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Pan-cancer analysis of RNA methyltransferases identifies FTSJ3 as a potential regulator of breast cancer progression

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ABSTRACT

RNA methylation, catalysed by a set of RNA methyltransferases (RNMTs), modulates RNA structures, properties, and biological functions. RNMTs are increasingly documented to be dysregulated in various human diseases, particularly developmental disorders and cancer. However, the genomic and transcriptomic alterations of RNMTs, as well as their functional roles in human cancer, are limited. In this study, we utilized an unbiased approach to examine copy number alterations and mutation rates of 58 RNMTs in more than 10,000 clinical samples across 32 human cancer types. We also investigated these alterations and RNMT expression level as they related to clinical features such as tumour subtype, grade, and survival in a large cohort of tumour samples, focusing on breast cancer. Loss-of-function analysis was performed to examine RNMT candidates with important roles in growth and viability of breast cancer cells. We identified a subset of RNMTs, notably *TRMT12, NSUN2, TARBP1*, and *FTSJ3*, that were amplified or mutated in a subset of human cancers. Several RNMTs were significantly associated with breast cancer aggressiveness and poor prognosis. Loss-of-function analysis indicated FTSJ3, a 2′-O-Me methyltransferase, as a candidate RNMT with functional roles in promoting cancer growth and survival. A subset of RNMTs, like FTSJ3, represents promising novel targets for anticancer drug discovery. Our findings provide a framework for further study of the functional consequences of RNMT alterations in human cancer and for developing therapies that target cancer-promoting RNMTs in the future.

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Introduction

RNA modifications, collectively termed the epitranscriptome, change the structural and chemical properties of RNA molecules, regulating their fundamental biological functions [1]. Most of the known RNA modifications are methylations, which add single or multiple methyl groups to one of four canonical RNA bases, the ribose moiety of the sugar backbone of RNA, or the 5′ Cap of mRNA transcripts [2]. RNA methylation can be categorized based on the nature of the RNA molecule modified (e.g., mRNA, tRNA, and rRNA) and the position of the methylation site [e.g. N^6-methyladenosine (m6A) and 2′-O-methylation (2′-O-Me)] [1–3]. Different positions of RNA methylations correspond to distinct chemical properties. The m6A methylation destabilizes pairing with uracil (U) by changing the energetics of the A-U pair through steric hindrance [4]. 2′-O-Me enhances hydrophobicity, protects against nucleolytic attack, and stabilizes RNA helices [5]. RNA methylation plays important roles in RNA metabolism, processing, stability, nuclear export, translation efficiency, and others [1–3,6]. For example, m6A is the most prevalently modified nucleotide in mRNA, and it is required for diverse cellular and physiological processes by regulating mRNA metabolism and gene expression [7–9]. 2′-O-Me is predominantly found in rRNA and tRNA of bacteria and eukaryotes, as well as in the 5′ mRNA Cap of higher eukaryotes [10,11]. Recent studies have suggested that 2′-O-Me is also present at internal sites in mRNA [11,12]. Generally, 2′-O-Me of rRNA regulates ribosome biogenesis and protein translation, while 2′-O-Me of tRNA affects its accurate and efficient decoding ability [11,13,14]. Furthermore, 2′-O-Me at the 5′ cap or internal sites of mRNA has a critical role in the innate immune response against RNA viral pathogens, as most viral RNAs lack this methylation [15]. Thus, accumulated studies demonstrated that functional roles of various RNA methylations are diverse and depend on the location and type of RNA molecule being modified.

RNA methylation is catalysed by a set of enzymes called RNA methyltransferases (RNMTs). RNMTs are S-adenosylmethionine (SAM)-dependent methyltransferases that transfer a methyl group from the cofactor SAM to a substrate [16,17]. More than 50 human RNMTs have been identified [16]. Based on the structure of their catalytic domain, RNMTs can be divided into two general families. The majority of RNMTs belong to the ‘Rossmann-fold’ methyltransferases, along with DNA methyltransferases (DNMTs), protein arginine methyltransferases, and DOT1L (DOT1-like histone lysine methyltransferase) [16,17]. The second family of RNMTs is the SPOUT (SpoU-TrmD) methyltransferase,

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Supplemental data for this article can be accessed here.

which is characterized by a catalytic domain with an unusual α/β fold forming a deep trefoil knot in the structure [18]. Additionally, based on their RNA molecule targets, RNMTs can be grouped as mRNA, tRNA, and rRNA RNMTs, among others. Notably, some of these enzymes may be able to methylate more than one type of RNA substrates, such as NSUN2 (NOP2/Sun RNA methyltransferase 2) that can catalyse the methylation of cytosine to 5-methylcytosine (m5C) of tRNAs, mRNAs, and non-coding RNAs [19–21].

Given the various functional roles of RNA methylation, mutation and dysregulation of several RNMTs have been directly linked to human diseases, especially developmental disorders and cancer [6,22]. Mutations of *FTSJ1* (FtsJ RNA 2′-O-methyltransferase 1) and *NSUN2* cause autosomal-recessive intellectual disability [23–25]. Defective mutation of mitochondrial rRNA methyltransferase *MRM2* (also known as *FTSJ2*) leads to disorders of mitochondrial respiration and MELAS (mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes)-like clinical syndrome [26]. Additionally, RNMTs belong to the superfamily of RNA binding proteins (RBPs) [27,28]. Several recent studies have examined various aspects of genomic aberrations of RBPs in human cancer [29–33]. For example, Neelamraju *et al*. determined the mutational landscape of ~1,300 experimentally confirmed RBPs in ~6,000 cancer genomes [30]. They identified 281 RBP genes to be enriched for mutations in at least one cancer type, five of which are RNMT including *FTSJ3* (FtsJ RNA 2′-O-methyltransferase 3) and *NSUN2*. By using the OncodriveFM approach (a bioinformatics approach to compute driver genes or gene modules), they identified 228 RBP candidate drivers with the majority of them specific to cancer types [30,34]. Among 228 RBPs, two are RNMTs: *NSUN2* in melanoma and uterine corpus endometrial carcinoma (UCEC), and *ALKBH8* (alkB homolog 8, tRNA methyltransferase) in stomach adenocarcinoma [30,34]. Another study performed genomic analysis of 1,542 RBPs in ~7,000 clinical specimens across 15 TCGA cancer types [29]. They revealed 76 potential driver RBPs that displayed copy number alterations. Two of 76 are RNMTs: *TARBP1* [TAR (HIV-1) RNA binding protein 1] in liver cancer and *METTL1* (methyltransferase like 1) in lung adenocarcinoma [29]. However, no previous studies have collectively examined the genomic and transcriptomic alterations of RNMTs as well as their functional roles in human cancer.

In this study, we hypothesized that RNMTs with recurrent genetic alterations might play important roles in cancer progression and can serve as novel therapeutic targets for cancer treatment. We utilized an unbiased approach to examine genetic alterations of 58 RNMTs in more than 10,000 clinical samples across 32 cancer types. We also investigated these alterations and RNMT expression levels as they related to clinical features such as tumour subtype, grade, and survival in a large cohort of tumour samples, focusing on breast cancer. Furthermore, loss-of-function analysis was performed to examine RNMT candidates with important roles in growth and viability of breast cancer cells.

Results

Genetic alterations of RNMTS across 32 human tumour types

To understand the biological importance of RNA methylation in cancer progression and development, it is vital to determine the somatic copy number alteration (CNA) and mutation profiles of RNMTs in different types of human cancer. Based on the current ChromoHub database (http://apps.thesgc.org), 58 RNMTs have been shown or are predicted to be involved in methylation of various types of RNAs at different positions (Table 1 and Supplementary Figure S1) [16,35–74]. We first performed CNA and mutation analyses in more than 10,000 tumour samples across 32 cancer types from the Pan-Cancer Atlas of The Cancer Genome Atlas (TCGA) via cBioPortal (Supplementary Table S1) [75,76]. The copy number for each RNMT was generated by the copy number analysis algorithm GISTIC (Genomic Identification of Significant Targets in Cancer) and categorized according to copy number level per gene. The five categories of gene copy number are high-level amplification, low-level gain, diploid, shallow deletion (possibly heterozygous deletion), and deep deletion (possibly a homozygous deletion) [77]. In the TCGA Pan-Cancer cohort, we found that *TRMT12* was the most frequently high-level amplified (6.25%) followed by *NSUN2, TARBP1, TFB2M, METTL1*, and *TRMT1L*, all between 2–3% (Fig. 1A and Supplementary Table S2). Two RNMT genes, *ELP3* and *TRMT9B*, exhibited homozygous deletions in more than 2% of Pan-Cancer samples (Fig. 1B and Supplementary Table S3).

Next, we performed copy number analysis of each RNMT in 32 TCGA individual tumour types and uncovered a considerable variation of CNA across different tumour types (Fig. 1A,B). We found that 26 RNMT genes were high-level amplified by more than 5% in at least one individual tumour type (Fig. 1A and Supplementary Table S2). Notably, *TRMT12* was amplified in 26.22% of ovarian cancer (OV), *NSUN2* in 12.11% of lung squamous cell carcinoma (LUSC), and *FTSJ3* in 6.26% of breast cancer (BRCA) (Fig. 1A and Supplementary Table S2). Five RNMTs exhibited deep deletion of more than 5% in at least one individual tumour type, and the highest percentage of deep deletion was *TRMT11* (10.42%) in diffuse large B-cell lymphoma (DLBC) (Fig. 1B and Supplementary Table S3).

For somatic mutation, eight RNMTs, including *TARBP1, FTSJ3*, and *NSUN2*, were mutated in more than 1% of the TCGA Pan-Cancer cohort (Fig. 1C and Supplementary Table S4). *TARBP1* demonstrated the highest rate (1.86%) of mutation in the Pan-Cancer cohort, with 179 missense, 20 nonsense, 13 splice, 10 frame-shift, and 7 fusion mutations (Fig. 1C and Supplementary Figure S2). The highest rate of mutation in individual tumour type was *TARBP1* in 8.12% of UCEC samples (Supplementary Table S4). Taken together among 58 RNMTs, several RNMT genes including *TRMT12, NSUN2, FTSJ3*, and *TARBP1* had relatively higher frequencies of genetic alterations in a spectrum of human tumours.

Table 1. List of 58 human RNMT proteins and their methylated RNA type and position.

RNMT	Gene Location	Full Name	RNA Type	Methylation Position	PubMed ID
ALKBH8	11q22.3	ALKB Homolog 8	rRNA/tRNA	mcm5U	20,123,966
BCDIN3D	12q13.12	BCDIN3 Domain Containing RNA Methyltransferase	tRNA/small RNAs (pre-miRNA)		23,063,121
BUD23	7q11.23	BUD23 rRNA Methyltransferase and Ribosome Maturation Factor	rRNA	m7G	18,332,120
CDK5RAP1	20q11.21	CDK5 Regulatory Subunit Associated Protein	tRNA	ms2	22,422,838
CDKAL1	6p22.3	CDK5 Regulatory Subunity Associated Protein 1 like 1	tRNA	ms2	21,841,312
CMTR1	6p21.2	Cap Methyltransferase 1	mRNA	2′Om	20,713,356
CMTR2	16q22.2	Cap Methyltransferase 2	mRNA	2′Om	21,310,715
DIMT1	5q12.1	DIMT1 rRNA Methyltransferase and Ribosome Maturation Factor	rRNA	m6A	25,851,604
ELP3	8p21.1	Elongator Acetyltransferase Complex Subunit 3	tRNA	m5C	15,769,872
EMG1	12p13.31	EMG1 N1-Specific Pseudouridine Methyltransferase	rRNA	m1	20,047,967
FBL	19q13.2	Fibrillarin	rRNA	2′Om	8,431,947
FBLL1	5q34	Fibrillarin like 1	rRNA	2′Om	26,566,070
FTSJ1	Xp11.23	FtsJ RNA 2′-O-Methyltransferase 1	tRNA	2′Om	11,927,565
FTSJ3	17q23.3	FtsJ RNA 2′-O-Methyltransferase 3	rRNA/mRNA	2′Om	30,626,973
HENMT1	1p13.3	HEN Methyltransferase Homolog 1	small RNAs (piRNA)	2′Om	18,029,764
MEPCE	7q22.1	Methlphosphate Capping Enzyme	small RNAs (snRNA)		29,425,494
METTL1	12q14.1	Methyltransferase like 1	tRNA/mRNA	m7G	31,031,084
METTL14	4q26	Methyltransferase like 14	mRNA	m6A	24,316,715
METTL3	14q11.2	Methyltransferase like 3	mRNA	m6A	24,316,715
METTL4	18p11.32	Methyltransferase like 4			26,566,070
MRM1	17q12	Mitochndrial rRNA Methyltransferase 1	rRNA	2′Om	25,074,936
MRM2	7p22.3	Mitochndrial rRNA Methyltransferase 2	rRNA	2′Om	25,074,936
MRM3	17p13.3	Mitochondrial rRNA Methyltransferase 3	rRNA	2′Om	25,074,936
NOP2	12p13.31	NOP2 Nucleolar Protein	rRNA	m5C	23,913,415
NSUN2	5p15.31	NOP2/Sun RNA Methyltransferase 2	tRNA	m5C	17,071,714
NSUN3	3q11.2	NOP2/Sun RNA Methyltransferase 3	tRNA	m5C	27,497,299
NSUN4	1p33	NOP2/Sun RNA Methyltransferase 4	rRNA	m5C	24,516,400
NSUN5	7q11.23	NOP2/Sun RNA Methyltransferase 5	rRNA	m5C	23,913,415
NSUN5P1	7q11.23	NSUN5 Pseudogene 1	rRNA		26,566,070
NSUN5P2	7q11.23	NSUN5 Pseudogene 2	rRNA		26,566,070
NSUN6	10p12.31	NOP2/Sun RNA Methyltransferase 6	tRNA		26,566,070
NSUN7	4p14	NOP2/Sun RNA Methyltransferase Family Member 7			26,774,474
RNMT	18p11.21	RNA Guanine-7 Methyltransferase	mRNA	m7G	27422871
RSAD1	17q21.33	Radical S-adenosyl Methionine Domain Containing 1			26566070
SPOUT1	9q34.11	SPOUT Domain Containing Methyltransferase 1			
TARBP1	1q42.2	TAR (HIV-1) RNA Binding Protein1	tRNA	2′Om	31019095
TFB1M	6q25.3	Transcription Facotr B1, Mitochondrial	rRNA	m6A	17031457
TFB2M	1q44	Transcription Facotr B2, Mitochondrial	rRNA	m6A	17031457
TGS1	8q12.1	Trimethylguaosine Synthase 1	small RNAs (snoRNA/snRNA)	m7G	11983179
THUMPD2	2p22.1	THUMP Domain Containing 2	tRNA		26566070
THUMPD3	3p25.3	THUMP Domain Containing 3	tRNA		26566070
TRDMT1	10p13	tRNA Aspartic Acid Mthyltransferase 1	tRNA	m5C	16424344
TRMT1	19p13.13	tRNA Methyltransferase 1	tRNA	mmG	28784718
TRMT10A	4q23	tRNA Methyltransferase 10A	tRNA	m1A	31292261
TRMT10B	9p13.2	tRNA Methyltransferase 10B	tRNA	m1A	31292261
TRMT10C	3q12.3	tRNA Methyltransferase 10C	tRNA	m1A	29880640
TRMT11	6q22.32	tRNA Methyltransferase 11 Homolog	tRNA	2′Om	15899842
TRMT112	11q13.1	tRNA Methyltransferase Subunit 11-2	rRNA	m7G	22493060
TRMT12	8q24.13	tRNA Methyltransferase 11 Homolog	tRNA		26566070
TRMT1L	1q25.3	tRNA Methyltransferase like 1	tRNA		26566070
TRMT2A	22q11.21	tRNA Methyltransferase 2 Homolog A	tRNA	m5U	31361898
TRMT2B	Xq22.1	tRNA Methyltransferase 2 Homolog B	tRNA	m5C	31361898
TRMT44	4p16.1	tRNA Methyltransferase 44 Homolog	tRNA	2′Om	26566070
TRMT5	14q23.1	tRNA Methyltransferase 5	tRNA	m1A	26189817
TRMT61A	14q32	tRNA Methyltransferase 61A	tRNA	m1A	30131402
TRMT61B	2p23.2	tRNA Methyltransferase 61B	tRNA	m1A	29107537
TRMT9B	8p22	tRNA Methyltransferase 9B	tRNA		23381944
TYW3	1p31.1	tRNA-yW Synthesizing Protein 3	tRNA		27932585

Molecular profiling of RNMT genes in different subtypes of breast cancer

Breast cancer is the leading cause of cancer diagnoses in women, with more than twice the number of new cases than any other individual cancer type [78]. Breast cancer has been classified into five molecular subtypes with distinct risks and underlying biology; these five subtypes are luminal A, luminal B, epidermal growth factor receptor 2–enriched (HER2+), basal-like, and normal-like breast cancers [79,80]. Both luminal A and luminal B breast cancers are oestrogen receptor positive, but luminal B cancers have poorer outcomes [81]. Furthermore, basal-like breast cancer usually occurs in young women and is a highly aggressive subtype associated with very poor prognosis [82]. Well-known breast cancer genes such as BRCA1 and MYC were mutated in 2.53% and amplified in 15.05% of TCGA breast cancer samples, respectively [75,76]. Here, we examined the genetic alteration and expression profiling of each RNMT as they relate to molecular subtypes and other clinical features of breast cancer. In TCGA breast cancer, seven RNMTs, including *TRMT12, TARBP1*, and *FTSJ3*, had high-level amplification in more than 5% of samples (Supplementary Table S2). Next, we analysed copy number, mutation and mRNA expression of RNMTs independently across five subtypes of breast cancer samples.

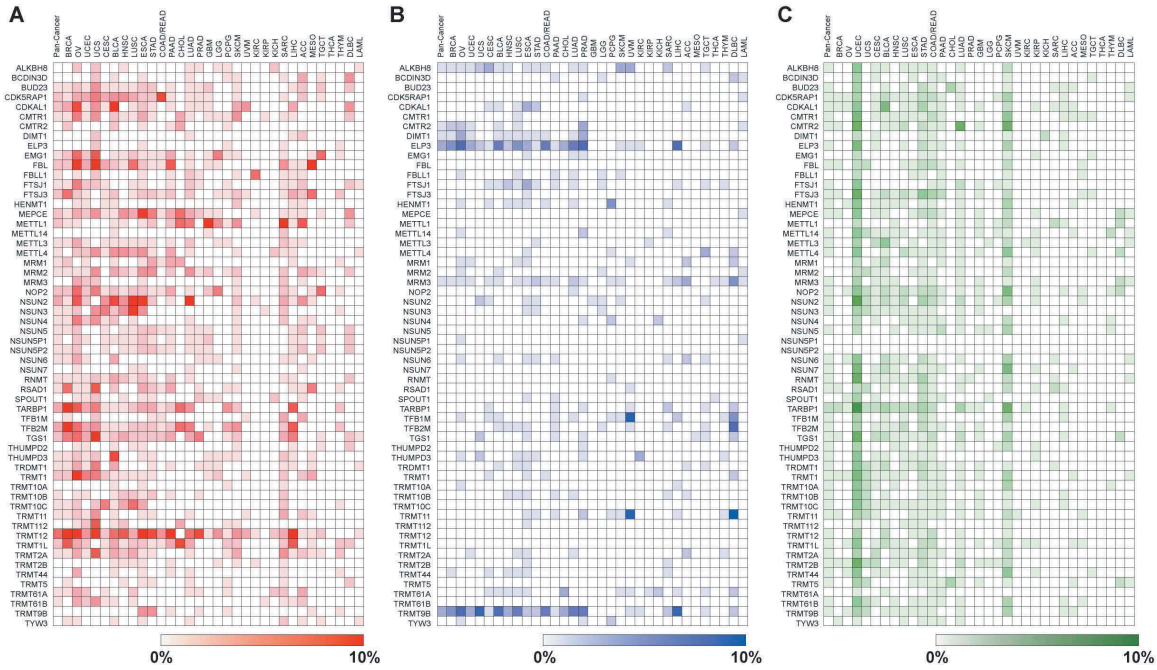

Figure 1. Heatmap showing the frequencies of (A) RNMT amplification (red), (B) deep deletion (blue) and (C) mutations (green) across all 32 TCGA tumour types. Heatmap was generated using Morpheus software from the Broad Institute (https://software.broadinstitute.org/morpheus/) .

The frequencies of high-level amplification, low-level gain, diploid, heterozygous deletion, homozygous deletion, and somatic mutation of 58 RNMT genes in five TCGA breast cancer subtypes are shown in Supplementary Table S5. Remarkably, we found that *TRMT12* exhibited high-level amplification in 27.49% of TCGA basal-like breast cancers. *FTSJ3* exhibited high-level amplification in 5.61% of luminal A, 11.17% of luminal B, and 12.82% of HER2+ breast cancer subtypes. *FTSJ3* also had the highest frequency of mutation (4.09%) in basal-like breast cancer (Supplementary Table S5). When we applied a binomial test to compare mutation rate of RNMTs with the median mutation rate of all genes (0.6%) in basal-like breast cancer, FTSJ3 was the only RNMT with statistical significance [$p < 0.001$ and false discovery rate (FDR) = 0.002].

Next, we analysed the correlation between copy number and mRNA level of 58 RNMTs from TCGA breast cancer specimens. As shown in Supplementary Table S6, all RNMT genes had positive correlations between DNA copy number and mRNA expression, and 23 of them had a Spearman correlation coefficient (r) greater than 0.5, with top three being *ELP3, FTSJ3*, and *TRMT12*. Similar results were also observed in other tumour types, suggesting that CNA may be a major factor in the dysregulation of RNMT mRNA in human cancer, particularly breast cancer. To determine how expression of each RNMT is associated with breast cancer subtypes, we assessed mRNA expression of each RNMT in breast cancer samples with molecular subtype data available. We found that the expression levels of three RNMTs (*FTSJ3, TRMT12*, and *TGS1*) were significantly higher ($p < 0.001$, mean Z score difference > 1.0) in luminal B compared to luminal A subtypes (Fig. 2A and Supplementary Table S7). Notably, *FTSJ3* and *HER2* genes are located within the same chromosome at 17q23.3 and 17q12 respectively, and luminal B breast cancer exhibited higher frequency of HER2 gain/amplification (67.51%) compared to that of luminal A breast cancer (22.65%). FTSJ3 was also highly expressed in HER2+ breast cancer subtype. Furthermore, 13 RNMTs, including *NSUN2, FBL*, and *NOP2*, were expressed significantly higher in basal-like compared to luminal subtype breast cancer (Fig. 2A and Supplementary Table S7).

To validate our findings from the TCGA breast cancer dataset regarding RNMT genetic alterations, we conducted an independent analysis using two additional breast cancer datasets: the METABRIC dataset with 2509 primary samples and the MBC (Metastatic Breast Cancer) with 237 samples [83]. In the METABRIC dataset, we found that seven RNMTs, including *TRMT12, TARBP1*, and *FTSJ3*, had high-level amplification in more than 5% of breast cancer samples. The frequencies of high-level amplification, low-level gain, diploid, heterozygous deletions, and homozygous deletions of 58 RNMT genes in five METABRIC breast cancer subtypes are shown in Supplementary Table S8. Particularly, *TRMT12* was found to be amplified in 40.19% of basal-like, *TARBP1* in 30.29% of luminal A, and *FTSJ3* in 17.05% luminal B of METABRIC breast cancers. We also analysed the frequencies of RNMT amplification, deep deletion and mutation in metastatic samples of breast cancer (MBC cohort). As shown in Supplementary Table S9, *TRMT12* and *FTSJ3* were the most amplified RNMTs, in 21.52% and 19.83% of metastatic breast cancer samples respectively. In summary, genomic profiling of 58 RNMTs in breast cancer revealed amplification of several RNMTs, notably *TRMT12* and *FTSJ3*, with higher frequencies

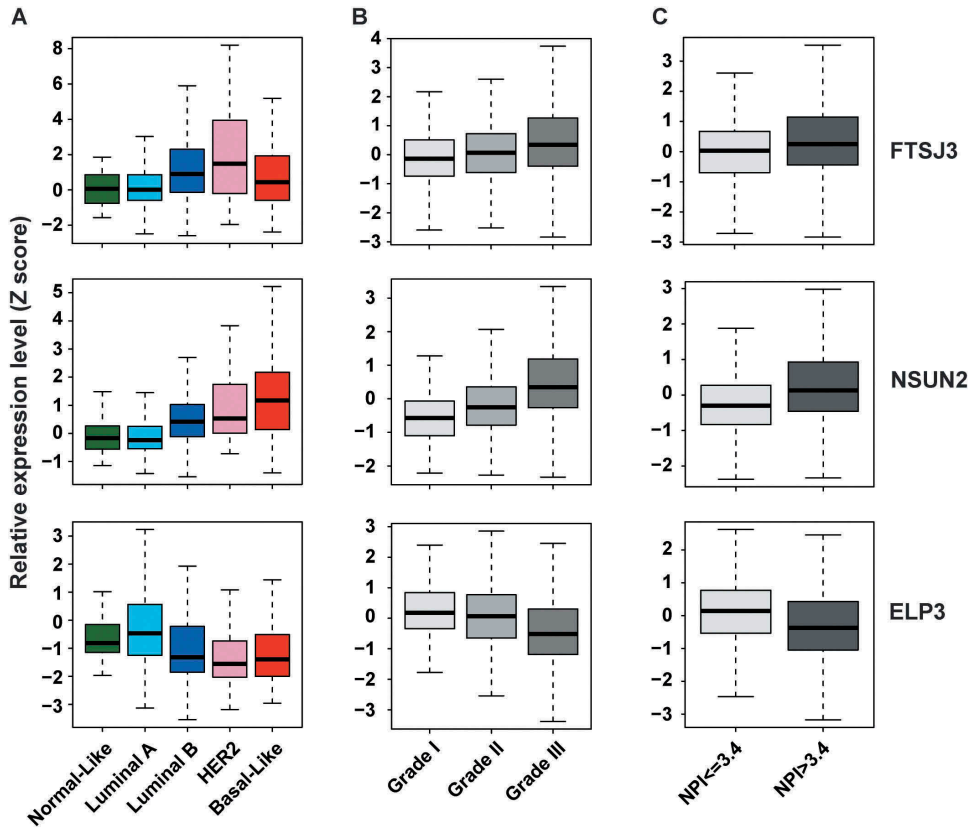

Figure 2. Expression levels of three RNMTs (*FTSJ3, NSUN2*, and *ELP3*) in breast cancer samples with different subtypes, grades, and NPIs. (A) Expression levels of three RNMTs across five subtypes of TCGA breast cancer samples. (B) Expression levels of three RNMTs in three grades of METABRIC breast cancer samples. (C) Expression levels of three RNMTs in METABRIC patients with poor prognosis (NPI > 3.4) and good prognosis (NPI ≤ 3.4) scores. High expressions of FTSJ3 and NSUN2, but lower expression of ELP3, were significantly ($p < 0.001$) associated with higher grade and NPI score of METABRIC breast cancers.

in primary and metastatic breast cancer. Moreover, different subtypes of breast cancer had different patterns of copy number and expression of each RNMT.

RNMT gene expression correlations with clinical breast cancer features

Since the METABRIC cohort contains approximately 2,000 breast cancer samples with histologic grade and long-term clinical follow-up data, we assessed RNMT copy number and expression by clinical features and disease-free survival in this cohort. Gene expression data of 52 RNMTs was available, while data for six RNMT genes, including *TRMT12*, was not available in the METABRIC cohort. We first examined expression levels of each RNMT gene at different grades of METABRIC breast cancer samples. The means of Z-score and *p*-value for each RNMT gene across grades 1–3 are shown in Supplementary Table S10. We found that ten RNMTs, including *FTSJ3, NSUN2, NOP2*, and *FBL*, were significantly highly expressed, while two genes (*ELP3* and *BCDIN3D*) were lower expressed in higher-grade breast cancers (T-test: Grade 3 vs 1 + 2; $p < 0.001$ and means difference > 0.3; Fig. 2B and Supplementary Table S10). The Nottingham Positivity Index (NPI), one of the primary prognostic tools used in assessing breast cancer aggressiveness in Europe, was also available in the METABRIC cohort [84,85]. Thus, we compared expression levels of RNMTs between patients with high NPI (>3.4) versus those with low NPI (≤ 3.4). As shown in Fig. 2C and Supplementary Table S10, eight RNMTs, including *FTSJ3* and *NSUN2*, were significantly over-expressed, while two RNMTs: *ELP3* and *BCDIN3D*, were under-expressed in samples with higher NPI ($p < 0.001$ and means Z-score difference > 0.3) compared with that in lower NPI samples.

Next, we analysed the relationship between RNMT copy number, mRNA expression, and disease-free survival of METABRIC breast cancer patients. We found that copy number increases (Amp and Gain) of seven RNMTs (e.g. *TRMT12* and *FTSJ3*) were significantly associated with shorter disease-free survival of breast cancer patients ($p < 0.001$) (Fig. 3A, Supplementary Figure S3 and Table S11). We also found that higher mRNA levels of four RNMTs (*NSUN2, FTSJ1, NOP2*, and *CDK5RAP1*) and lower expression of three RNMTs (*ELP3, ALKBH8*, and *DIMT1*) were significantly associated with shorter disease-free survival in METABRIC breast cancer patients ($p < 0.001$) (Fig. 3A and Supplementary Table S11). Higher expression of *FTSJ3* was moderately associated with shorter survival in METABRIC breast cancer patients (Fig. 3A). Combined with genetic profiling of RNMTs in breast cancer, our results indicate several RNMTs that might contribute to breast cancer development and progression.

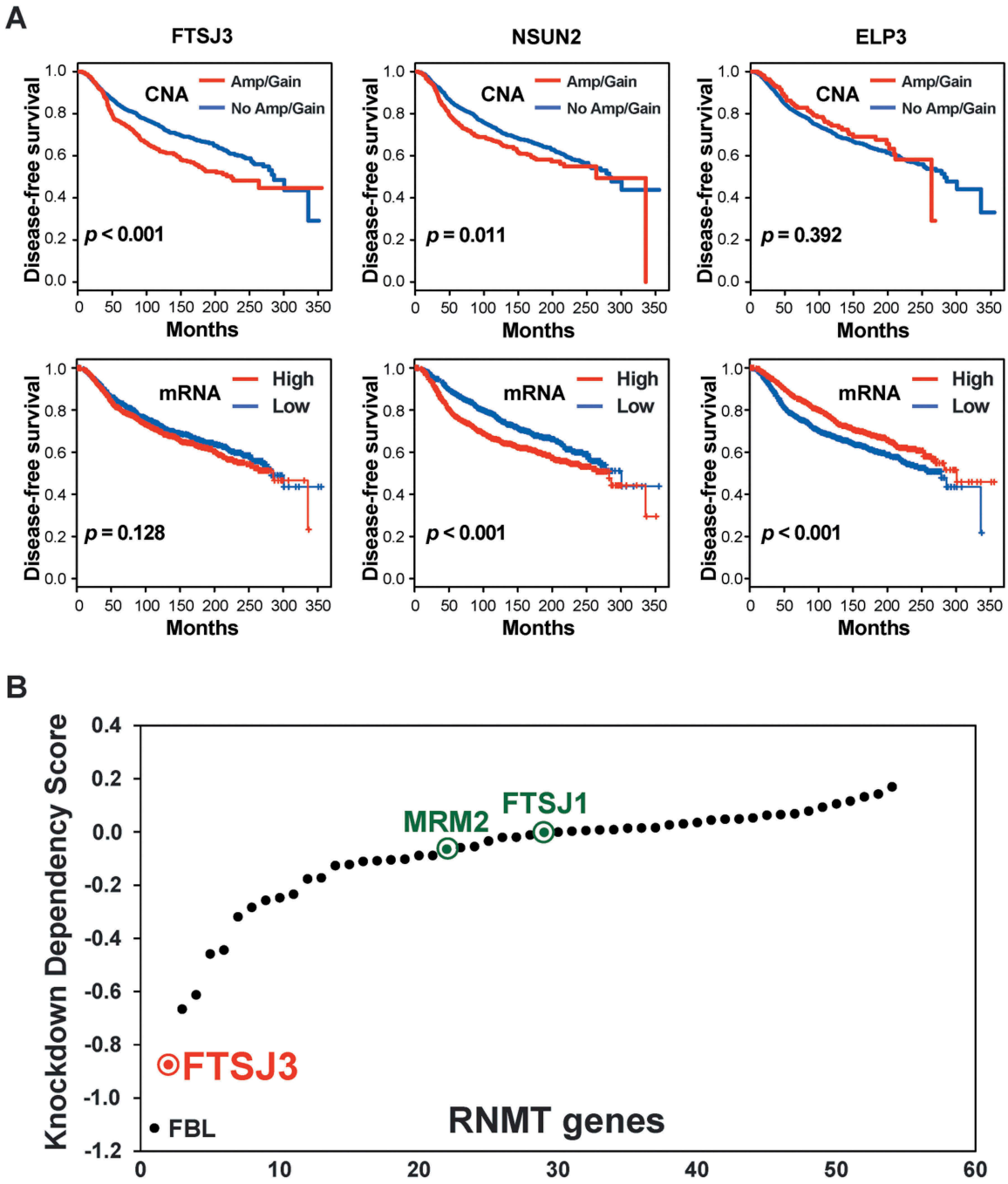

Figure 3. RNMTs associate with disease-free survival of breast cancer and contribute to growth and viability of tumour cells *in vitro*. (A) Kaplan-Meier plots of disease-free survival associated with copy number and mRNA expression levels of three RNMTs (*FTSJ3, NSUN2*, and *ELP3*) in METABRIC breast cancers. (B) Scatter plot showing mean of each RNMT dependency score in genome-scale loss-of-function screens of 712 tumour lines.

Loss-of-function analysis of RNMTS for cancer dependency

The availability of genome-wide loss-of-function screening in a large panel of tumour lines presents new opportunities for understanding cancer vulnerabilities [86,87]. Based on our genotranscriptomic meta-analysis, we hypothesized that several of these dysregulated RNMTs may act as genetic vulnerabilities in various cancers and represent potential therapeutic targets. To investigate potential RNMT dependencies in various tumours, particularly breast cancer, we analysed genome-wide shRNA screen data in 712 tumour lines [86,88]. Accordingly, we ranked average dependency (DEMETER2) scores of 54 RNMTs that were examined in genome-wide shRNA screens of 712 tumour lines [86,88,89]. Examining the impact of the shRNA knockdown of 54 RNMTs revealed that *FBL, FTSJ3, NOP2*, and *EMG1* had the lowest cancer dependency scores among all RNMT genes assessed (Fig.

3B). The lower the score, the more critical that gene is to tumour cell growth and survival. Previous studies revealed that knockdown of FBL inhibits cell proliferation *in vitro* and reduces the frequency of tumour formation and the tumour volumes *in vivo* [90,91]. The second hit is FTSJ3, which together with its homologues FTSJ1 and MRM2 (also known as FTSJ2) are orthologues of bacterial FtsJ/RrmJ, a highly conserved 2′-O- methyltransferase [11,37,54,92]. However, FTSJ1 and MRM2/FTSJ2 had no impact on the shRNA *in vivo* screen of most tumour cells (Fig. 3B). Given that *FTSJ3* is commonly amplified and overexpressed in breast cancer, we performed a more detailed analysis of FTSJ3 shRNA screen data in breast cancer cell lines. Among 81 breast cancer lines, 18 lines have FTSJ3 DEMETR2 scores of less than −1.0. Altogether, this data places FTSJ3 as a candidate RNMT with functional roles in promoting cancer growth and progression.

Knockdown of FTSJ3 decreases cancer cell proliferation in breast cancer cells

In order to find the most suitable cell line models for further work that recapitulates *FTSJ3* amplification and over-expression observed in primary tumours, we examined the copy number and expression of FTSJ3 in 13 breast cancer lines. Inspection of comparative genomic hybridization (CGH) array data that was previously published by us and others revealed *FTSJ3* high-level amplification in SUM52, MCF7, and ZR75-1 luminal subtype breast cancer lines [93–96]. Notably, these three lines were established from pleural and ascetic effusions of patients with metastatic breast cancers, and their cell line data were consistent with that in clinical samples observed in MBC cohort [97–99]. Next, we assessed FTSJ3 levels in breast cancer cell lines by qRT-PCR and western blotting assays. Indeed, we revealed that mRNA and protein expression of FTSJ3 were dramatically higher in a subset of breast cancer cell lines, remarkably SUM52, MCF7, and ZR75-1 (Fig. 4A).

Next, we further assessed the functional role of FTSJ3 by knocking down FTSJ3 using siRNA oligos in breast cancer cell lines with high FTSJ3 expression (SUM52: luminal B subtype; and MCF7: luminal A subtype) and in immortalized breast epithelial cells (MCF10A). To perform siRNA knockdown experiments, we obtained three siRNAs targeting different regions of FTSJ3, and all three siRNAs attenuated the expression of FTSJ3 in breast cancer cells. As shown in Fig. 4B, qRT-PCR and western blot assays revealed that two selected potent siRNAs significantly decreased the expression of FTSJ3 at mRNA and protein levels. FTSJ3 knockdown dramatically slowed SUM52 and MCF7 cell growth compared with that in the negative control cells (Fig. 4C). Conversely, in breast epithelial cells (MCF10A), no significant inhibitory effects were observed (Fig. 4C). We also found that FTSJ3 depletion in SUM52 cells led to marked induction of apoptosis, as determined by Annexin V staining (Fig. 4D). Additionally, we knocked down FTSJ3 in two non-breast cancer cell lines (HepG2: liver cancer and KYSE150: oesophageal cancer) in which FTSJ3 is highly expressed (Supplementary Figure S4A). FTSJ3 knockdown significantly slowed HepG2 and KYSE150 cell growth compared to that of the negative control cells (Supplementary Figure S4). Together, this data demonstrates that targeting FTSJ3 can attenuate the growth of FTSJ3 amplified/over-expressed cancer cells.

Discussion

In this study, we performed integrated genotranscriptomic and clinicopathological analyses of 58 RNMTs in a large cohort of primary tumours and cell lines. We identified that a subset of RNMT genes, including *TRMT12, NSUN2, TARBP1*, and *FTSJ3*, has high frequencies of genomic amplification and/or mutation in a spectrum of human cancers, particularly in breast cancer. Different subtypes of breast cancer had different patterns of copy number and expression of each RNMT. We revealed that expressions of several RNMTs, including *NSUN2* and *FTSJ3*, were associated with aggressive subtype and poor prognosis of breast cancer patients. Loss-of-function analysis indicated FTSJ3, a 2′-O-Me methyltransferase, as a candidate RNMT with functional roles in promoting breast cancer growth and survival.

Based on their RNA molecule targets, RNMTs could be grouped into mRNA, tRNA, rRNA, small RNA methyltransferases. In this study, we found that mRNA methyltransferase group has the highest frequency of mutation (ANOVA test, $p < 0.001$), with most mutations in UCEC (5.00%, $p < 0.001$) and SKCM (3.03%, $p < 0.005$). Among 58 RNMTs, the most mutated RNMT in TCGA Pan-cancer is *TARBP1*, which is also amplified more than 5% in five TCGA tumour types (BRCA 10.09%, OV 6.99%, LIHC 6.81%, CHOL 5.56%, and UCS 5.36%), suggesting it likely has oncogenic potential. TARBP1 belongs to the SPOUT methyltransferase family, and it facilitates the activation of HIV gene expression [100,101]. Previous immunohistochemistry assays revealed that over-expression of TARBP1 proteins is significantly associated with advanced grade, advanced clinical stage, and shorter overall survival of lung cancer [102]. TARBP1 is also over-expressed in skin and liver cancers [103,104]. Despite the known functions of TARBP1 in regulating HIV transcription and innate immune responses, the functional consequences of TARBP1 amplification and mutation in cancer are worth further investigation [105].

A finding of particular interest from our current study is that FTSJ3 is amplified in a subset of human cancer, particularly in breast cancer, and promotes cancer growth and survival. The FTSJ3 protein, together with its two close homologues FTSJ1 and MRM2/FTSJ2, contains a conserved 2′-O-methyltransferases domain: FtsJ/RrmJ [24,37,54,92]. The bacterial FtsJ/RrmJ protein is responsible for 2′-O-Me of 23S rRNA U2552 (Um2552), which is located in the Peptidyl Transferase Centre (PTC) [106,107]. Deletion of FtsJ/RrmJ in *E.coli* results in a severe growth defect with cold sensitivity and accumulation of the 45S precursor [108]. In addition, FTSJ3 is a homologue of yeast Spb1 that contains an Spb1 C-terminal domain and a nuclear localization signal in the Spb1 domain [37]. Indeed, based on COMPARTMENTS (subcellular localization database) and SubCellBarCode database, the three human FTSJ proteins have different subcellular localizations [109,110]. FTSJ1 is the primarily cytosolic

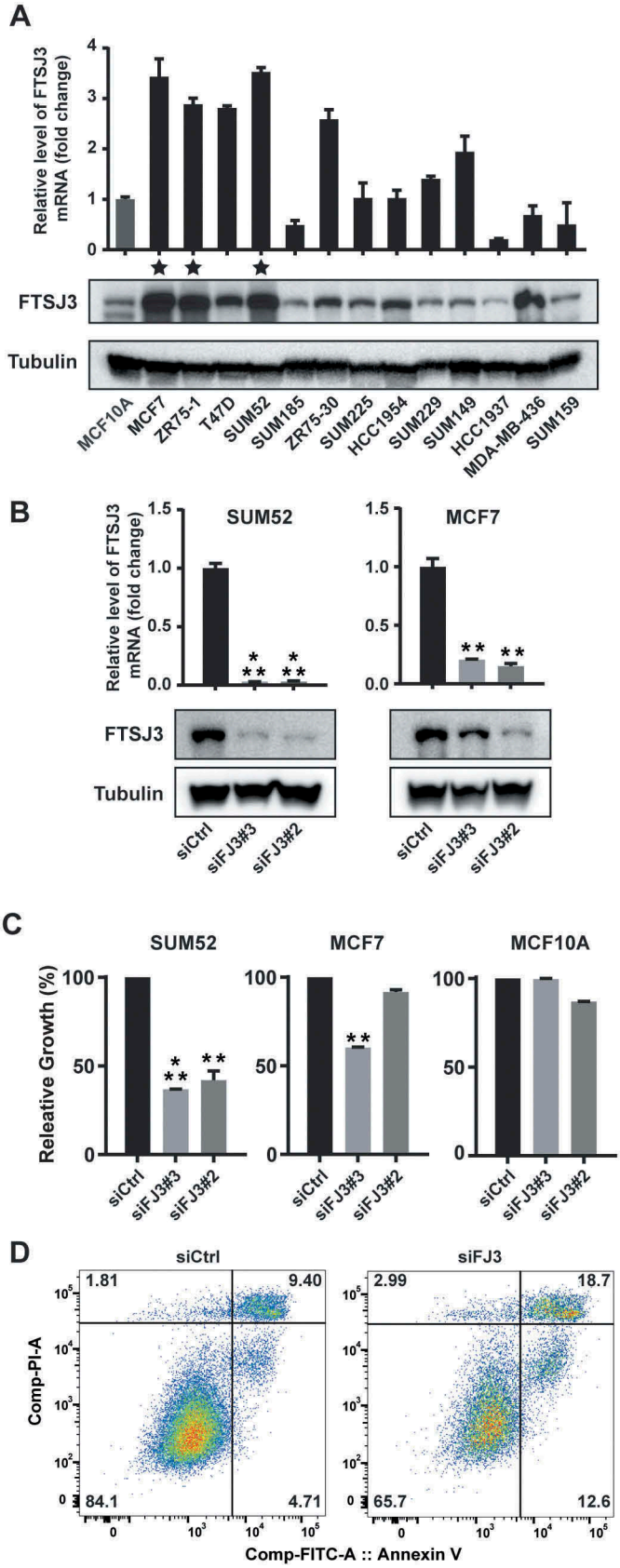

Figure 4. Knockdown of FTSJ3 inhibits cell proliferation and survival in breast cancer cell lines. (A) Expression levels of *FTSJ3*, measured by qRT-PCR and western blot assays, in a panel of 13 breast cancer cell lines plus MCF10A. mRNA expression levels in the immortalized but nontumorigenic breast epithelial cell line MCF10A cells were arbitrarily set as 1. Three stars indicate the cell lines with FTSJ3 gene amplification. (B) Knockdowns of FTSJ3 in SUM52 and MCF7 cells with two different siRNAs were confirmed by qRT-PCR and western blot assays. (FJ3 = FTSJ3). (C) Bar graph shows relative cell growth after knocking down FTSJ3 in SUM52 and MCF7 breast cancer cells (** $p < 0.01$, *** $p < 0.001$). Data are expressed as mean ± SD. (D) FTSJ3 depletion results in cell death 2 days after siRNA transfection in SUM52 cells, measured by flow cytometry and double staining with Annexin V-FITC and PI. These experiments were repeated independently three times with similar results.

protein that methylates the 2′-O-ribose of nucleotides at positions 32 and 34 of the tRNA anticodon loop [111]. FTSJ2/MRM2 is a mitochondrial protein that modifies U1369 at the PTC of human mitochondrial 16S rRNA, the equivalent position (U2791) in the yeast mitochondrial 21S rRNA [54,112]. FTSJ3 is a nuclear protein that contains a C-terminal SPB1 domain in addition to the N-terminal FtsJ/RrmJ enzymatic domain. The yeast methyltransferase, Spb1, methylates both U2921 and G2922 residues in the late 27S pre-rRNA precursor [113]. Markedly, about 70% of 2′-O-Me sites are conserved from yeast to human, particularly those located within functional regions of rRNAs such as PTC [114]. Yeast U2921 (U2552 in E. coli) and G2922 are conserved to U4498 and G4499 in humans. Genetic studies in yeast have demonstrated that 2′-O-Me is important for the translational capacity of ribosomes [13]. Furthermore, previous studies have revealed that human FTSJ3 is involved in pre-rRNA processing *via* its interaction with NIP7, a transacting factor required for ribosome biogenesis [115,116]. Thus, FTSJ proteins are highly conserved methyltransferases from bacteria to human, indicating their functional importance in many aspects of RNA processing including ribosome biogenesis and translation in normal and pathological conditions.

Recent studies demonstrated that the rRNA 2′-O-Me pattern modification is associated with changes in translational control during tumorigenesis [90,91]. For example, FBL catalyzes the 2′-O-Me of rRNAs at specific positions guided by small nucleolar RNAs (snoRNAs) from the C/D box family, which carry a sequence complementary to the target rRNA. In cellular models of cancer, forced FBL up- or down-regulation modulated tumour progression [90,91]. In breast cancer, FBL over-expression alters rRNA 2′-O-Me patterns, triggers changes in translational fidelity, and promotes translation of subsets of mRNAs (e. g. MYC) involved in tumorigenesis and cell survival [90]. Here, analysis of the genome-wide loss-of-function screen in a large panel of tumour cell lines also revealed FBL as the top hit of RNMTs for cancer cell survival. The second RNMT hit from loss-of-function screening in tumour cell lines is FTSJ3. Furthermore, our siRNA knockdown assays reveal that depletion of FTSJ3 induces apoptosis and inhibits breast cancer growth and survival *in vitro*. Thus, we speculate that modulation of the rRNA 2′-O-Me pattern at specific positions induced by changes in FTSJ3 expression, at least partly, might favour cancer cell growth and progression.

Interestingly, recent studies also revealed that FTSJ3 and yeast Spb1 are involved in 2′-O-Me of internal sites of mRNA [11,12]. Ringeard *et al.* identified FTSJ3 as the interacting partner of TAR RNA-binding protein (TRBP also known as TARBP2), and TARBP2 plays significant roles in many biological and pathological conditions, including viral expression of HIV-1, microsatellite instability, cancer stem cell properties, and tumour progression [37,117–119]. Furthermore, they found that FTSJ3 catalyzes 2′-O-Me of HIV RNAs and leads to the inhibition of innate immune sensing and response [37]. A recent study deposited in BioRxiv claimed that yeast Sbp1 has 2′-O-MTase activity and is responsible for methylation of thousands of sites in yeast mRNA, suggesting that human FTSJ3 may have the same function [120]. More interestingly, gene ontology (GO) enrichment analysis of Spb1-methylated yeast mRNAs identified that GO terms related to ribosomes, ribosome biogenesis, and translation were exclusively over-represented [120]. It is largely assumed that cancer cells become addicted to ribosomes and translation owing to their enhanced need for protein production to sustain their unrestricted growth. Thus, even though the exact role of FTSJ3 in cancer remains to be established and better characterized, our results suggest that FTSJ3 is a critical 2′-O methyltransferase that targets rRNA and mRNA in ribosome biogenesis and translation, subsequently contributing to cancer cell proliferation and survival.

In summary, integrated genomic, transcriptomic, and clinicopathological data in a large cohort of primary tumours and cell lines identified a subset of RNMTs, especially *TRMT12, NSUN2, TARBP1*, and *FTSJ3*, that was amplified or mutated in a subset of human cancer. Several RNMTs were significantly associated with breast cancer aggressiveness and poor prognosis. Loss-of-function analysis revealed that FTSJ3 had important roles in promoting breast cancer cell growth and survival. To date, DNMT and protein methyltransferase inhibitors have been successfully identified and several inhibitors have been approved or tested for cancer treatment, suggesting that the RNMTs represent a new avenue for anticancer drug discovery [121]. Our findings provide a framework for further study of the functional consequences of RNMT alterations in human cancer and for developing therapies that target RNMTs in the future.

Materials and methods

Genomic and clinical data of cancer samples

Genetic and expression alteration data from 10,967 tumour samples spanning 32 tumour types in The Cancer Genome Atlas (TCGA) Pan-Cancer studies were obtained from the cBio Cancer Genomics Portal (*http://www.cbioportal.org*) [75,76,122–124]. In the cBioPortal, the copy number for each RNMT gene was generated by the GISTIC algorithm and categorized as copy number level per gene: '-2' is a possible homozygous deletion, '-1' is a heterozygous deletion, '0' is diploid, '1' indicates a low-level gain, and '2' is a high-level amplification. The relative expression of an individual gene and the gene's expression distribution in a reference population were analysed in mRNA expression data. The reference population consists of tumours that are diploid for the gene in question. The Z score represents the number of standard deviations the expression of a gene is from the reference population gene expression. Somatic mutation data were obtained by exome sequencing [75,76]. Breast cancer subtype and clinicopathologic information in the TCGA cohort was obtained from a previous publication and extracted via the cBioPortal [76,79]. Among the 1084 breast cancer samples, 981 had intrinsic subtype data available, including 36 normal-like, 499 luminal A, 197 luminal B, 78 HER2+, and 171 basal-like breast cancers [76,123]. A detailed description of the METABRIC dataset can be found in the original publication [83]. The CNAs and normalized expression data from the METABRIC database were downloaded with permission from the European Genome-phenome Archive (https://www.ebi.ac.uk/ega) under accession number

EGAC00000000005 as well as from the cBio Cancer Genomics Portal [76]. In the METABRIC dataset, 1974 samples had subtype data available, including 199 normal-like, 718 luminal A, 488 luminal B, 240 HER2+, and 329 basal-like breast cancers [83]. The CNA and mutation data from 237 MBC (Metastatic Breast Cancer) samples were also obtained from the cBio Cancer Genomics Portal.

Semiquantitative PCR reactions

To assess gene expression at the mRNA level, RNA was prepared from human breast cancer cell lines and the MCF10A cell line by using an RNeasy Plus Mini Kit (QIAGEN) [122]. RNA was mixed with qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD, USA) and then converted to cDNA through a reverse-transcription (RT) reaction. The cDNA was then used for real-time PCR reactions. Primer sets were obtained from Life Technologies (Carlsbad, CA, USA). A PUM1 primer set was used as a control. Semiquantitative RT-PCR was performed using the FastStart Universal SYBR Green Master (Roche Diagnostics, Indianapolis, IN, USA) as described earlier [122,123].

Immunoblotting and antibodies

Immunoblot assays were performed as previously described [122,123]. Whole-cell lysates were prepared by scraping cells from the dishes into cold RIPA lysis buffer. Centrifugation protein content was estimated by the Bradford method. A total of 20–50 μg of total cell lysate was resolved by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane. Antibodies used for the western blot in the study included anti-FTSJ3 (A304-199, Bethyl Laboratories, Montgomery, TX, USA) and anti-β-Tubulin (Sigma-Aldrich T8328, St. Louis, MO, USA).

Cell culture and growth assays

The SUM cell lines were obtained from Dr. Stephen P. Ethier, and the remaining cell lines were obtained from American Type Culture Collection (Manassas, VA, USA). All cell lines were tested routinely and authenticated using cell morphology, proliferation rate, a panel of genetic markers, and contamination checks. To determine the effect of FTSJ3 overexpression on the growth of human cancer *in vitro*, FTSJ3 expression was knocked down using small interfering RNA (siRNA) in breast, liver, and oesophageal cancer lines as well as the control line, MCF10A. The FTSJ3 and negative control siRNAs were purchased from Sigma-Aldrich (St. Louis, MO, USA). For the transfection procedure, cells were seeded in appropriate cell culture plates and maintained overnight under standard conditions. Plate sizes, cell densities, and siRNA quantities were dependent on the cell line and the experimental setup; siRNA was transfected using the MISSION siRNA transfection reagent according to the manufacturer's protocol (Sigma-Aldrich). Five days after siRNA transfection, CellTiter-blue cell viability assays (Promega) were performed according to the manufacturer's guideline.

Apoptosis assays

FTSJ3 siRNA pools and negative control siRNA were used for apoptosis assays in SUM52 cells. Cells were collected and stained with FITC–Annexin V and propidium iodide (PI) according to the manufacturers' instructions (FITC Annexin V Apoptosis Detection Kit I, BD Biosciences, San Jose, CA). Apoptotic cells were detected by flow cytometry using an LSR II (BD Biosciences; excitations at 488, 561, 640 nm), and data were analysed with FlowJo software. Cells were gated by FSC-A x SSC-A to exclude debris and then by FSC-H × FSC-A to exclude cell aggregates.

Statistical analysis

Statistical analyses were performed using R software (http://www.r-project.org) and Graphpad Prism software [122,123]. Statistical significance of the differences in mRNA expression level for each RNMT among different subtypes, grades, and NPI score of breast cancer samples was determined using ANOVA and Welch's *t*-test as described earlier [122,123]. Spearman, Kendall, and Pearson correlation tests were used to correlate copy numbers and mRNA levels of each RNMT from TCGA breast cancer specimens. We used the 'cor' function in R for computation, specifying which type of test we wanted (Spearman, Kendall, or Pearson). Relationships between RNMT copy number, mRNA expression, and disease-free survival in METABRIC breast cancer were analysed by dividing samples into amp/gain (amplification plus gain) and no amp/gain, or high and low expression groups for each RNMT.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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