation (RIP)-qPCR, cross-linking immunoprecipitation (CLIP)-qPCR, (B) dual luciferase reporter assay, (C) site-specific cleavage, and radioactive-labeling followed by ligation-assisted extraction and thin-layer chromatography (SCARLET) technologies. m^{6}A, N^6-methyladenosine; IP, immunoprecipitation; qPCR, quantitative polymerase chain reaction; LUC, luciferase; CMV, cytomegalovirus promoter; A, adenosine; TLC, thin-layer chromatography.

Site-specific cleavage and radioactive-labeling followed by ligation-assisted extraction and thin-layer chromatography (SCARLET)

SCARLET was established to accurately decide whether an m^{6}A is presented at a given site of mRNA/lncRNA [74]. The method uses a chimeric DNA oligonucleotide with 2'-OMe/2'-H modifications to guide RNase H to create a site-specific cleavage at the 5' end of candidate m^{6}A site. The cleaved site is radiolabeled with ^{32}P and splint-ligated to a 116 nt ssDNA oligonucleotide. The resulted fused RNA-DNA oligonucleotide is purified and digested to generate mononucleotides with 5' phosphate and determined

by thin-layer chromatography [74]. The method can confirm any m^{6}A site at mRNA or lncRNA from a total-RNA sample without additional purification step (Fig. 3C).

Potential targeted therapeutic strategies based on m^{6}A modification in breast cancer

Several new drugs targeting DNA methylases or histone modifying enzymes have been approved for the treatment of cancer, and epigenetic chemical intervention to influence gene expression has become an active

field of international research on new drug development [75,76]. Given the critical role of m6A regulatory proteins in breast cancer, they are expected to be promising drug targets for cancer treatment. Metformin, which was previously regarded as anti-diabetic medicine, is capable of inhibiting breast cancer cell proliferation by down-regulating the expression of METTL3. This study suggests that drug repurposing is a valuable approach for the screening of m6A inhibitors, due to the possibility that FDA approved medications may exert m6A regulatory functions [77]. Recently, the researchers developed small molecule inhibitors CS1 and CS2, which target m6A "eraser" FTO in cancer [78]. The inhibitor could occupy FTO catalytic "pocket" to stop the m6A modified oligonucleotides into function, thus inhibiting the demethylation function of FTO. The inhibitors showed effective anti-leukemia effect in PDX mouse model, and the clinical trials showed a satisfactory anti-cancer effect. Moreover, it is worth noting that the anti-cancer effect of CS1 and CS2 in diverse solid tumors was verified, including breast cancer [78]. Their study suggests that to develop applicable selective and effective inhibitors of m6A regulatory protein for clinical use may well provide more effective strategies to treat breast cancer, especially when combined with other therapeutic agents for cancers that are resistant to existing therapies.

Conclusion

Although m6A has been the focus of many studies in recent years, our knowledge about it is far from complete. From the current studies, METTL3, METTL14, FTO, ALKBH5, YTHDF3 and IGF2BP1 are proved to exert functions dependent on m6A modification in breast cancer. However, whether the other m6A modulators regulate breast cancer progression replying on m6A modification is unanswered (Table 1).

Given that cancer is proven to be driven by epigenetic alteration, and alteration of mRNA controls the expression of oncogenes, targeting m6A regulatory proteins can serve as a new approach for precisely modifying epitranscriptome of cancer and benefiting cancer treatment. Development of enzyme inhibitor of m6A "writer" and "eraser" can control the addition and removal of m6A marks which control RNA life fate. Development of competence of m6A reader can specify the RNA to be regulated. Combined interventions of "writer", "eraser" and "reader" may make specific control of RNA life fate come true.

Author contributions

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