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Original Research

Epigenetic regulator KDM4A activates Notch1-NICD-dependent signaling to drive tumorigenesis and metastasis in breast cancer

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ABSTRACT

Background: Altered epigenetic reprogramming and events contribute to breast cancer (Bca) progression and metastasis. How the epigenetic histone demethylases modulate breast cancer progression remains poorly defined. We aimed to elucidate the biological roles of KDM4A in driving Notch1 activation and Bca progression.
Methods: The KDM4A expression in Bca specimens was analyzed using quantitative PCR and immunohistochemical assays. The biological roles of KDM4A were evaluated using wound-healing assays and an *in vivo* metastasis model. The Chromatin Immunoprecipitation (ChIP)-qPCR assay was used to determine the role of KDM4A in Notch1 regulation.
Results: Here, we screened that targeting KDM4A could induce notable cell growth suppression. KDM4A is required for the growth and progression of Bca cells. High KDM4A enhances tumor migration abilities and *in vivo* lung metastasis. Bioinformatic analysis suggested that KDM4A was highly expressed in tumors and high KDM4A correlates with poor survival outcomes. KDM4A activates Notch1 expressions via directly binding to the promoters and demethylating H3K9me3 modifications. KDM4A inhibition reduces expressions of a list of Notch1 downstream targets, and ectopic expressions of ICN1 could restore the corresponding levels. KDM4A relies on Notch1 signaling to maintain cell growth, migration and self-renewal capacities. Lastly, we divided a panel of cell lines into $KDM4A^{high}$ and $KDM4A^{low}$ groups. Targeting Notch1 using specific LY3039478 could efficiently suppress cell growth and colony formation abilities of $KDM4A^{high}$ Bca.
Conclusion: Taken together, KDM4A could drive Bca progression via triggering the activation of Notch1 pathway by decreasing H3K9me3 levels, highlighting a promising therapeutic target for Bca.

Introduction

As the most common solid tumor, breast cancer has become the leading reason of cancer-related deaths worldwide. As reported by the authoritative statistics, the incidence and mortality of breast cancer are 24.2% and 15.0%, individually [1]. The main treatment strategy of Bca is surgery, along with chemotherapy, radiotherapy, endocrine therapy, targeted therapy, as well as immunotherapy [2–4]. Nearly 80% of Bca patients were divided as ER^+ due to ERα positive expressions, and these patients have a 5-year overall survival (OS) rate of nearly 90% [5,6]. Given that ERα is the essential oncogenic driver of ER^+ tumors, the endocrine-based therapeutic strategies like ERα-blockade, estrogen synthesis inhibition, and selective ERα degradation, were largely explored and highlighted [7,8]. Owing to the apparent heterogeneity of breast cancer, Bca could be divided into luminal A, luminal B, HER2-positive and basal-like subtypes [9].

As is well documented, methyltransferases (KMTs) and demethylases (KDMs) were demonstrated to participate in histone lysine methylation [10]. There are two subgroups of KDMs, including the KDM1 or LSD1 family and the JmjC family that relies on 2-oxoglutarate (2-OG) to exert

Abbreviations: KDM4A, lysine demethylase 4A; Notch1, notch receptor 1; TCGA, The cancer genome atlas; IHC, immunohistochemistry; BRCA, breast cancer; ChIP, Chromatin Immunoprecipitation; GSEA, Gene Set Enrichment Analysis.

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the demethylase activity [11]. Among them, the JmjC domain-containing KDMs constitute the larger KDM category with 20 members that are further divided into five subfamilies, like KDM2/7, KDM3, KDM4, KDM5, and KDM6 [12,13]. Of note, the histone demethylase jumonji C domain 2 A (JMJD2A), also named with lysine-specific demethylase 4 A (KDM4A), is regarded to be a potential oncogene and is found to be highly expressed in various human tumors [14]. Previous studies have reported that KDM4A could serve as an AR coactivator via H3K9 demethylation at the promoters of AR targets [15]. Meanwhile, KDM4A could also modulate DNA damage repair genes and regulate genomic instability to exert functions in potentiating tumorigenesis. Previous studies have reported that overexpression of KDM4A was found in 60% of Bca tumors with both mRNA and protein levels. KDM4A overexpression could activate a list of estrogen-dependent genes, whereas KDM4A depletion could reduce the transcription of ERα downstream targets, including CCND1 or JUN [16]. Eric Metzger et al. have reported that the selective and potent KDM4 inhibitor (QC6352) is effective to suppress the progression of breast cancer stem-like cells (BCSC) [17]. However, the specific relationships between KDM4A and Bca tumorigenesis or metastasis remain to be unclear. Apart from the classical ERα pathway, it is meaningful to elucidate whether KDM4A could modulate other biological crosstalk to initiate the progression of Bca.

Distal metastasis is an essential problem for cancer treatment and accounts for nearly 90% of deaths in patients with breast cancer [18,19]. Many dysregulated oncogenes and biological events contribute to the specific phenotypic trait of tumor progression and metastasis in breast cancer [20]. Among these underlying mechanisms, epigenetic reprogramming has an essential role in dysregulation of these genes and promotes breast tumor progression and metastasis [21,22]. Many epigenetic writers, erasers or writers, including YTHDF3, EZH2, histone deacetylase 3 (HDAC3), and E2F1, have been reported to induce abnormal expressions of specific transcriptome in breast cancer cells and enhance the epithelial-mesenchymal transition, a key cellular program in the initiation of metastasis, thereby triggering breast tumor metastasis to other organs [23–25]. Meanwhile, it is still unclear about the underlying mechanisms that KDM4A regulates to enhance tumor metastasis.

Here, in this study, we demonstrated that KDM4A was overexpressed in breast cancer and high levels of KDM4A could predict inferior outcomes of patients. KDM4A was demonstrated to enhance breast cancer proliferation, metastasis and self-renewal abilities. Moreover, we uncovered the KDM4A/Notch1 axis that sustain malignant features of Bca. The efficacy of Notch1 inhibitor (LY3039478) in KDM4A^{high} and KDM4A^{low} groups was also evaludated. Taken together, our findings suggested that KDM4A could be regarded as a novel therapeutic target for breast cancer treatment.

Methods and materials

Cell culture

MCF-7, T47D, MDA-MB-231, MDA-MB-453, BT-474, BT-20 and HEK-293 T cell lines were obtained from the Institute of Biochemistry and Cell Biology at the Chinese Academy of Sciences (Shanghai, China). The cells were maintained in DMEM or RPMI 1640 medium (HyClone, USA) and cultured in a humidified incubator at 37°C. All cells were incubated in an environment containing 5% carbon dioxide.

Patient specimens and immunohistochemical (IHC) staining

Paired breast cancer specimens ($n = 50$) were obtained from the First Affiliated Hospital of Anhui Medical University from November 2014 to June 2020. Before surgery, the patient did not receive chemotherapy or radiotherapy. This study was approved by the Ethics Committee of the First Affiliated Hospital of Anhui Medical University. Immunohistochemistry (IHC) analysis was conducted with a GT Vision III Kit (Genetech, Shanghai, China) according to the manufacturer's instructions. The final stainings were determined as follows: staining intensity score, 0 (no staining), 1 (weak), 2 (moderate), or 3 (strong); staining area score, 0 (≤10% positive staining), 1 (11–25% positive staining), 2 (26–50% positive staining), 3 (51–75% positive staining), and 4 (≥75% positive staining). Staining intensity and staining area were calculated to give a final score.

CCK8 assay and colony formation assay

KDM4A^{high} (MCF-7, MDA-MB-231 and BT-474) and KDM4A^{low} (BT-20, MDA-MB-453 and T47D) cells were transiently transfected and were seeded into 96-well plates with 3×10^3 cells per well. After one day, the cells were treated with ADR (Sigma Chemical Co, St. Louis, MO) for 48 h. Next, all cells were incubated with 10 μl of CCK-8 reagent (Dojindo, Japan) for 1 h before the absorbance was measured at 450 nm on a spectrophotometer. For the colony formation assay, 500 cells/well were seeded in a 6-well culture dish. After 2 weeks, the cells were fixed in 4% paraformaldehyde, stained with crystal violet (Beyotime Biotechnology, CAT#C0121) and counted microscopically.

Wound healing and Transwell invasion assays

For wound healing assay, BT-20 or MCF-7 cells were cultured in six-well plates coated with 0.1% gelatin. When 70% confluency is reached, the cells were starved overnight, wound was scratched in the center of the cell monolayer by a sterile plastic pipette tip, and debris was removed by PBS washing. The wound was photographed at indicated time. For the Transwell invasion assay, 5×10^4 cells suspended in medium without FBS were plated on the upper chamber membranes (8 μm pore size, 6.5 mm diameter, Corning) coated with Matrigel (BD Biosciences). The insert was incubated in 500 μl medium with 10% FBS. To evaluate the invasive ability, non-invasive cells were removed by swiping the top of membrane with cotton swabs and invasive cells were stained with crystal violet and counted.

Tumor sphere formation assay

Breast cancer cells were harvested and re-suspended in sphere formation medium, containing RPMI-1640 or DMEM supplemented with 20 ng/mL fibroblast growth factor-basic and 20 ng/mL epidermal growth factor (Thermo Fisher Scientific, Waltham, MA, USA). Cells were plated in a 48-well Clear Flat Bottom Ultra-Low Attachment Microplate (CORNING, Corning, NY, USA) at a density of 2000, 1000, 500, 250, or 100 cells/well. After 7 days (for MCF-7 cells and MDA-MB-453 cells) or 10 days (for MDA-MB-231 and T-47D cells), the spheres (defined as >20 cell/spheroid) were recorded using Olympus BX51 Epifluorescent microscopy (Olympus, Tokyo, Japan).

RNA isolation and quantitative RT-PCR

Total RNA was isolated using Trizol reagent RNAiso Plus (Takara). Total mRNA was reversely transcribed into cDNA using the 5 × Primescript RT Master Mix (Takara). Quantitative RT-PCR was performed using 2 × SYBR Green Mix (Takara) in Bio-Rad detection system.

Western blot analysis

SDS loading buffer was added to samples and boiled for 5 min at 95 °C. Cell samples were loaded on 10% SDS-PAGE gels and performed with electrophoresis followed by transfer to polyvinylidene fluoride membrane subjected to immunoblotting with different antibodies. After incubation with HRP-conjugated secondary antibodies (CST) at room temperature for 2 h, immunoreactions were visualized using ECL Plus (Thermo Scientific, USA). Antibodies used in this study were listed as the

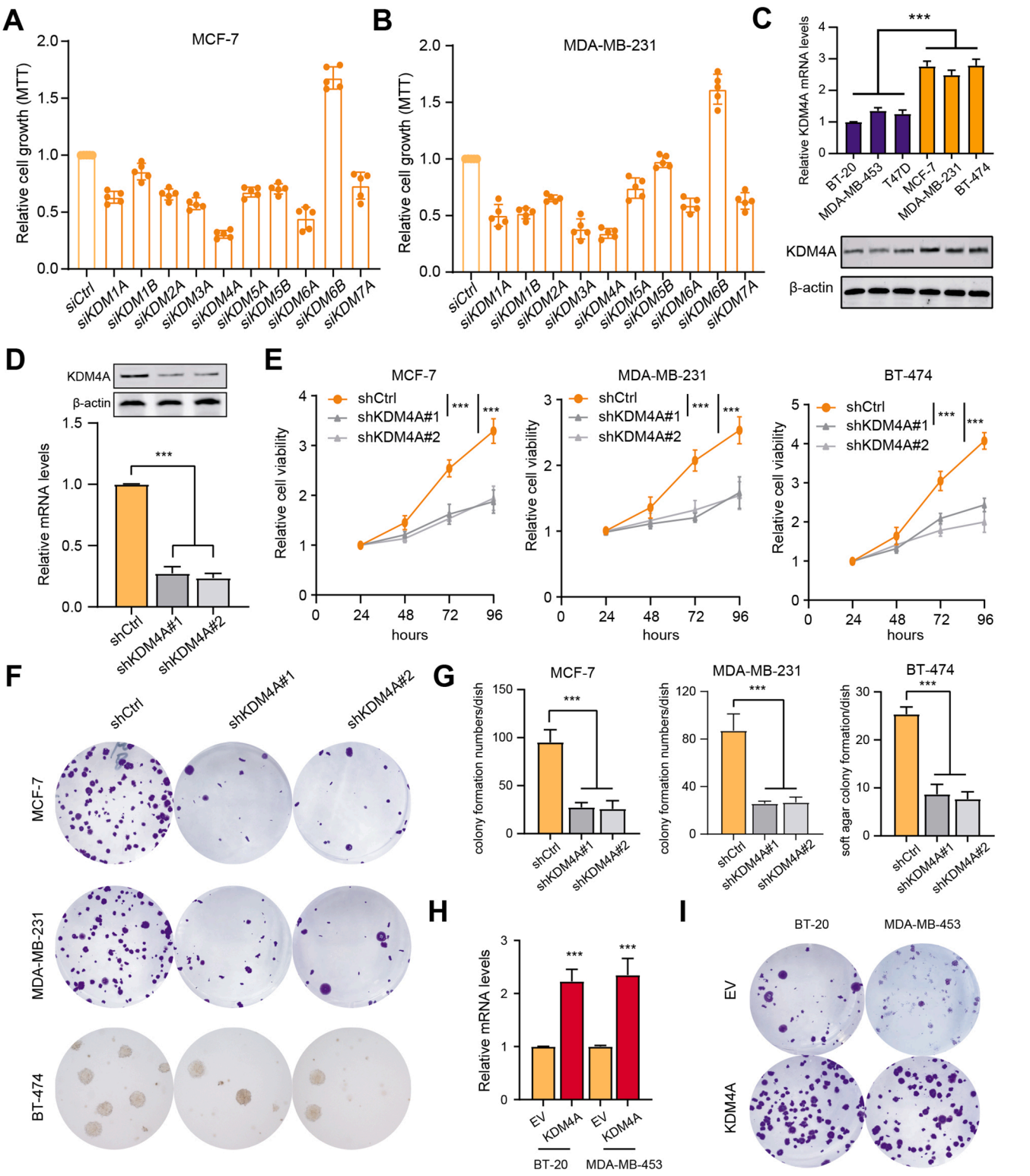

Fig. 1. *MTT assay screened that KDM4A is required for the growth and progression of Bca.* **(A-B)** The siRNA KD of 10 candidate KDM genes and their effects on the growth of MCF-7 and MDA-MB-231 cells. Quantitative results shown are representative of 4 experiments. **(C)** The KDM4A^{low} (BT-20, MDA-MB-453 and T47D) and KDM4A^{high} (MCF-7, MDA-MB-231 and BT-474) cell lines were categorized by RT-qPCR and western blotting assays. **(D)** The RT-qPCR and western blotting assays showed the decreased levels of KDM4A in shCtrl and shKDM4A MCF-7 cells. **(E)** The CCK-8 assays revealed that KDM4A KD could significantly reduce the cell growth as compared to shCtrl cells in three independent cell lines (MCF-7, MDA-MB-231 and BT-474). **(F)** The colony formation assay and soft agar anchorage-independent assay revealed that KDM4A inhibition could significantly reduce the cell proliferation of Bca cells. **(G)** Quantitative statistical data of colony formation assays was shown. **(H)** The RT-qPCR assay showed the KDM4A mRNA levels in cells transfected with KDM4A and EV. **(I)** KDM4A overexpression could promote the colony formation assays as compared to control cells. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

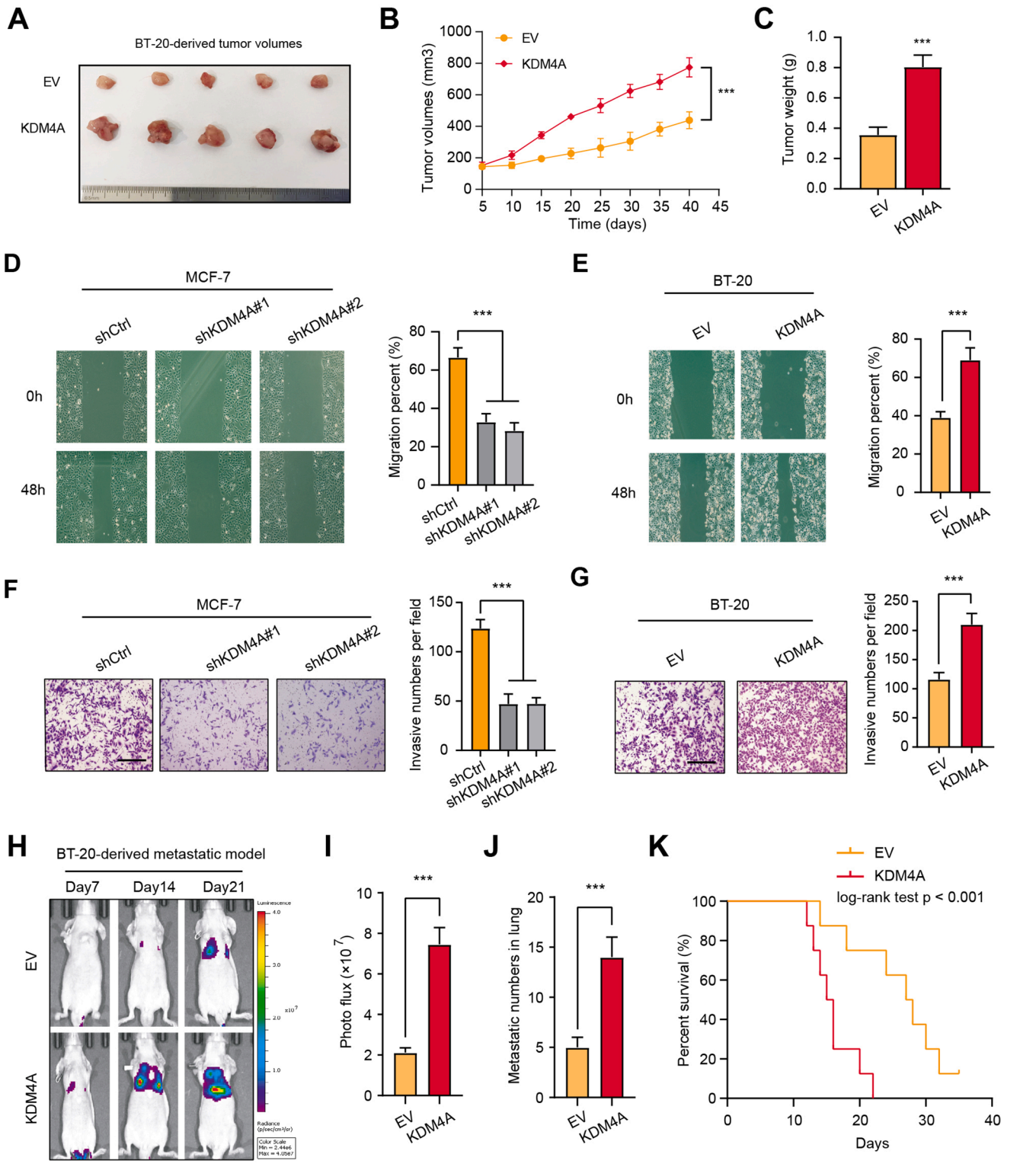

Fig. 2. *High KDM4A promotes Bca cell invasion and metastasis in vitro and in vivo*. **(A)** The subcutaneous tumor model showed the *in vivo* tumor growth in the EV and KDM4A-OE group. **(B)** The tumor growth curve showed the serial tumor volumes in the EV and KDM4A-OE group. **(C)** Quantification of tumor weight in the EV and KDM4A-OE group. **(D)** The wound-healing assay showed that targeting KDM4A with shRNAs could significantly impede the migratory ability of MCF-7 cells. The quantitative data was shown on the right. **(E)** The wound-healing assay showed that KDM4A overexpression could significantly promote the migratory ability of BT-20 cells. The quantitative data was shown on the right. **(F)** The Transwell matrigel invasion assay revealed that the invasive ability of MCF-7 cells was markedly suppressed in response to KDM4A knockdown. **(G)** In contrast, KDM4A overexpression could enhance the invasive ability of BT-20 cells. **(H)** Representative mice injected with modified KDM4A expressing BT-20 cells revealed that overexpression of KDM4A increased Bca lung metastases. **(I-J)** The quantitative results of luciferase signals and lung metastases were shown. **(K)** Kaplan-Meier survival analysis revealed that mice bearing high KDM4A cells suffered from shorter survival months relative to those bearing control cells. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

following: KDM4A (abcam, ab191433), Notch1 (abcam, ab52627), CCND3 (abcam, ab183338), β-actin (abcam, ab8226).

ChIP-qPCR assay

Bca cells were cross-linked with 1% fresh formaldehyde for 10 min at room temperature, neutralized with glycine for 5 min and lysed in SDS lysis buffer. The cross-linked DNA was then sheared into fragments ~200-1000 bp in length with UCD-300 (Bioruptor). ChIP was performed with a Chromatin Immunoprecipitation Kit according to manufacturer's instructions (Millipore, 17-371) to obtain ChIP-enriched DNA. The ChIP grade antibodies were listed as the following: H3K27ac (abcam, ab4729); H3K9me3 (abcam, ab8898).

Xenograft models of breast cancer

The female BALB/c nude mice (4-weeks old) were housed under pathogen-free conditions in the Centre of Laboratory Animals at the Medical College of Anhui Medical University. Animal experiments were performed according to the protocols approved by the Ethics Review Committee of the Medical College of Anhui Medical University. Mice were randomly grouped ($n = 6$ mice per group, the sample size is based on experience from previous studies using the same animals) by random number method with no blinding. A mouse model was established via injecting Bca cells (3×10^6) subcutaneously into the flank of the mice, and we determined the tumor volume for each mouse every three days using 0.5 × length × width × width. The mice were sacrificed after 3 weeks and the tumor specimens were harvested for further experiments. The pulmonary metastatic model was established by injecting Bca cells (1×10^6) into the tail vein. After 6 weeks, the lung tissues were collected and subjected to hematoxylin and eosin staining, followed by examination microscopically.

Bioinformatic analysis

The *limma* package was utilized to compare the differential KDM4A levels between tumor and normal samples. Wilcoxon rank sum test was used to compare the differences. The *survival* package was used to conduct the Kaplan-Meier analysis between the KDM4A-high and KDM4A-low patients. The log-rank test was used to conduct the survival analysis and $p < 0.05$ was considered to be significant. The GSEA (http://www.broadinstitute.org/gsea/index.jsp) was conducted to determine if the identified sets had significant differences between the KDM4A-high and KDM4A-low groups. Gene sets with a p value of < 0.05 and false discovery rate (FDR) of <0.25 after 1000 permutations were considered significantly enriched. GSEA was performed in javaGSEA v. 3.0 based on the Molecular Signatures Database v. 6.2. C2 (curated gene sets), C5 (GO gene sets), and C6 (oncogenic signatures) were searched to identify enriched KEGG pathways, biological processes, cellular components, molecular functions, and dysregulated oncogenic signatures. We used the normalized $P < 0.05$ to define statistical significance. Correlation analysis was conducted by the *Pearson* correlation analysis.

Statistical analysis

All experiments were carried out with at least three replicates. The data were exhibited by Mean ± S.D. or mean ± S.E.M. as indicated in the figure legends. For comparison of central tendencies, normally distributed data sets were analyzed by unpaired two-sided Student's t-tests under assumption of equal variance. The non-normally distributed data sets were analyzed by non-parametric Mann–Whitney U-tests. $\chi 2$-test was utilized to analyze the relationship between KDM4A levels and clinical variables. Differences were considered as statistically significant with $P < 0.05$.

Results

KDM4A is required for the growth and proliferation of Bca cells

To identify the specific members of histone demethylases that are required for Bca growth, we utilized the MTT assay to screen the potential targets. As shown in Fig. 1A-B, we observed that KDM4A inhibition could significantly restrict the cell growth of MCF-7 and MDA-MB-231 cells compared with other KDM family members. In line with the previous studies, KDM6B is a tumor suppressor for Bca and targeting KDM6B induce cell growth (Fig. 1A-B). The knockdown efficacy of siRNAs for specific members of histone demethylases was confirmed by RT-qPCR in Fig. S1A.

Given that KDM4A was little investigated in Bca, we intended to focus on KDM4A for further analysis. First of all, we detected the expressions of KDM4A by RT-qPCR and western blot methods in a panel of Bca cell lines and divided them into $KDM4A^{low}$ (BT-20, MDA-MB-453 and T47D) and $KDM4A^{high}$ (MCF-7, MDA-MB-231 and BT-474) groups (Fig. 1C). Next, we validated two independent shRNA (shRNA1 and shRNA2) specific to human KDM4A, which could notably decrease KDM4A levels (Fig. 1D). Then, the CCK-8 assays revealed that knockdown of KDM4A by both shRNA1 and shRNA2 significantly decreased the cell growth abilities of Bca cells (MCF-7, MDA-MB-231 and BT-474) as compared to shCtrl cells (Fig. 1E). Accordingly, we performed the 2D colony formation and soft-agar colony formation assays to validate that targeting KDM4A significantly suppressed the colony formation abilities of cells (Fig. 1F-G). Conversely, we generated the KDM4A-overexpressing cells and observed that KDM4A overexpression could enhance cell colony formation abilities as compared to EV control group (Fig. 1H-I). Last of all, we generated the subcutaneous tumor model using the KDM4A-overexpressing BT-20 cells and observed that KDM4A overexpression could significantly enhance *in vivo* tumor growth, as reflected by the tumor volumes and tumor weight. Taken together, these results suggested that KDM4A is an epigenetic regulator that is required for maintaining Bca cell growth and proliferation.

KDM4A promotes the migration capacities and metastasis of Bca in vitro and in vivo

To confirm whether KDM4A-mediated effects are indispensible for Bca progression, we decided to further investigate the effects of KDM4A on cell growh and motility. First of all, we generated the subcutaneous tumor model using the KDM4A-overexpressing BT-20 cells and observed that KDM4A overexpression could significantly enhance *in vivo* tumor growth, as reflected by the tumor volumes and tumor weight (Fig. 2A-C). The wound healing assays suggested that KDM4A inhibition remarkably impeded the migratory ability of MCF-7 cells (Fig. 2D). In contrast, KDM4A overexpression could increase cell migration abilities compared with control cells (Fig. 2E). Moreover, transwell matrigel invasion assay confirmed that the invasive ability of Bca cells was notably inhibited with KDM4A knockdown, whereas it was remarkably enhanced with the ectopic expression of KDM4A (Fig. 2F-G). Lastly, we generated the metastatic model in which BT-20 cells with modified KDM4A expression were injected into the tail vein of BABL/c nude mice. We detected the lung luciferase signals at the regular time points to monitor the location and growth of distal metastases in the lung. Notably, we observed that KDM4A overexpression significantly promoted the lung metastases burden of BT-20 cells relative to mice derived from the EV control group, as revealed by metastatic luciferase signals and metastatic nodes (Fig. 2H-J). Mice from the KDM4A-overexpressing group suffered from shorter survival time as compared to mice derived from the control group with log-rank test $p < 0.001$ (Fig. 2K).

KDM4A is an independent factor for predicting prognosis of Bca patients

In addition, we queried the cancer genome atlas (TCGA) datasets and

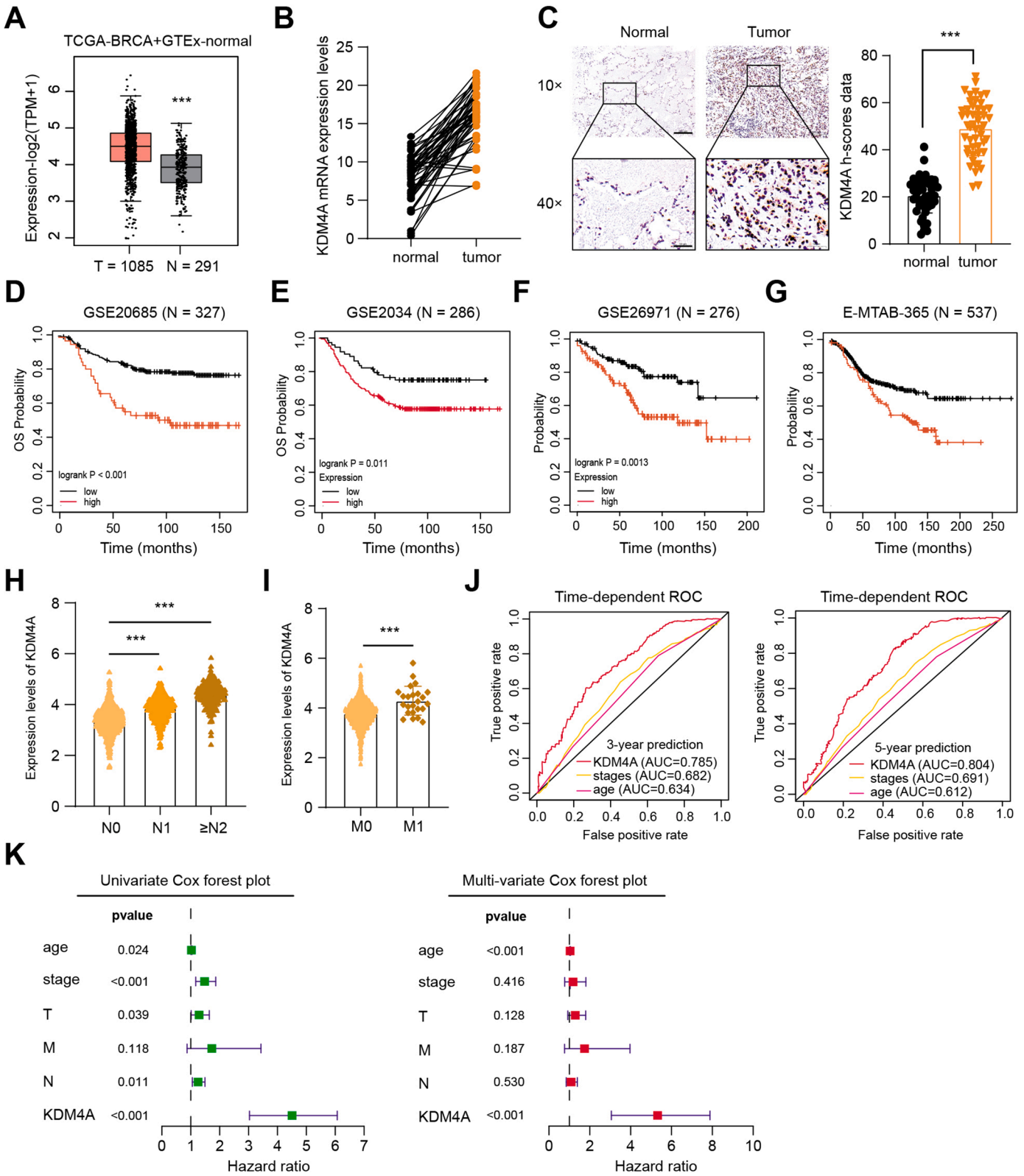

Fig. 3. *KDM4A is highly expressed in breast cancer and predicts poor prognosis of breast cancer patients in TCGA-Bca cohort and other public datasets.* **(A)** The expression level of KDM4A in breast cancer tissues or normal control tissues in TCGA-Bca cohort was analyzed. **(B)** The relative expression of KDM4A in breast cancer tissues or normal control tissues collected from hospital was analyzed by qRT-PCR ($n = 50$, $p < 0.001$). **(C)** The IHC assay confirmed the high KDM4A levels in tumor versus normal tissues. Scale bar of upper panel is 200μm, and the scale bar of lower panel is 50μm. **(D-G)** Kaplan-Meier survival analysis implicated that patients with high KDM4A levels had shorter OS months relative to those with low KDM4A levels in four independent datasets. Log-rank test was calculated in two groups. **(H-I)** High KDM4A correlated positively with NM stages. **(J)** The ROC curves showed the 3-, and 5-year AUC values of predictive efficiency for three variables, including KDM4A levels, clinical stages and age. **(K)** The forest plot showed that KDM4A is an independent factor associated with Bca prognosis. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

B

KDM4A positively associated genes

Pathway	p.geomen	stat.mean	p.val	set.zise
hsa04327 Notch signaling pathway	0.00009	-3.817	1E-04	41
hsa04512 ECM-receptor interaction	0.00011	-3.254	4E-04	26
hsa04972 Pancreatic secretion	0.00023	-3.008	0.0013	17
hsa04360 Axon guidance	0.00146	-2.734	0.0025	38
hsa04350 TGF-beta signaling pathway	0.00272	-2.518	0.0033	29
hsa04630 Jak-STAT signaling pathway	0.00318	-2.419	0.0037	24

(caption on next page)

Fig. 4. *KDM4A activates Notch signaling pathway in Bca cells.* **(A)** Overall distribution of KDM4A levels in TCGA-Bca samples. **(B)** Pathway enrichment by KEGG according to KDM4A expressions. Noted a significant correlation between high level of KDM4A and Notch signaling. **(C)** The correlation analysis and coefficient between KDM4A and Notch1 levels were calculated in TCGA-Bca cohort. **(D)** The RT-qPCR assays revealed the decreased Notch1 mRNA levels in shCtrl and shKDM4A cells (MCF-7 and MDA-MB-231). **(E)** The RT-qPCR assays revealed that KDM4A overexpression could increase Notch1 mRNA levels in EV and KDM4A-overexpressing cells. **(F)** The RT-qPCR assays revealed that KDM4A inhibition could reduce expressions of Notch1 downstream genes, whereas ectopic expression of ICN1 could restore the levels of Notch1-signature. **(G)** Coversely, KDM4A overexpression could elevate the mRNA levels of Notch1 downstream genes, which could completely abolished by Nothch1 inhibition. **(H)** The western blotting assay detected the Notch1 signaling in shCtrl and shKDM4A cells. **(I)** Notch1 restoration could completely rescue the self-renewal abilities of Bca cells that inhibited by KDM4A knockdown. The quantitative results of sphere formation assays were shown on the right. Scale bar = 200 μm. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

found that KDM4A was highly expressed in Bca samples relative to normal tissues (Fig. 3A). Besides, we collected 50 paired Bca samples to find that KDM4A mRNA levels were significantly higher in tumors versus normal groups, as revealed by the RT-qPCR assay (Fig. 3B). In addition, the immunohistochemistry (IHC) assay also suggested that KDM4A expressed highly in Bca than normal tissues (Fig. 3C). Moreover, patients with high KDM4A suffered from worse overall survival (OS) months as compared to those with low KDM4A levels in four independent datasets, as revealed by Kaplan-Meier analysis (Fig. 3D-G). Meanwhile, we conducted the correlation analysis in TCGA-Bca cohort and found that KDM4A levels were positively associated with positive lymphatic nodes and metastatic stages (Fig. 3H-I). The Receiver Operating Characteristic (ROC) curve analysis was conducted in TCGA-Bca cohort and we observed that the 3-, and 5-year AUC of KDM4A levels were 0.785 and 0.804, respectively (Fig. 3J). Compared with age and stages, KDM4A levels have superior predictive efficiency, implicating that KDM4A could predict prognosis of patients well (Fig. 3J). Last of all, we integrated KDM4A and other clinical variables to conduct the univariate Cox regression analysis, in which we found that KDM4A is an independent factor associated with prognosis of Bca patients (Fig. 3K). Collectively, we concluded that KDM4A is a prognostic factor that could predict the survival outcomes of Bca samples.

KDM4A modulates Notch signaling pathway to maintain malignant features of Bca

According to the KDM4A expressions levels and an online omic tool, we conducted the KEGG analysis based on the RNA-seq obtained from the 1097 individuals diagnosed of invasive breast carcinoma. We illustrated the overall distribution of KDM4A levels in Bca samples and thus identified the high-, middle- and low-groups (Fig. 4A). Besides, we also observed a positive association between KDM4A and an enrichment of pathways, including Notch signaling pathway (p = 1e-04), ECM-receptor interaction (p = 4e-04), TGF-beta signaling pathway (p = 0.0033) and Jak-STAT signaling pathway (p = 0.0037) (Fig. 4B). Considering the tightest relationship between KDM4A and Notch signaling crosstalk, we queried the TCGA-Bca datset and confirmed the positive correlation between KDM4A and Notch1 mRNA levels with the correlation coefficient $r = 0.39$ (Fig. 4C). Accordingly, we utilized the shRNA-1 and shRNA-2 that target KDM4A to suppress KDM4A levels and observed that Notch1 levels were consistently decreased with KDM4A-KD (Fig. 4D). In contrast, we also constructed the KDM4A-overexpressing cells and observed an increase of Notch1 mRNA levels in Bca cells (Fig. 4E). Based on these data, we thus speculated that KDM4A could activate Notch1 signaling in Bca. To explore whether the Notch pathway is an essential downstream hit of KDM4A in Bca, we thus conducted the rescue assay with forced activation of NOTCH1 in the KDM4A-depleted cells. Overexpression of the active form of Notch1 (Intracellular domain of NOTCH1 [ICN1]) could effectively elevate the expression of NOTCH1 in KDM4A-knockdown cells. Although KDM4A could consistently activate a list of Notch1 downstream targets, like Myc, p21, SOX2, or HES1, forced activation of the NOTCH1 by ICN1 could completely restore the expressions of Notch1 signature (Fig. 4F). Conversely, KDM4A could elevate the levels of Notch1 downstream genes, whereas Notch1 knockdown could completely suppress the downstream genes (Fig. 4G). In addition, we validated that KDM4A knockdown could inhibit Notch signaling by western blotting assay (Fig. 4H). Previsou studies have highlighted that Notch signalings is essential to maintain self-renewal abilities of tumors. We therefore confirmed that KDM4A knockdown could significantly attenuate the stemness capacities of MCF-7 and MDA-MB-231 cells, which could be completely rescued by ectopic expression of Notch1 (Fig. 4I). Collectively, we concluded that KDM4A modulates Notch signaling to sustain the stemness features of Bca tumors.

KDM4A demethylates H3K9me3 to epigenetically induce Notch1 expressions

To thoroughly elucidate the epigenetic regulations of KDM4A on Notch1, we firstly conducted the ChIP-qPCR assays. The independent ChIP-qPCR experiments validated that KDM4A was directly present at the Notch1 promoter loci, along with other reported targets like HIF-1, SLC7A11 and MyoD (Fig. 5A). ChIP-qPCR showed that KDM4A overexpression could markedly suppress H3K9me3 levels at the indicated genes (Fig. 5A). Given that the H3K27ac marker could distinguish active from poised and inactive chromatin, we thus found the simultaneous increases of H3K27ac at the same loci with KDM4A overexpression (Fig. 5A). These results thus indicated that KDM4A favors an active chromatin state to maintain Notch1 and other gene expressions. To further figure out the underlying transcriptional regulations of Notch1 by KDM4A, we thus generated a luciferase-based reporter containing the promoter region of Notch1 (TSS: -2000 ~ + 50 bp). The luciferase-based reporter was co-transfected into BT-20 and MCF-7 cells with the pcDNA3.1-YAP and Renilla plasmids, respectively. The dual luciferase reporter assay suggested that the KDM4A overexpression could notably elevate the Notch1 promoter activity (Fig. 5B). Conversely, KDM4A knockdown could notably suppress the Notch1 promoter activity as compared to shCtrl group (Fig. 5C). Therefore, we further cloned a list of fragments of the Notch1 promoter, and found KDM4A could increase the luciferase activities of promoter fragments of P3, P4 and P5, but not the P1 or P2 (Fig. 5D). Of note, the Luc is cloned downstream of the indicated NOTCH1 promoter regions, individually. Thus, we determined that only the region, ranging from -280 to -150 within the Notch1 promoter, is the required sequence that was binded and regulated by KDM4A. Lastly, we also utilized the shRNA-1 to knock down Notch1 in the KDM4A-overexpressing cells. We thus found that KDM4A overexpression possessed the abilities to activate cell growth, colony formation and *in vitro* migration, which could be largely inhibited by Notch1 KD (Fig. 5E-F). Taken together, these data suggested that KDM4A could demethylate H3K9me3 to elevate Notch1 expressions and depend on Notch1 to promote Bca proliferation and migration.

Notch1 inhibitor (LY3039478) is effective to suppress KDM4A^{high} Bca, but not the KDM4A^{low} group

Considering that there exists great tumor heterogeneity in Bca, we thus wondered whether KDM4A expressions or status would impact tumor responses to Notch1 inhibitor (LY3039478). Besides, to confirm whether Notch1 is specifically required in KDM4A^{high} cells, we thus utilized two different shRNA constructs to deplete Notch1 expression. We accordingly observed that Notch1 KD could notably suppress the growth of KDM4A^{high} cells, including MCF-7, MDA-MB-231 and BT-474

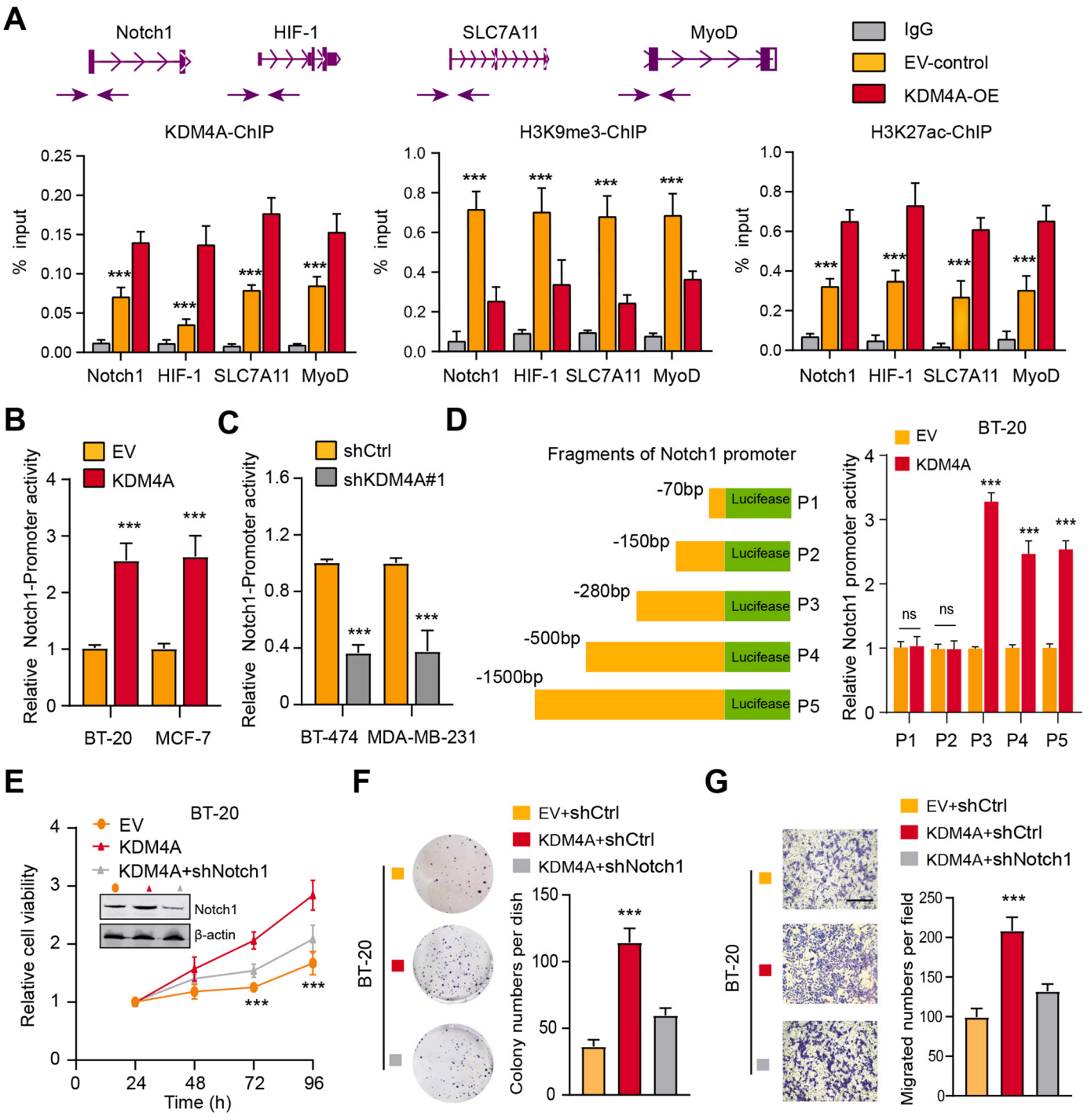

Fig. 5. *KDM4A demethylates H3K9me3 to epigenetically induce Notch1 expressions*. **(A)** The ChIP-qPCR assays of KDM4A binding, H3K27ac, and H3K9me3 in genes, as indicated. Arrows indicate primer locations. **(B)** The luciferase reporter assay revealed that KDM4A could enhance the transcriptional activity of Notch1 promoter in MCF-7 and BT-20 cells. **(C)** The luciferase reporter assay revealed that KDM4A inhibition could suppress the transcriptional activity of Notch1 promoter in BT-474 and MDA-MB-231 cells. **(D)** The luciferase reporter gene assay was utilized to determine the activities of corresponding fragments of Notch1 promoter in BT-20 cells, respectively. Cells were transiently transfected with EV and KDM4A plasmids (Mean±SD, $n = 3$). **(E)** The CCK-8 assays revealed that KDM4A could potentiate cell growth, whereas Notch1 inhibition could largely inhibit the increase of growth. **(F)** The colony formation assay showed that KDM4A could potentiate the growth abilities of cell clones, which could be largely suppressed by Notch1 inhibition. **(G)** The invasion ability of BT-20 cells was enhanced with KDM4A overexpression, but the increase was largely suppressed with Notch1 inhibition. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

(Fig. 6B). However, Notch1 inhibition failed to alter the cell growth of KDM4A^{low} cells (BT-20, MDA-MB-453 and T47D) in Fig. 6B. A similar dependency on Notch1 of KDM4A^{high} cells was further obseerved in colony formation growth assays, in which Notch1 inhibitor (LY3039478) could inhibit growth capacities of MCF-7 cells (KDM4A-^{high}) in a dose-dependent manner, but not the BT-20 (KDM4A^{low}) cells (Fig. 6C). Lastly, we also strengthened our *in vitro* findings on the xenograft models. As revealed by the tumor volumes and tumor weight, MCF-7 (KDM4A^{high}) cells were more sensitive to Notch1 inhibition with LY3039478, but BT-20 cells exhibited resistant to Notch1 inhibition (Fig. 6D-E). Taken together, these results highlighted that Notch1 inhibition is more effective for KDM4A^{high} cells, but not the KDM4A^{low} Bca.

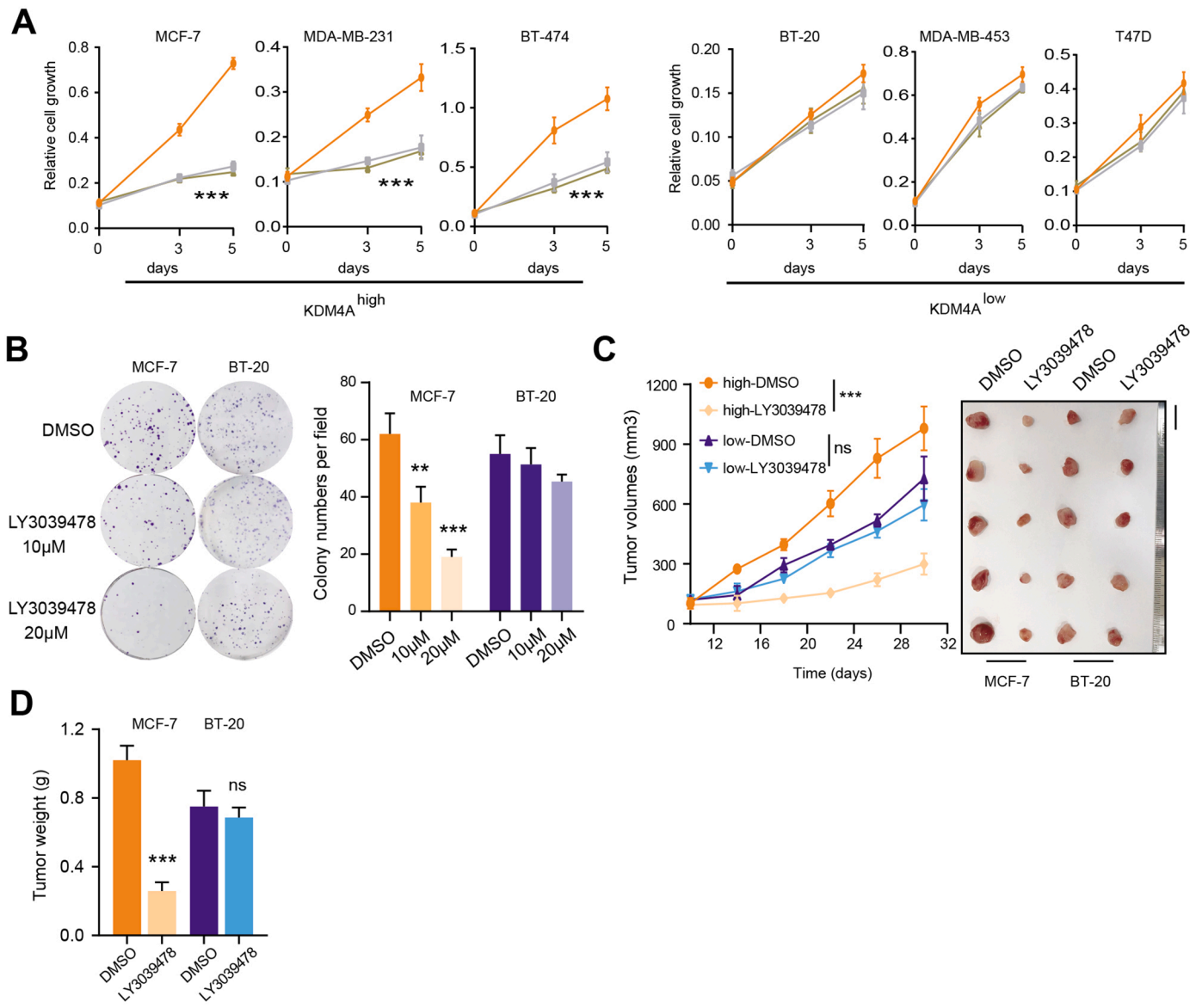

Fig. 6. *Notch1 inhibitor (LY3039478) is effective to suppress KDM4A^{high} Bca, but not the KDM4A^{low} group*. **(B)** CCK-8 assays were used to compare the differences of cell growth with Notch1 KD between KDM4A^{high} and KDM4A^{low} cells. **(C)** The colony formation assay was used to compare the differences of Notch1 inhibitor (LY3039478) in treating KDM4A^{high} and KDM4A^{low} cells. **(D)** Measurement of subcutaneous tumor growth of KDM4A^{high} and KDM4A^{low} cells cells with or without LY3039478 treatment ($n = 6$, 2-way ANOVA followed by Tukey's multiple comparisons test). The representative image is shown on the right. Scale bar = 1 cm. **(E)** The tumor weight of tumors derived from mice in four groups was recorded and compared. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Discussion

Epigenetics refers to the molecular features that modulate gene expressions without disturbing the DNA sequences [26]. Apart from the disorders in the genetic landscape, dysregulation of the epigenetic landscape could occur in every process of tumors, including cancer initiation, proliferation, drug resistance and metastasis [27,28]. Of note, DNA methylation, histone modifications, and chromatin remodeling are some of the essential epigenetic features that are commonly altered during breast cancer progression and resistance and are, therefore, explored to be potential therapeutic targets for epigenetic therapies [29, 30]. As is well known, histone modifications exert essential functions in cell fate determination, terminal differentiation and X inactivation [31, 32]. KDM4A, a member of Jumonji domaincontaining proteins, belongs to a histone demethylase and is an important regulator in multiple cellular processes, such as DNA replication stress, cell ferroptosis, genomic stability and cell self-renewal process. More importantly, intensive studies have reported the association of KDM4A abnormality with tumor progression. KDM4A is highly expressed in non-small cell lung cancer (NSCLC) that promotes the growth of NSCLC by enhancing the expression of Myc via DLX5 through the Wnt/β-catenin signaling pathway [33]. In prostate cancer, USP1 is a deubiquitinase that regulates KDM4A K48-linked deubiquitin and stability to drive tumor cells proliferation and enzalutamide resistance [34].

In this study, we utilized the MTT assay to find that KDM4A inhibition reduce the most decrease of cell growth as compared to other KDM family members. *In vitro* and *in vivo* functional assays demonstrated that KDM4A is a required epigenetic regulator for Bca growth and metastasis. In addition, KDM4A overexpression significantly enhance cell migration and *in vivo* lung metastasis. KDM4A is highly expressed in Bca samples versus normal tissues, which were validated in TCGA-Bca and collected samples. Patients with high KDM4A suffered from worse survival outcomes relative to those with low KDM4A levels in four independent Bca datasets. KDM4A correlated positively with NM stages and could predict

prognosis of patients, which is an independent factor. Bioinformatic analysis revealed that KDM4A correlated tightly with Notch1 signaling and modulated Notch downstream signature. Mechanistically, KDM4A binds directly to the promoter region of Notch1 to activate its transcriptions and expressions by demethylating histone H3K9. Functional assays demonstrated that KDM4A relied on Notch1 to maintain cell proliferation, migration and self-renewal capacities. Last of all, we categorized a panel of KDM4A^{high} and KDM4A^{low} cell lines to determine the cell responses to Notch1 inhibitor (LY3039478). The LY3039478 was effective to treat KDM4A^{high} cell lines, whereas it did not work in KDM4A^{low} cell lines, as revealed by CCK-8 and colony formation assays. Importantly, we also demonstrated our results in the xenograft models to discuss the differential efficacy of LY3039478 in Bca with distinct KDM4A status, implicating the intrinsic tumor heterogeneity.

The Notch family consists of four highly conserved transmembrane receptors. The release of the active intracellular domain requires the enzymatic activity of γ-secretase. Notch participates in embryonic development and many physiologic processes of normal cells, including cell growth, apoptosis, and differentiation. Notch1 belongs to the NOTCH family of receptors (Notch1-4), which is part of an evolutionarily conserved signaling pathway innate to all multicellular organisms and plays a crucial role in embryogenesis and tissue homeostasis. Notch receptors regulate essential cellular functions linked with cell fate specification, including proliferation, differentiation, apoptosis, and stem cell maintenance. Notch1 is the most extensively studied and characterized NOTCH family member because its mutation prevalence among human cancers is higher than that for other members. Aberrant Notch1 signaling is implicated in the progression of various cancer types including breast cancer, leukemias, HNSCC and squamous cancers of the skin, esophagus, cervix, and lung [35,36]. Notch1 can function as either a tumor promoter or suppressor largely depending on the cellular context. Gang Deng et al. revealed that Notch1 suppresses prostate cancer cell invasion via the metastasis-associated 1-KiSS-1 metastasis-suppressor pathway [37]. In contrast, oncogene APOL1 promotes proliferation and inhibits apoptosis via activating NOTCH1 signaling pathway in pancreatic cancer [38]. These findings suggested the distinct roles of Notch1 in regulating tumor progression. In breast cancer, intensive studies have revealed that aberrant activation of Notch1 signaling could contribute to tumorigenesis, metastatic progression and chemotherapy resistance. Shuxuan Zhu et al. found that Stabilization of Notch1 and β-catenin in response to ER^{-} breast cancer-specific up-regulation of PSAT1 mediates distant metastasis [39]. Besides, the tumor microenvironment (TME) could also contribute to aberrant Nocth1 signaling. For instance, TME-derived endothelial cells provide the Notch ligand Jagged1 (Jag1) to neighboring breast CSCs, leading to Notch1-dependent upregulation of Zeb1 [40]. In this study, we determined that abnormal epigenetic regulators could lead to Notch1 activation to drive Bca progression. Intriguingly, previous studies have investigated the efficacy of Notch1 inhibitors in treatment of Bca and the responses exhibit variously. We accordingly proposed that KDM4A expressions may determine the tumor responses to LY3039478.

Also, we still have some concerns in the current study. Firstly, we are still uncertain about the appropriate cutoff to categorize the KDM4A^{high} and KDM4A^{low} groups. Large patient samples might be warranted to perform the IHC assays to obtain the standard criteria. Secondly, apart from Notch signaling, we have to thoroughly explore the associations between KDM4A and other pathways, including Jak-STAT signaling, ECM-receptor interaction, TGF-beta signaling pathway. Last of all, more pre-clinical models, like patient-derived xenografts (PDXs) and patient-derived organoids (PDOs), were needed to assess the clinical efficacy of LY3039478 in KDM4A^{high} and KDM4A^{low} groups.

In conclusion, our study revealed that KDM4A contributes to the proliferation and metastasis of Bca. High KDM4A correlated with inferior outcomes of patients. KDM4A activates Notch1 signaling to maintain Bca stemness and progression. Notch1 inhibition is effective to suppress growth of KDM4A^{high} tumors, implicating a therapeutical vulnerability for translational treatment.

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Author contributions

Jing Pei and Jing Zhang designed and conceived this project. Jing Pei, ShengQuan Zhang and Xiaowei Yang conducted the experimental assays. Chunguang Han, Yubo Pan and Jun Li conducted the bioinformatic analysis. Zhaorui Wang and Chenyu Sun collected the clinical samples. Jing Pei worte and revised this manuscript. All authors have reviewed and approved the final version of paper.

Declaration of Competing Interest

The authors have no conflicts of interest to declare.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2022.101615.

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