

Loss function of tumor suppressor FRMD8 confers resistance to tamoxifen therapy via a dual mechanism

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eLife Assessment

The research presents **valuable** findings on the impact of FRMD8 loss on tumor progression and resistance to tamoxifen therapy. Through a series of **convincing** and systematic experiments, the author thoroughly investigates the role of FRMD8 in breast cancer and its underlying regulatory mechanisms. The study confirms that FRMD8 holds potential as a therapeutic target for reversing tamoxifen resistance, offering helpful insights for future treatment strategies.

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Abstract

Approximately 40% ER α -positive breast cancer patients suffer from therapeutic resistance to tamoxifen. Although reduced ER α level is the major cause of tamoxifen resistance, the underlying mechanisms remain elusive. Here, we report that FRMD8 raises the level of ER α at both transcriptional and post-translational layers. FRMD8 deficiency in *MMTV-Cre*⁺; *Frmd8*^{fl/fl}; *PyMT* mice accelerates mammary tumor growth and loss of luminal phenotype, and confers tamoxifen resistance. Single-cell RNA profiling reveals that *Frmd8* loss decreases the proportion of hormone-sensing differentiated epithelial cells and downregulates the levels of ER α . Mechanically, on one hand, loss of FRMD8 inhibits *ESR1* transcription via suppressing the expression of FOXO3A, a transcription factor of *ESR1*. On the other hand, FRMD8 interacts both with ER α and UBE3A, and disrupts the interaction of UBE3A with ER α ,

thereby blocking UBE3A-mediated ER α degradation. In breast cancer patients, *FRMD8* gene promoter is found hypermethylated and low level of FRMD8 predicts poor prognosis. Therefore, FRMD8 is an important regulator of ER α and may control therapeutic sensitivity to tamoxifen in ER α -positive breast cancer patients.

Impact Statement

Tumor suppressive protein FRMD8 inhibits breast cancer progression by regulating the level of ER α and has the possibility to be a potential target for overcoming tamoxifen treatment resistance.

Introduction

Breast cancer is the most commonly diagnosed cancer worldwide(Sung et al., 2021 [↗](#)) and more than 70% of breast cancer are estrogen receptor α (ER α)-positive(Habara & Shimada, 2022 [↗](#)). Although endocrine therapy is the most common systemic treatment for ER α -positive breast cancer in clinical practice, approximately 40% of patients still develop primary or secondary resistance to endocrine therapy(Badia et al., 2007 [↗](#); Légaré & Basik, 2016 [↗](#); Rondón-Lagos et al., 2016 [↗](#)). Therefore, it is urgent and necessary to explore the mechanisms of endocrine therapy resistance and search for new therapeutic targets.

ER α is a ligand-activated transcription factor that is activated by oestrogen, and promotes cell proliferation during breast cancer development (Harbeck et al., 2019 [↗](#)). Tamoxifen (TAM), a selective estrogen receptor antagonist, is the most widely used medicine in endocrine therapy. Tamoxifen competes with estrogen to bind with ER α and changes the conformation of ER α , thereby preventing the interaction between co-activators and ER α and inhibiting activation of ER α (Katzenellenbogen et al., 2018 [↗](#)). Thus, the level of ER α is strongly correlated with reactivity and resistance of endocrine therapy. Uncovering the mechanisms by which ER α expression is regulated is essential for overcoming endocrine therapy resistance. Multiple transcription factors, such as AP-2 γ , FOXO3, FOXM1, and GATA3, have been reported to bind to the promoter region of *ESR1*, the gene encoding ER α , and participate in transcriptional regulation of *ESR1*(Jia et al., 2019 [↗](#); Koš et al., 2001 [↗](#)). In addition, post-translational modifications of ER α , including phosphorylation, acetylation, and ubiquitination, also have effects on subcellular localization, transcriptional activity, and stability of ER α (Rogatsky et al., 1999 [↗](#); Williams et al., 2009 [↗](#); Zhou & Slingerland, 2014 [↗](#)). However, the mechanisms underlying the regulation of ER α expression are still not clear and require further investigation.

FERM domain-containing proteins are widely involved in processes such as the formation of macromolecular complexes, subcellular localization, functional activation, and signal transduction, thereby regulating the occurrence and development of tumors(Frame et al., 2010 [↗](#); Moleirinho et al., 2013 [↗](#); Zhan & Zhang, 2018 [↗](#)). FRMD8, as a member of FERM domain-containing proteins, has been reported to bind with iRhom and enhance the stability of the iRhom/TACE complex on the cell surface, thereby preventing iRhom/TACE degradation mediated by lysosomes. TACE is responsible for cleaving and releasing TNF, and the absence of FRMD8 impairs the production of TNF(Künzel et al., 2018 [↗](#); Oikonomidi et al., 2018 [↗](#)). Additionally, FRMD8 expressions in both microenvironment and tumor cells promote lung tumor growth(Badenes et al., 2023 [↗](#)). FRMD8 inhibits colon cancer growth by preventing cell cycle progression. FRMD8 disrupts the interaction of CDK7 with CDK4, subsequently inhibiting CDK4 activation. Furthermore, FRMD8 competes with MDM2 to bind RB, thereby attenuating MDM2-mediated RB degradation(Yu et al., 2023 [↗](#)). However, the roles of FRMD8 in breast tumorigenesis and progression need further exploration.

In this study, we found that loss of *Frmd8* in luminal epithelial cells of *MMTV-PyMT* mice accelerates mammary tumor progression and luminal epithelial phenotype loss, and confers tamoxifen resistance. Single-cell RNA profiling reveals that the number of hormone-sensing differentiated cells is diminished and the level of ER α is decreased in *Frmd8*-knocked-out mammary tumors. FRMD8 not only increases *ESR1* expression, but also prevents ER α degradation by interrupting the interaction between ER α and E3 ligase UBE3A. Further, a low FRMD8 level predicts poor prognosis in human breast cancer patients. Thus, we demonstrated that FRMD8 is an important ER α regulator and a vital tumor suppressive protein in breast cancer growth and drug resistance.

Results

Loss of *Frmd8* promotes mammary tumor growth and generates tamoxifen resistance *in vivo*

To examine whether FRMD8 plays a role in breast tumorigenesis, we established luminal epithelium-specific *Frmd8* knockout mice (*MMTV-Cre⁺; Frmd8^{fl/fl}*) (Fig. 1A and 1B) and further generated a *Frmd8*-deletion breast cancer mouse model (*MMTV-Cre⁺; Frmd8^{fl/fl}; PyMT*) by crossing *MMTV-Cre⁺; Frmd8^{fl/fl}* mice with *MMTV-PyMT* (*PyMT*) mice, a widely used transgenic mouse model of mammary tumorigenesis (Fig. 1C, 1D, S1A and S1B). Compared with *MMTV-Cre⁺; Frmd8^{fl/fl}; PyMT* mice, *Frmd8* depleted in mice significantly promotes mammary tumor development (Fig. 1E and 1F). The total tumor weight (Fig. 1G) and number of tumors (Fig. 1H) were markedly higher in *MMTV-Cre⁺; Frmd8^{fl/fl}; PyMT* mice than the control mice. Histological examination of the breast tumors from *PyMT* mice using haematoxylin-eosin (H&E) staining revealed that the luminal epithelium was poorly differentiated in *MMTV-Cre⁺; Frmd8^{fl/fl}; PyMT* mice tumors compared with the control mice (Fig. 1I). Furthermore, immunohistochemical staining showed that the percentage of Ki67-positive cells was significantly elevated in mammary tumors of *Frmd8*-depleted mice (Fig. 1J and 1K). Consistently, 5-ethynyl-2'-deoxyuridine (EdU) incorporation assays demonstrated that FRMD8 depletion promoted human breast cancer cell MCF7 and T47D proliferation (Fig. S1C and S1D). These findings indicated that *Frmd8* deficiency in the luminal epithelium accelerates mammary tumor growth in *MMTV-PyMT* mice and promotes cell proliferation.

Since tamoxifen is commonly used for the treatment of ER α ⁺/HER2⁻ breast cancer, we thus investigated whether loss of *Frmd8* affects sensitivity of mammary tumors to tamoxifen treatment in mice. To this end, *MMTV-Cre⁺; Frmd8^{fl/fl}; PyMT* mice and *MMTV-Cre⁺; Frmd8^{fl/fl}; PyMT* mice were injected intraperitoneally with tamoxifen or corn oil as control every two days. The results showed that tamoxifen significantly prevents mammary tumor progression in the control mice (Fig. 1L and 1M). However, mammary tumors of *MMTV-Cre⁺; Frmd8^{fl/fl}; PyMT* mice showed no response to tamoxifen treatment (Fig. 1L and 1M). Consistently, the total tumor weight (Fig. 1N) and number of tumors (Fig. 1O) of *MMTV-Cre⁺; Frmd8^{fl/fl}; PyMT* mice were markedly decreased after tamoxifen treatment, whereas there was no difference in *MMTV-Cre⁺; Frmd8^{fl/fl}; PyMT* mice (Fig. 1N and 1O). Taken together, these findings demonstrated that loss of *Frmd8* accelerates mammary tumor growth and generates resistance to tamoxifen therapy in *Frmd8*-depleted mice.

Frmd8 knockout decreases the proportion of the hormone-sensing differentiated epithelial cells

To investigate the mechanism through which *Frmd8* loss promotes mammary tumor growth and leads to tamoxifen resistance, we then performed single-cell RNA sequencing (scRNA-seq) analysis. Mammary tumors from 4-month-old *MMTV-Cre⁺; Frmd8^{fl/fl}; PyMT* and *MMTV-Cre⁺; Frmd8^{fl/fl}; PyMT* mice were harvested and analyzed using the Chromium Single Cell 3' Reagent Kitsv3 (10 $\times$ Genomics). Cells that passed quality control (QC) filter totaled 24,320, of which 11,606 cells were

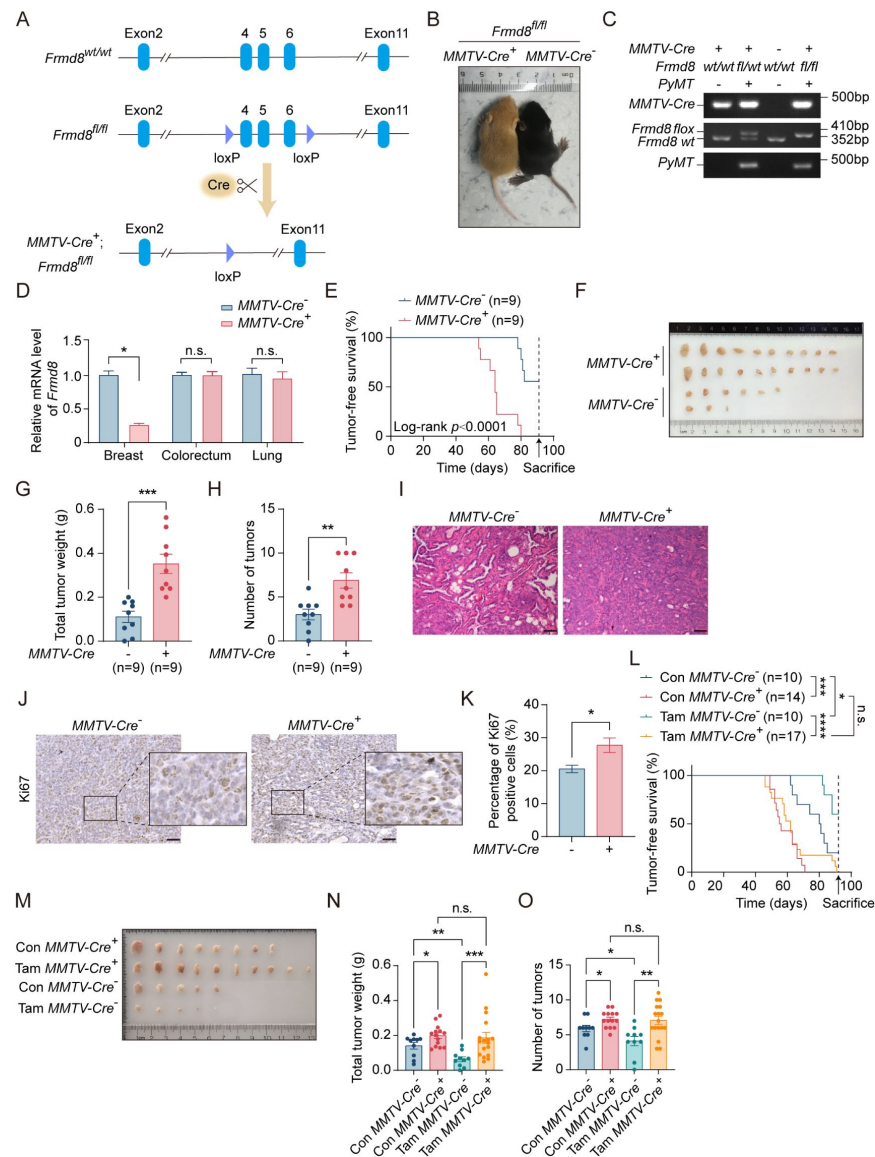


Figure 1.

Loss of *Frmd8* promotes mammary tumor growth and generates tamoxifen resistance *in vivo*

(A) A diagram of the *Frmd8* targeted alleles. Exons 4, 5 and 6 are flanked by loxP sites. (B) Distinguishing the genotype of littermate mice by mice coat color. Yellow represents *MMTV-Cre*⁺; *Frmd8*^{fl/fl}; *PyMT* genotype and black represents *MMTV-Cre*⁺; *Frmd8*^{fl/fl}; *PyMT* genotype. (C) Representative PCR genotyping of mouse tail DNA. (D) Relative mRNA level of *Frmd8* in 7-week-old mammary glands from *PyMT* mice was analyzed by qRT-PCR. *Gapdh* was used as an internal reference. **p*<0.05 by unpaired Student's t-test. (E) Kaplan-Meier plot showing the appearance of palpable tumors in *MMTV-Cre*⁺; *Frmd8*^{fl/fl}; *PyMT* (n=9) and *MMTV-Cre*⁺; *Frmd8*^{fl/fl}; *PyMT* (n=9) mice (Log-rank test). (F) Representative images of tumors from *MMTV-Cre*⁺; *Frmd8*^{fl/fl}; *PyMT* and *MMTV-Cre*⁺; *Frmd8*^{fl/fl}; *PyMT* mice. (G-H) Total tumor weight (G) and number of tumors (H) per mice were measured. ***p*<0.01, ****p*<0.001 by unpaired Student's t-test. (I) Representative H&E staining of tumors from *MMTV-Cre*⁺; *Frmd8*^{fl/fl}; *PyMT* and *MMTV-Cre*⁺; *Frmd8*^{fl/fl}; *PyMT* mice. Scale bar, 50 μm. (J) Immunohistochemistry (IHC) staining for Ki67 expression in mammary tumors from *PyMT* mice. The black boxes represent the magnified typical staining of the original images. Scale bar, 50 μm. (K) Quantification of Ki67-positive cell percentage in (J). **p*<0.05 by unpaired Student's t-test. (L) Kaplan-Meier plot showing the appearance of palpable tumors in *MMTV-Cre*⁺; *Frmd8*^{fl/fl}; *PyMT* and *MMTV-Cre*⁺; *Frmd8*^{fl/fl}; *PyMT* mice, with or without tamoxifen treatment (Log-rank test, **p*<0.05, ****p*<0.001, *****p*<0.0001). (M) Representative images of tumors from *MMTV-Cre*⁺; *Frmd8*^{fl/fl}; *PyMT* and *MMTV-Cre*⁺; *Frmd8*^{fl/fl}; *PyMT* mice, with or without tamoxifen treatment. (N-O) Total tumor weight (N) and number of tumors (O) per mice from (L) were measured. **p*<0.05, ***p*<0.01, ****p*<0.001 by unpaired Student's t-test or Mann Whitney test.

from *MMTV-Cre⁺; Frmd8^{fl/fl}; PyMT*, and 12,714 cells were from *MMTV-Cre⁺; Frmd8^{fl/fl}; PyMT* mice (Fig. 2A). After that, we profiled the three major cell lineages, including epithelial cells, immune cells and stromal cells by the UMAP visualization (Fig. 2B). Their associated top-expressed and canonical markers were shown in Figure S2A and S2B, respectively. Based on the expression of known markers, a total of 12 clearly separated cell lineages were finally identified (Fig. 2C and 2D). In particular, they were as follows: B cells highly expressing *Cd19*, *Cd79a*, *Cd79b*; CD4⁺ T cells characterized with high *Cd3g* and *Cd4* expression; CD8⁺ T cells highly expressing *Cd3g* and *Cd8a*; dendritic cells (DCs) expressing *Cd74* and *Cd14*; endothelial cells specifically expressing the markers *Pecam1* and *Emcn*; epithelial cells expressing *Epcam* and *Krt8*; fibroblast cells high expressing *Col1a1* and *Col3a1*; granulocyte cells specifically expressing the markers *S100a9*; macrophage cells highly expressing *Cd14*, *Cd68* and *C1qa*; monocyte cells highly expressing *Cd14* and *Ccr2*; natural killer (NK) cells specifically expressing *Nkg7* and *Ncr1* (Fig. S2C). The dot plots compared the proportion of cells expressing cluster-specific markers and their scaled relative expression levels (Fig. S2D).

To further explore whether the proportions of epithelial cells in mammary tumors were affected by *Frmd8* loss, we subset and re-identified four epithelial cell clusters (Valdes-Mora et al., 2021), including alveolar progenitor (Avp), basal (Bsl), hormone-sensing differentiated (Hsd) and luminal progenitor (Lp) epithelial cells (Fig. 2E). As expected, the epithelial cells were composed of luminal epithelial cells and basal epithelial cells, which were consistent with the cellular characteristics of the mammary glands. In contrast to control mice, the proportion of the Hsd epithelial cells was significantly decreased in *MMTV-Cre⁺; Frmd8^{fl/fl}; PyMT* mice (Fig. 2F).

To define the tumor cells in the mouse mammary tumors, we applied inferCNV algorithm to calculate the copy number variations (CNVs) of the single cells (Fig. 2G). To this end, we analyzed CNV scores of epithelial subclusters, immune and stromal cells, revealing immune cells with low CNV score (Fig. 2H). Given that Hsd epithelial cells which specifically expressed *Esr1* and *Pgr* had lower CNV score than alveolar progenitor (Avp), basal (Bsl) and luminal progenitor (Lp) epithelial cells, we defined the low CNV score Hsd epithelial cells as normal cells (Fig. 2H and 2I). Furthermore, we defined the high CNV score epithelial cells as tumor cells, and plotted the UMAP visualization (Fig. 2J). The results also showed that the expression of *Frmd8* was decreased in tumor cells compared with normal cells (Fig. 2K and S2E). In addition, we observed that loss of *Frmd8* significantly decreased the expression of *Esr1* and *Pgr* in normal cells of mammary tumors, and decreased expression of *Frmd8* in tumor cells accompanied with low expression of *Esr1* and *Pgr* compared with normal cells (Fig. 2L and S2E). Taken together, these findings indicated that *Frmd8* depletion in *PyMT* mice leads to decreases of the Hsd epithelial cells proportion and the expression of *Esr1* and *Pgr*.

FRMD8 promotion of *ESR1* expression is mediated by FOXO3A

Given that scRNA-seq results suggested that loss of *Frmd8* reduced the proportion of Hsd epithelial cells and the expression of *Esr1* and *Pgr*, multiple immunofluorescence staining analyses were then performed to examine the change of ERα and PR at the protein levels *in situ* in mammary tumors of 4-month-old *Frmd8*-depleted mice. Consistent with the scRNA-seq results, deficiency of *Frmd8* dramatically decreased the levels of ERα, PR and CK8, a marker of mammary luminal epithelium, in normal tissues adjacent to tumors (Fig. 3A and 3B). *In situ* tissue flow cytometry analysis demonstrated that in normal tissues adjacent to tumor of the control mice, the proportion of ERα-positive cells among CK8⁺ cells were 45.49%, whereas this proportion was just 9.28% in *MMTV-Cre⁺; Frmd8^{fl/fl}; PyMT* mice (Fig. S3A). Similarly, the proportion of PR-positive cells among CK8⁺ cells was also reduced in normal tissues adjacent to tumor of *MMTV-Cre⁺; Frmd8^{fl/fl}; PyMT* mice compared with the control mice (Fig. S3B). FRMD8^{high}ERα^{high} and FRMD8^{high}PR^{high} cells were mainly present in normal tissues adjacent to tumor of *MMTV-Cre⁺; Frmd8^{fl/fl}; PyMT* mice, whereas *Frmd8* depletion led to FRMD8^{low}ERα^{low} and FRMD8^{low}PR^{low} cells markedly increased (Fig. S3C and S3D). In mammary tumor tissues from both control mice and *MMTV-Cre⁺; Frmd8^{fl/fl}; PyMT* mice, the expressions of ERα and PR were almost negative (Fig. 3A and 3B).

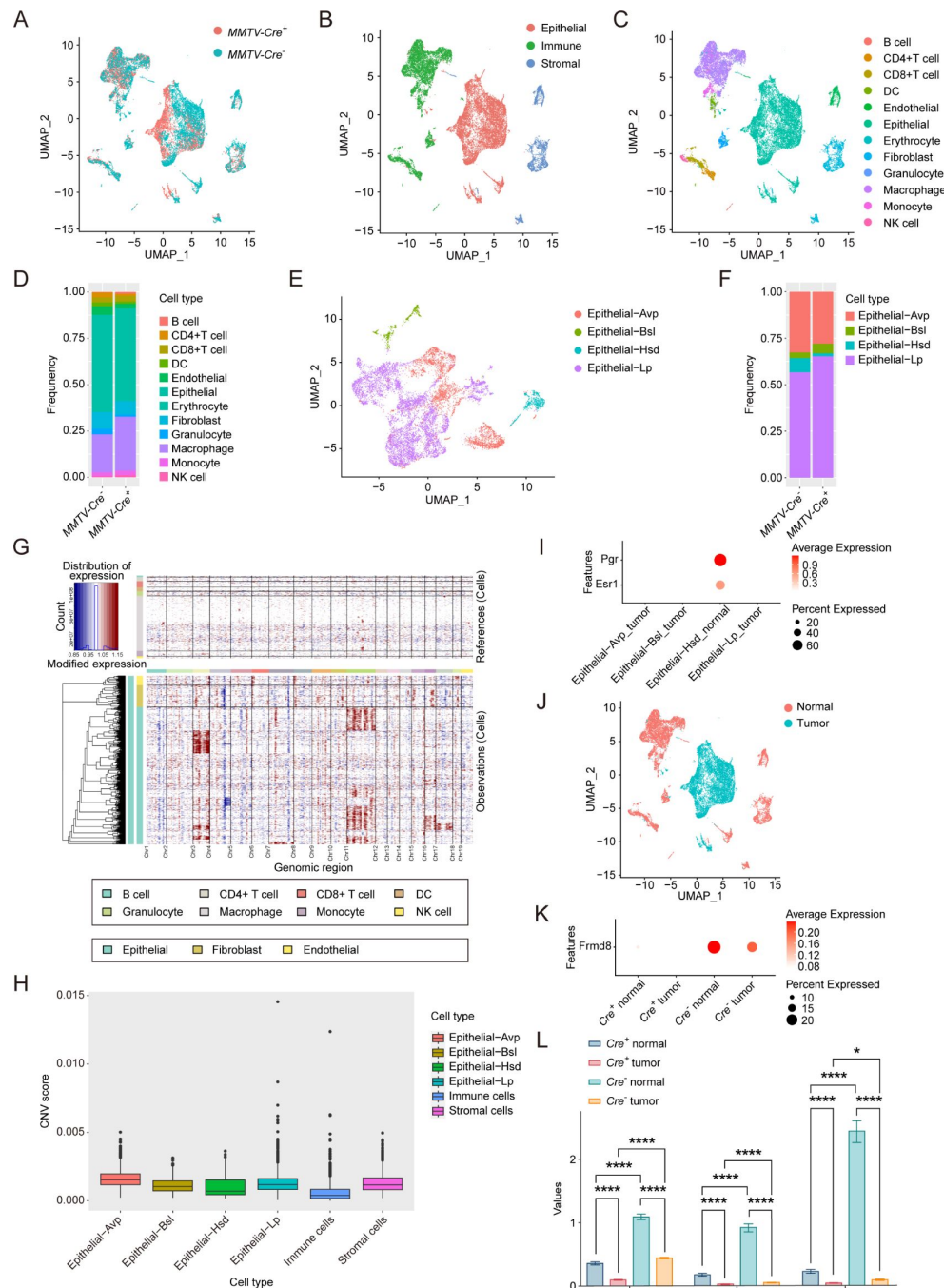


Figure 2.

Frmd8 knockout decreases the proportion of the hormone-sensing differentiated epithelial cells

(A) T-SNE plot showing the distribution of cells from *MMTV-Cre⁺; Frmd8^{fl/fl}; PyMT* and *MMTV-Cre⁺; Frmd8^{fl/fl}; PyMT* mice. (B) T-SNE plot showing the distribution of epithelial, immune and stromal cells. (C-D) T-SNE plot showing the distribution of main cell lineages (C) and their relative percentage (D). (E-F) T-SNE plot showing the distribution of epithelial cell lineages (E) and their relative percentage (F). (G) Heatmap showing distinct features of each cell lineages. Rows, genes. Columns, cells. The color key from blue to red indicates low to high gene expression. (H) Boxplot showing CNV score of main cell lineages. (I) Dot plot showing the expression of *Esr1* and *Pgr* in epithelial cell lineages. (J) T-SNE plot showing the distribution of normal cells and tumor cells. (K) Dot plot showing the expression of *Frmd8* in normal and tumor cells from *MMTV-Cre⁺; Frmd8^{fl/fl}; PyMT* and *MMTV-Cre⁺; Frmd8^{fl/fl}; PyMT* mice. (L) Statistical analysis of *Frmd8*, *Esr1* and *Pgr* expression in normal and tumor cells from *MMTV-Cre⁺; Frmd8^{fl/fl}; PyMT* and *MMTV-Cre⁺; Frmd8^{fl/fl}; PyMT* mice. **p* < 0.05, *****p* < 0.0001 by Mann-Whitney test.

Since the expression of ER α is lost with tumor progression, to further clarify the regulation of Frmd8 on ER α , we performed immunohistochemistry (IHC) staining of mammary glands of 7-week-old *PyMT* mice, which had no palpable tumors. The results showed that Frmd8 depletion also markedly decreased the expression of ER α in normal and atypical hyperplasia breast tissues (Fig. S3E). Taken together, these data suggested that deficiency of FRMD8 downregulates the protein levels of ER α and PR in mammary tissues of *MMTV-PyMT* mice, and accelerates the loss of luminal phenotype in mammary gland.

It was known that the common endocrine treatment for non-metastatic breast cancer relies on the expression of ER α (Waks & Winer, 2019) and our results demonstrated that loss of Frmd8 promotes mammary tumor growth and confers tamoxifen resistance in mice, we thus aim to further examine whether FRMD8 regulates ER α expression. To this end, we transiently transfect Flag-FRMD8 vector or FRMD8 siRNA in human breast cancer cells. Our results showed that FRMD8 overexpression drastically increased the levels of ER α , while ER α expression was greatly downregulated when FRMD8 was knocked down (Fig. 3C and 3D). Moreover, qRT-PCR results indicated that depletion of FRMD8 significantly decreased the mRNA level of *ESR1* (Fig. 3E).

FOXO3A is a crucial transcription factor for ER α (Jia et al., 2019). To answer whether FOXO3A is involved in the regulation of FRMD8 on ER α , we examined the expression of FOXO3A after silencing FRMD8 through transfecting siRNA into MCF7 and T47D cells. The results showed that FRMD8 silencing dramatically decreased the level of FOXO3A (Fig. 3F). Consistently, overexpression of FRMD8 in MCF7 and T47D cells markedly raised the level of FOXO3A (Fig. 3G). To examine whether FRMD8 promotes *ESR1* transcription through FOXO3A, HA-tagged FRMD8 as well as FOXO3A siRNA were co-transfected into MCF7 cells. Although exogenous FRMD8 significantly upregulated ER α expression, depletion of endogenous FOXO3A greatly reduced the effect of FRMD8 on ER α (Fig. 3H and 3I). Besides, chromatin immunoprecipitation (ChIP)-qPCR was performed and revealed that depletion of FRMD8 significantly decreased the occupancy of FOXO3A at the *ESR1* promoters (Fig. 3J). Altogether, these data indicated that FRMD8 promoted upregulation of *ESR1* is mediated by FOXO3A (Fig. 3K).

FRMD8 stabilizes ER α via prevention of its degradation

Although our results suggest that FRMD8 depletion inhibits the mRNA level of *ESR1*, unexpectedly, we also observed that decreased expression of FRMD8 led to a decreased level of exogenous Flag-ER α (Fig. 4A). This result suggested that in addition to inhibiting mRNA expression of ER α , FRMD8 may also regulate ER α protein expression at the post-translational level. To this end, T47D cells were treated with cycloheximide (CHX), a protein synthesis inhibitor. Depletion of FRMD8 greatly increased the ER α turnover rate and the half-life of ER α decreased from 6 h to approximately 2 h (Fig. 4B and 4C). Furthermore, MCF7 and T47D cells were treated with MG132, a proteasome inhibitor, and chloroquine (CQ), a lysosome inhibitor, to determine whether ER α degradation was mediated by the proteasome or the lysosome. The results showed that the reduction of ER α levels by FRMD8 depletion was blocked by treatment with MG132 but not CQ (Fig. 4D). Altogether, these data suggested that FRMD8 stabilizes ER α protein via a proteasome-mediated degradation pathway.

FRMD8 inhibits ER α degradation by blocking UBE3A binding with ER α

Given that FERM domain containing proteins play roles via regulating protein-protein interaction, we wonder whether there is an interaction between FRMD8 and ER α . To this end, co-immunoprecipitation (co-IP) assays were performed using Flag-FRMD8 in MCF7 and T47D cells to examine the association of exogenous FRMD8 with endogenous ER α . The results showed that FRMD8 interacts with ER α (Fig. 5A and 5B). Importantly, an interaction between endogenous FRMD8 and endogenous ER α was also observed in MCF7 cells (Fig. 5C). Furthermore, a

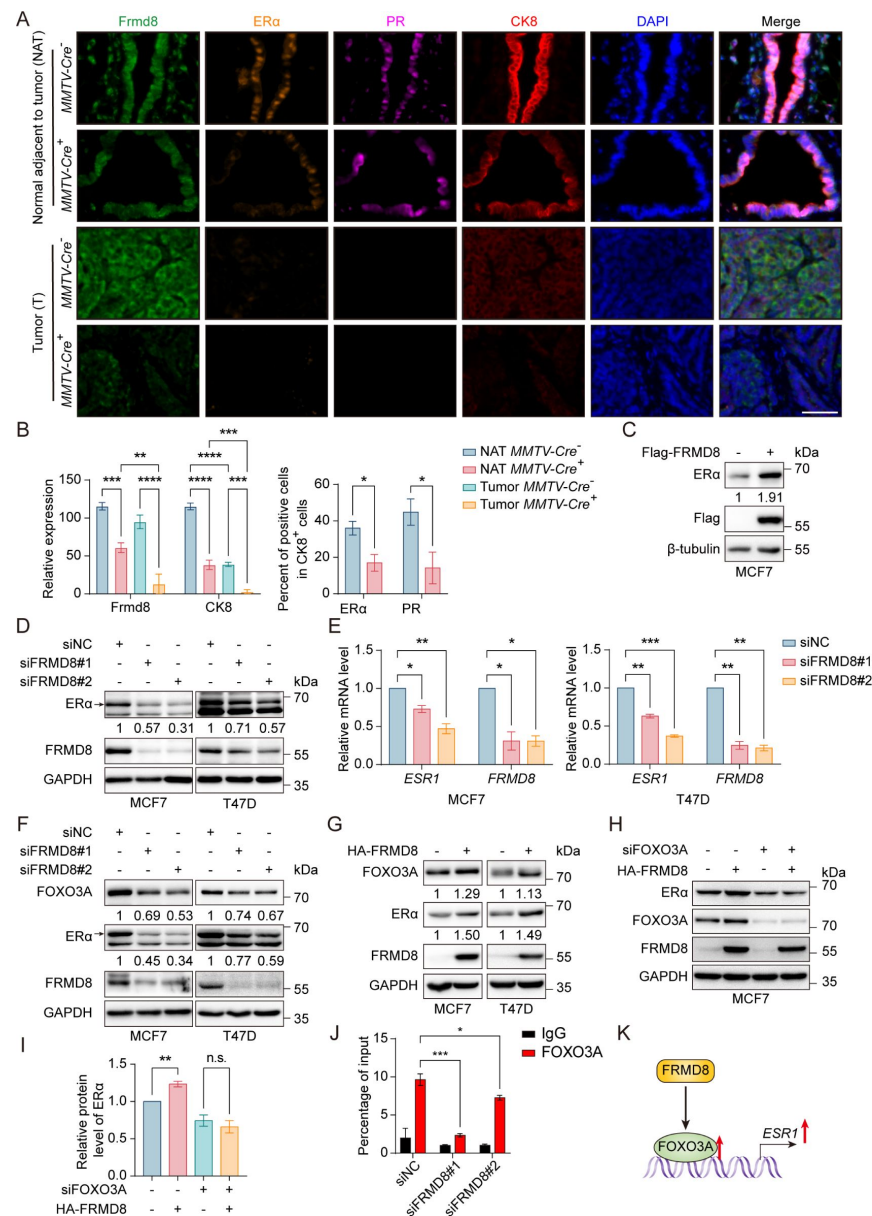


Figure 3.

FRMD8 promotion of *ESR1* expression is mediated by FOXO3A

(A) Representative multiplex immunofluorescence images of tumor tissues and tissues adjacent to tumor from *MMTV-Cre⁺; Frmd8^{fl/fl}; PyMT* and *MMTV-Cre⁺; Frmd8^{fl/fl}; PyMT* mice. Scale bar, 50 μ m. (B) Qualification of Frmd8 and CK8 expression (left panel) and ER α and PR-positive cell percentage in CK8⁺ cells (right panel) in (A). * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001 by one-way ANOVA (left panel) or unpaired Student's t-test (right panel). (C) Lysates from MCF7 cells transiently transfected with Flag or Flag-FRMD8 were immunoblotted. (D) Lysates from MCF7 and T47D cells transiently transfected with control or FRMD8 siRNA were immunoblotted. In this and subsequent figures, specific bands are marked with an arrow. (E) Relative mRNA levels of *ESR1* and *FRMD8* from MCF7 and T47D cells transiently transfected with control or FRMD8 siRNA were analyzed by qRT-PCR. *GAPDH* was used as an internal reference. * p <0.05, ** p <0.01, *** p <0.001 by one-way ANOVA. (F) Lysates from MCF7 and T47D cells transiently transfected with control or FRMD8 siRNA were immunoblotted. (G) Lysates from MCF7 and T47D cells transiently transfected with HA or HA-FRMD8 were immunoblotted. (H-I) Lysates of MCF7 cells co-transfected with HA-FRMD8 and FOXO3A siRNA as indicated were immunoblotted (H). ER α protein levels were quantified by normalizing to the intensity of the GAPDH band (I). ** p <0.01 by unpaired Student's t-test. (J) Lysates of T47D cells transfected with control or FRMD8 siRNA were subjected to anti-FOXO3A ChIP-qPCR. * p <0.05, *** p <0.001 by one-way ANOVA. (K) Working model for FRMD8 promotes *ESR1* transcription via upregulating FOXO3A expression.

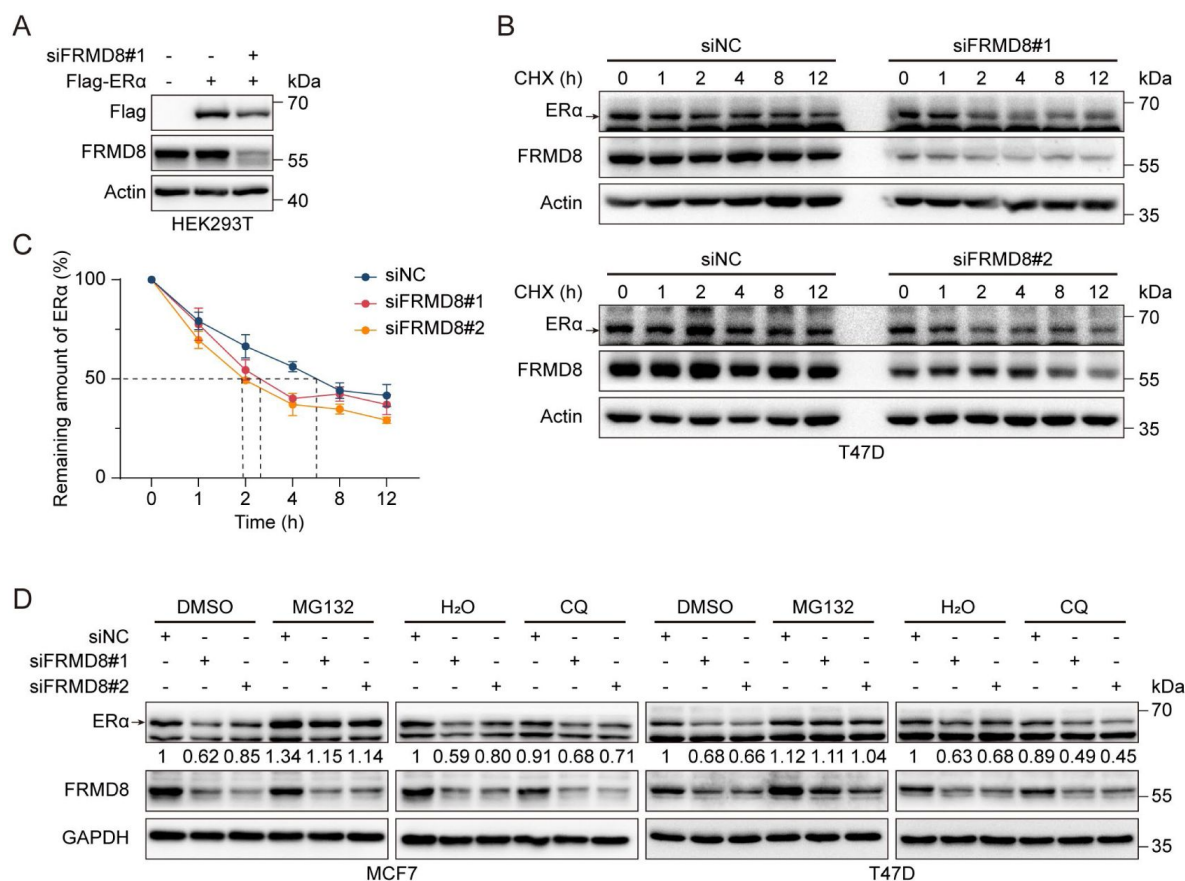


Figure 4.

FRMD8 stabilizes ERα via prevention of its degradation

(A) Lysates of HEK293T cells co-transfected with Flag-ERα and FRMD8 siRNA as indicated were immunoblotted. (B-C) Lysates from T47D cells transiently transfected with control or FRMD8 siRNA were subjected to immunoblotting. Cells were treated with 100 μg/ml CHX for the indicated times (B). ERα protein levels were quantified by normalizing to the intensity of the Actin band (C). (D) Immunoblot analysis of ERα in MCF7 (D) and T47D (E) cells transiently transfected with control or FRMD8 siRNA. The cells were treated with MG132 (25 μM) or chloroquine (50 μM) for 6 h.

glutathione S-transferase (GST) pull-down assay using purified recombinant GST-FRMD8 and Flag-tagged ER α proteins was also performed. The results indicated that FRMD8 interacts directly with ER α *in vitro* (Fig. 5D). Taken together, these findings suggest that FRMD8 is a binding partner of ER α in human cells.

The aforementioned findings demonstrated that FRMD8 prevents ER α degradation via proteasome pathway. To explore the mechanism by which FRMD8 inhibits ER α degradation, co-IP assay and mass spectrometry (MS) analysis were performed in HEK293A cells transiently expressing Flag-FRMD8 (Table S1). We next searched for the FRMD8-interacting proteins identified by MS matched with the known E3 ubiquitin ligases of ER α . Interestingly, UBE3A, a ubiquitin ligase for ER α (Sun et al., 2012), is the only matched protein that interacts with FRMD8 (Fig. 5E). We thus examined the interaction between FRMD8 and UBE3A through co-IP. The results indicated an interaction between FRMD8 and UBE3A (Fig. 5F and 5G). Further, we assumed that FRMD8 may interfere with the interaction between ER α and UBE3A. To test this idea, co-IP assays were performed in HEK293T and T47D cells. In both cell types, the level of ER α ubiquitination and the interaction between ER α and UBE3A were markedly increased by FRMD8 depletion (Fig. 5H and 5I). Intriguingly, FRMD8 depletion led to a marked decrease of ER α expression, while depletion of UBE3A dramatically rescued the effect of FRMD8 depletion (Fig. 5J and 5K). Taken together, these results strongly demonstrated that FRMD8 binds to ER α and UBE3A, and prevents UBE3A interaction with ER α , thereby blocking UBE3A-mediated ER α ubiquitination and degradation (Fig. 5L).

FRMD8 promoter is methylated and low FRMD8 level predicts poor prognosis in breast cancer patients

Given that *Frmd8* prevents mammary tumor growth and inhibits tumor cell proliferation in mice (Fig. 1), we would like to investigate whether the expression of FRMD8 is downregulated in mammary tumors. ScRNA-seq results demonstrated that the level of *Frmd8* in normal Hsd epithelial cells was significantly higher than other tumor cells in the mammary tumors from *PyMT* mice (Fig. 6A). To further verify the expression of FRMD8 in human breast cancer cell lines, whole cell lysates from normal mammary epithelial cells and various subtypes of breast cancer cell lines were subjected to Western blot analysis. Results showed that FRMD8 expression was lower in claudin-low breast cancer cell lines compared with normal mammary epithelial cell line or ER α -positive cell lines (Fig. 6B).

Hypermethylation of tumor suppressor genes is one of the major causes for tumorigenesis. Therefore, we investigated whether the promoter region of FRMD8 is hypermethylated in breast cancer patients. An analysis based on TCGA database showed that the *FRMD8* promoter was highly methylated in primary breast tumors (Fig. 6C). Subsequently, breast cancer cells were treated with the DNA methyltransferase inhibitor 5-aza-2-deoxycytidine (5-Aza-dC). The results showed that 5-Aza-dC treatment significantly upregulated both mRNA and protein levels of FRMD8 in claudin-low but not luminal breast cancer cell lines (Fig. 6D, 6E and S4A), which was consistent with the lower expression of FRMD8 in claudin-low breast cancer cell lines (Fig. 6A). These findings demonstrated that *FRMD8* gene promoter is hypermethylated, which could be the reason for the reduced FRMD8 expression in triple-negative breast cancer cells.

To further investigate the clinical significance of FRMD8 in breast cancer patients, we performed immunohistochemical staining of FRMD8 in a tissue microarray of breast cancer patients and evaluated the level of FRMD8 (Fig. 6F). The results suggested that patients with lower level of FRMD8 showed poor overall survival ($p=0.0409$) (Fig. 6G). Moreover, we also found that a decreased FRMD8 level was associated with poor recurrence free survival in breast cancer patients according to Kaplan-Meier Plotter analysis (Fig. 6H, 6J, S4B and S4C). Collectively, these data indicated that the promoter of *FRMD8* is hypermethylated, and the low FRMD8 level predicts poor prognosis of breast cancer patients.

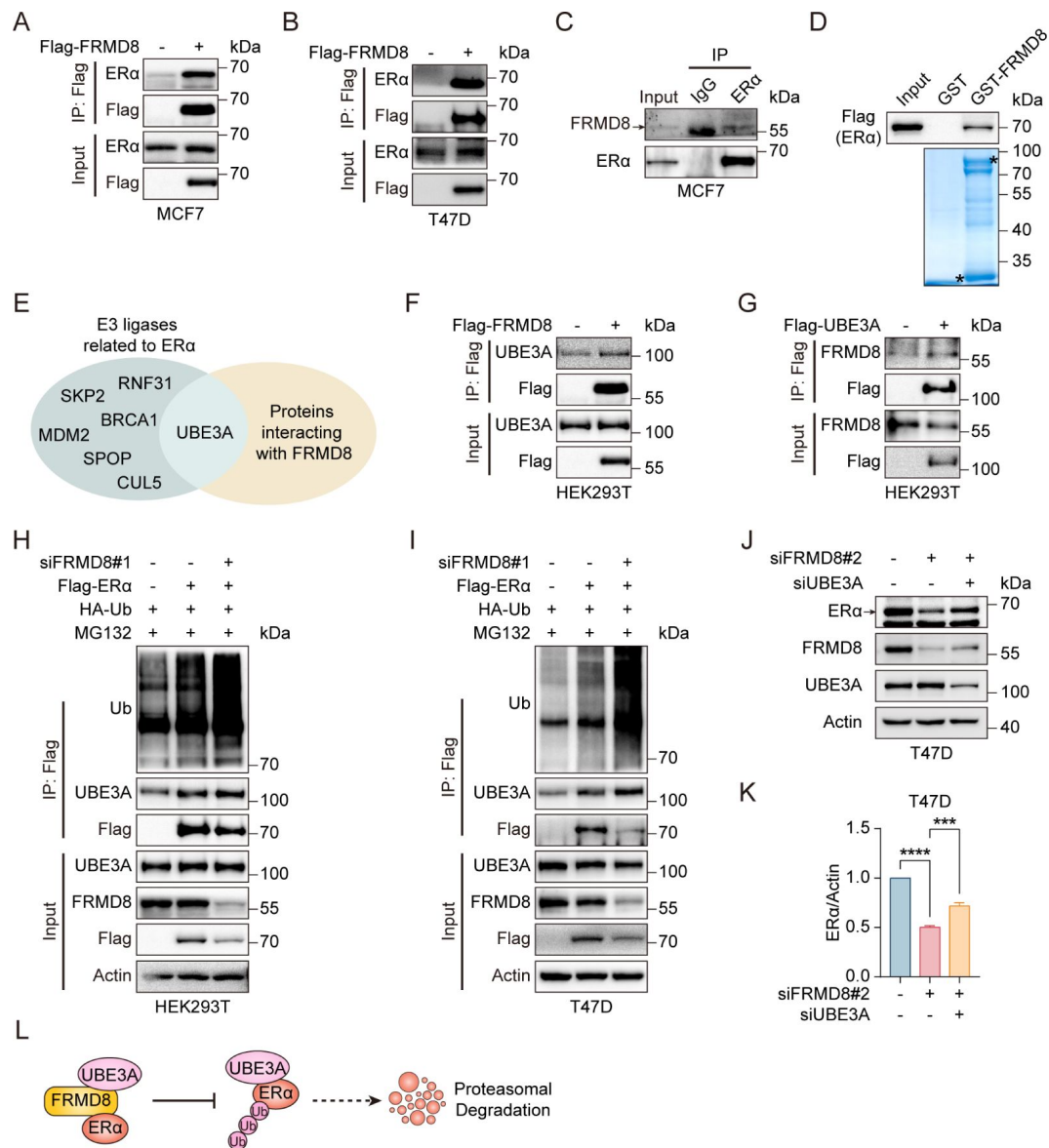


Figure 5.

FRMD8 inhibits ERα degradation by blocking UBE3A binding with ERα

(A-B) Lysates of MCF7 (A) and T47D (B) cells transfected with Flag or Flag-FRMD8 were anti-Flag immunoprecipitated and immunoblotted for ERα and Flag. (C) Lysates from MCF7 cells were immunoprecipitated with IgG or anti-ERα, then immunoblotted for FRMD8 and ERα. (D) HEK293T cells were transiently transfected with Flag-ERα. ERα proteins in HEK293T whole cell lysates (WCL) pulled down by GST or GST-FRMD8 recombinant proteins were subjected to Western blot. Asterisks indicate proteins at the expected molecular weight. (E) Venn diagram showing overlap of E3 ligases related to ERα and proteins interacting with FRMD8. (F) Lysates of HEK293T cells transfected with Flag or Flag-FRMD8 were anti-Flag immunoprecipitated and immunoblotted. (G) Lysates of HEK293T cells transfected with Flag or Flag-UBE3A were anti-Flag immunoprecipitated and immunoblotted. (H-I) HEK293T (H) and T47D (I) cells were co-transfected with Flag-ERα, HA-Ub and FRMD8 siRNA as indicated. Cells were treated with MG132 (25 μM) for 6 h. WCL were immunoprecipitated with anti-Flag and then immunoblotted for ubiquitinated ERα. (J-K) Lysates of T47D cells co-transfected with FRMD8 and UBE3A siRNA as indicated were immunoblotted (J). ERα protein levels were quantified by normalizing to the intensity of the Actin band (K). *** $p < 0.001$, **** $p < 0.0001$ by one-way ANOVA. (L) Working model for FRMD8 disrupts the interaction between ERα and UBE3A, and protects ERα from UBE3A-mediated degradation.

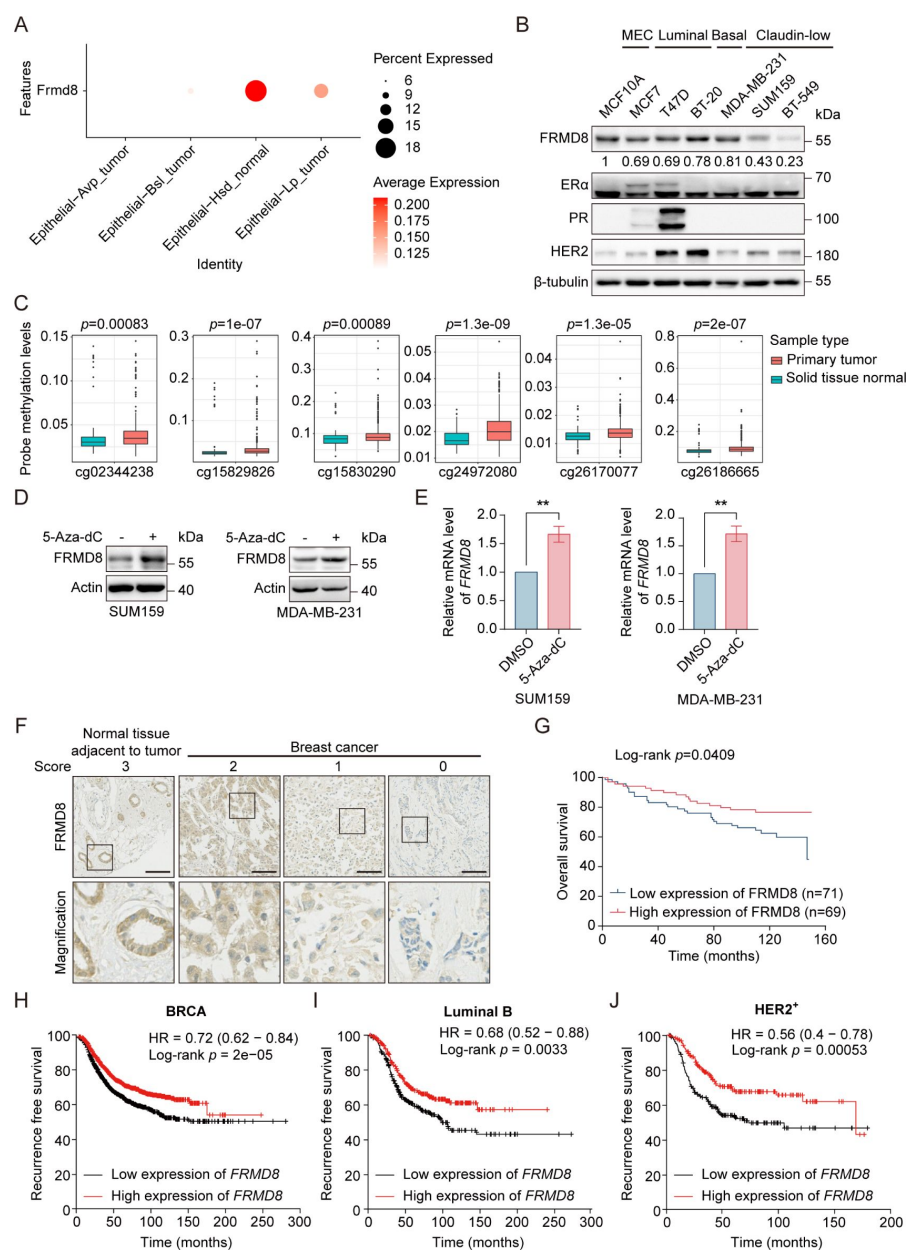


Figure 6.

***FRMD8* promoter is methylated and low *FRMD8* level predicts poor prognosis in breast cancer patients**

(A) Dot plot showing the expression of *Frmd8* in epithelial cell lineages from *PyMT* mice. (B) Lysates from human mammary epithelial cell (MEC) and breast cancer cells were subjected to immunoblotting. (C) Methylation of *FRMD8* promoter region in breast cancer according to the University of California Santa Cruz (UCSC) database (<http://xena.ucsc.edu/>). (D) SUM159 and MDA-MB-231 cells were treated with 5-Aza-dC (10 μM) for 48 h. Protein expression of *FRMD8* was examined by Western blot. (E) SUM159 and MDA-MB-231 cells were treated with 5-Aza-dC (10 μM) for 48 h. *FRMD8* mRNA levels was examined by quantitative reverse transcription PCR (qRT-PCR) *GAPDH* was used as an internal reference. $**p<0.01$ by unpaired Student's t-test. (F) IHC analysis of *FRMD8* expression in human breast carcinoma TMA was performed. Representative examples (scale bar, 100 μm) of normal tissue adjacent to tumor and breast cancer with different levels of *FRMD8* expression are shown, with the magnification of selected areas inserted. (G) Kaplan-Meier analysis for the overall survival of breast cancer patients according to *FRMD8* expression (Log-rank test). (H-J) Recurrence free survival of breast cancer patients according to *FRMD8* expression were analyzed according to Kaplan-Meier plotter (<http://kmplot.com/analysis/>).

Discussion

In this study, we found that FRMD8 plays a tumor suppressive role in breast cancer progression. We demonstrated that loss of FRMD8 promotes mammary tumor growth, accelerates the loss of mammary luminal phenotype, and confers tamoxifen resistance via downregulating ER α level. FRMD8 depletion not only suppresses transcription of *ESR1* through decreasing the level of FOXO3A, but also promotes the E3 ligase UBE3A binding with ER α to disrupts ER α protein stability. Moreover, the promoter of *FRMD8* is hypermethylated and a decreased FRMD8 level predicts poor outcomes in breast cancer patients (Fig. 7C).

As a FERM-domain containing protein, FRMD8 is necessary for releasing of TNF and EGFR ligands (Künzel et al., 2018; Oikonomidi et al., 2018). FRMD8 regulates lung cancer cell growth by regulating tumor microenvironment (Badenes et al., 2023). Frmd8-deficient mice are defective in intestinal epithelial barrier repair function and deletion of Frmd8 promotes colorectal tumorigenesis induced by azoxymethane/ dextran sodium sulfate in mice (Badenes et al., 2023; Yu et al., 2023). FRMD8 acts as a scaffold protein, inhibiting CDK4 activation mediated by CDK7 and preventing MDM2-mediated RB degradation (Yu et al., 2023). In this study, our results suggested that FRMD8 inhibits mammary tumor progression in *MMTV-PyMT* mice. Besides, FRMD8 also acts as a scaffold molecule, which interacts simultaneously with ER α and UBE3A, and renders UBE3A unable to bind with ER α , thus stabilizing ER α .

In *MMTV-PyMT* mice, early-stage mammary tumors express ER α and PR, but these receptors are gradually lost as the tumor progresses (Lapidus et al., 1998). Our scRNA-seq results revealed that mammary tumor epithelial cells in *MMTV-PyMT* mice fall into four clusters, with only Hsd epithelial cells showing ER α and PR expression. Additionally, Hsd epithelial cells exhibited the lowest CNV score, indicating a closer resemblance to normal epithelial cells. The loss of Frmd8 reduced the proportion of Hsd epithelial cells and led to a downregulation of ER α and PR expression, implying that Frmd8 deficiency promotes the loss of luminal features in the mammary gland and accelerates mammary tumor progression.

In this study, we identified that loss of FRMD8 inhibiting *ESR1* expression through downregulating the level of FOXO3A. FOXO3A locates in nucleus and blocks cell cycle progression via activating the cell cycle blocking protein p27^{KIP1} (Seoane et al., 2004). In addition, FOXO3A contributes to cell death through a Fas ligand-dependent mechanism (Seoane et al., 2004). Phosphorylated FOXO3A mediated by AKT binds to 14-3-3 protein and remains in the cytoplasm, resulting in loss of activity (Arden, 2004). Thus, FRMD8 may also be involved in the regulation of cell cycle or apoptosis in an ER α -independent manner by regulating FOXO3A. Furthermore, FOXO3A is closely associated with the stemness of breast cancer cells. FOXO3A suppresses breast cancer stem cell properties and tumorigenicity via inhibition of FOXM1/SOX2 signaling (Liu et al., 2020; Yan et al., 2017), suggesting that FRMD8 may have an effect on breast cancer stem cells. However, the mechanisms by which FRMD8 promotes FOXO3A expression remain unclear and need to be further investigated.

The proportion of luminal subtype is increasing in new cases of breast cancer (Waks & Winer, 2019). Tamoxifen therapy is one of the most important systemic treatment for ER α ⁺/HER2⁻ subtype breast cancer patients (Waks & Winer, 2019). Unfortunately, primary or secondary tamoxifen resistance occurs in about 40% of patients treated with tamoxifen therapy, and tamoxifen resistance often leads to the development of resistance to other selective estrogen receptor modulators (Badia et al., 2007; Légaré & Basik, 2016; Rondón-Lagos et al., 2016). As a regulator of ER α , FRMD8 may be a therapeutic target for rescue tamoxifen resistance.

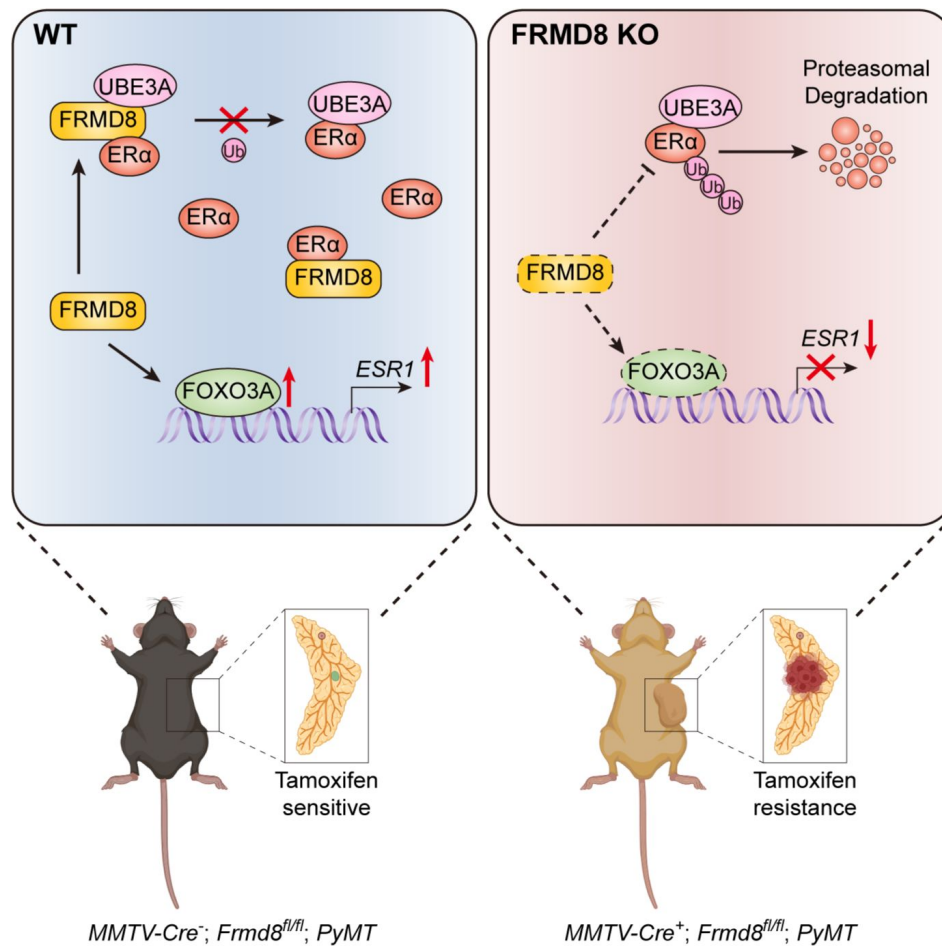


Figure 7.

A working model shows that loss of FRMD8 promotes mammary tumor progression and confers tamoxifen resistance by downregulating ERα expression at both transcriptional and post-translational levels.

In summary, we identified FRMD8 as a prognostic marker in breast cancer. FRMD8 regulates ER α level through a dual mechanism. Loss of FRMD8 inhibits *ESR1* transcription via downregulating FOXO3A expression. FRMD8 also stabilizes ER α protein by preventing UBE3A from binding to ER α . Deficiency of FRMD8 promotes mammary luminal features loss and confers tamoxifen resistance. Our findings indicated that targeting *FRMD8* promoter methylation may provide a novel therapeutic approach for reversing tamoxifen resistance.

Materials and Methods

Mice

Both *Frmd8* floxed mice and *MMTV-PyMT* mice were purchased from Shanghai Model Organisms Center Inc (SMOC). *MMTV-Cre*⁺ mice were from the Nanjing Biomedical Research Institute of Nanjing University.

Frmd8 floxed (*Frmd8*^{fl/fl}) mice were crossed with *MMTV-Cre*⁺; *PyMT* transgenic mice to generate *Frmd8* heterozygous (*MMTV-Cre*⁺; *Frmd8*^{fl/wt}; *PyMT*) mice. *MMTV-Cre*⁺; *Frmd8*^{fl/wt}; *PyMT* mice were further backcrossed with *Frmd8*^{fl/fl} mice to obtain littermate *MMTV-Cre*⁺; *Frmd8*^{fl/fl}; *PyMT* and *MMTV-Cre*⁺; *Frmd8*^{fl/fl}; *PyMT* mice.

Mice were housed in a pathogen-free animal facility at Laboratory Animal Center of Peking University Health Science Center with a 12 h light/dark cycle, constant temperature and humidity, and fed standard rodent chow and water ad libitum. All animal experiments were approved by the Peking University Biomedical Ethics Committee and the approval number is BCJB0104. Genomic DNA extracted from mouse tail biopsies was subjected to standard genotyping PCR using the primers specified in [Table S3](#). The reaction conditions were: 5 minutes at 94°C; 35 cycles of 30 s at 94°C, 30 s at 56°C and 1 minute at 72°C; followed by 5 minutes at 72°C and hold at 4°C.

Tamoxifen treatment

7-week-old female mice were given intraperitoneal injections of either corn oil or tamoxifen (50 mg/kg) every two days. Tumor formation was assessed every two days, and the time point at which the tumor first became palpable was recorded as the tumor-free survival time. At the end of the treatment, mice were euthanized and the total number of tumors were counted. Tumors were measured using calipers, and tumor volume was calculated using the formula: $V = (\text{length} \times \text{width} \times \text{height} \times 0.5) \text{ mm}^3$. Tumors were subsequently fixed in 4% paraformaldehyde (PFA) and housed in individual cassettes for paraffin embedding. 5 μm sections were stained for hematoxylin-eosin (H&E) or immunohistochemistry.

Antibodies and reagents

Antibodies against ER α (#8644), Ubiquitin (#3936) and PR (#8757) were purchased from Cell Signaling Technology. Antibodies specific for ER α (#ab32063), CK8 (#ab53280), FOXO3A (#ab109629), UBE3A (#ab272168) and HER2 (#ab134182) were from Abcam. Anti-PR (#YM3348) antibody was from Immunoway. Anti-FRMD8 (#HPA002861) antibody was from Atlas Antibodies. Anti-Flag (#F3165) antibody was from Sigma-Aldrich. Antibodies against GAPDH (#AC002) was purchased from ABclonal Technology. Anti-Actin (#sc-58673) antibodies was from Santa Cruz Biotechnology. Antibodies specific for β -tubulin (#TA-10) was from Zhong Shan Jin Qiao (ZSGB-Bio).

MG132 (#S2619) was purchased from Selleck. Chloroquine (CQ, #C6628), cycloheximide (#C7698) and 5-aza-2-deoxycytidine (5-Aza-dC; #A3656) were purchased from Sigma-Aldrich. Tamoxifen (#HY-13757A) and estradiol (E2, #HY-B0141) were purchased from MedChemExpress.

Plasmids

The recombinant vectors encoding human FRMD8 and ER α were constructed by PCR-based amplification, and then subcloned into the p3×Flag-CMVTM-10 expression vector. The GST-tagged FRMD8 expression plasmid was generated by inserting PCR-amplified fragments into pGEX-4T-1 vector. All constructs were confirmed by DNA sequencing.

Cell culture

Human embryonic kidney cell lines HEK293T and HEK293A and human breast cancer cell lines MCF7, BT-20, MDA-MB-231 and SUM159 were cultured in Dulbecco's Modified Eagle Medium (DMEM). Human breast cancer cell lines T47D and BT-549 were cultured in RPMI 1640 medium. All media were supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin. Medium for culturing T47D was supplemented with 0.2 U/ml insulin. Human breast cancer cell line MCF10A was cultured in DMEM/F12 medium supplemented with 10% horse serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin, 20 ng/ml epidermal growth factor, 0.5 μ g/ml hydrocortisone, 10 μ g/ml insulin and 100 ng/ml cholera toxin. All cell lines were maintained in a humidified atmosphere at 37°C with 5% CO₂ and passaged using 0.25% trypsin containing 0.02% EDTA for dissociation at 80% confluence.

Plasmid and specific siRNA transfection

For transient transfection, cells at 50%-60% confluence were transfected with plasmids via polyethylenimine (PEI; Polyscience, #24765). For RNA interference experiments, cells at 60-70% confluence were transfected with siRNA using Lipo8000 (Beyotime, # C0533). The specific sequences of siRNA are listed in [Table S2](#).

Extraction of proteins and immunoblotting

Cells or tissues were lysed using RIPA buffer (1×PBS, pH 7.4, 0.5% sodium deoxycholate, 1% NP-40, and 0.1% SDS) complemented with an EDTA-free cocktail of protease inhibitors (Roche). This was followed by centrifugation at 12,000 rpm for over 15 minutes at a temperature of 4°C to collect the supernatant. Protein concentrations were ascertained using a bicinchoninic acid (BCA) protein assay kit (Applygen, #P1511). Protein samples underwent electrophoresis via SDS-PAGE and were then transferred to a polyvinylidene fluoride (PVDF) membrane employing conventional methods. The membranes were treated with 5% non-fat milk for one hour, then incubated with primary antibodies at 4°C overnight, and subsequently exposed to HRP-conjugated goat anti-mouse or rabbit IgG secondary antibodies for one hour at 4°C. The bound antibodies were visualized using the EZ-ECL Chemiluminescence Detection Kit for HRP (Biological Industries, # 20-500-1000) through ChampChemiTM (SageCreation).

Chromatin immunoprecipitation

The chromatin immunoprecipitation (ChIP) assay was performed according to the manufacturer's instructions of Sonication ChIP Kit (ABclonal, #RK20258). The purified DNA was analyzed by quantitative reverse transcription PCR (qRT-PCR). All primers are shown in [Table S2](#).

Immunoprecipitation

IgG, serving as controls, or indicated antibodies were added to pre-cleared lysates and allowed to incubate overnight at 4°C with sustained rocking. Following this, lysates were subjected to a 4-hour incubation with 35-50 μ l of either protein A or protein G Sepharose beads (Santa Cruz Biotechnology) at 4°C. Subsequently, the Sepharose beads were washed thrice with RIPA buffer and heated in SDS-loading buffer at 99°C for 10 minutes. The immunoprecipitation results were assessed via immunoblotting.

Mass spectrometry analysis

Flag-FRMD8 and empty vector were transiently transfected in HEK293A cells using PEI, and cell lysates were harvested after 48 h. The cell lysates were pre-cleared and then incubated with pre-washed anti-Flag M2 agarose beads (Yeasten, #20584ES08) overnight at 4°C. Beads were washed three times with PBS. Protein samples were then eluted by boiling with sodium dodecyl sulfate (SDS)-loading buffer at 99°C for 10 min. Ten percent of the samples were saved for immunoblotting. The other 90% of samples were separated by SDS-PAGE, visualized by colloidal coomassie blue staining, destained and subjected to mass spectrometry (MS) analysis.

For MS analysis, the protein in the gel was subjected to trypsin digestion. In the LC-MS/MS analysis, the digested products were separated using a 120-minute gradient elution at a flow rate of 0.300 µL/min, utilizing the Thermo Ultimate 3000 nano-UPLC system directly interfaced with the Thermo Fusion LUMOS mass spectrometer. The analytical column used was an Acclaim PepMap RSLC (75 µm ID, 250 mm length, C18). Mobile phase A consisted of 0.1% formic acid, while mobile phase B was 100% acetonitrile with 0.1% formic acid. The Fusion LUMOS mass spectrometer operated in data-dependent acquisition mode, employing Xcalibur 4.1.50 software. It started with a single full-scan mass spectrum in the Orbitrap (375-1500 m/z, 60,000 resolution), followed by data-dependent MS/MS scans. The MS/MS spectra from each LC-MS/MS run were analyzed against the selected database using Proteome Discovery software (version 2.4).

Recombinant protein expression and GST pull-down assay

For the expression of Glutathione S-transferase (GST) fusion proteins, GST-tagged recombinant vectors were transfected into *Escherichia coli* Rosetta (DE3) cells. Upon the cell culture attaining an OD₆₀₀ between 0.6 and 0.8, the cells were subjected to overnight incubation with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma, #92320) at 20°C. For the purification of GST fusion proteins, cells were collected and sonicated.

For GST-pull down assays, HEK293T cells were transfected and subsequently lysed on ice for over 15 minutes. Purified GST or GST fusion proteins were anchored onto Glutathione Sepharose 4B beads (Pharmacia Biotech). These beads were then further incubated with cellular extract at 4°C for an extended period of over 4 hours, followed by washes with ice-cold PBS for three times. The process concluded with Western blot analysis.

RNA extraction and qRT-PCR

Total RNA was isolated using Trizol reagent (Invitrogen) as per the manufacturer's guidelines. 1 µg of the isolated RNA was converted to cDNA using HiScript II Q RT SuperMix for qPCR (+gDNA wiper) (Vazyme). Quantitative real-time PCR, carried out in triplicate, was used to assess relative mRNA levels and was standardized to *GAPDH* expression. The cDNA products were amplified using the LightCycler 96 (Roche) platform, and data were subsequently analyzed through LightCycler 96 (Roche) along with GraphPad Prism 7.0 software. All relevant primers are listed in [Table S2](#).

Histology and immunohistochemistry staining

Tissues were preserved in 4% paraformaldehyde, embedded in paraffin, and then sliced into 5 µm sections prior to staining. Following deparaffinization and rehydration, these sections were subject to H&E staining for structural examination. Immunohistochemistry staining was executed using the streptavidin-biotin-peroxidase complex method, with subsequent detection of 3'3'-diaminobenzidine (DAB) as guided by the manufacturer's instructions (Dako, Agilent pathology solutions). Observations and imaging were performed using an Olympus BX51 microscope coupled with an Olympus DP73 CCD photography system.

Tumor tissue microarray (TMA) immunohistochemistry analysis

Human breast carcinoma TMA (HBre-Duc140Sur-01) was purchased from Shanghai Biochip. Immunohistochemistry staining for FRMD8 in TMA was performed as described above. The stained microarrays were evaluated by a pathologist blind to cancer outcomes. Based on histological evaluations, staining reactivity was categorized into four levels: absence of reactivity (score=0), weak reactivity (score=1), moderate reactivity (score=2), and intense reactivity (score=3 or 4).

Multiple immunohistochemistry staining and analysis

Multicolor immunohistochemistry was conducted using the TissueGnostics Multiple IHC Assay Kit (TissueGnostics, TGF550) on mammary tumors from mice. Tumors were preserved in 4% paraformaldehyde, embedded in paraffin, and then sliced into 5 μ m sections prior to staining. The sections were then deparaffinized and hydrated, followed by antigen retrieval in a Tris-EDTA buffer via microwave. Slides underwent treatment with 3% H_2O_2 at ambient temperature to eliminate endogenous peroxidase activity. For iterative rounds of cyclic staining, slides were treated with blocking solution, then incubated with the primary antibody either overnight at 4°C or for 2 hours at 37°C, and subsequently with HRP-linked secondary antibody at 37°C for 30 minutes. Signal was enhanced using Tyramide Signal Amplification (TSA) reagents. Antigen retrieval buffer was applied once more, and the aforementioned steps were repeated for additional staining. Lastly, DAPI was used for 10 minutes to stain nuclei. Tissue images were captured using the tissue faxes platform (TissueGnostics).

Immunofluorescence image quantification was carried out via StrataQuest v7.0.158 software (TissueGnostics). Cells were identified based on DAPI staining, and the expression levels of Frmd8, ER α , PR, and CK8 were calculated by the software, measuring fluorescence intensity and area to enumerate positively-stained cells. The threshold for identifying positive cells was set by inspecting cell recognition on the original image using the View Backward Data function.

EdU (5-ethynyl-2'-deoxyuridine) incorporation assay

The EdU incorporation assay was performed according to the manufacturer's instructions of BeyoClick™ EdU Cell Proliferation Kit with Alexa Fluor 488 (Beyotime Biotechnology, #C0071) for cell proliferation.

Single-cell RNA sequence (scRNA-seq)

Single-cell RNA sequencing libraries were meticulously prepared utilizing the Chromium Single Cell 3' Reagent Kits v3 according to the protocols recommended by 10× Genomics. Briefly, an initial quantity of approximately 1×10^5 cells, which had been sorted through fluorescence-activated cell sorting (FACS), underwent a triple wash process in DPBS containing 0.04% BSA. These cells were subsequently resuspended to a final concentration of 700 to 1,200 cells/ μ l, with a minimum viability threshold of 85%. During the library preparation process, cells were encapsulated within droplets to facilitate a precise targeted recovery rate. After the reverse transcription phase, the generated emulsions were disrupted, and the barcoded cDNA was isolated using Dynabeads technology. This was followed by PCR amplification to enrich the cDNA pool. The amplified cDNA served as the template for constructing the 3' gene expression libraries. Specifically, for this construction phase, 50 ng of the amplified cDNA was fragmented and subjected to end repair. This material was then subjected to a double-size selection process using SPRIselect beads. The prepared libraries were sequenced on NovaSeq system provided by Illumina, generating 150 bp paired-end reads.

Single-cell RNA sequence original data process

Prior to commencing the analysis, cellular data underwent a stringent filtering process. Cells with unique molecular identifier (UMI) counts below 30,000 and those exhibiting gene counts within the range of 200 to 5,000 were excluded. Additionally, cells displaying a mitochondrial content exceeding 20% were also removed from further analysis. Following this preprocessing step, the Seurat package (version 2.3) was employed for both dimensionality reduction and cluster identification. The normalization and scaling of gene expression data were accomplished using the `NormalizeData` and `ScaleData` functions, respectively. Subsequently, the `FindVariableFeatures` function facilitated the selection of the 2,000 genes demonstrating the most significant variation for principal component analysis (PCA). Cluster identification was achieved through the `FindClusters` function, which partitioned the genes into distinct groups. To mitigate batch effects across samples, the Harmony package was utilized. The refined dataset was then visualized in a two-dimensional space employing Uniform Manifold Approximation and Projection (UMAP) techniques, thereby providing insightful representations of the underlying cellular heterogeneity.

Chromosomal copy-number variations estimation

The estimation of chromosomal copy-number variations (CNVs) was conducted utilizing the ‘inferCNV’ R package. A diverse reference panel comprised of B cells, T cells, NK cells, macrophages and other immune cells facilitated this analysis. Quantification of CNV scores for each subcluster was achieved by aggregating the CNV levels observed across the constituent cells.

Quantification and statistical analysis

Statistical comparisons were performed using unpaired two-tailed Student’s *t* tests, Mann Whitney test, one-way ANOVA or two-way ANOVA. Statistical analyses were performed using GraphPad Prism 9.0 software (GraphPad Software, La Jolla, CA, USA). Data are presented as mean $\pm$ the standard error of the mean (SEM). Statistical significance was defined as $p < 0.05$.

Data Availability

The accession number for the scRNA-seq data reported in this paper is Gene Expression Omnibus (GEO): GSE244582.

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Additional information

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Additional files

Supplemental information [↗](#)

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Reviewer #1 (Public review):

Summary:

Tamoxifen resistance is a common problem in partially ER-positive patients undergoing endocrine therapy, and this manuscript has important research significance as it is based on clinical practical issues. The manuscript discovered that the absence of FRMD8 in breast epithelial cells can promote the progression of breast cancer, thus proposing the hypothesis that FRMD8 affects tamoxifen resistance and validated this hypothesis through a series of experiments. The manuscript has certain theoretical reference value.

Strengths:

At present, research on the role of FRMD8 in breast cancer is very limited. This manuscript leverages the MMTV-Cre⁺;Frmd8^{fl/fl};PyMT mouse model to study the role of FRMD8 in tamoxifen resistance, and single-cell sequencing technology discovered the interaction between FRMD8 and ESR1. At the mechanistic level, this manuscript has demonstrated two ways in which FRMD8 affects ER α , providing some new insights into the development of ER-positive breast cancer in patients who are resistant to tamoxifen.

Limitations:

Whether FRMD8 can become a biomarker should be verified in large clinical samples or clinical data.

Reviewer #2 (Public review):**Summary:**

The manuscript presents a valuable finding on the impact of FRMD8 loss on tumor progression and the resistance to tamoxifen therapy. The author conducted systematic experiments to explore the role of FRMD8 in breast cancer and its potential regulatory mechanisms, confirming that FRMD8 could serve as a potential target to reverse tamoxifen resistance.

The research is logically coherent and persuasive. The results support their conclusions and have achieved the research objectives.

<https://doi.org/10.7554/eLife.101888.2.sa1>

Author response:

The following is the authors' response to the original reviews.

Public Reviews:**Reviewer #1 (Public review):****Summary:**

Tamoxifen resistance is a common problem in partially ER-positive patients undergoing endocrine therapy, and this manuscript has important research significance as it is based on clinical practical issues. The manuscript discovered that the absence of FRMD8 in breast epithelial cells can promote the progression of breast cancer, thus proposing the hypothesis that FRMD8 affects tamoxifen resistance and validating this hypothesis through a series of experiments. The manuscript has a certain theoretical reference value.

Strengths:

At present, research on the role of FRMD8 in breast cancer is very limited. This manuscript leverages the MMTV-Cre+;Frm8fl/fl;PyMT mouse model to study the role of FRMD8 in tamoxifen resistance, and single-cell sequencing technology discovered the interaction between FRMD8 and ESR1. At the mechanistic level, this manuscript has demonstrated two ways in which FRMD8 affects ERα, providing some new insights into the development of ER-positive breast cancer in patients who are resistant to tamoxifen.

Weaknesses:

This manuscript repeatedly emphasizes the role of FRMD8/FOXO3A in tamoxifen resistance in ER-positive breast cancer, but the specific mechanisms have not yet been fully elucidated. Whether FRMD8 can become a biomarker should be verified in large clinical samples or clinical data.

We appreciate your recognition and valuable suggestions. The proliferation of ERα-positive breast cancer cells is contingent upon the expression of ERα. Tamoxifen, a selective estrogen receptor modulator, competitively binds to ERα, thereby inhibiting the activation of the proliferation signaling pathway. Previous studies have demonstrated that the downregulation

of ER α expression results in a reduction in the sensitivity of breast cancer cells to tamoxifen (PMID: 15894097; PMID: 922747). Our study revealed the molecular mechanism by which FRMD8 regulates ER α expression through FOXO3A and UBE3A, and thus FRMD8 deficiency is a cause of tamoxifen treatment resistance.

In this study, our results showed that low expression of FRMD8 predicts poor prognosis in breast cancer patients. We agree with this reviewer and will validate the role of FRMD8 in more patient samples and expand its application in different cancer types.

Reviewer #2 (Public review):

Summary:

The manuscript presents a valuable finding on the impact of FRMD8 loss on tumor progression and the resistance to tamoxifen therapy. The author conducted systematic experiments to explore the role of FRMD8 in breast cancer and its potential regulatory mechanisms, confirming that FRMD8 could serve as a potential target to reverse tamoxifen resistance.

Strengths:

The majority of the research is logically clear, smooth, and persuasive.

Weaknesses:

Some research in the article lacks depth and some sentences are poorly organized.

Thank you for your helpful suggestion. We have carefully revised the manuscript again.

Recommendations for the authors:

Reviewer #1 (Recommendations for the authors):

This manuscript suggests that the resistance of tamoxifen in breast cancer is linked to the loss of function of FRMD8. This is a relatively good and valuable contribution. However, there are several points that confused me.

(1) The subfigures with important conclusions should include quantitative analysis, for example, Figure 4D, 4E, and 6A. In Figure 6F, which subtypes of normal and tumor tissues were investigated.

Thank you for your helpful suggestions. We have quantified the bands in Figure 4D, 4E, and 6A and labelled them in the figures.

We have also provided details of the tumor samples in Table S3 and the “Materials and Methods” section. The majority of tumor tissues are invasive ductal carcinomas.

(2) In the luminal epithelium-specific Frmd8 knockout mice (MMTV-Cre⁺; Frmd8^{fl/fl}), the authors demonstrated that the loss of FRMD8 promotes the growth of breast tumors. In Figure 3A, the expression of ER α and PR in tumors is nearly negative. However, why was the validation of the mechanism performed in breast tumor cell lines and not in epithelial cells?

Thanks for the question. Early-stage mammary tumors in MMTV-PyMT mice express ER α , while ER α is negative in advanced tumors of MMTV-PyMT mice. Figure 3A shows the results of tumors from four-month-old mice. Meanwhile, our supplementary results showed that loss of Frmd8 decreased ER α expression also in normal and atypical hyperplasia mammary

tissues from 7-week-old *MMTV-PyMT* mice, when the mice had no palpable tumors and ERα is positive (Fig. S3E). We believe that the absence of FRMD8 contributes to the acceleration of the malignant progression during the dynamic evolution of breast cancer. Limited by the difficulty of transfection in breast normal epithelial cell line (MCF10A), we explored the subsequent mechanisms mainly in breast cancer cells and HEK293, a human embryonic kidney cell line. Besides, Figure S3E also showed the regulation of ERα expression by Frmd8 in mouse mammary

epithelial cells.

(3) To explore the mechanism by which FRMD8 inhibits ERα degradation, what is the reason for choosing HEK293A?

Thank you for the good question. HEK293 cell line is commonly used in mechanistic studies. We also employed the breast cancer cell line T47D to verify the observations in HEK293 cells. Furthermore, the mass spectrometry result of HEK293A cells presented in Figure 5E was an additional experiment performed when we were exploring the regulation of the cell cycle by FRMD8, which is published in Cell Reports (PMID: 37527040). Based on the mass spectrometry result, we assumed that FRMD8 may influence ERα degradation mediated by UBE3A.

Reviewer #2 (Recommendations for the authors):

Introduction

(1) In order for the reader to better understand the content of the article, it is better to briefly describe the role of ERα in the progression of breast cancer.

Thank you for your suggestion. We have provided a brief description of the role of ERα in the introduction of revised manuscript:

“ERα is a ligand-activated transcription factor that is activated by oestrogen, and promotes cell proliferation during breast cancer development (Harbeck et al., 2019).”

(2) As ESR1 is mentioned in the second paragraph, a brief description of the relationship between ESR1 and ERα can make the article more logical.

Thank you for the suggestion. We have added the description in the introduction:

“Multiple transcription factors, such as AP-2γ, FOXO3, FOXM1, and GATA3, have been reported to bind to the promoter region of *ESR1*, the gene encoding ERα, and participate in transcriptional regulation of *ESR1* (Jia et al., 2019; Koš et al., 2001).”

(3) In the text, there are two variations of the term FRMD8: 'FRMD8' and 'Frmd8'. It is best to standardize on one form throughout the document.

We apologize for any confusion. The terms "FRMD8" and "Frmd8" are used to indicate proteins derived from human and mouse, respectively.

Results

(4) In Figure 2L, there is no noticeable difference in the expression levels of Pgr and Esr1 between the Cre+ tumor and Cre- tumor groups. Figure S2E is more suitable for inclusion in the main text compared to Figure 2L.

Thank you for this suggestion. ERα and PR are positive in early-stage mammary tumors of *MMTV-PyMT* mice, while ERα and PR are gradually lost as the tumor progresses. In figure 2,

mammary tumors from 4-month-old *MMTV-PyMT* mice were subjected to scRNA-seq analysis. Since the expression of ERα was very low in tumor cells at this time, there appears to be no difference between the two groups. We have exchanged Figure 2L and Figure S2E in the manuscript.

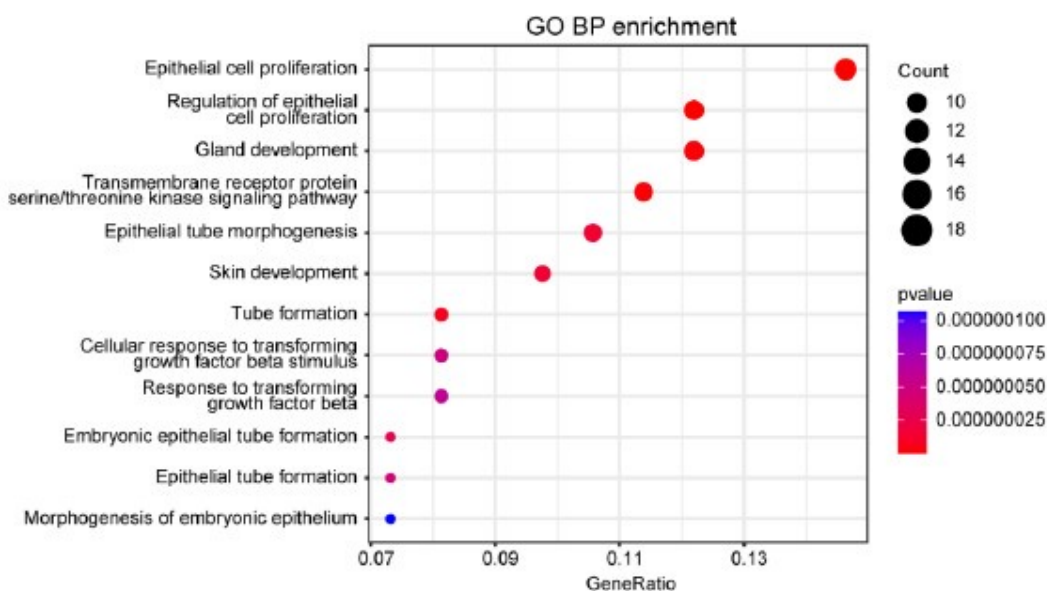
(5) The CNV score can be used to assess the malignancy of cells, it would be better to compare the malignancy levels between the two groups.

This is a very good suggestion. However, copy number variations usually occur randomly and have a high degree of heterogeneity. Due to the limited sample size in our study, we did not compare the difference between the two groups.

(6) Enrichment analysis is crucial for single-cell sequencing studies. It is recommended to perform differential gene analysis and enrichment analysis between the *Cre+* and *Cre-* groups to further explore the impact of *FRMD8* deficiency on the functions of malignant cells.

Thank you for your suggestion. We have performed differential gene analysis and biological process enrichment analysis on the results of scRNA sequence using the gene ontology (GO) database. Our results showed that upregulated genes in luminal progenitor (Lp) epithelial cells were enriched in epithelial cell proliferation and transmembrane receptor protein serine/threonine kinase signaling pathways, suggesting that *Frmd8* deficiency significantly promotes epithelial cells proliferation in *MMTV-PyMT* mice.

Author response image 1.



(7) The coherent logic in lines 300 to 308 should be that *FRMD8* is expressed at higher levels in normal *Hsd* epithelial cells in mice, hence further verification was conducted to examine the expression levels of *FRMD8* in various human breast cancer cell lines.

We have revised the figures and text as suggested.

Discussion

(8) *In lines 352 to 360, the background narrative in the first half seems to have little connection with the research findings in the second half; it is suggested to reorganize the language of this section.*

Thank you for the advice. We have rewritten this paragraph in the manuscript:

“In *MMTV-PyMT* mice, early-stage mammary tumors express ERα and PR, but these receptors are gradually lost as the tumor progresses (Lapidus et al., 1998). Our scRNA-seq results revealed that mammary tumor epithelial cells in *MMTV-PyMT* mice fall into four clusters, with only Hsd epithelial cells showing ERα and PR expression. Additionally, Hsd epithelial cells exhibited the lowest CNV score, indicating a closer resemblance to normal epithelial cells. The loss of *Frmd8* reduced the proportion of Hsd epithelial cells and led to a downregulation of ERα and PR expression, implying that *Frmd8* deficiency promotes the loss of luminal features in the mammary gland and accelerates mammary tumor progression.”

(9) *As stated in the result section, the depletion of *FRMD8* may lead to the decrease of the Hsd epithelial cells proportion, it might be beneficial to discuss the significance of this finding.*

We have added a discussion of the Hsd epithelial cell proportion in the third paragraph of this section (please refer to the above question (8)).

Figures

(10) *The structural layout of Figure 4 should be reorganized to make it more aesthetically pleasing.*

Thank you for this suggestion. We have rearranged Figure 4 as suggested.

<https://doi.org/10.7554/eLife.101888.2.sa0>