EPIGENOMICS
2025, VOL. 17, NO. 14, 1009–1028
https://doi.org/10.1080/17501911.2025.2542113

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REVIEW

The functional and therapeutic potential of epitranscriptomics in breast cancer

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ABSTRACT

Breast cancer (BCa) is one of the most commonly diagnosed malignancies worldwide and is clinically heterogenous. BCa is classified into distinct histopathological and molecular subtypes that inform diagnosis, treatment, and prognosis. Although therapeutic advances, particularly targeted therapies, have improved outcomes, metastatic BCa remains an unmet clinical need. In addition, treatment options remain limited especially for triple negative BCa (TNBC). There is an urgent need to develop novel approaches that can prevent, delay, or reverse disease progression in these patients. The roles of genetic and epigenetic alterations in BCa are well established. Emerging evidence highlights the dysregulation of epitranscriptomic mechanisms involving covalent RNA modifications as a contributing factor in BCa pathogenesis. Notably, recent evidence supports functional crosstalk between epigenetic and epitranscriptomic processes with potential clinical and therapeutic relevance. This review explores key epitranscriptomic RNA modifications, m^6A, m^6A_m, m^5C, m^7G, and m^1A in the context of BCa. The functional consequences of the epitranscriptomic regulators ("writers," "erasers", and "readers") are discussed alongside the accumulating evidence of their contribution to cancer development and progression. This review considers how RNA modifications and their regulators might serve as biomarkers or therapeutic targets and offers new directions for translational research and clinical intervention in BCa.

ARTICLE HISTORY
Received 20 May 2025
Accepted 28 July 2025

KEYWORDS
RNA methylation; epicapome; N6-methyladenosine; 5-methylcytosine; N7-methylguanosine; N1-methyladenosine; RNA therapeutics; clinical applications

1. Overview of BCa and current treatment options

BCa is one of the most commonly diagnosed cancers and is clinically and pathologically heterogeneous [1,2]. In the US, it is estimated that one in eight women will be diagnosed with invasive BCa during their lifetime [2]. There are several known risk factors for BCa which include aging, estrogen levels, lifestyle, and hereditary factors including genetic variants affecting the *BRCA1* and *BRCA2* genes [3,4].

BCa is classified into biologically and clinically distinct subgroups based on histological phenotype as well as molecular characterization approaches [5,6]. There are four main subgroups: luminal A, luminal B, human epidermal growth factor receptor 2 (HER2) positive and TNBC which are characterized by the expression of hormone receptors (estrogen receptor; ER and progesterone receptor; PR) and HER2 [7,8]. Following diagnosis, treatment for localized BCa commonly involves surgery and adjuvant and neo-adjuvant treatments such as chemo- and radio-therapies [9]. Molecular classification is used to inform the treatment decision for BCa patients [9,10].

For patients with ER positive BCa, treatments include endocrine therapies which block the ER function in BCa cells (selective ER modulators (SERMs) such as tamoxifen and selective ER degraders (SERDs) e.g., fulvestrant) or inhibit biosynthesis production of estrogens (aromatase inhibitors such as anastrazole) [9,11]. Recent advances have seen the development of nonsteroidal, oral SERDs including elacestrant that improves outcomes as a second- and third-line endocrine therapy, including in patients with ER mutations [10,12]. While hormone therapy is generally effective, treatment resistance arises frequently which poses a major clinical challenge [13–15]. Hormone therapy response is influenced by alterations of the estrogen receptor pathway such as ER mutations or signaling pathways including the PI3K/Akt/mTOR pathway. Furthermore, epigenetic factors and regulators, notably p160 and other histone-associated coregulators, which can contribute to treatment resistance may represent druggable targets themselves [16–18].

For patients with advanced, hormone receptor positive, HER2 negative BCa, CDK4/CDK6 inhibitors are used in

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Supplemental data for this article can be accessed online at https://doi.org/10.1080/17501911.2025.2542113

Article highlights

- Breast cancer is one of the most commonly diagnosed malignancy worldwide and is clinically heterogenous.
- Epitranscriptomic modifications are dynamically regulated, added by writers, removed by erasers and interpreted by readers.
- These regulators are dysregulated in many diseases including breast cancer.
- RNA therapeutics remains an emerging clinical field with the leading METTL3 inhibitor, STC-15, in clinical trials.
- Future research is needed to determine the clinical applications of epitranscriptomics to improve patient outcome.

combination with aromatase inhibitors [10]. CDK4 and CDK6 are required for the transition into S-phase of the cell cycle so when inhibited trigger cell cycle arrest in the G1-phase inhibiting tumor growth [19]. The use of CDK4/6 within the clinic and potential for combination therapies was recently reviewed by Yarlagadda and colleagues [20].

For HER2 positive BCa, monoclonal antibody and antibody-drug conjugates (ADCs) targeting HER2 function are utilized [9,10,14]. ADCs allow for the targeting of toxic payloads, for example, trastuzumab deruxtecan is a HER2 targeting antibody conjugated to chemotherapeutic drug. Patients with HER2 positive BCa can also receive a tyrosine-kinase inhibitor (e.g., tucatinib) [10].

There is currently a critical clinical need to develop improved treatments for TNBC, given that TNBCs lack the hormone receptors and HER2 that common BCa treatments target. There is considerable interest in targeting distinct molecular pathways including DNA repair and the cell cycle. Poly (ADP-ribose) polymerase 1 (PARP1) is involved in DNA-damage repair of single stranded breaks and when inhibited in cancers with a homologous recombination repair (HRR) deficiency, results in the accumulation of DNA double stranded breaks and induced synthetic lethality [21–23]. PARP inhibitors, such as olaparib, have been shown to be effective in tumors with *BRCA1* and *BRCA2* germline mutations [22]. Talazoparib, another PARP inhibitor, is also given to patients with HER2-negative, locally advanced or metastatic BCa with germline *BRCA1* or *BRCA2* mutations following progression with second-line therapy [10,14]. Resistance to PARP inhibitors can arise due to multiple mechanisms as reviewed previously [24].

Recent advances in treatments that benefit patients with TNBC include immune checkpoint inhibitors and ADCs. The binding of PD-L1, which can be expressed by cancer cells, to the PD-1 receptor on T-cells results in the suppression of immune response, thus blocking this interaction can enhance anti-tumor immune activity [25]. Pembrolizumab, an anti – PD-1 monoclonal antibody, improved outcomes in both early and advanced TNBCs and is recommended for subsets of patients (clinicaltrials.gov identifier: NCT03036488 and NCT02819518) [9,14,26,27]. Sacituzumab govitecan, an ADC targeting cell surface antigen TROP-2, has provided clinical benefit to patients with metastatic TNBC [28]. Also offering promise in TNBC are PI3K/AKT/mTOR signaling inhibitors. PI3K/AKT/mTOR inhibitors include the PI3Kα inhibitor Alpelisib and AKT inhibitor capivasertib that have been approved for hormone receptor positive BCa and have ongoing clinical trials to assess their benefit for TNBC (clinicaltrials.gov identifier: NCT04251533, NCT03997123) [10,14].

Despite several therapeutic avenues available in a subset of BCa, novel therapies that target epitranscriptomic regulators could benefit patients that are underserved due to limited treatment options. Whilst genetic and epigenetic contributions to BCa risk have been well established, there is now increasing evidence that epitranscriptomic mechanisms affecting covalent modifications of RNA are also dysregulated in cancer. The regulators of the epitranscriptomic modifications may represent potential novel therapeutic targets for BCa treatment. This review gives an overview of epitranscriptomic regulators and the role they play in BCa.

2. The role of epitranscriptomics in BCa

Messenger RNA (mRNA), transfer RNA (tRNA), ribosomal RNA (rRNA), micro RNAs (miRNAs), long non-coding RNA (lncRNAs) as well as mitochondrial RNA all undergo covalent chemical modifications. The understanding of the biological and clinical significance of these epitranscriptomic modifications is rapidly increasing. Epitranscriptomic modifications are dynamically regulated, and are added by enzymatic writers, and removed by distinct enzymes, termed erasers. The resulting chemical modification of RNA influences its interaction with proteins, termed readers, which impact the processing of the RNA. This review focuses on the function of the most common and best understood modifications N^6-methyladenosine (m^6A), N^6-2′-O-methyladenosine (m^6A_m), 5-methylcytosine (m^5C), N^7-methylguanosine (m^7G) and N^1-methyladenosine (m^1A), their regulatory proteins and their associations with BCa (Figure 1).

2.1. m^6A function, regulators, and association with BCa

The methylation at the N^6 position of adenosine (m^6A) (Figure 1A) is an abundant internal mRNA modification in eukaryotic cells [29]. m^6A is evolutionarily conserved in eukaryotes and predominantly occurs on the GG(m^6A)C consensus sequence [29,30]. The presence of m^6A influences the processing of several RNAs including mRNA, miRNA and lncRNAs [30–32]. Within mRNAs, m^6A modifications are enriched near stop codons and in 3′ untranslated regions (UTRs) [30,31]. There is now considerable interest in m^6A in human diseases including cancer due to its crucial roles in gene regulation through splicing, RNA stability, and translational regulation [33–35]. Depending on the type of RNA species, the addition of m^6A is performed by specific methyltransferases as discussed later. There are different regulators for the m^6A modification dependent on the target RNA species.

2.1.1. Writers of m^6A

Methyltransferase-like 3 (METTL3) and METTL14 form the heterodimeric component of the RNA methyltransferase complex (MTC), which adds a methyl group to adenosine. METTL3 contains a S-adenosylmethionine (SAM)-binding domain and catalyzes the transfer of the methyl group from SAM to RNA adenosine [36]. METTL14 is reported to have little/no intrinsic methyltransferase activity but has a structural role in substrate

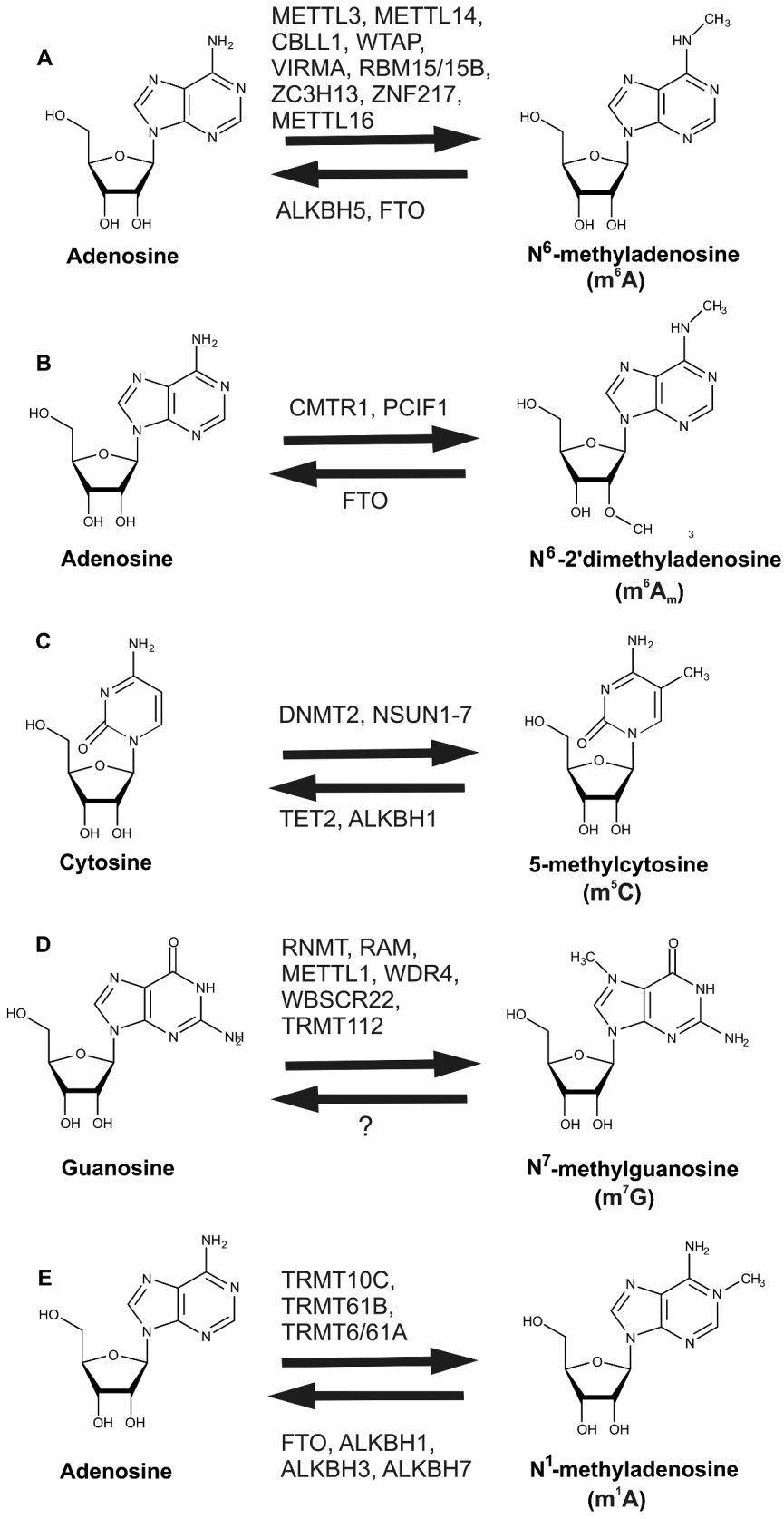

Figure 1. Structure of epitranscriptomic modifications. Structure of the epitranscriptomic modifications discussed in this review (A) N^6-methyladenosine (m^6A) and (B) N^6-2′-O-methyladenosine (m^6A_m), (C) 5-methylcytosine (m^5C), (D) N^7-methylguanosine (m^7G), (E) N^1-methyladenosine (m^1A). The arrow from left to right indicates the methyltransferases (writers) and the arrow from right to left indicates the demethylases (erasers) for each RNA modification.

recognition [37]. In BCa, METTL3 is upregulated and drives cancer growth via several different mechanisms [38,39]. METTL3 and m^{6}A regulated alternative splicing of tumor associated transcripts which may promote breast tumorigenesis, although further work is needed to determine the biological role of these splice variants [39]. This splicing was regulated both directly, through m^{6}A modification of these BCa associated mRNAs, and indirectly, through the m^{6}A regulation encoding splicing factors and transcriptional regulators such as c-MYC [39]. The study reported that m^{6}A deposition often occurred near splice junction sites, which supports findings in other studies, and that METTL3 depletion predominantly resulted in increased exon inclusion [39].

There is emerging evidence that METTL3 can promote treatment resistance in BCa. In the MCF-7 BCa cell line, m^{6}A has been implicated in promoting the maturation of *miR-221-3p* conferring resistance to adriamycin/doxorubicin chemotherapy and promotion of tamoxifen resistance through the regulation of AK4 [40,41] Consistent with these studies, METTL3 and m^{6}A have been associated with DNA damage repair and promoted doxorubicin and cisplatin resistance [42,43]. In addition, METTL3 was found to have a protective role in hormone receptor positive and HER2 negative BCa and in an m^{6}A-dependent manner regulated the CDKN1A/EMT and BAX/caspase3/8/9 axes [44]. In contrast to previous studies, they also suggest that reduced METTL3 expression may have a role in chemoresistance in hormone receptor positive, HER2 negative BCa [44].

METTL3 has also been implicated in BCa metastasis. Reduced METTL3 expression promoted a metastatic phenotype in TNBC cell lines and was associated with shorter overall survival in patients with TNBC [45]. It was proposed that the m^{6}A mediated upregulation of COL3A1 is a key effector in promoting metastasis [45]. In contrast to this, other studies have associated the increased m^{6}A methylation of *KRT7*, *GPRC5A* and *PRMT7* by METTL3 as drivers of metastasis [46–48]. The acetylation of METTL3 has been shown to result in METTL3 retention in the cytoplasm, reducing both m^{6}A transcript methylation and lung metastasis [49]. This suggests that the nuclear m^{6}A catalyzing role of METTL3 promotes metastasis over the cytoplasmic translational role of METTL3.

METTL3 mediated m^{6}A methylation has also been implicated in tumor immune response in BCa. METTL3 inhibition increased cellular dsRNA which stimulated an anti-cancer cell-intrinsic interferon response in the mouse mammary triple negative tumor cell line AT3 [50]. Furthermore, pretreatment of cancer cells with a METTL3 inhibitor prior to co-culture augmented antigen-dependent T-cell killing *in vitro*. A greater anti-tumor response *in vivo* was observed when a METTL3 inhibitor was given alongside anti-PD1 as compared with anti-PD1 alone [50]. METTL3 has been proposed to m^{6}A methylate *PD-L1* mRNA contributing to transcript stabilization and increased protein expression [51]. A positive correlation between METTL3 and PD-L1 expression in BCa tissue was observed in a patient cohort, further supporting a role in tumor immune evasion [51].

Both tumor suppressor and tumor promoting roles have been reported for METTL14 in BCa. These studies also reported conflicting findings on the differential expression of METTL14 in nonmalignant breast tissue compared to BCa tissue. Some studies reported METTL14 was elevated in BCa [52,53], whereas others reported reduced METTL14 expression in BCa [54,55]. A limitation of these studies however was their small patient cohort size.

WT1 associated protein (WTAP) has been shown to recruit several adapter proteins to the methyltransferase complex including VIRMA, RBM15, and ZC3H13 [56]. WTAP interaction with METTL3 and METTL14 is required for the localization and accumulation of these two MTC components into nuclear speckles [57]. WTAP expression was also significantly elevated in BCa compared with nonmalignant breast tissue [58]. Furthermore, WTAP promoted BCa cell migration and N-cadherin and vimentin expression, indicating a role in epithelial-to-mesenchymal transition (EMT) [59].

Cbl proto-oncogene like 1 (CBLL1) was originally identified as an E3 ubiquitin ligase, playing a role in E-cadherin degradation [60]. Later, CBLL1 was found to be involved in m^{6}A regulation as part of the METTL3-METTL14-WTAP complex [56]. In BCa cells, CBLL1 was found to directly bind ER alpha (ERα), competing with ER coactivators and inhibiting the ERα transcriptional activity [61]. Elevated CBLL1 expression in BCa has been associated with better prognosis [62]. In contrast, in BCa cell lines, due to its role in E-cadherin degradation, increased CBLL1 has been associated with promoting invasion and metastasis [63,64].

Vir-like m^{6}A methyltransferase associated protein (VIRMA) has been found to mediate mRNA m^{6}A methylation in 3′UTR and near the stop codon [65]. It is associated with the CPSF5 and CPSF6 polyadenylation cleavage factors in an RNA-dependent manner [65]. Increased VIRMA expression was observed in BCa tumor tissue compared to nonmalignant tissue and was also shown to be present in the cytosol, despite its known nuclear function [66]. This mis-localization of VIRMA was found to be involved in BCa cell proliferation, migration, and invasion via its role in stabilizing *HAS2* mRNA expression in the cytosol [66]. The Cancer Genome Atlas – Breast Invasive Carcinoma (TCGA-BRCA) cohort revealed several *VIRMA* gene amplifications in BCa (Figure 2).

RNA-binding motif protein 15 (RBM15) and its paralogue RBM15B, are RNA binding proteins that recruit other methyltransferase components to guide m^{6}A deposition [67]. Increased RBM15 expression has been observed in BCa tissues and cell lines [68,69]. RBM15 has demonstrated regulation of serine and glycine metabolism in basal-like BCa and TNBC and increased RBM15 has been associated with poor prognosis due to this reprogramming of cancer metabolism [68].

Zinc finger CCCH-type containing 13 (ZC3H13), and Zinc finger protein 217 (ZNF217) are zinc-finger containing components of the MTC [70]. Evidence from *in vivo* mouse studies indicated Zc3h13 functions to retain the Zc3h13-WTAP-VIRMA-CBLL1 complex in the nucleus [70]. ZC3H13 expression was lower in BCa, and was negatively correlated with overall survival (OS) and progression-free survival (PFS) of BCa patients [55]. ZNF217 is a putative oncogene. In BCa, ZNF217 expression stimulated migration and invasion *in vitro* and was reported to be a marker of poor prognosis [71]. In embryonic

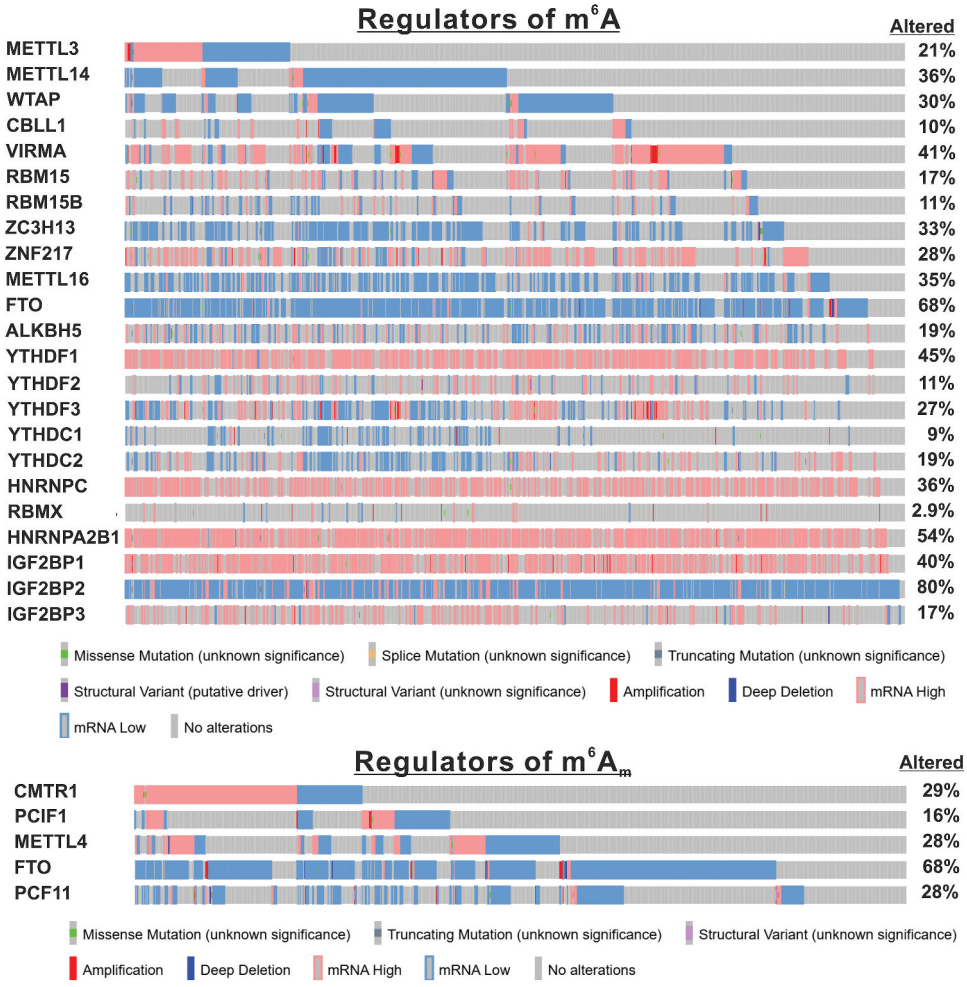

Figure 2. Genetic alterations in m^{6}A and m^{6}A$_m$ regulators. Cbioportal was used to identify the genetic alterations in m^{6}A and m^{6}A$_m$ regulators. The TCGA PanCancer Atlas Breast Invasive Carcinoma cohort ($n = 1084$), including subtypes Breast Invasive Mixed Mucinous Carcinoma, Breast Invasive Carcinoma (NOS), Breast Invasive Lobular Carcinoma and Breast Invasive Ductal Carcinoma was obtained from cBioPortal [250,251]. Plot displays genetic alterations obtained from mutation, structural variant, putative copy-number alteration and mRNA expression data. mRNA expression z-scores relative to normal samples (log RNA Seq V2 RSEM) were selected with a z-score threshold of ±2.0.

stem cells, Zfp217 (mouse homolog to human ZNF217) interacted with METTL3 to inhibit the m^{6}A methylation of the *Nanog mRNA*, resulting in upregulation of the Nanog pluripotency marker in ES cells and BCa [72,73].

Unlike the METTL3:METTL14 methyltransferase complex which preferentially modifies mRNA, the METTL16 m^{6}A methyltransferase is believed to selectively modify U6 small nuclear RNA (snRNA) and other non-coding RNAs, and has been found to regulate SAM biosynthesis and homeostasis [74,75]. METTL16 has been observed to regulate the splicing of SAM synthetase pre-mRNA *MAT2A* [74,76]. In the TCGA-BRCA cohort *METTL16* was altered in 11% of tumors, showing copy number variations of deletions and amplifications (Figure 2). Wang and colleagues reported METTL16 was overexpressed in BCa tissue at both mRNA and protein levels [77]. In BCa, METTL16 regulated *FBXO5* mRNA stability in an m^{6}A-dependent manner [77]. FBXO5, a cell cycle regulation factor, subsequently increased the proliferation and invasion in METTL16 knockdown cell lines [77]. FBXO5 has also been found to be overexpressed in BCa tissue and associated with poorer prognosis [77,78].

2.1.2. Erasers of m^{6}A

To date, two m^{6}A demethylases have been identified, (i) fat mass and obesity-associated protein (FTO) and (ii) α-ketoglutarate – dependent dioxygenase alkB homolog 5 (ALKBH5) [79,80]. FTO was originally found to be associated with obesity in humans, later studies showed it demethylates m^{6}A [79,81]. FTO expression was found to be increased in BCa and correlated with decreased survival [82,83]. It has been proposed that this was due to the negative regulation of pro-apoptotic and tumor-suppressor gene *BNIP3* [82]. Another study suggested FTO promoted ARL5B driven cell invasion and migration through the regulation of *miR-181b-3p* [83]. FTO knockdown resulted in the inhibition of the C/EBPβ-LIP oncogene expression, which can act as an oncogene and regulate EMT genes in TNBC [84]. C/EBPβ-LIP correlated with ER/PR expression and with high Ki-67 proliferative index [84–86]. Under hypoxic conditions in BCa, FTO has been shown to promote immunosuppression in BCa by enhancing PD-L1 expression via activation of the PDK1/AKT/STAT3 axis [87]. Preclinically, FTO inhibitors have demonstrated suppression of tumor

growth *in vivo*, including in a BCa model [88–90]. Similar to METTL3 inhibition, FTO inhibition has been found to enhance anti-tumor immune response and complement anti-PD1 therapy [89].

In a large well-characterized BCa patient specimen cohort low ALKBH5 protein expression was associated with unfavorable clinical parameters [91]. ALKBH5 knockdown in MDA-MB -231 TNBC xenograft tumors, impaired tumor formation, reduced breast cancer stem cells (BCSC) and decreased metastasis to lungs [92]. In a TNBC cohort, increased ALKBH5 expression correlated with chemoresistance and poor prognosis [93]. Demethylation by ALKBH5 was found to increase forkhead box protein O1 (*FOXO1*) mRNA stability and subsequent doxorubicin (DOX) resistance [93]. This was due to FOXO1 being the master regulator and reducing cellular reactive oxygen species in DOX-resistant TNBC cells [93].

2.1.3. Nuclear readers of m^{6}A

When reader proteins bind to m^{6}A modifications, diverse RNA processing effects are initiated. These reader proteins are classified as nuclear or cytoplasmic readers based on their subcellular location. Nuclear readers YTH N6-methyladenosine RNA binding protein C1 (YTHDC1), and heterogeneous nuclear ribonucleoproteins (HNRNPs), HNRNPA2B1, HNRNPC, HNRNPG have exhibited roles in nuclear export and RNA splicing.

YTHDC1 has been associated with BCa progression through m^{6}A dependent regulation of splicing and metabolism [33,94]. YTHDC1 regulated mRNA splicing by the promotion of exon inclusion in targeted mRNAs and via the recruitment of pre-mRNA splicing factor SRSF3 and blocking SRSF10 mRNA binding [33]. In addition to regulating splicing events, YTHDC1 is also reported to mediate nuclear export of targeted mRNAs [95]. Although *YTHDC1* expression was most frequently low in the TCGA BRCA cohort (Figure 2), YTHDC1 has been reported to promote proliferation and invasion of TNBC so recent efforts to identify and characterize YTHDC1 inhibitors may have application in BCa.

HNRNPC mediated mRNA splicing with interactions between HNRNPC and HNRNPG resulting in changes to gene expression and splicing, particularly exon inclusion [96,97]. In BCa, the repression of HNRNPC inhibited cell proliferation and xenograft growth [98]. HNRNPG (RBMX) binds RNA via RNA recognition motifs in its C-terminal low-complexity region and binds directly to RNA polymerase II (RNAPII) through its Arg-Gly-Gly (RGG) motif [96,97]. This association between HNRNPG and RNAPII resulted in the transcriptome-wide regulation of alternative splicing [97]. Although HNRNPG is not well characterized in BCa, it has mechanistic links to ER, with increased *HNRNPG* levels associated with improved clinical outcome in endometrial cancer [99]. HNRNPA2B1 interacts with m^{6}A and is thought to influence pri-microRNA processing and alternative splicing [100]. HNRNPA2B1 has been linked to tumor growth and tamoxifen resistance [101]. In BCa tissue, HNRNPA2B1 expression was significantly higher than in normal breast tissues [102]. These expression levels significantly correlated with tumor, node, metastasis (TNM) stage, therefore indicating poor prognosis in BCa [102].

2.1.4. Cytoplasmic readers of m^{6}A

YTHDF1–3, YTHDC2 and insulin-like growth factor 2 mRNA-binding protein 1–3 (IGF2BP1–3) are cytoplasmic m^{6}A readers which have roles in mediating RNA degradation, stability, and storage. YTHDF1 recruits eIF3 and mediates translation initiation [103]. In BCa YTHDF1 has been found to have a tumor promoting role. YTHDF1 promoted BCa cell growth *in vitro* and *in vivo* and was overexpressed in BCa tissues with higher expression associated with shorter survival [104,105]. YTHDF1 promoted FOXM1 translation and in-turn metastasis in an m^{6}A-dependent manner [105]. In BCa, FOXM1 promoted progression and metastasis as well as having a direct involvement in PI3Kα inhibitor resistance in ER positive BCa [106]. In BCa, YTHDF1 has also been found to facilitate S-phase entry, DNA replication and DNA damage repair [104]. G protein-coupled receptor class C group 5 member A (GPRC5A) was shown to promote the proliferation and migration of TNBC *in vitro* and accelerate growth of xenograft tumors and liver metastasis *in vivo* [47]. GPRC5A was observed to be regulated via the METTL3/YTHDF1 axis [47].

YTHDF2 mediates degradation of selectively recognized mRNAs [107]. For example, YTHDF2 facilitated the recruitment of the CCR4–NOT complex which resulted in de-adenylation and subsequent degradation of mRNA [108]. The m^{6}A mediated destabilization of mRNAs by YTHDF2 is important for tumor cell survival [109]. Consistent with this, BCa cell growth and survival in both cell lines and xenografts was reduced by YTHDF2 depletion, particularly in BCa reliant on elevated MYC expression [110]. Furthermore, YTHDF2 mediated the degradation of CDKN1A through the binding to processing of precursors 1 (POP1), an RNase which localizes in the nucleus and acts in pre-RNA processing [111]. This RNase in turn was observed to degrade CDKN1A [111]. In TNBC, POP1 promoted proliferation by down regulating CDKN1A in an m^{6}A-dependent manner [111].

YTHDF3 is associated with EMT and metastasis. YTHDF3 has been found to interact with YTHDF1 to promote protein synthesis and with YTHDF2 to regulate RNA decay [112]. In TNBC, YTHDF3 expression was associated with poorer survival potentially due to its regulation of EMT transcriptional factor *ZEB1* mRNAs [113]. YTHDF3 has also been associated with promoting BCa brain metastasis by mediating increased expression of EGFR and VEGFA [114].

YTHDC2 is reported to be an RNA-helicase-containing m^{6}A reader which promoted translation efficiency by resolving mRNA secondary structures of coding regions [115]. In BCa, YTHDC2 expression showed a positive correlation with progression [116]. Following the knockdown of YTHDC2 in BCa cell lines, a reduction of the mRNA levels of *SOX2*, *c-MYC* and *NANOG* was observed [116].

IGF2BPs have known roles in tumorigenesis, promoting the stability and storage of target mRNAs in an m^{6}A-dependent manner [34]. Specifically, binding of RNA-binding regulatory peptide (RBRP) to IGF2BP1 specifically targeted *c-MYC* mRNA, which subsequently increased cMYC expression thereby promoting BCa tumorigenesis [34,117]. In TNBC, IGF2BP2 was found to be highly expressed and also promoted the proliferation via the direct regulation of CDK6 in an m^{6}A-directed

manner through the recruitment of eIF4A1 by IGF2BP2 [118]. In BCa, the METTL3-mediated stabilization of *PD-L1* mRNA was m^6A-IGF2BP3-dependent presenting a potential approach to target tumor immune surveillance [51].

2.2. m^6A_m function, regulators, and association with BCa

m^6A_m (Figure 1B) can be found as part of the epicapome on RNAPII transcribed RNAs and at internal sites of non-coding RNAs [119–121]. There is conflicting evidence about the function of m^6A_m on mRNA. It has been shown to promote and repress translation and have a role in mRNA stabilization without affecting translation [122–126]. These contrasting findings may be a result of different experimental design including diverse cell lines used, *in vitro* and *in vivo* experiments, and global mapping methods, this has been reviewed previously [127]. The 5′ region of mRNA is highly modified, and the abundance of these other "epicapome" marks is not easily measured but may confound m^6A_m studies [128].

2.2.1. Writers of m^6A_m

mRNA cap adjacent m^6A_m methylation is only found on the first nucleotide of the transcript [129]. The methylation occurs co-transcriptionally, after the guanine cap has been added and the adenosine has been methylated at the 2′O ribose by cap methyltransferase 1 (CMTR1) [124,130]. A recent study analyzed the CMTR1 levels in a pan-cancer study, for BCa CMTR1 was upregulated compared to nonmalignant tissue, with CMTR1 knockdown leading to growth inhibition and downregulation of ribosomal protein genes [131].

Phosphorylated CTD interacting factor 1 (PCIF1), also known as cap-specific adenosine-N^6-MTase (CAPAM), methylates the nucleotide at the N^6 position in a SAM-dependent manner [123–125,132]. Following pan-cancer analysis, PCIF1 protein expression was observed to have no significant difference between normal and tumor tissues in BCa [133]. However, further studies are needed to identify the role PCIF1 plays in breast cancer.

At internal positions of the U2 snRNA, m^6A_m which is catalyzed by METTL4, has been implicated in pre-mRNA splicing events [134,135]. METTL4 knockout altered splicing with a higher number of alternative 3′ splice sites as compared with 5′ splice sites [135]. The role of METTL4 in BCa remains unknown.

2.2.2. Erasers of m^6A_m

FTO is a m^6A_m demethylase, in addition to demethylating m^6A and m^1A. Studies have suggested that m^6A_m is the preferred substrate of FTO *in vitro* and in the absence of vitamin C. However, FTO does not demethylate internal m^6A_m on U2 snRNAs [81,121,129,136,137].

2.2.3. Readers of m^6A_m

Currently there is only one known m^6A_m reader, the premature cleavage factor II (PCF11). When m^6A_m is present it can sequester PCF11, which functions as a cleavage and polyadenylation factor, preventing premature transcription termination [138,139]. Currently there is limited knowledge on the role of PCF11 in BCa, however *Pcf11* knockout in a mouse mammary tumor-derived cell line (4T1) resulted in reduced migration and invasion [138].

2.3. m^5C function, regulators, and association with BCa

The m^5C modification (Figure 1C) has been detected in many RNA species, including tRNA, mRNA, rRNA and mitochondrially encoded tRNA [140]. Until recently the absence of tools to accurately analyze transcriptome-wide m^5C distribution has limited understanding of the mechanistic and clinical significance of this epitranscriptomic modification. However recently described tools might allow accurate transcriptome wide m^5C mapping [141,142].

2.3.1. Writers of m^5C

Both DNA methyltransferase 2 (DNMT2 or TRDMT1) and NOP/sun methyltransferase 2 (NSUN2) have a role in tRNA methylation and stability through m^5C methylation [143,144]. DNMT also recruited NSUN2 to facilitate m^5C methylation on mRNA [145]. DNMT2 is known to methylate tRNAs and DNMT knockout cells had homologous recombination defects therefore suggesting this writer and m^5C may play a role in DNA damage [146].

NSUN1–7 are involved in m^5C on different species of RNA. NSUN1, also known as NOP2, catalyzes the deposition of the m^5C mark at the C4447 position of 28S rRNA [147,148]. NSUN1 was originally identified as p120, a proliferating-cell nucleolar antigen overexpressed in BCa [147–149]. As compared with nonmalignant breast epithelial cells and tissue, NSUN2 had significantly higher mRNA and protein expression in BCa cell lines and tissue and was associated with poor prognosis [150].

NSUN3 and NSUN4 both function in the mitochondria, with NSUN3 required for the deposition of m^5C in mitochondrially encoded tRNA, and NSUN4 required for the methylation of 12S rRNA [151–153]. NSUN5 functions as a methyltransferase on the 28S rRNA at position C3782 [154,155]. The function of NSUN5 remains unclear in BCa, however, *NSUN5* RNA is highly expressed in the TCGA BRCA cohort (Figure 3). NSUN6 functions on tRNA and mRNA, showing strong substrate specificity primarily targeting the 3′UTR [156–158]. In TNBC lower *NSUN6* expression has been associated with higher grade TNBC (histological grades 3 and 4 compared to normal tissue) and decreased overall survival [159]. Although there was no significant difference in *NSUN6* expression between normal tissue and primary tumors [159]. NSUN7 has been shown to be a close homolog of NSUN2, and when *NSun7* was depleted, this resulted in the loss of m^5C associated enhancer RNAs [160].

2.3.2. Erasers of m^5C

Ten-eleven translocation protein 2 (TET2) can function as an m^5C demethylase on tRNAs which has been shown to promote translation *in vitro* [161]. In the MCF-7 BCa cell line, TET2 has been observed to be overexpressed at mRNA and protein levels [162].

m^5C on the mitochondrial $tRNA^{Met}$ is reported to be oxidized by ALKBH1 to form 5-formylcytidine (f5C) [153]. These

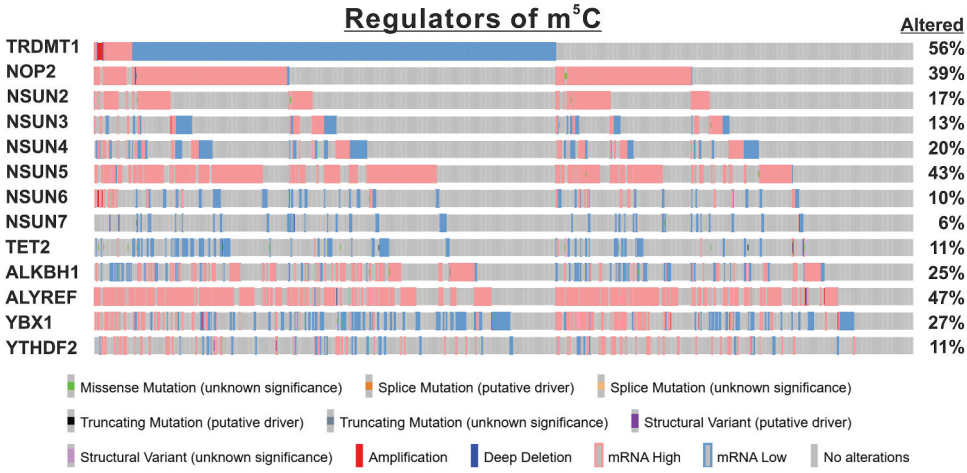

Figure 3. Genetic alterations in m^{5}C regulator. Cbioportal was used to identify the genetic alterations in m^{5}C regulators. The TCGA PanCancer Atlas Breast Invasive Carcinoma cohort ($n = 1084$), including subtypes Breast Invasive Mixed Mucinous Carcinoma, Breast Invasive Carcinoma (NOS), Breast Invasive Lobular Carcinoma and Breast Invasive Ductal Carcinoma was obtained from cBioPortal [250,251]. Plot displays genetic alterations obtained from mutation, structural variant, putative copy-number alteration and mRNA expression data. mRNA expression z-scores relative to normal samples (log RNA Seq V2 RSEM) was selected with a z-score threshold of ±2.0.

modifications were shown to affect mitochondrial translation by altering codon recognition [153].

2.3.3. Readers of m^{5}C

Aly/REF export factor (ALYREF) is an RNA binding protein and m^{5}C reader, which plays an important role in transcriptional regulation and nuclear mRNA export [163–165]. ALYREF was found to be involved in breast tumorigenesis [165]. In BCa tissue ALYREF mRNA expression was observed to be higher as compared to normal breast tissue [164] (Figure 3). Furthermore, knockdown of ALYREF induced apoptosis in TNBC cell lines and reduced growth of xenografts, which alludes to its potential as a therapeutic target in this molecular subtype of BCa [164].

Y-box binding protein 1 (YBX1) belongs to the cold shock domain (CSD) protein family, which was originally identified as a transcription factor and, more recently, shown to be an m^{5}C reader protein [166,167]. In BCa YBX1 has been linked to cell proliferation, changes in metabolism and therapy resistance [168,169]. It was demonstrated that YBX1 and NSUN2 worked together, which resulted in enhanced stability of *HGH1* mRNA, subsequently aiding BCa progression [170]. In BCa, YBX1 also regulated the lncRNA SBF2-AS1 to promote cell proliferation and tamoxifen resistance by regulating PI3K/AKT/mTOR signaling [168]. Furthermore, interaction between YBX1 and WAVE3 in TNBC cells was required for nuclear translocation of YBX1, and transcriptional activation of cancer stem cell-specific genes, which in turn regulated therapy resistance in TNBC [171].

YTHDF2 has also been identified as an m^{5}C reader [172]. Five critical amino acids (Y418, D422, W432, W486, and W491), that are responsible for binding with m^{5}C, have been identified in the RNA binding pocket of YTHDF2 [173].

2.4. m^{7}G function, regulators, and association with BCa

The m^{7}G modification (Figure 1D) can be found at multiple sites within mRNA transcripts both at the 5′ guanine cap as well as in internal AG-rich regions [174,175]. m^{7}G has also been observed on rRNAs and tRNAs [175–177]. Methylation of the cap has an important role in the translation of mRNA and has been well studied [175,178,179]. Internal m^{7}G methylation on mRNA has been linked to regulatory roles in mRNA translation [180]. Similarly, the m^{7}G modification has been found to play a key role in the stabilization of tRNA$^{Val(AAC)}$ [181]. There are many proteins that interact with the guanine cap including the cap binding complex (CBC), eIF4A, and several of the methyltransferases of the epicapome including CMTR1 (HMTr1) and PCIF1 [124,125,182–185]. There are currently no identified erasers for m^{7}G.

2.4.1. Writers of m^{7}G

RNA guanine-7 methyltransferase (RNMT) is the methyltransferase responsible for the modification associated with RNA guanine-7 methyltransferase activating subunit (RAM or RAMAC) [186–188]. Activated T-cells upregulated RNMT, increasing translational efficacy of various RNAs (mRNA, rRNA and small nucleolar RNAs (snoRNAs)) via the m^{7}G cap to drive ribosome biogenesis [189]. RNMT was shown to be the catalyst of the stable mRNA methyltransferase complex RNMT-RAM, which methylates the guanine cap at the N(7) position, promoting mRNA maturation [190]. A subset of BCa cell lines displayed a reduction in proliferation and increased apoptosis when RNMT was experimentally reduced by 50% [191]. Most of these cell lines also exhibited *PIK3CA* mutations as well as an increase in PI3K p110α activity [191]. This suggested that RNMT may prove a promising target for BCa patients who harbor mutant PIK3CA [191]. RAM is the regulatory subunit of RNMT-RAM methyltransferase complex promoting cap maturation and maintains mRNA levels for translation and cell survival [188,190,192]. Inhibition of RAM *in vivo* resulted in a reduction of methyl caps found on endogenous cellular transcripts [188].

Internal m^{7}G methylation on mRNAs and tRNAs are catalyzed by METTL1, for the methylation of tRNAs, METTL1 and

the scaffold protein WD repeat domain 4 (WDR4) cooperate to act as an m^7G methyltransferase [175,193]. Conflicting evidence has been described as to whether METTL1 may also mediate m^7G in miRNA [176,194]. METTL1 and WDR4 were both reduced in BCa tissues at both the protein and mRNA levels, suggesting a tumor suppressor role in BCa [195]. In contrast, WDR4 was upregulated in all BCa subtypes but had the highest expression in TNBC [196]. WDR4 promoted BCa tumor progression via the regulation of mTORC1 signaling and its involvement in cell cycle pathways [196]. Knockdown of WDR4 decreased proliferation and migration of BCa, and this inhibition significantly impaired BCa progression both *in vitro* and *in vivo* [196]. METTL1 has been shown to have high mRNA expression in the TCGA BRCA cohort (Figure 4).

On rRNAs, m^7G is catalyzed by Williams-Beuren syndrome chromosomal region 22 (WBSCR22) regulated by its stabilizing interaction with tRNA methyltransferase activator subunit 11–2 (TRMT112) [197]. WBSCR22 (BUD23) is an S-adenosyl-L-methionine-dependent methyltransferase observed to be required for ribosomal biogenesis and the methylation of m^7G in 18s rRNA [198]. WBSCR22 was also shown to play a role in 40S ribosome synthesis, independent of its m^7G methyltransferase activity [199]. Whilst WBSCR22 was highly expressed in BCa, knockdown mouse models resulted in reduced cell migration and metastatic formation [200]. WBSCR22 expression also negatively correlated with the tumor suppressor Zac1 [200]. WBSCR22 suppressed Zac1/p53-dependent apoptosis via histone H3 methylation at Lys (9) in the Zac1 promoter region, resulting in decreased apoptosis within the vasculature which consequently enhanced the metastatic progression of BCa [200].

TRMT112 was found to be an activator of both rRNA/tRNA and protein methyltransferases. TRMT112 and WBSCR22 are observed to form a heterodimeric methyltransferase complex, stabilizing the metabolic activity of WBSCR22, as well as functioning in the pre-rRNA processing for the synthesis and consequent methylation of 18s rRNA at the G1639 position [198]. TRMT112 expression has been upregulated in many tumor types which is consistent with findings in BCa (Figure 4) [201]. A positive correlation was observed between TRMT112 expression and tumor-related immune infiltrates in BCa including tumor-related fibroblasts, dendritic cells, CD8+ and CD4+ T-cells, macrophages, neutrophils, and B-cells [201]. Interactions between TRMT112 and tumor-infiltration immune cells might influence BCa tumorigenesis and hence influence the survival of patients [201]. TRMT112 was also positively linked to SART1 expression which is essential for BCa cell division [201,202].

2.4.2. Readers of m^7G

Two of the best studied m^7G readers are eIF4E and CBC. eIF4E, a eukaryotic initiation factor, is a reader of m^7G located at the cap structure at the 5′ end of mRNA and is essential for m^7G-dependent translation [203]. eIF4E mRNA expression was observed to be higher in the TCGA-BRCA cohort (Figure 4). Given their co-location, cap-adjacent modifications (i.e., the epicapome) may influence these interactions, a phenomenon supported by recent works [204]. In BCa, high expression of eIF4E was observed to enhance the translation of ERα as well as having a significant association with tamoxifen resistance [205]. Ribavirin has been shown to inhibit eIF4E-dependent Akt survival signaling by competing for m^7G cap binding [206].

The CBC consists of two proteins, NCBP1 and NCBP2, which play key roles in gene expression via nonsense-mediated decay, mRNA splicing, 3′-end processing and nuclear export [207,208]. In BCa, NCBP1 played a role in the infiltration of several immune cells [209]. In several cancers, excluding BCa, high *NCBP2* expression was correlated with poorer overall survival, although increased expression of *NCBP2* was observed in BCa compared to normal breast tissue [208]. Furthermore, NCBP2 was also associated with immune pathways, showing a positive correlation with diverse immune cells [208].

2.5. m^1A function, regulators, and association with BCa

The m^1A modification is positioned at the first nitrogen of the modified adenosine (Figure 1E) [210]. On mRNA, m^1A is typically enriched in the 5′ UTR region as well as being situated near translation initiation sites [211,212]. m^1A can be found on tRNA, rRNA, lncRNA, mitochondrial RNAs but rarely on mRNAs [213–218].

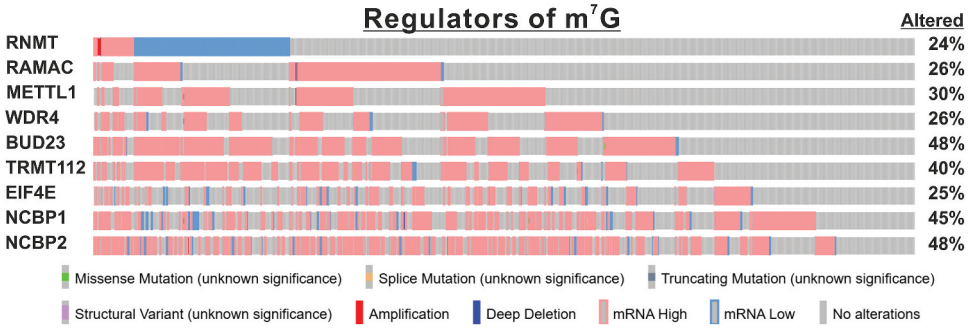

Figure 4. Genetic alterations in m^7G regulators. Cbioportal was used to identify the genetic alterations in m^7G regulators. The TCGA PanCancer Atlas Breast Invasive Carcinoma cohort ($n = 1084$), including subtypes Breast Invasive Mixed Mucinous Carcinoma, Breast Invasive Carcinoma (NOS), Breast Invasive Lobular Carcinoma and Breast Invasive Ductal Carcinoma was obtained from cBioPortal [250,251]. Plot displays genetic alterations obtained from mutation, structural variant, putative copy-number alteration and mRNA expression data. mRNA expression z-scores relative to normal samples (log RNA Seq V2 RSEM) was selected with a z-score threshold of ±2.0.

2.5.1. Writers of m^1A

tRNA methyltransferase 10C (TRMT10C) m1A modifies mitochondrial mRNA and TRMT61B m1A modifies mitochondrial tRNA and mitochondrial 16S rRNA [214,219,220]. TRMT10C, originally termed mitochondrial RNase P protein 1 (MRPP1), has been shown to catalyze the m^1A site on the mitochondrial gene *ND5* [214]. TRMT61B has been demonstrated to form a homo-oligomer responsible for m^1A modification in tRNAs [219].

TRMT61A and TRMT6 have been shown to work together in a complex and function on mRNA and tRNA [214]. The TRMT6/61 (TRMT6-TRMT61A) complex is known to methylate adenosine 58 of the initiator methionine tRNA [221]. This tRNA was crucial for the translation-initiation process and therefore when TRMT6/61 was depleted, decreased proliferation was detected [221]. Furthermore, PKCα, a serine/threonine kinase, correlated with tumor aggression and treatment resistance [221]. In the TCGA BCa cohort, high *TRMT6* and *TRMT61A* mRNA expression were common in BCa (Figure 5). TRMT6 and TRMT61A have been observed to contribute to liver tumorigenesis [222].

2.5.2. Erasers of m^1A

Several erasers of m^1A have been reported; FTO and ALKBH1 which both demethylate m^1A on tRNA, ALKBH3 which functions on mRNA, and ALKBH7 which functions on mitochondrial tRNA [81,217,223,224]. As noted earlier, FTO can demethylate m^6A and m^6A_m, but it has also demethylated m^1A in the tRNA context [81]. In these tRNAs, FTO was able to mediate both nuclear and cytoplasmic m^1A demethylation [81].

ALKBH1 was found to be a tRNA demethylase in both the cytoplasm and mitochondria [223,225]. ALKBH1 knockdown resulted in elevated $tRNA^{iMet}$ which promotes translation initiation and cell proliferation [223].

ALKBH3 has been found to demethylate m^1A [212], which included m^1A *Aurora A* mRNA, and played a key role in development processes including ciliogenesis and cilia-associated developmental events in vertebrates [224]. In BCa, following the demethylation of m^1A by ALKBH3, the half-life of *CSF-1* mRNA was increased [226]. Increased expression of CSF-1 has been associated with metastasis and poorer survival in BCa [227]. The invasive phenotype of BCa cell line, BT-20, was increased by overexpression of ALKBH3 [226].

ALKBH7 has been found to demethylate m^1A and regulate mitochondrial RNA [217]. Following ALKBH7 depletion, reduced mitochondrial-tRNA and reduced mitochondrial-protein levels were observed, subsequently leading to decreased mitochondrial activity [217].

2.5.3. Readers of m^1A

The YTH family, as described above, are known readers of m^6A, however they have also been found to recognize the m^1A methylation mark [228]. YTHDF1–3 and YTHDC1, but not YTHDC2, were shown to bind directly to m^1A in RNA [228]. Overall, these readers had a stronger binding affinity toward m^6A than m^1A [228]. In the m^1A context, the understanding of the function of these readers and their association, if applicable, to BCa remains unclear.

Furthermore, YTHDF1 recognized the m^1A methylated *SP100A* transcript [229]. In this study, YTHDF1 demonstrated a tumor suppressive role in ocular melanoma, in contrast to the demethylase ALKBH3 that is elevated in ocular melanoma and promotes progression through the same mechanism [229]. With eRF3, YTHDF1 is shown to regulate glycolysis in cancer cells through the m^1A mediated regulation of ATP5D [230]. Following biochemical analysis of YTHDF2 m^1A binding, it has been suggested that YTHDF2 can also recognize and facilitate the degradation of m^1A modified RNA [231]. The two modifications m^1A and -m^6A might also be mechanistically associated. For example, HRSP12 recognition of m^1A promoted the association of YTHDF2 with m^6A to promote the degradation of mRNA [232]. YTHDF3 is a cytoplasmic m^1A reader which can bind directly to the RNA carrying the m^1A modification [233]. In m^1A-mediated function, YTHDC1 was revealed to work

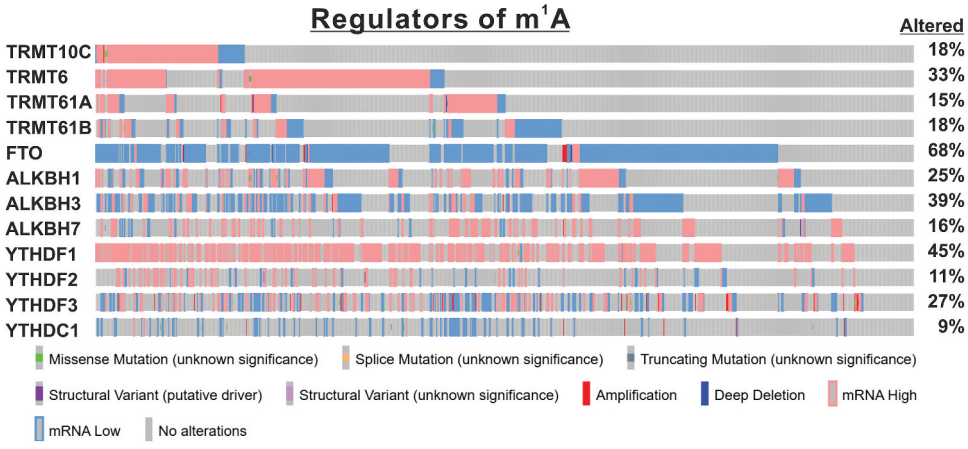

Figure 5. Genetic alterations in m^1A regulator. Cbioportal was used to identify the genetic alterations in m^1A regulators. The TCGA PanCancer Atlas Breast Invasive Carcinoma cohort ($n = 1084$), including subtypes Breast Invasive Mixed Mucinous Carcinoma, Breast Invasive Carcinoma (NOS), Breast Invasive Lobular Carcinoma and Breast Invasive Ductal Carcinoma was obtained from cBioPortal [250,251]. Plot displays genetic alterations obtained from mutation, structural variant, putative copy-number alteration and mRNA expression data. mRNA expression z-scores relative to normal samples (log RNA Seq V2 RSEM) were selected with a z-score threshold of ±2.0.

alongside the THO complex to prevent RNA damage induced DNA breaks [234].

3. Therapeutic potential of the epitranscriptome

The field of epitranscriptomic therapies is emerging and there have been rapid advancements in the biological understanding and clinical relevance of epitranscriptomic modifications in the last 10 years. Research into the feasibility of translating this understanding into the clinic through patient stratification, biomarkers, and therapeutic targeting is an area of intense interest. Current research has focused primarily on identifying inhibitors of m^6A regulator proteins, specifically METTL3 and FTO. Given the development of epigenetic therapy research over the last two decades, as more research into the other epitranscriptome regulator proteins is undertaken, it can be anticipated there will be increased interest in developing additional inhibitors.

m^6A is currently the best characterized RNA modification, and several inhibitors have been developed to target the regulatory proteins [235,236]. Increased METTL3 expression has been reported in many cancers, and in recent years, pharmacological inhibitors have been developed to counteract the pro-oncogenic functions of METTL3 and m^6A. This includes the STM2457 small molecule inhibitor, which has exhibited specificity for the methyltransferase activity of the METTL3-METTL14 complex when screened against many other methyltransferases, and shows no adverse effect on the body weight of mice in the *in vivo* study [235]. STM2457 reduced cell proliferation and migration in TNBC cell lines and enhanced sensitivity to chemotherapy and PARP inhibitors [39,237,238]. A pharmacologically optimized small molecule METTL3 inhibitor, STC-15, recently entered Phase I clinical trials as a monotherapy for patients with advanced malignancies, including BCa patients (NCT05584111). A recent update on this trial suggested any observed adverse effects were manageable. In addition, further clinical trials are currently recruiting to evaluate STC-15 in combination with the anti-PD-1 therapeutic, Toripalimab (NCT06975293).

There is also active investigation of m^6A demethylase inhibitors, in particular for FTO given its putative role in obesity. Rhein, the first identified FTO inhibitor, competitively binds to the nucleic acid binding site in FTO [239]. Since then, a number of other inhibitors for FTO have been identified including Entacapone, a drug long-approved for the treatment of Parkinson's disease [240]. Brequinar and bisantrene, which have other mechanisms of action, were shown to be FTO inhibitors in AML preclinical models [241]. Additionally, a series of small molecule inhibitors targeting FTO, such as FB23, FB23–2 [88] and Dac51 [89] are now developed through continuous drug optimization. Recently an ALKBH5 inhibitor has been investigated preclinically for TNBC [242]. This ALKBH5 inhibitor reduced tumor growth and metastasis of MDA-MB-231 xenografts and demonstrated a favorable tolerability [242].

Recent research has also aimed to identify inhibitors targeting the m^6A readers. These include the small molecule ebselen that inhibits YTHDF proteins, the YTHDF1 inhibitor tegaserod, and YTHDC1 inhibitor YL-5092 [243–245]. YTHDF2 inhibitors are currently being investigated preclinically [173,246].

There are also active programs to develop novel candidate therapeutics targeting other epitranscriptomic enzymes including inhibitors of the DNMT2 and NSUN2 m^5C methyltransferases and the METTL1 m^7G methyltransferase [243,247,248].

Given the complexity of the epitranscriptome and its regulators, and the often disease and context-specific oncogenic functions, further work is required to understand the exact clinical context that epitranscriptomic therapies may offer most benefit. While ongoing epitranscriptomic targeting therapy clinical trials are early stage, results so far have been encouraging, with recent data showing promising therapeutic potential with limited side effects. Further studies are required to determine if resistance to these therapies will emerge in patients. Potential mechanisms of resistance could include functional redundancy of some of the epitranscriptomic regulatory proteins, changes in epigenetics to compensate for epitranscriptomic targeting, or changes in downstream regulatory networks.

4. Crosstalk between epigenetics and epitranscriptomics in BCa

Epigenetic dysregulation has a well-established role in BCa carcinogenesis, progression, drug resistance, and stem cell like characteristics [249–251]. Epigenetic modifications affect chromatin function by modifying genomic DNA and associated histones and are involved in gene expression activation, repression, and silencing. Numerous epigenetic drugs are currently being evaluated clinically for cancer applications. However, to date many have demonstrated limited efficacy as monotherapies in solid tumors and/or resulted in adverse events in patients, as recently reviewed by Rossi and colleagues and Suraweera and colleagues [252,253]. The recently described functional cross talk between epitranscriptomic and epigenetic mechanisms may offer a novel strategy to target epigenetic transcriptional regulation with fewer side effects and higher efficacy.

The best characterized crosstalk is between m^6A and the epigenetic modifications, DNA methylation, and histone modifications [254–256]. There is also evidence to support the concept that m^5C functionally interacts with DNA methylation [257,258]. m^6A regulators interact with the regulators of DNA methylation. The m^6A reader YTHDC2 was found to bind to LTR7/HERV-H primate-specific transposable elements (TE) through its interaction with m^6A methylated HERV-H RNAs. YTHDC2 then recruited TET1 to demethylate the TE enabling transcription [254]. Additionally, a study identified that the m^6A reader YTHDC1 recruited KDM3B to m^6A-associated chromatin regions, promoting H3K9me2 demethylation and gene expression [259].

Emerging evidence also suggests that the epitranscriptomic landscape is encoded by epigenetics. H3K36me3 is recognized and bound directly by METTL14 which guides the co-transcriptional m^6A modification via the METTL3 component of the m^6A MTC [260]. There are several other histone

modifications that crosstalk with m^{6}A, these include H3K4me3, H3K27ac, H3K9me2 and H3K9me3 [255,259,261,262].

The RNA and DNA methylation modulators are known to regulate expression of each other. The ALKBH5 m^{6}A demethylase has been shown to demethylate *DMNT3B* RNA preventing its degradation [263]. Recently, the HDAC2 histone demethylase has been found to deacetylate METTL3 thereby enhancing its methyltransferase activity in TNBC [43].

This epitranscriptomic-epigenetic crosstalk is known to play a role in the development and progression of cancer. In colorectal cancer, lysine-specific histone demethylase 5C (KDM5C) mediated H3K4me3 demethylation resulted in METTL14 inhibition [264]. It is currently unknown whether there is a link between KDM5C and TNBC. In pancreatic cancer m^{6}A methylation of super enhancer RNAs resulted in YTHDC1 recruiting MLL1, promoting H3K4me3 deposition on oncogenes facilitating their transcription [265]. Further studies are needed to identify additional mechanistic, and clinically relevant, links between epigenetics and epitranscriptomics in BCa.

5. Future research and directions

Therapeutically targeting epitranscriptomic regulators is a relatively new and expanding area of research. These modifications and their regulators have been shown to play a role in the development, progression, and treatment resistance in various cancers, with combination therapies offering a potential therapeutic strategy. The development of new technologies will enable a better understanding of the transcriptome-wide distribution and combinatorial function of all RNA modifications and will reveal opportunities for improved therapeutic interventions.

Until recently detection of novel RNA modifications has been a limiting factor. But new developments in mass spectrometry, methylated RNA immunoprecipitation sequencing (MeRIP-seq) and direct RNA sequencing (DRS) are enabling a better understanding of these modifications. A more detailed summary of these detection methods is reviewed in Mongan et al. [266]. Liquid chromatography tandem mass spectrometry (LC-MS/MS) is increasingly used for global quantification of RNA modifications. These approaches have been used in BCa research. Fang and coauthors developed the hydrophilic interaction liquid chromatography tandem mass spectrometry (HILIC-MS/MS) to identify m^{6}A marks in serum samples collected from BCa patients [267]. The ability to detect and map epitranscriptomic modifications in biospecimens and liquid biopsies may enable the identification of patterns of RNA modifications that may hold prognostic potential in cancer and may also inform the development of novel therapeutic strategies.

MeRIP-seq, a commonly used transcriptome wide profiling method, uses an anti-m^{6}A antibody to enrich modified RNA fragments which are then sequenced. The results provide transcript-specific information, however the large amount of input material required has been a limiting factor. However recent advances in low input MeRIP-seq have allowed for the sequencing of primary tissue samples [268,269].

Despite their advantages, LC-MS/MS and MeRIP-seq do not offer insight into the transcriptome-wide distribution of RNA modifications. The development of DRS allows the RNA molecules to be sequenced without the need to convert them into cDNA through reverse transcription [270]. Directly sequencing the RNA molecules provides base level resolution, isoform-specific distribution, and potential heterogeneity of the modification. In addition, RNA-epimodification detection and base-recognition, also known as RedBaron, reveals the methylation status of specific residues using ssDNA oligonucleotides and radiolabeling [271]. This method has revealed that the topology of m^{6}A is crucial for m^{6}A reader proteins to bind [271]. Further research using DRS and RedBaron may offer insights into m^{6}A positioning in BCa which could clinically provide information as a potential biomarker for BCa patient stratification and therapeutic decision making.

Using these technologies, comprehensive studies can be undertaken in BCa specimens to understand the role of the modifications and their regulators in the heterogenous context of BCa and its subtypes. Here the Breast Cancer Gene-Association of Expression Miner v5.2 was used to identify the mRNA expression of the methyltransferases for each RNA modification across different BCa subtypes. All methyltransferases investigated were expressed in all PAM50 BCa subtypes (including TCGA ($n = 743$) and SCAN-B ($n = 3,678$), total ($n = 4421$) datasets, Supplementary Figures S1–S5). However, the expression of the methyltransferases varied between the subtypes. For example *METTL3* expression was significantly higher in luminal B as compared with Basal-like, HER2-E, luminal A and normal breast like (Supplementary Figure S1). mRNA expression of *RAMAC* showed significantly greater expression in luminal B as compared to basal-like, HER2-E and luminal A but showed significantly lower expression compared with normal breast-like (Supplementary Figure S4). A better understanding of the heterogenous landscape of the epitranscriptome, and its cross talk with epigenetics, will aid in prognosis and personalized treatments, and indicate the most effective combinations for different subtypes.

6. Conclusions

BCa remains a critical clinical challenge with a pressing need for novel therapeutics. Epigenetic and epitranscriptomic dysregulation plays a role in BCa development and progression, with epigenetic and, more recently, epitranscriptomic targeting drugs being explored for application in BCa. The understanding of RNA epitranscriptomics is rapidly advancing and targeting epitranscriptomic regulators may provide promising treatment avenues. This is especially evident following the results reported for the STC-15 METTL3 inhibitor clinical trials (clincaltrials.gov identifiers: NCT05584111, NCT06975293).

There is increasing evidence of cross regulation between epitranscriptomic and epigenetic mechanisms. Further research is necessary for the biological and clinically relevant mechanisms to be determined. This will provide the rationale for utilizing epigenetic and epitranscriptomic targeting therapies in BCa.

Disclosure statement

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Funding

The authors gratefully acknowledge the financial support of the BBSRC Doctoral Training Program (BB/I024291/1, BB/T008369/1; to AEH, JLR, RLT, DV, MH, CLW, CSR, JNJ, NPM), the University of Nottingham, and the Stanyard, Moody, and Kenyon families for unrestricted support for cancer research at the University of Nottingham. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript; Biotechnology and Biological Sciences Research Council [BBSRC Doctoral Training Program]; Philanthropic donation [Stanyard, Moody and Kenyon families].

Reviewer disclosures

Peer reviewers on this manuscript have no relevant financial or other relationships to disclose.

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Ethical declaration

The project was reviewed and approved by the local Committee on Animal and Research Ethics at the School of Veterinary Medicine and Science, University of Nottingham (approvals # 2304 180,604; 3075 200,109).

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Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

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