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Targeted Profiling of Epitranscriptomic Reader, Writer, and Eraser Proteins Accompanied with Radioresistance in Breast Cancer Cells

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Abstract

Epitranscriptomic reader, writer, and eraser (RWE) proteins recognize, install, and remove modified nucleosides in RNA, which are known to play crucial roles in RNA processing, splicing, and stability. Here, we established a liquid chromatography-parallel-reaction monitoring (LC–PRM) method for high-throughput profiling of a total of 152 epitranscriptomic RWE proteins. We also applied the LC–PRM method, in conjunction with stable isotope labeling by amino acids in cell culture (SILAC), to quantify these proteins in two pairs of matched parental/radioresistant breast cancer cells (i.e., MDA-MB-231 and MCF-7 cells and their corresponding radioresistant C5 and C6 clones), with the goal of assessing the roles of these proteins in radioresistance. We found that eight epitranscriptomic RWE proteins were commonly altered by over 1.5-fold in the two pairs of breast cancer cells. Among them, TRMT1 (an $m^{2,2}G$ writer) may play a role in promoting breast cancer radioresistance due to its clinical relevance and its correlation with DNA repair gene sets. To our knowledge, this is the first report of a targeted proteomic method for comprehensive quantifications of epitranscriptomic RWE proteins. We envision that the LC–PRM method is applicable for studying the roles of these proteins in the metastatic transformation of cancer and therapeutic resistance of other types of cancer in the future.

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The authors declare no competing financial interest.

All the LC–MS/MS raw files and Skyline PRM library were deposited to the ProteomeXchange Consortium via the PRIDE[44] partner repository with the data set identifier PXD030387.

ASSOCIATED CONTENT

Supporting Information

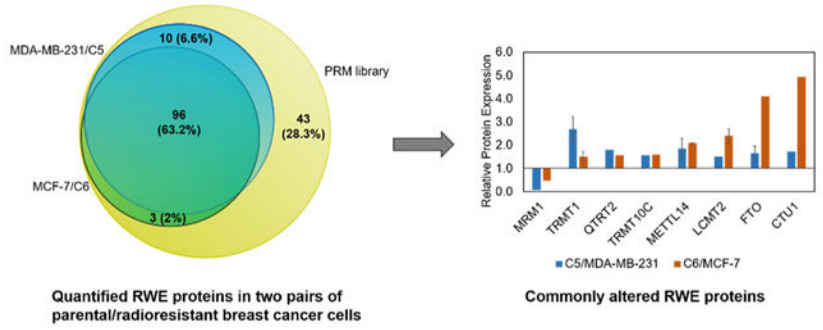
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.1c05441.

Detailed experimental procedures; Figure S1, Gene ontology (GO) biological pathway (BP) analysis and Kaplan–Meier survival analysis; and Table S1, list of epitranscriptomic RWE proteins included in the PRM library (PDF)

Table S2, expression ratios of epitranscriptomic RWE proteins in MDA-MB-231/C5 and MCF-7/C6 cells (XLSX)

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.analchem.1c05441

Graphical Abstract



Unlike the extensively studied DNA methylation and histone post-translational modifications, the investigations about RNA modifications did not gain wide attention in the scientific community until the availability of a high-throughput sequencing method rendered transcriptome-wide profiling of N^6-methyladenosine (m^6A) in 2012.[1] RNA is known to contain more than 170 types of modifications, among which m^6A is the most abundant internal modification in mRNA.[2] m^6A-modifying enzymes ("writers" and "erasers") install or remove m^6A, whereas m^6A-binding proteins ("readers") recognize m^6A to confer downstream effects. m^6A is involved in regulating various cellular processes, including mRNA stability, splicing, translation, and decay.[3–6] Aside from m^6A, other RNA modifications also regulate biological processes through their reader, writer, and eraser (RWE) proteins. For instance, ALYREF and YTHDF2, which are 5-methylcytidine (m^5C) reader proteins, modulate mRNA export and rRNA maturation, respectively.[7,8] In addition, NSUN2 (m^5C writer) and YBX1 (m^5C reader) drive the pathogenesis of human bladder urothelial carcinoma by targeting the m^5C site in the mRNA of the *HDGF* gene.[9]

Breast cancer represents the second most common cancer among women in the United States. Radiation therapy harnesses ionizing radiation to eliminate local malignant cells and prevent cancer recurrence. It delivers high-energy X-rays to target tissues and elicits DNA damage in rapidly dividing cancer cells. Although more than 83% of breast cancer patients benefit from radiation therapy,[10] some patients suffer from tumor recurrence due to the development of resistance to radiation therapy.[11] Many genes involved in DNA damage repair and cell cycle checkpoints have been documented to modulate radioresistance, including *AKT, HER2, BRCA2, CDK1*, and *CHK1*.[12–15]

Several studies also unveiled the functions of m^6A RWE proteins in modulating radioresistance ofcancer cells. METTL3, the catalytic subunit of the major m^6A writer complex, promotes radioresistance in glioblastoma by regulating m^6A modification of *SOX2* mRNA and enhancing its stability.[16] m^6A eraser, ALKBH5 augments radioresistance by modulating homologous recombination in glioblastoma.[17] m^6A reader YTHDC2 promotes radioresistance of nasopharyngeal carcinoma via enhancing *IGF1R* mRNA and activating the IGF1R-AKT/S6 i signaling pathway.[18] Little, however, is known about the roles of other epitranscriptomic RWE proteins, such as those for N^1-methyladenosine (m^1A), m^5C, and pseudouridine (Ψ) in RNA, in modulating the sensitivity of cancer cells to radiation therapy.

Parallel-reaction monitoring (PRM)-based targeted proteomics, which can be performed on hybrid quadrupole-Orbitrap or quadrupole time-of-flight (TOF) mass spectrometers, can be used to quantify hundreds of peptides in complex sample matrixes in a single LC–MS/MS run.[19] Since the MS/MS are acquired on a high-resolution mass analyzer, PRM offers highly selective and reliable identification and quantification of target peptides. Moreover, the mass spectrometer can be programed to collect MS/MS of precursor ions in predefined retention time windows with the use of normalized retention time (iRT), which provides improved throughput of the LC–PRM method.[20]

To investigate systematically the roles of epitranscriptomic RWE proteins in modulating radioresistance in breast cancer, we established an LC–PRM method, coupled with stable isotope labeling by amino acids in cell culture (SILAC), to examine the differences in expression levels of the proteins in MDA-MB-231 and MCF-7 breast cancer cells relative to their corresponding radioresistant C5 and C6 clones (Figure 1a). We first developed a Skyline[21] PRM library, which includes all the 68 human epitranscriptomic RWE proteins deposited in the Modomics database[2] and another 84 RWE proteins retrieved from several recent review articles (Figure 1b, Table S1).[22–27] Each RWE protein is represented by two or three unique peptides, whose MS/MS were acquired from previously published shotgun proteomic analyses and imported into the Skyline library.[28]

To achieve high-throughput analysis of these proteins, we employed scheduled LC–PRM with a 7 min retention time window and a maximum of 40 concurrent precursor ions. In this vein, iRT of each peptide was derived from the linear regression of RT with iRT by analyzing a tryptic digestion mixture of bovine serum albumin (BSA) under the same chromatographic conditions. With this method, the 152 epitranscriptomic RWE proteins (i.e., 444 unique peptides, and 888 precursor ions for SILAC) could be monitored in three LC–MS/MS runs with a 125 min gradient. The LC–PRM analysis enabled the quantifications of 106 and 99 epitranscriptomic RWE proteins from two forward and two reverse SILAC experiments in the MDA-MB-231/C5 and the MCF-7/C6 pairs, respectively, accounting for approximately 70% and 65% of proteins in the PRM library (Figure 1c). The quantification result of each RWE protein was calculated from the average ratios of all detected tryptic peptides of the protein, where the ratio of each peptide was calculated in Skyline based on LC–PRM results from the four replicates of SILAC experiments. A total of 96 epitranscriptomic RWE proteins were commonly quantified in the two pairs of cell lines. We also performed hierarchical clustering analysis to illustrate the differential expression of the quantified epitranscriptomic RWE proteins in the radioresistant C5 and C6 lines relative to the corresponding parental MDA-MB-231 and MCF-7 lines (Figure 2). Such analysis revealed similarities and differences in alterations in expression of epitranscriptomic RWE proteins accompanied by the development of radioresistance in the two breast cancer cell lines 1 (Figure 2).

Our LC–PRM data revealed that 8 and 11 epitranscriptomic RWE proteins were down-regulated by more than 1.5-fold, and 18 and 27 epitranscriptomic RWE proteins were up-regulated by over 1.5-fold in the radioresistant C5 and C6 lines relative to their corresponding parental lines, respectively (Figure 3a,b). Gene ontology (GO) analysis of these differentially expressed proteins showed that the up-regulated epitranscriptomic RWE

proteins are mainly involved in tRNA modification, tRNA processing, and rRNA base methylation (Figure S1a). The down-regulated epitranscriptomic RWE proteins play roles in tRNA methylation, the oxidation-reduction process, and tRNA 1 dihydrouridine synthesis (Figure S1a). In this context, it is worth noting that over 100 types of modifications have been detected in tRNA,[2] including Ψ, m^1A, N1-methylguanosine (m^1G), and N^6-threonyl-carbamoyl-adenosine (t^6A), where many tRNA modifications regulate the stabilities of tRNA.[29,30]

Among the epitranscriptomic RWE proteins that are up- or down-regulated by at least 1.5-fold, eight were commonly altered in both pairs of breast cancer cell lines (Figure 3c,d). For instance, MRM1 was pronouncedly down-regulated, whereas FTO and CTU1 were markedly up-regulated in radioresistant lines compared to parental lines in both pairs of breast cancer cells (Figure 2). Figure 4a illustrates the PRM traces of representative peptides from TRMT1 and FTO, two ofthe eight commonly altered proteins, in two pairs of matched radioresistant/parental breast cancer cells. The up-regulations of TRMT1 and FTO in the radioresistant cells were further validated by Western blot analysis (Figure 4b).

Our proteomic results showed that the established LC–PRM method coupled with SILAC affords a highly efficient, selective, sensitive, and reproducible peptide quantification. The efficiency of the method is manifested by its high throughput, where 888 precursor ions of 444 tryptic peptides derived from the 152 epitranscriptomic RWE proteins could be monitored in three LC–MS/MS runs. Additionally, the high consistency of the quantification results of TRMT1 and FTO obtained from PRM and Western blot analyses underscores the high accuracy of the method. Moreover, the relatively high coverage (i.e., 70% and 65%) of the epitranscriptomic RWE proteins in the library indicates the high sensitivity of the PRM method. The PRM method is also highly reproducible, as reflected by the small mean relative standard deviations of the quantification results obtained from two forward and two reverse SILAC experiments, i.e., 11.7% and 9.1% in the MDA-MB-231/C5 and MCF-7/C6 and pairs of breast cancer cells, respectively (Table S2). In this context, it is worth noting that our PRM method does not take into account post-translational modifications (PTMs) in the peptides employed for the quantifications of the epitranscriptomic RWE proteins. Hence, differences in PTMs between the radioresistant and parental breast cancer cells may contribute, in part, to variations in quantification results obtained from different peptides of the same protein (Table S2).

Considering that the above-mentioned proteomic results were acquired from breast cancer cell lines derived from two patients, we next asked if the findings could be extended to breast cancer patients in general. To this end, we performed Kaplan-Meier survival analyses in two breast cancer patient cohorts, i.e., The Cancer Genome Atlas-Breast Invasive Carcinoma (TCGA-BRCA) and Molecular Taxonomy of Breast Cancer International Consortium (METABRIC). We placed our emphasis on patients who received radiation therapy and explored the correlation between the mRNA expression level of each commonly altered epitranscriptomic RWE protein and patient survival. Our results showed that a higher level of mRNA expression of *TRMT1* is significantly correlated with poorer survival of breast cancer patients who received radiation therapy in both TCGA-BRCA and METABRIC cohorts (Figure 4c). This result is in keeping with our proteomic data showing

that TRMT1 is up-regulated in C5 and C6 cells compared with parental MDA-MB-231 and MCF-7 cells. For the other commonly altered epitranscriptomic RWE proteins in both pairs, only the Kaplan-Meier survival analysis of *CTU1* gene in the METABRIC cohorts who received radiation therapy corroborates with proteomics results (Figure S1b). The lack of correlation for other proteins may be due to the differences in the mRNA and protein expression levels of epitranscriptomic RWE proteins and/or the heterogeneity of breast cancer.[31]

To explore the potential mechanism of TRMT1 in radioresistant breast cancer, we carried out gene set enrichment analysis (GSEA). TCGA-BRCA data set was stratified by the high and low mRNA expression of *TRMT1* using its median value as a cutoff. Upon performing GSEA between the stratified TCGA data set and the hallmark gene sets downloaded from the GSEA Molecular Signatures Database,[32] we observed that, among 23 gene sets, 4 are significantly (at FDR < 25%) up-regulated in the high-TRMT1-expression group. The DNA repair gene set is the most significantly enriched (Figure 4d). Since radioresistance is known to be associated with the enhanced ability to repair radiation-induced DNA damage,[11] this finding again suggests a role of TRMT1 in promoting radioresistance. Additionally, two other hallmark gene sets, i.e., Myc_target_V2 (Figure 4d) and Myc_target_V1 (Figure S1c), were also enriched significantly with the high-TRMT1-expression group; hence, TRMT1 may be associated with Myc target genes. Moreover, the hallmark gene set UV_response_up, i.e., up-regulated in response to ultraviolet (UV) radiation, was also associated with high expression of TRMT1 (Figure S1d).

Radiation therapy is known to enhance cancer metastasis through activating epithelial-mesenchymal transition (EMT) transcription factors, including Snail, Slug, ZEB1, and ZEB.[33] Additionally, radioresistant breast cancer cells exhibit increased metastatic potential,[34] and breast cancer distant metastasis was shown to promote resistance to radiation therapy.[35] Based on the observed co-occurrence between metastasis and radioresistance, several reports interrogated their cross-regulation and revealed several common pathways, including PI3K/AKT/mTOR, MAPK, Wnt/β-catenin, NF-κB, EMT, and reactive oxygen species scavenging.[36–40]

TRMT1 dimethylates the N^2 position of guanosine 26 in most tRNAs to give $m^{2,2}G$. It was documented that the urinary level of $m^{2,2}G$ was elevated in 35.1% or 57% in two cohorts of metastatic breast cancer patients.[41,42] TRMT1 is the only known writer of $m^{2,2}G$ in humans;[43] thus, the augmented levels of $m^{2,2}G$ in metastatic breast cancer patients also suggest a role of TRMT1 in the metastatic transformation of breast cancer.

In summary, we established, for the first time, a high-throughput scheduled LC–PRM method for profiling simultaneously a total of 152 epitranscriptomic RWE proteins. We also employed this method to explore the roles of these proteins in radioresistance in breast cancer cells, and we found that eight epitranscriptomic RWE proteins were commonly altered by over 1.5-fold in the MDA-MB-231/C5 and MCF-7/C6 pairs of breast cancer cells. Among them, TRMT1 may play a role in promoting radioresistance in breast cancer and be involved in breast cancer metastatic transformation. Thus, TRMT1 could be a target for overcoming radioresistance in breast cancer therapy. In addition, other

differentially expressed epitranscriptomic RWE proteins in matched radioresistant/parental breast cancer cell lines revealed from this study may provide a comprehensive understanding of epitranscriptomic RWE proteins in modulating radiation sensitivity in breast cancer. Moreover, we envision that the LC–PRM method developed in this study can also be employed to examine, in the future, the roles of epitranscriptomic RWE proteins in the metastatic transformation of cancer and therapeutic resistance of other types of cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

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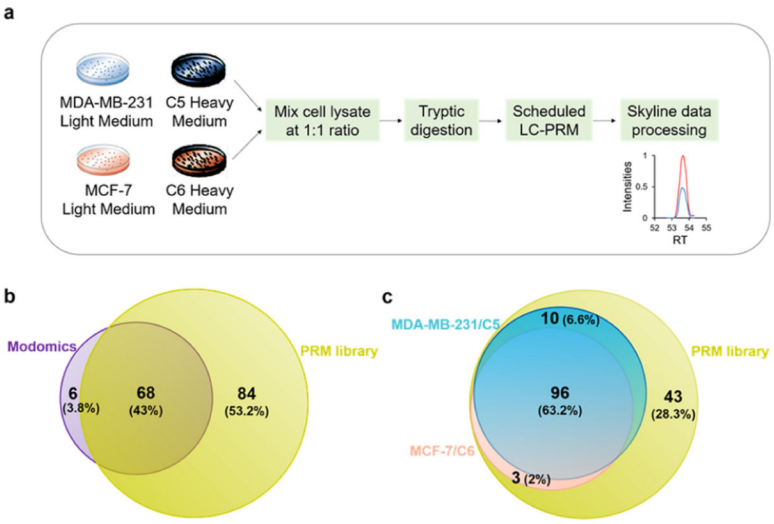

Figure 1.

LC–PRM method for uncovering alterations in expression of epitranscriptomic RWE proteins associated with the development of radioresistance. (a) A SILAC-based LC–PRM workflow. The parental cells (i.e., MDA-MB-231 and MCF-7) and their radioresistant counterparts (i.e., C5 and C6) were labeled in light- or heavy- amino acid-containing media for over six cell doubling times. In the forward SILAC labeling experiments, light-isotope-labeled C5 and C6 cell lysates were mixed at a 1:1 ratio (by mass) with heavy-isotope-labeled MDA-MB-231 and MCF-7 cell lysates, respectively. In the reverse SILAC labeling experiments, light-isotope-labeled MDA-MB-231 and MCF-7 cell lysates were mixed at a 1:1 ratio (by mass) with heavy-isotope-labeled C5 and C6 cell lysates, respectively. The mixed cell lysate was tryptic digested and subjected to LC–PRM analysis. Data were processed using Skyline. (b,c) Venn diagrams showing the number and percentage of human epitranscriptomic RWE proteins deposited in the Modomics database (purple) compared with those included in the PRM library of this study (yellow) (b) and illustrating the number and percentage of quantified epitranscriptomic RWE proteins in MDA-MB-231/C5 and MCF-7/C6 pairs of breast cancer cells from LC–PRM analyses, compared with those deposited in the PRM library (c). Blue and pink circles in parts b and c designate the numbers of quantified epitranscriptomic RWE proteins in MDA-MB-231/C5 and MCF-7/C6 pairs of breast cancer cells, respectively.

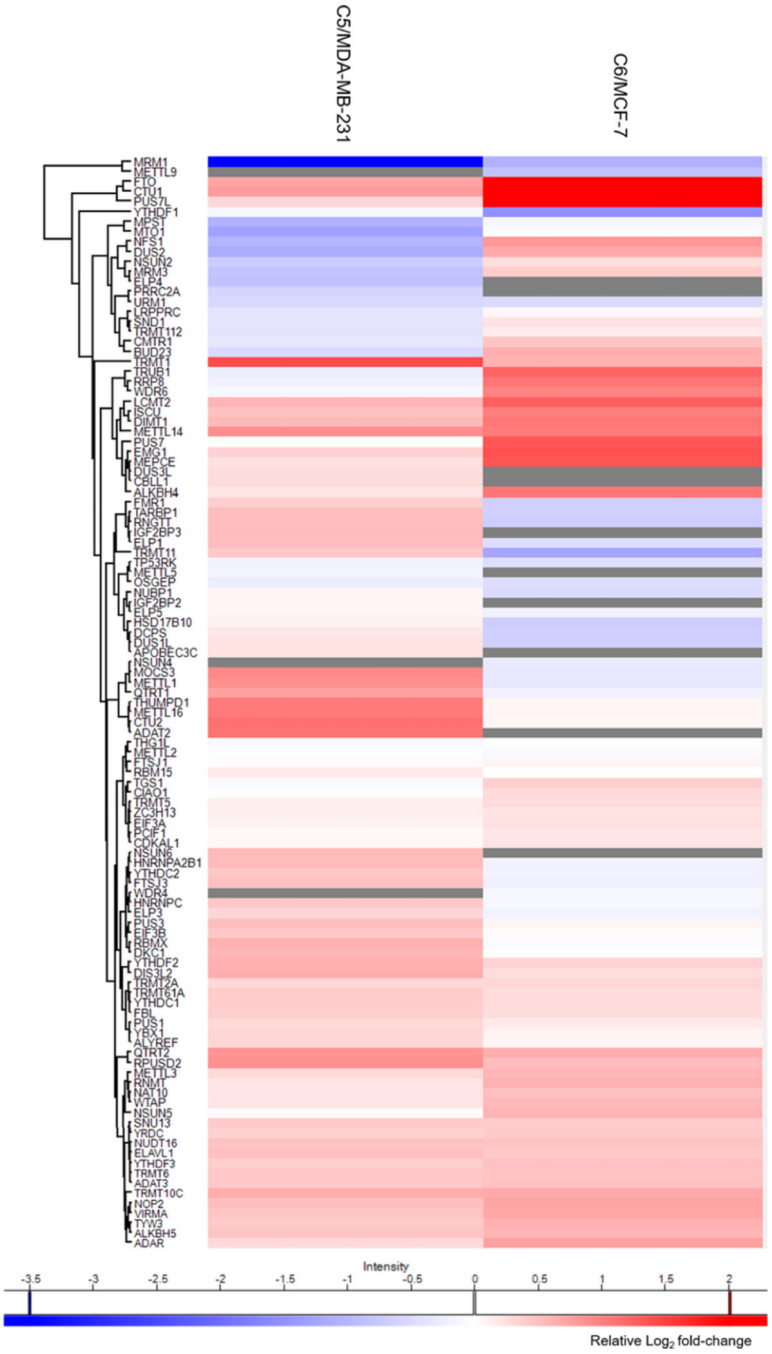

Figure 2.

Hierarchical clustering displaying the Log_2 transformed expression fold differences of epitranscriptomic RWE proteins in radioresistant C5 cells relative to parental MDA-MB-231 cells and radioresistant C6 cells relative to parental MCF-7 cells. The expression fold differences were averaged from two forward and two reverse SILAC experiments. Hierarchical clustering was generated using Perseus, where red and blue boxes designate proteins up- and down-regulated in radioresistant breast cancer cells compared with the

corresponding parental lines, respectively; gray boxes represent missed data. Genes were clustered using the Euclidean distance.

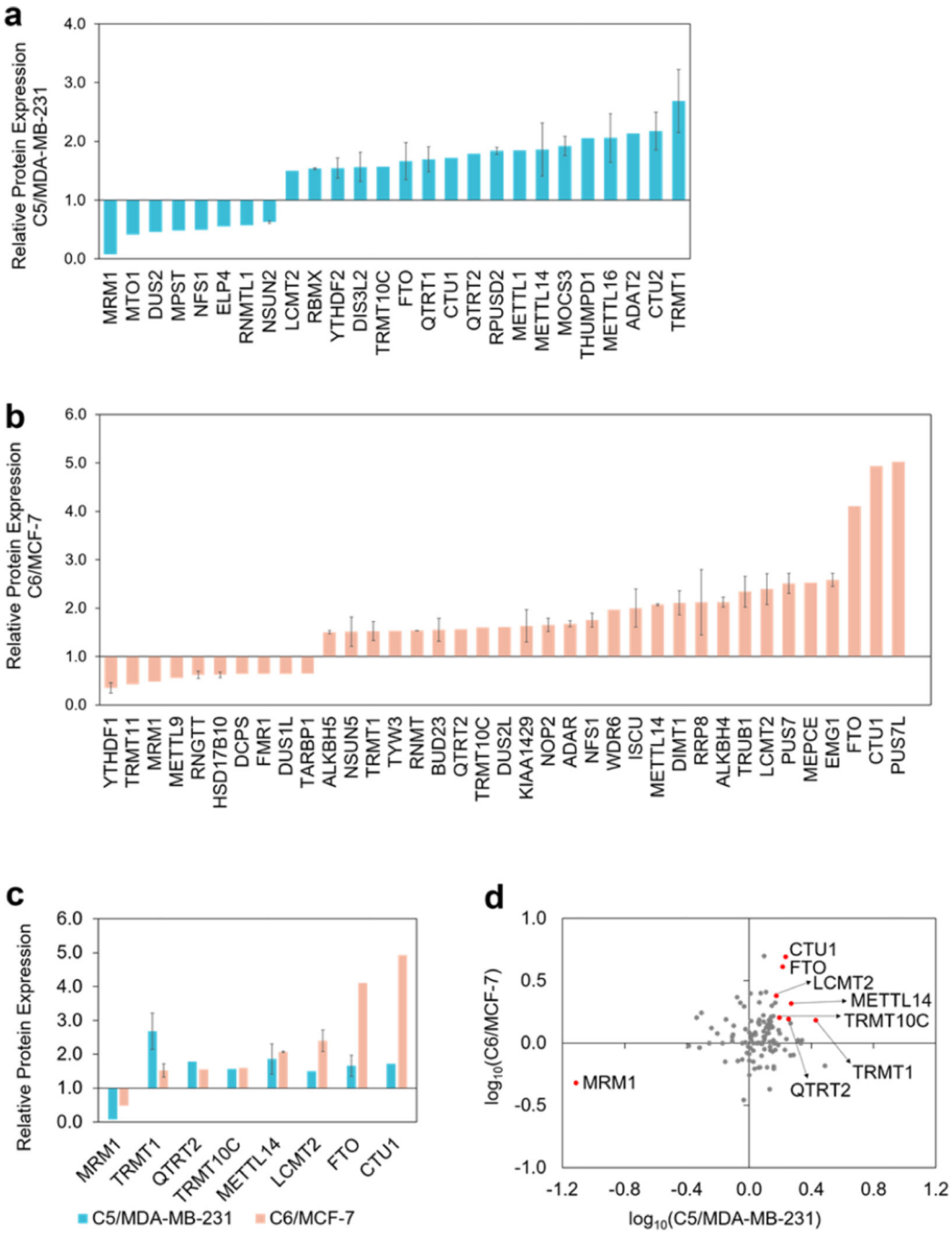

Figure 3.

(a,b) Bar graphs depicting the LC–PRM results for those epitranscriptomic RWE proteins with expression differences of over 1.5-fold or less than 0.67-fold in radioresistant cells relative to the corresponding parental cells. (c) Bar graphs illustrating epitranscriptomic RWE proteins that were commonly altered by over 1.5-fold in the two pairs of matched breast cancer cells. (d) Scatter plot displaying $\log_{10}$ transformed expression ratios of the quantified epitranscriptomic RWE proteins in the two pairs of matched breast cancer cells. Eight commonly altered RWE proteins from both pairs by over 1.5-fold were labeled in red

dots. The data in parts a-c display the means and standard deviations of the quantified ratios of different peptides representing a specific epitranscriptomic RWE protein, where the ratio of each peptide was averaged from the quantification results of two forward and two reverse SILAC experiments. Error bars were displayed for those epitranscriptomic RWE proteins with more than one peptide being quantified.

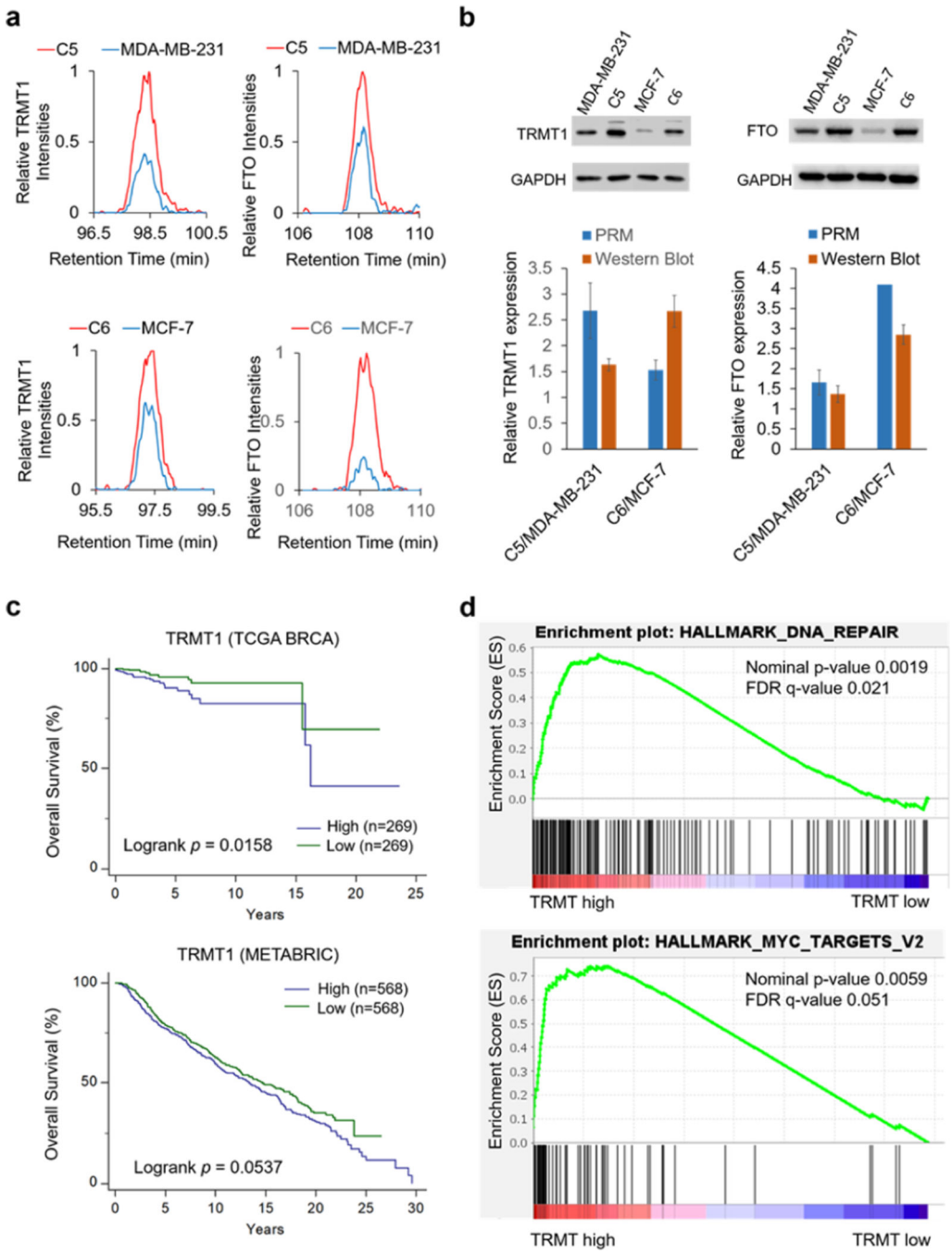

Figure 4.

(a) PRM traces of representative peptides, FALEVPGLR from TRMT1 and FTVPWPVK from FTO, in C5/MDA-MB-231 and C6/MCF-7 pairs of breast cancer cells. (b) Western blots of TRMT1 and FTO proteins in MDA-MB-231 and MCF-7 pairs of radioresistant/ parental breast cancer cells. Relative quantification results of TRMT1 and FTO obtained from PRM and Western blot analysis were shown. The PRM results represent the mean and standard deviation of quantification results of different peptides from a given epitranscriptomic RWE protein, where the ratio of each peptide in the radioresistant over

parental cells was averaged from the quantification data of two forward and two reverse SILAC experiments. Western blot data represent the mean and standard deviation ofresults obtained from three separate experiments. (c) Kaplan-Meier survival analysis of *TRMT1* gene in the TCGA-BRCA and METABRIC cohort of patients who received radiation therapy. Breast cancer patients were stratified by the mRNA expression of *TRMT1* using its median value as a cutoff. The survival plots and log-rank *p*-values were generated and calculated by using MedCalc software. (d) GSEA enrichment plots were generated using GSEA 4.1.0, where the number of permutations was set at 1000.