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NOTCH3 promotes docetaxel resistance of prostate cancer cells through regulating TUBB3 and MAPK signaling pathway

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Abstract

Docetaxel is the preferred chemotherapeutic agent in patients with castrate-resistant prostate cancer (CRPC). However, patients eventually develop docetaxel resistance and in the absence of effective treatment options. Consequently, it is essential to investigate the mechanisms generating docetaxel resistance and develop novel alternative therapeutic targets. RNA sequencing was undertaken on docetaxel-sensitive and docetaxel-resistant prostate cancer (PCa) cells. Subsequently, chemoresistance, cancer stemness, and lipid metabolism were investigated. To obtain insight into the precise activities and action mechanisms of NOTCH3 in docetaxel-resistant PCa, immunoprecipitation, mass spectrometry, ChIP, luciferase reporter assay, cell metabolism, and animal experiments were performed. Through RNA sequencing analysis, we found that NOTCH3 expression was markedly higher in docetaxel-resistant cells relative to parental cells, and that this trend was continued in docetaxel-resistant PCa tissues. Experiments in vitro and in vivo revealed that NOTCH3 enhanced stemness, lipid metabolism, and docetaxel resistance in PCa. Mechanistically, NOTCH3 is bound to TUBB3 and activates the MAPK signaling pathway. Moreover, NOTCH3 was directly regulated by MEF2A in docetaxel-resistant cells. Notably, targeting NOTCH3 and the MEF2A/TUBB3 signaling axis was related to docetaxel chemoresistance in PCa. Overall, these results demonstrated that NOTCH3 fostered stemness, lipid metabolism, and docetaxel resistance in PCa via the TUBB3 and MAPK signaling pathways. Therefore, NOTCH3 may be employed as a prognostic biomarker in PCa

Abbreviations: CHX, cycloheximide; CRPC, castrate-resistant prostate cancer; CSCs, cancer stem cells; DFS, disease-free survival; DR, docetaxel resistant; DTX, docetaxel; ECAR, extracellular acidification rate; IF, immunofluorescence; NC, negative control; OCR, oxygen consumption rate; PCa, prostate cancer; sh, short hairpin.

Xianchao Sun and Ying Zhang contributed equally to this study.

patients. NOTCH3 could be a therapeutic target for PCa patients, particularly those who have developed docetaxel resistance.

KEYWORDS
docetaxel resistance, MAPK, NOTCH3, prostate cancer, TUBB3

1 | INTRODUCTION

The incidence of PCa is quickly rising, posing a global threat to men's health. Patients with metastatic PCa have a relatively low long-term survival rate compared with patients with localized PCa.[1] Androgen deprivation therapy (ADT) remains the standard treatment for advanced PCa. In spite of an initial favorable response, an alarmingly high proportion of patients will experience recurrences, be resistant to ADT, and develop CRPC.[2] DTX is a member of the taxane family and is regarded as the first-line treatment for metastatic CRPC.[3] Despite the fact that the use of DTX has led to improved clinical results and a longer life expectancy, many patients still developed resistance to the therapy.[4] This underscores the urgent need for more research into the chemoresistance of PCa and the development of new possible therapeutic targets in order to enhance the outcomes of treatment.

The NOTCH signaling pathway is one of the most essential cell fate-determining processes. NOTCH receptor-mediated signaling between adjacent cells can govern cell differentiation, proliferation, and apoptotic processes.[5] NOTCH receptors are single transmembrane proteins transcribed by the NOTCH gene, and four NOTCH receptors have been found in humans (NOTCH 1, 2, 3, and 4).[6] NOTCH3 is encoded on chromosome 19p13.12, encompassing 33 exons (location 15,159,038–15,200,995).[7] Multiple investigations have demonstrated that aberrant NOTCH3 expression is frequent in human cancer tissues.[8–10] NOTCH3 is essential for maintaining the stemness of CSCs. CSCs, a population of self-renewing cells with substantial oncogenic capability, are identified to be activated by NOTCH3 in multiple cancer types and contribute to the progression of cancer.[11,12] Inducing tumor resistance to many chemotherapeutic treatment classes is another hallmark of NOTCH3.[13]

Lipid molecules mainly include three types of fatty acids, glycerides, and lipids, which are key components of biological membranes and participate in a variety of important life activities.[14,15] It has been shown that fatty acid oxidation (FAO) is linked to chemotherapy resistance.[16] Drug-resistant tumor cells exhibit a dependence on lipid metabolism, which may stem from the fact that energy metabolism compensates for increased cellular ATP and produces intermediates to support rapid cell growth, eliminate potentially toxic lipids, and inhibit apoptotic pathways.[17,18]

Taxanes inhibit cell mitosis and cause tumor cells to undergo apoptosis by focusing their attention on the functioning of microtubules.[19] Microtubules are formed of polymers of heterodimeric α-tubulin and β-tubulin. The microtubule protein III-tubulin encoded by the *TUBB3* gene is usually expressed in neural cells.[20] Recent research has indicated that TUBB3 is linked to the advancement of CRPC.[21] In addition, evidence has shown that overexpression of TUBB3 contributes to DTX resistance in certain malignancies, including PCa.[22]

The current study aimed to evaluate the difference in NOTCH3 expression between parental and DR cell lines. The expression of NOTCH3 was discovered to be associated with DTX resistance in PCa. Further investigation revealed that NOTCH3 was associated with lipid metabolism and cancer stemness. According to functional investigations, NOTCH3 is linked directly to TUBB3 and activates the MAPK signaling pathway, and its activity was controlled by MEF2A.

2 | MATERIALS AND METHODS

Additional methodologies are detailed in Appendix S1.

2.1 | Patients and tissue samples

Tissue microarrays (TMAs) incorporating PCa tissues taken from patients with PCa at Shanghai Tenth People's Hospital between 2014 and 2020 were collected after receiving informed consent from each participant. DFS was calculated from the date of initial surgery to the date of disease progression. The Ethics Committee of Shanghai Tenth People's Hospital approved our study. The characteristics of PCa patients are shown in Table S1.

2.2 | Cell lines and culture

The human PCa cell lines PC3 and DU145 were obtained from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences. Cells were cultured in DMEM (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin–streptomycin (Gibco) and were cultured at 37°C in a humidified atmosphere containing 5% CO_2. DTX (HY-B0011), AZD6244 (ARRY-142886) and DAPT (HY-13027) were purchased from MedChemExpress (Shanghai, China) and prepared according to the manufacturer's instructions.

2.3 | Cell transfection

Lentiviruses containing control shRNA (NC) and specific shRNAs against NOTCH3 (sh-NOTCH3-1/sh-NOTCH3-2), TUBB3

(sh-TUBB3), or MEF2A (sh-MEF2A; Genomeditech, Shanghai, China) were transduced into PCa cells according to the manufacturer's instructions to generate stable cell lines. The sequences of NC and shRNAs are described in Table S2. Briefly, the cells were infected for 24 h with medium containing virus and 1 ng/mL polybrene (Sigma, USA). The transduced cells were then screened for 3 days with puromycin (Sigma) at a concentration of 2 mg/mL. The plasmid was transfected into cells with lipofectamine 3000 (Invitrogen, USA).

2.4 | Statistical analysis

Student's *t*-test was used to analyze differences between two groups. One-way ANOVA was used to compare three or more groups, followed by Dunnett's or Tukey's multiple comparison tests. For the statistical analysis, GraphPad Prism 8 (GraphPad Software, Inc.) was used. A p-value <0.05 was set as the significance threshold.

3 | RESULTS

3.1 | Establishment of docetaxel-resistant cells

To examine the underlying mechanisms driving DTX chemoresistance in PCa, we established two DR PCa cell lines and confirmed DTX chemosensitivity. Parental cells were selected from the CRPC cell lines PC3 and DU145. In the presence of different concentrations of DTX, the parental cells were tested for drug sensitivity. Since DR cells (PC3-DR, DU145-DR) emerged in culture, we investigated the cytotoxicity of DTX with the resistant cell lines and found that DTX exposure resulted in weak inhibition cellular viability as compared to the control group. PC3-DR and DU145-DR cells showed a high proliferative capacity along with DTX treatment (Figure S1A). EdU assay and colony formation verified chemoresistance of PC3-DR and DU145-DR (Figure S1B,C). After 24 h of DTX exposure, the early, and late apoptosis rates of the drug-resistant groups were considerably lower than those of the control groups, as detected by flow cytometry (Figure S1D).

3.2 | Elevated expression of NOTCH3 in docetaxel-resistant PCa cells

RNA sequencing indicated co-expression of upregulated and downregulated genes in both resistant cell and parental cell types. NOTCH3, which was expressed at the highest level among these genes, attracted our attention (Figure 1A). Consistent with the RNA-seq results, NOTCH3 was markedly upregulated in DR PCa cells compared with parental cells (Figure 1B,C). We also showed that prolonged DTX treatment (0–30 days) increased NOTCH3 expression in both PC3 and DU145 cells (Figure 1D; Table S3). Our findings were similar to the results of NOTCH3 expression in several other docetaxel-resistant cell lines from the GSE33455 and GSE158494 public datasets (Figure 1E). Moreover, we demonstrated that patients with higher NOTCH3 expression exhibited worse DFS in The Cancer Genome Atlas (TCGA; 50% cut-off) and Memorial Sloan Kettering Cancer Center (MSKCC; 50% cut-off) datasets (Figure 1F,G).

3.3 | NOTCH3 promotes PCa cell survival and contributes to docetaxel-resistant tumor progression

To determine whether elevated NOTCH3 expression in PCa cells contributes to docetaxel-resistant development, we have applied a lentivirus infection experiment to deplete NOTCH3 expression in PC3-DR and DU145-DR cells, while overexpressed NOTCH3 in PC3-Parental and DU145-Parental cells (Figure S2A,B). Cell viability assays showed that NOTCH3 knockdown in docetaxel-resistant cells markedly resensitized them to DTX (Figure 2A,B). Cell migration assays showed that the migration ability of cells in both sh-NOTCH3-1 and sh-NOTCH3-2 groups was reduced compared with the sh-NC group, indicating that knockdown of NOTCH3 could inhibit the migration ability of drug-resistant cells (Figure S2C,D). In contrast, overexpression of NOTCH3 significantly reduced the inhibitory activity of DTX on PC3 and DU145 cells (Figure 2C,D). Cell proliferation was significantly suppressed upon NOTCH3 knockdown in PC3-DR and DU145-DR cells, which was more evident after DTX treatment (Figure 2E–H). However, NOTCH3 overexpression increased cell proliferation of PC3-Parental and DU145-Parental cells (Figure 2I–L). Additionally, prolonged DTX treatment also increased NOTCH3 expression in androgen receptor (AR)-positive LNCaP cells (Figure S2E). NOTCH3 overexpression also increased cell proliferation and DTX resistance of LNCaP cells (Figure S2F–H).

The in vivo tumor-promoting and chemoresistance-inducing activities of NOTCH3 were then investigated. PC3-DR cells harboring NOTCH3 knockdown or empty vector were injected subcutaneously into nude mice, followed by intraperitoneal DMSO or DTX treatment. Similar results were obtained in vivo; NOTCH3 knockdown decreased tumor growth speed and overall tumor weight and compromised the DTX resistance of PC3-DR cells (Figure 2M–O; Figure S2I,K). The expression level of NOTCH3 in different groups was confirmed by western blotting (Figure 2P). Furthermore, the immunohistochemistry (IHC) assay showed that Ki-67 and NOTCH3 expression were significantly reduced in the sh-NOTCH3 group after DTX treatment compared with the control group (Figure 2Q).

3.4 | NOTCH3 can affect cancer stemness and lipid metabolism

To determine whether NOTCH3 could serve as a therapeutic target in DR PCa, we discovered that the number of apoptotic cells was higher in the sh-NOTCH3 group than in the NC group. Moreover, the number of apoptotic cells was considerably higher in the sh-NOTCH3 + DTX group than in the NC + DTX group following DTX

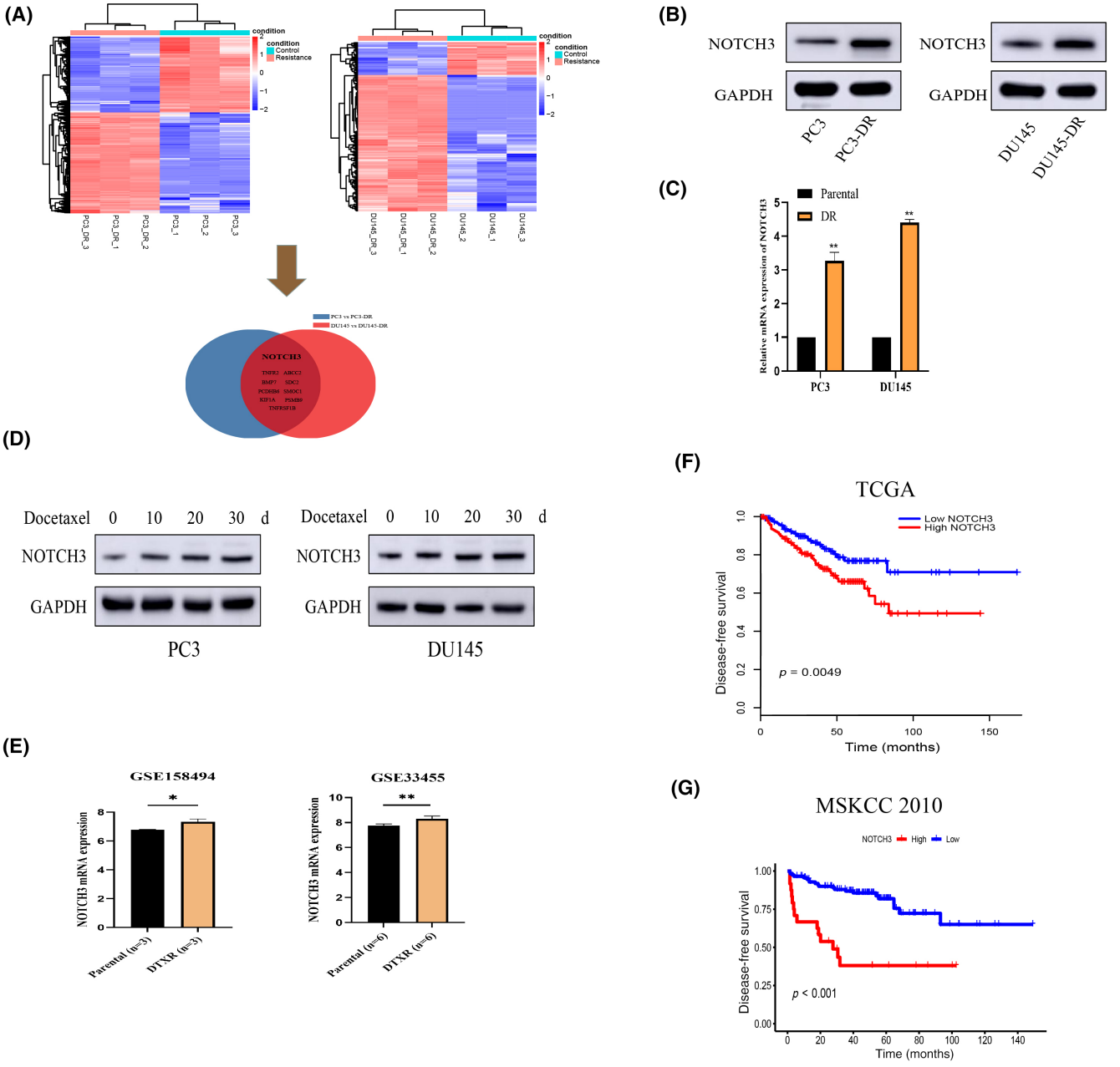

FIGURE 1 Elevated expression of NOTCH3 in docetaxel-resistant PCa cells. (A) Heat map depiction of the upregulated and downregulated genes upon PC3/PC3-DR and DU145/DU145-DR groups. The expression level of NOTCH3 was confirmed in PC3/PC3-DR and DU145/DU145-DR by western blotting (B) and qRT-PCR (C). (D) PC3 and DU145 cells were treated with 1 nM docetaxel for 0–30 days. NOTCH3 protein levels were detected at the indicated time points by western blotting. d, days. (E) NOTCH3 expression in GSE158494 and GSE33455 datasets. (F, G) PCa patients in TCGA and MSKCC datasets were stratified based on NOTCH3 expression levels and analyzed for disease-free survival. DR, drug resistance. $^{*}p < 0.05$; $^{**}p < 0.01$.

treatment (Figure 3A). Abnormal activation of NOTCH3 signaling is observed in CSCs. In the present study, the knockdown of NOTCH3 not only reduced expression levels of stemness-related genes (*CD133*, *CD44*, *SOX2*, and *Nanog*) but also decreased the sphere numbers (Figure 3B–D). Next, we tested the proportion of CSCs among PC3-DR and DU145-DR cell populations. The percentage of the $CD133^{high}CD44^{high}$ cells population in the sh-NOTCH3-1 and sh-NOTCH3-2 groups was markedly reduced compared with the NC group (Figure 3E).

We have successfully constructed DTX chemoresistant cell lines PC3-DR and DU145-DR for transcriptome sequencing. The sequencing results included differential gene expression and analysis of the Kyoto Encyclopedia of Genes and Genomes (KEGG) database enrichment for the differential genes. Lipid metabolism was significantly changed in drug-resistant cells (Figure S3A,B). As shown in Figure 3F, NOTCH3-deficient resistant cells displayed reduced lipid accumulation, as measured by the lipophilic dye Bodipy 505/515 staining. qRT-PCR experiments consistently showed that

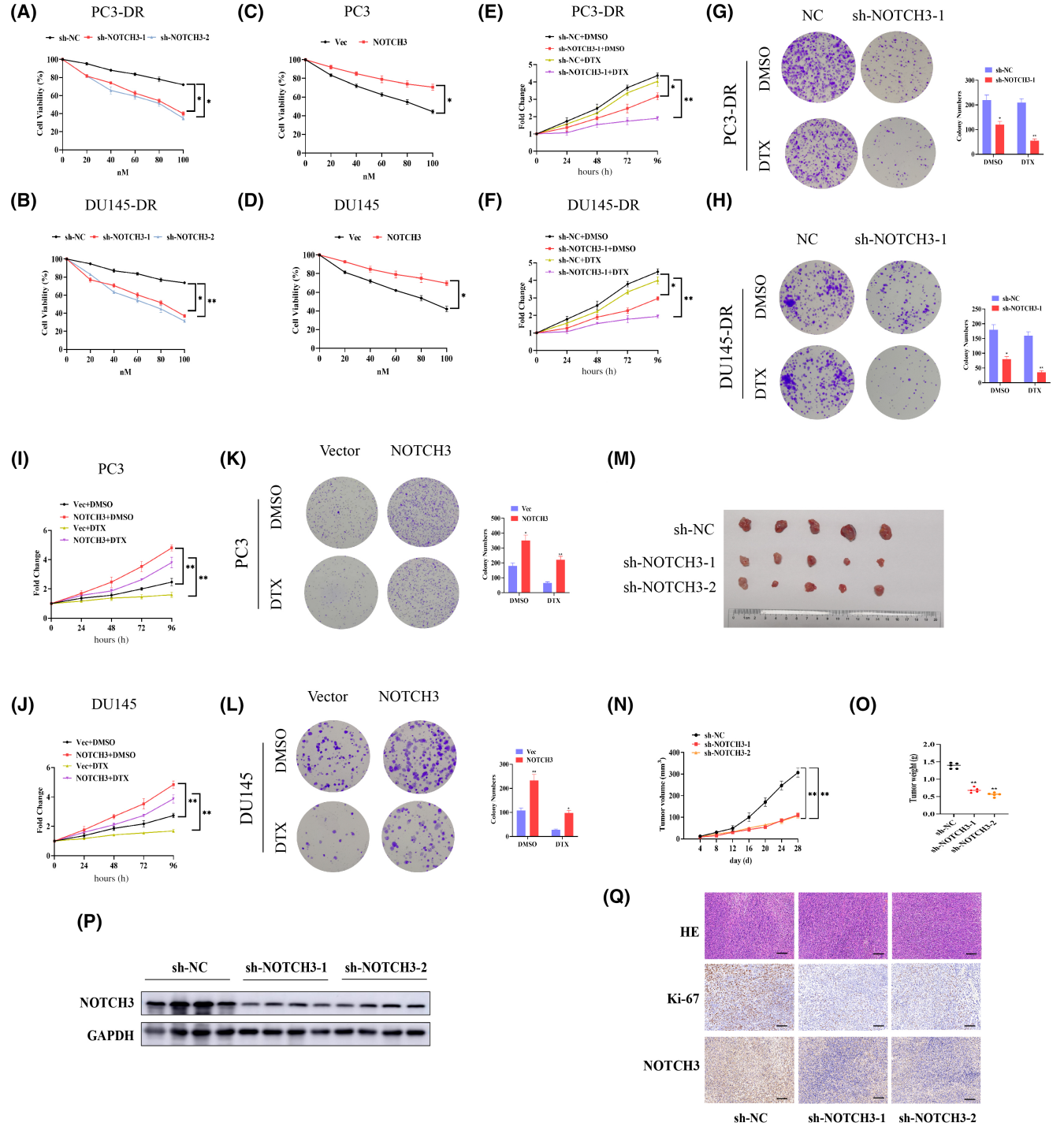

FIGURE 2 NOTCH3 contributes to docetaxel-resistant tumor progression. Cell viability is measured in the indicated cell lines under docetaxel treatment. PC3-DR, DU145-DR (A, B), and PC3-Parental, DU145-Parental (C, D) cells were transfected as indicated and treated with docetaxel. Cell viability was quantified by the CCK-8 assays. Cell proliferation was assessed in PC3-DR and DU145-DR cells transfected with sh-NC or sh-NOTCH3-1 (E–H) or in PC3-Parental and DU145-Parental cells transfected with empty vector or NOTCH3 overexpression plasmid (I–L). (M) Subcutaneous injection of PC3-DR cells expressing sh-NOTCH3 or control was performed in nude mice ($n = 5$). Every 4 days, tumor size was measured (N). Tumor weights are shown (O). Western blotting confirmed NOTCH3 protein levels (P). IHC staining of Ki-67 and NOTCH3 staining on tumor slide from each group are shown (Q). Scale bars: 100 μm. $^{*}p < 0.05$; $^{**}p < 0.01$.

NOTCH3 affected the mRNA expression levels of genes associated with lipid metabolism (Figure 3G; Table S4). To further understand whether lipid metabolism is reprogrammed, we examined the oxygen consumption rate and extracellular acidification rate of PC3-DR and DU145-DR cells. As shown in Figure S3C,D, the glycolysis level and reserve capacity of sh-NOTCH3-1 and sh-NOTCH3-2 cells were lower than those of control cells. NOTCH3-sh1/sh2 cells had lower basal oxygen consumption, ATP-linked OCR, peak respiration, and

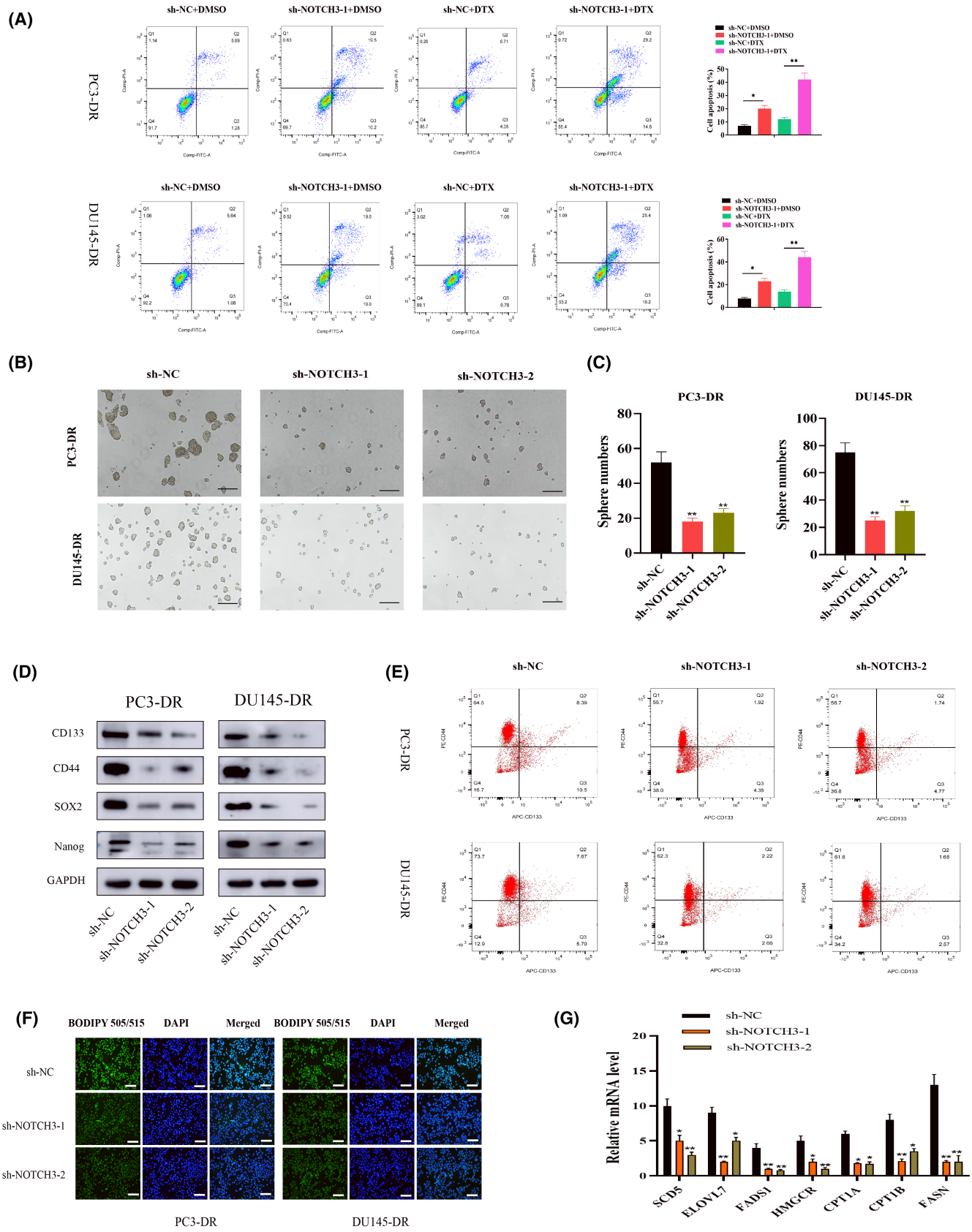

FIGURE 3 NOTCH3 can affect cancer stemness and lipid metabolism. Cell apoptosis results in the NC, sh-NOTCH3-1, NC + DTX, and sh-NOTCH3-1 + DTX groups (A). (B, C) Cells transfected with sh-NC or sh-NOTCH3 were used to perform the tumor sphere assay. Scale bars: 100 μm. (D) The protein levels of stemness-related genes (*CD133*, *CD44*, *SOX2* and *Nanog*) were detected by western blotting. (E) The percentage of the $CD133^{high}CD44^{high}$ cells population in different groups are shown. (F) Lipid accumulation was measured by the lipophilic dye Bodipy 505/515. Scale bars: 100 μm. (G) qRT-PCR experiments show that NOTCH3 affects mRNA expression levels of genes associated with lipid metabolism. DTX, docetaxel. $^{*}p < 0.05$; $^{**}p < 0.01$.

reserve capacity compared with control cells (Figure S3E,F). The association between NOTCH3 expression and lipids was then further investigated. We performed untargeted absolute quantitative lipidomic assays using sh-NC and sh-NOTCH3-1 cells. Significantly different lipids from 14 lipid classes were found in sh-NOTCH3-1 cells compared with control cells (Figure S3G). As shown in Figure S3H, a heat map depicts the significant variable differences between the control group and the sh-NOTCH3-1 group. Figure S3I displays the findings of the correlation study of the lipid classes.

3.5 | NOTCH3 regulates TUBB3 ubiquitination

To investigate the potential binding proteins associated with NOTCH3, we performed immunoprecipitation (IP) and mass spectrometry analyses (Figure 4A,B). We found that NOTCH3 was correlated with TUBB; the type III beta microtubule protein encoded by TUBB3 was most closely associated with anti-microtubule chemotherapeutic drugs. Subsequently, we performed a co-IP assay and the results showed that NOTCH3 was directly bound to TUBB3 (Figure 4C). IF assays detected the relationship between NOTCH3 and TUBB3 in PC3-DR and DU145-DR cell lines (Figure 4D). TUBB3 was also upregulated in DR PCa cells compared with parental cells and the prolonged DTX treatment increased TUBB3 expression in both PC3 and DU145 cells (Figure S4A,B). NOTCH3 silencing led to reduced expression of TUBB3 (Figure 4E,F; Figure S4C). Next we overexpressed TUBB3 in NOTCH3-knockdown cells (Figure S5A). Accordingly, TUBB3 overexpression could reverse the inhibiting effect of NOTCH3 knockdown on cell growth (Figure 4G,H). TUBB3 overexpression also increased the resistance to DTX treatment (Figure 4I,J). In order to determine the influence of NOTCH3 on TUBB3 activity, we analyzed the effect of NOTCH3 on TUBB3 stability in PC3-DR and DU145-DR cells treated with the protein synthesis inhibitor CHX. We discovered that TUBB3 stability is diminished following NOTCH3 depletion (Figure 4K–M). The effect of NOTCH3 on the ubiquitination of TUBB3 protein was then determined. Immunoprecipitation based on ubiquitination showed that NOTCH3 reduced the global polyubiquitination of TUBB3 (Figure 4N). We also detected the TUBB3 expression/stability and lipid accumulation in PC3 and DU145 parental cells. The results showed that NOTCH3 knockdown in the PC3 and DU145 parental cells did not affect TUBB3 expression and stability (Figure S5B,C). We further found that lipid accumulation also had no significant change after NOTCH3 knockdown in PC3 and DU145 parental cells (Figure S5D).

3.6 | NOTCH3 activates the MAPK pathway in PCa cells to promote DTX resistance

We then compared the transcriptomes between PC3-DR cells with control (sh-NC) and NOTCH3 knockdown (sh-NOTCH3-1) by RNA sequencing. KEGG analysis showed that the MAPK signaling pathway was affected by NOTCH3 knockdown (Figure 5A). Indeed, phosphorylated ERK1/2 (p-ERK1/2), phosphorylated JNK (p-JNK) and phosphorylated p38 (p-p38) were significantly elevated in NOTCH3 overexpression parental cells (Figure 5B). The results also showed that stable knockdown of NOTCH3 decreased the expression levels of p-ERK1/2, p-JNK and p-p38 (Figure 5C). To further validate the findings that increased NOTCH3 levels stimulate the MAPK pathway in docetaxel-resistant cells, in vivo assay demonstrated that NOTCH3 expression depleted by shRNA, or NOTCH3 activity inhibited by DAPT in the tumor xenografts resulted in significantly reduced p-ERK1/2 expression (Figure 5D,E). In addition, p-ERK1/2 inhibitor AZD6244 reversed the NOTCH3 overexpression-induced proliferation effects of PC3 and DU145 cells (Figure 5F). Furthermore, the chemoresistance of PC3-DR and DU145-DR cells can be reduced by either NOTCH3 silencing or AZD6244 therapy (Figure 5G).

3.7 | MEF2A regulated NOTCH3 at the transcriptional level in docetaxel-resistant cells

We endeavor to identify the transcription factors that regulate NOTCH3 gene expression alterations in DR cells. The GeneCards database (http://www.genecards.org) was utilized to screen 222 transcription factors, whereas the PROMO database (http://alggen.lsi.upc.es/) was used to screen 79 transcription factors (Figure S6A). Based on the two databases, NOTCH3 shared eight transcription factors (YY1, FOXP3, VDR, MEF2A, SRY, IRF1, ELF1, and GATA2). JASPAR (http://jaspar.genereg.net/) predicted the MEF2A binding sites inside the NOTCH3 promoter sequence (Figure 6A). The GEPIA database (http://gepia.cancer.pku.cn/) suggested that MEF2A expression was positively associated with NOTCH3 expression in PCa (Figure 6B). To confirm the specific MEF2A binding location within the NOTCH3 promoter, promoter segments of varying lengths were cloned and analyzed for *cis*-acting components (Figure 6C). ChIP analysis of PC3-DR cell lines revealed a single binding site upstream of the transcription start point (Figure 6D). The results of dual luciferase reporter assays demonstrated that mutation of the binding site alone abolished luciferase activity (Figure 6E). These findings confirmed that MEF2A is bound to the promoter region of NOTCH3 and that binding events stimulate NOTCH3 transcription. Next, we overexpress or knock down MEF2A and examine the mRNA and protein expression of NOTCH3 and TUBB3 (Figure S6B,C). We also demonstrated that both the mRNA and protein expression of NOTCH3 and the protein expression of TUBB3 were upregulated after overexpressing MEF2A and downregulated after silencing MEF2A (Figure S6D–G). Confocal microscopy was applied to show that NOTCH3 was co-localized with MEF2A and TUBB3 in PC3-DR and DU145-DR cell lines (Figure 6F). Apoptosis assays revealed that the sh-MEF2A group had a considerably higher number of apoptotic cells compared with the NC group. After treatment with DTX, the number of apoptotic cells increased significantly in the sh-MEF2A + DTX group as compared with the control group (Figure 6G).

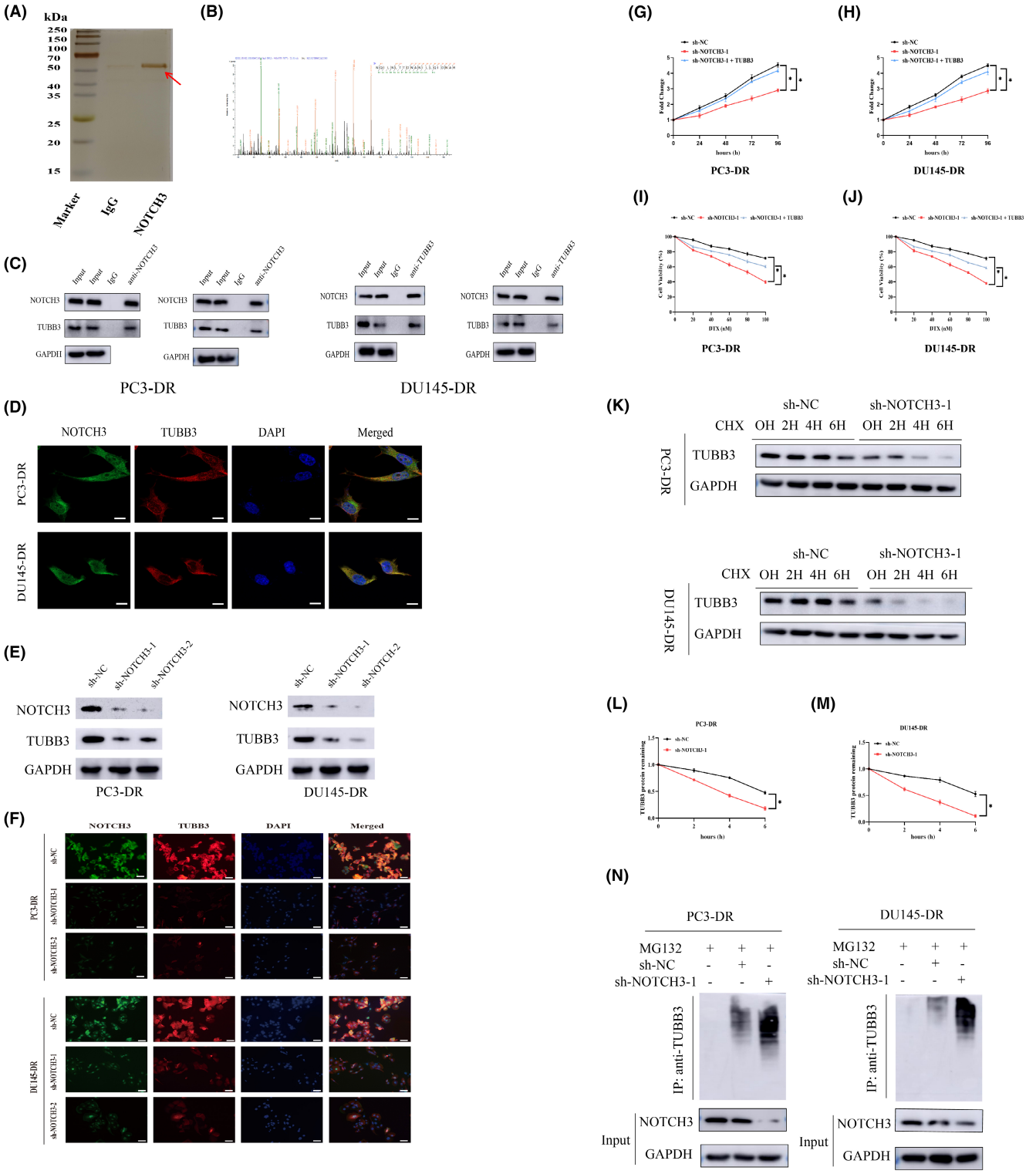

FIGURE 4 NOTCH3 regulates TUBB3 ubiquitination. (A, B) Immunoprecipitation and mass spectrometry analysis identifies that TUBB3 is a potential NOTCH3-interacting protein in PC3-DR cells. (C) Co-IP analysis demonstrates an interaction between NOTCH3 and TUBB3 protein in PC3-DR and DU145-DR cells. (D) Immunofluorescence assay for determining the localization between NOTCH3 and TUBB3. Scale bars: 20 μm. (E, F) Western blotting and immunofluorescence assays are used to detect the expression of NOTCH3 and TUBB3 in sh-NC and sh-NOTCH3 groups. Scale bars: 100 μm. (G, H) PC3-DR and DU145-DR cells stably expressing sh-NC or sh-NOTCH3 were transfected with the vector or TUBB3 and cell proliferation was then determined using the CCK-8 assay. (I, J) Cell viability was observed in different groups after docetaxel treatment. (K–M) TUBB3 protein was detected with NOTCH3 depletion. (N) Ubiquitination of TUBB3 was confirmed by western blot analysis. CHX, cycloheximide; DTX, docetaxel. $^{*}p < 0.05$; $^{**}p < 0.01$.

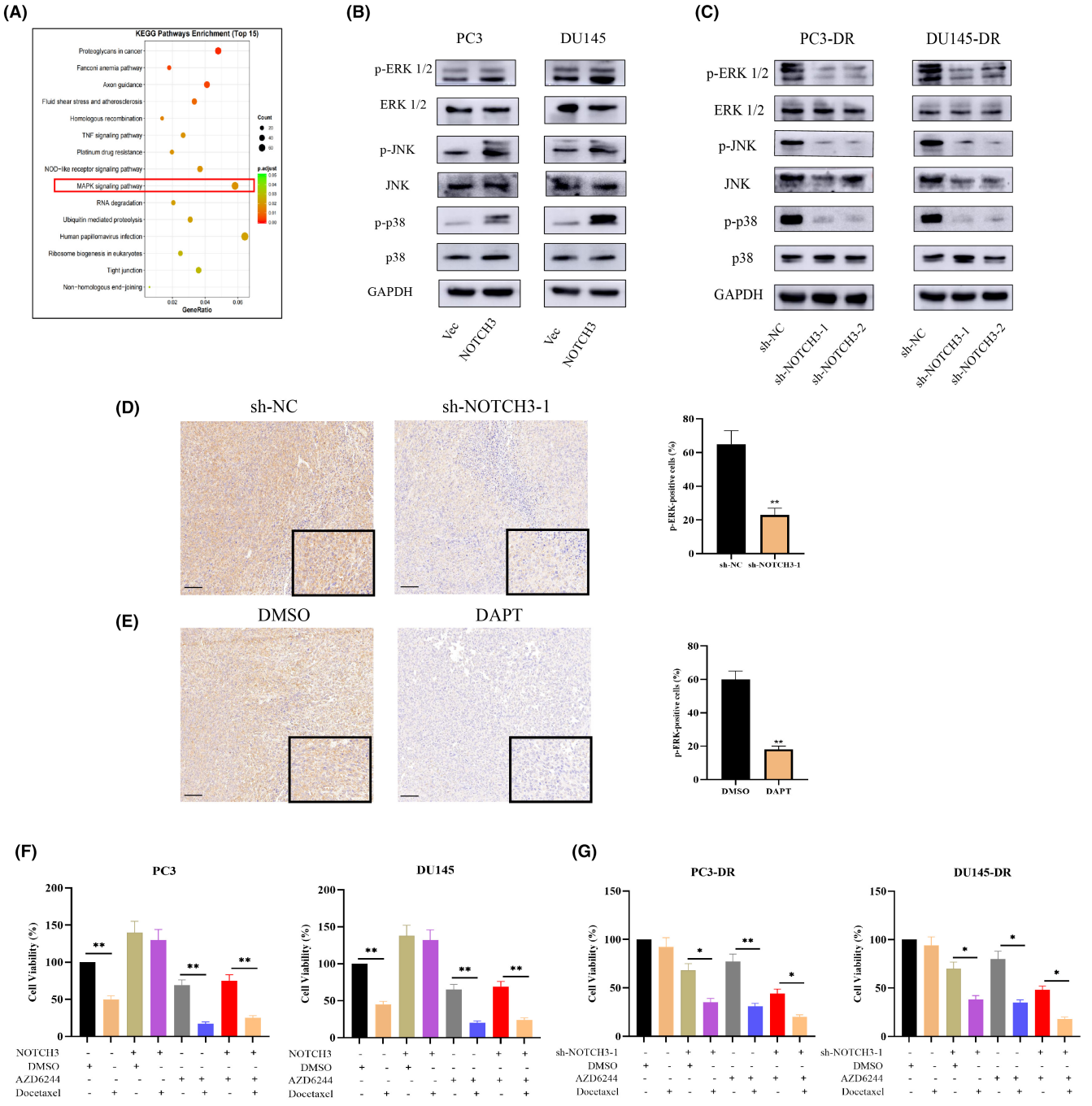

FIGURE 5 NOTCH3 activates the MAPK pathway in PCa cells to promote docetaxel resistance. (A) KEGG analysis was performed using a NOTCH3 knockdown cell line and the control. (B) PC3-Parental and DU145-Parental cells were transfected with the indicated plasmid and detected by western blotting. (C) Cells were transfected and then observed by western blotting. (D, E) IHC images of p-ERK expression in xenografts. (F, G) Docetaxel or AZD6244 were used to measure cell viability in the indicated cell lines. Scale bars: 100 μm. $^{*}p < 0.05$; $^{**}p < 0.01$.

3.8 | NOTCH3 inhibitor inhibits the progression of PCa and sensitizes cancer cells response to DTX in vivo

As increased NOTCH3 expression promotes the development of DTX resistance, pharmacological inhibition of NOTCH3 may provide a viable treatment alternative for DR patients. *N*-[*N*-(3,5-Difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester (DAPT) is a γ-secretase inhibitor that is previously referred to as a NOTCH inhibitor. As shown in Figure 7A, DAPT impaired cell viability in PC3-DR and DU145-DR, and the suppressive effect was enhanced by the addition of DTX. Markedly, DAPT significantly enhanced the sensitivity of resistant cells to DTX (Figure 7B). To determine the effect of DAPT on tumor growth, we have established PC3-DR cell harboring control or NOTCH3-sh1 xenografts in nude mice. Mice were also grouped and treated with DMSO, DTX (5 mg/kg), DAPT

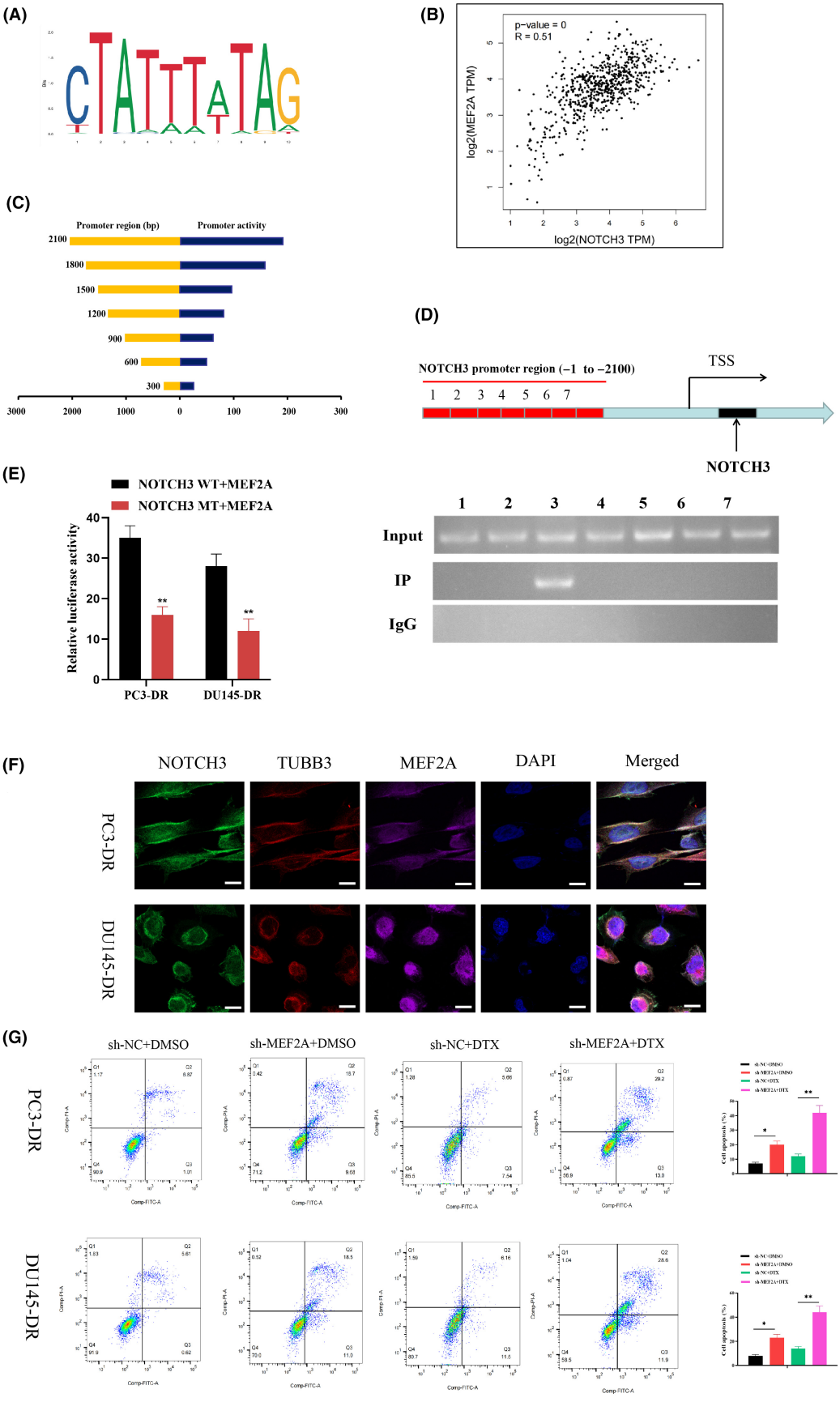
(A)
(B)
p-value = 0
R = 0.51
log2(MEF2A TPM)
log2(NOTCH3 TPM)
(C)
Promoter region (bp)
Promoter activity
2100
1800
1500
1200
900
600
300
3000
2000
1000
0
100
200
300
(D)
NOTCH3 promoter region (−1 to −2100)
TSS
1 2 3 4 5 6 7
NOTCH3
(E)
NOTCH3 WT+MEF2A
NOTCH3 MT+MEF2A
Relative luciferase activity
PC3-DR
DU145-DR
1 2 3 4 5 6 7
Input
IP
IgG
(F)
NOTCH3
TUBB3
MEF2A
DAPI
Merged
PC3-DR
DU145-DR
(G)
sh-NC+DMSO
sh-MEF2A+DMSO
sh-NC+DTX
sh-MEF2A+DTX
PC3-DR
DU145-DR

FIGURE 6 MEF2A regulates NOTCH3 at the transcriptional level in docetaxel-resistant cells. (A) JASPAR indicates the MEF2A binding sites inside the NOTCH3 promoter sequence. (B) The GEPIA database suggested that MEF2A expression is positively associated with NOTCH3 expression. (C, D) Map of MEF2A binding that sits in the promoter region of NOTCH3; the results of ChIP analysis showed that MEF2A can bind to the NOTCH3 promoter region. (E) A luciferase reporter assay was used to detect mutant sites in the promoter region of NOTCH3. (F) Immunofluorescence showing NOTCH3 (green), TUBB3 (red) and MEF2A (rose red) localizations in PC3-DR and DU145-DR cells. (G) Cell apoptosis results in the NC, sh-MEF2A, NC + DTX, and sh-MEF2A + DTX groups. DTX, docetaxel. Scale bars: 20 μm. $^{*}p < 0.05$; $^{**}p < 0.01$.

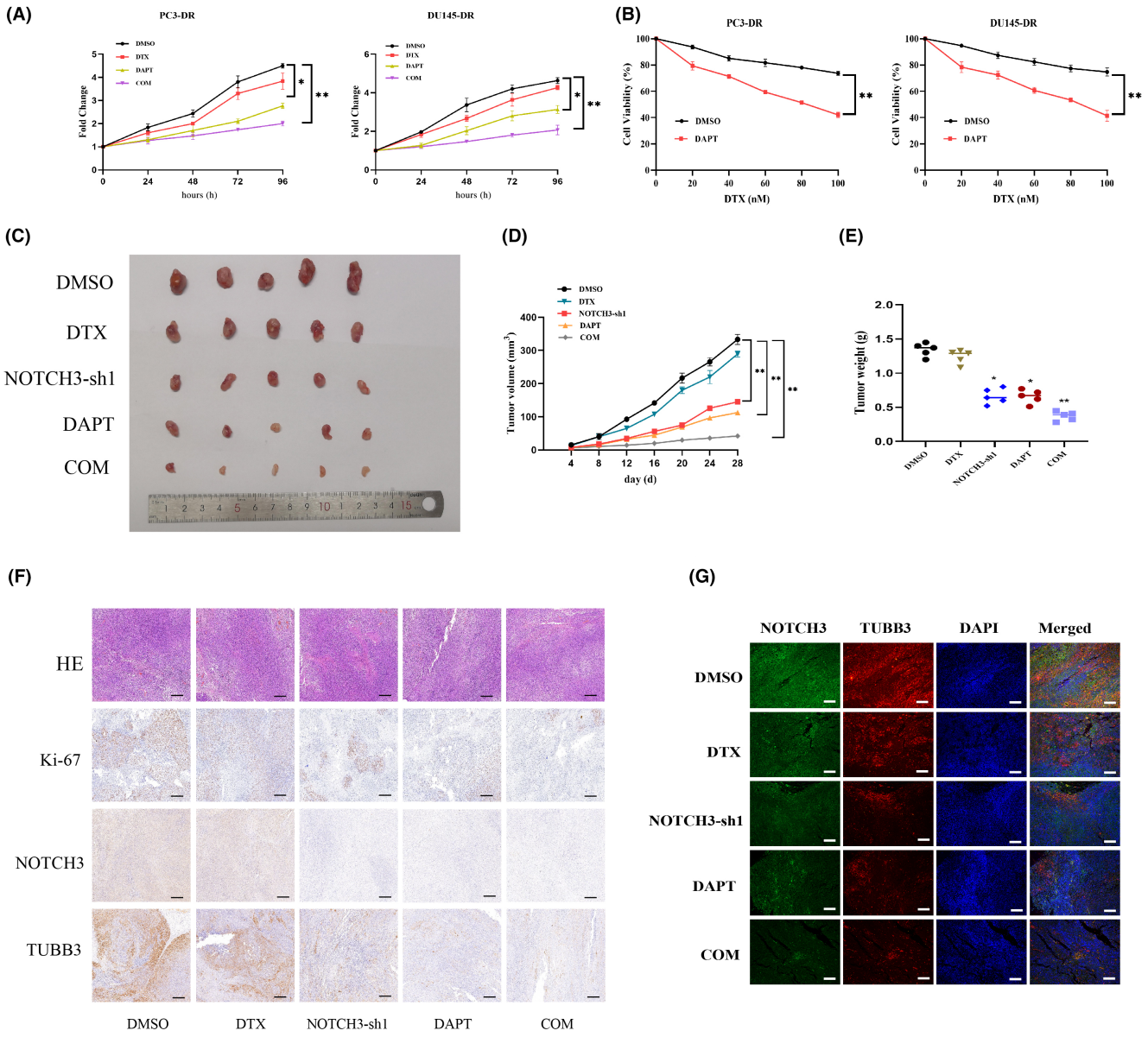

FIGURE 7 NOTCH3 inhibitor inhibits the progression of prostate cancer and sensitizes cancer cells response to docetaxel in vivo. (A) PC3-DR and DU145-DR cells were treated with DMSO, DTX, DAPT or in combination for 0–96 h. Cell viability was quantitated by CCK-8 assays. (B) Cell viability is measured in the indicated cell lines under docetaxel treatment. These cells were exposed to docetaxel with or without DAPT. Cell viability was measured by CCK-8 assays. (C–E) Mice bearing PC3-DR xenografts generated by NOTCH3-sh1 or control cells. Then the mice grouped and treated with DMSO, docetaxel, DAPT, or their combination for 4 weeks ($n = 5$/group). Tumor volumes were measured every 4 days. Tumors were collected, photographed, and weighed. (F) IHC staining of Ki-67, NOTCH3, and TUBB3 on tumor slide from each group were shown. (G) IF analysis showed the relationship between NOTCH3 and TUBB3 in tissues. Scale bars: 100 μm. DTX, docetaxel. $^{*}p < 0.05$; $^{**}p < 0.01$.

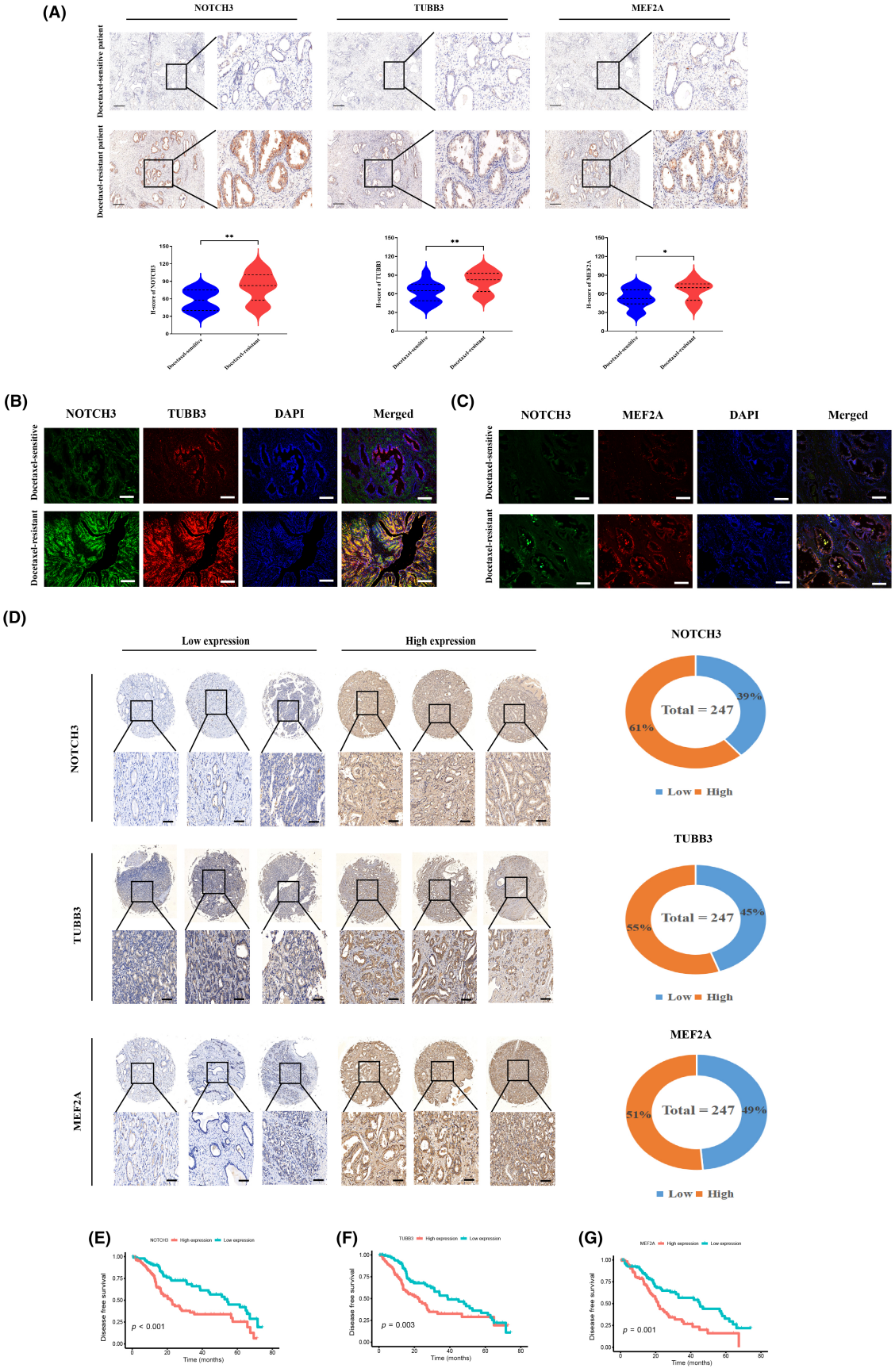

FIGURE 8 Expression of NOTCH3, TUBB3, and MEF2A is associated with poor survival in prostate cancer patients. The expression level of NOTCH3, TUBB3, and MEF2A in docetaxel-sensitive and docetaxel-resistant prostate cancer tissues were detected by IHC (A) and IF (B, C). (D) IHC staining of NOTCH3, TUBB3, and MEF2A on TMA. (E–G) DFS of PCa patients with different expression levels of NOTCH3, TUBB3, and MEF2A. Scale bars: 100 μm. $^{*}p < 0.05$; $^{**}p < 0.01$.

(10 mg/kg), and DTX (5 mg/kg) plus DAPT (10 mg/kg) for 4 weeks. In accordance with the above results in Figure 2M, NOTCH3 depletion resulted in a reduction in tumor growth speed. DTX treatment alone group had no discernible effect on tumor sizes as compared to the control group. However, treatment with DAPT decreased tumor volume and tumor weight, and the combination of DTX and DAPT further inhibited tumor growth (Figure 7C–E). No tumor group exhibited major morphological abnormalities in key organs, including the liver and kidney (Figure S6H). The proliferation index of PC3-DR xenografts inhibited by DAPT was also confirmed by IHC and the expression levels of NOTCH3 and TUBB3 were also decreased upon DAPT and combination groups (Figure 7F). Similarly, we performed IF assay and indicated that NOTCH3 inhibition group exhibited lower expressions of TUBB3 compared with the control group (Figure 7G).

3.9 | Expression of NOTCH3, TUBB3, and MEF2A is associated with poor survival in PCa patients

To determine the clinical significance of NOTCH3, TUBB3, and MEF2A in PCa, their expression profiles in DTX-sensitive and DR PCa tissues were evaluated. IHC staining for NOTCH3, TUBB3, and MEF2A was performed on the serial sections. High expressions of NOTCH3, TUBB3, and MEF2A were detected in DR PCa samples (Figure 8A). Immunofluorescence assay also showed that NOTCH3, TUBB3, and MEF2A were downregulated in docetaxel-sensitive PCa tissues (Figure 8B,C). In addition, we investigated their expression levels using TMA (Figure 8D; Figure S7A). Positive correlations between NOTCH3 and TUBB3 ($r=0.6376$, $p<0.01$) and NOTCH3 and MEF2A ($r=0.4825$, $p<0.01$) were observed in the PCa tissues (Figure S7B,C). High expression of NOTCH3, TUBB3, and MEF2A was associated with poor DFS in PCa patients (Figure 8E–G). Figure 9 is a hypothetical schematic diagram explaining the involvement of NOTCH3 on DTX resistance in PCa.

4 | DISCUSSION

DTX-based chemotherapy has been widely employed as a standardized first-line treatment strategy for patients with metastatic CRPC. However resistance to conventional chemotherapy develops rapidly and is associated with a poor prognosis.[23] Understanding the mechanism of action of DTX in CRPC is crucial for optimizing existing therapy approaches.

The impact of the NOTCH family on PCa has been extensively studied. In PCa, the knockdown of NOTCH1 inhibits growth and increases chemosensitivity. Elevated NOTCH1 staining is observed in aggressive prostate tumors.[24] NOTCH4 silencing could inhibit PCa growth via the NF-κB pathway.[25] Recent studies demonstrate that NOTCH3 is involved in prostate development and contributes to luminal cell differentiation.[26] Moreover, elevated NOTCH3 expression positively correlates with PCa progression and drug resistance.[27] NOTCH3 promotes PCa-induced bone lesion development through MMP-3.[28] The transcriptome sequencing technique offers unique advantages for cancer diagnosis and prognosis assessment.[29] We found that the expression of NOTCH3 was greatly upregulated in DR PCa

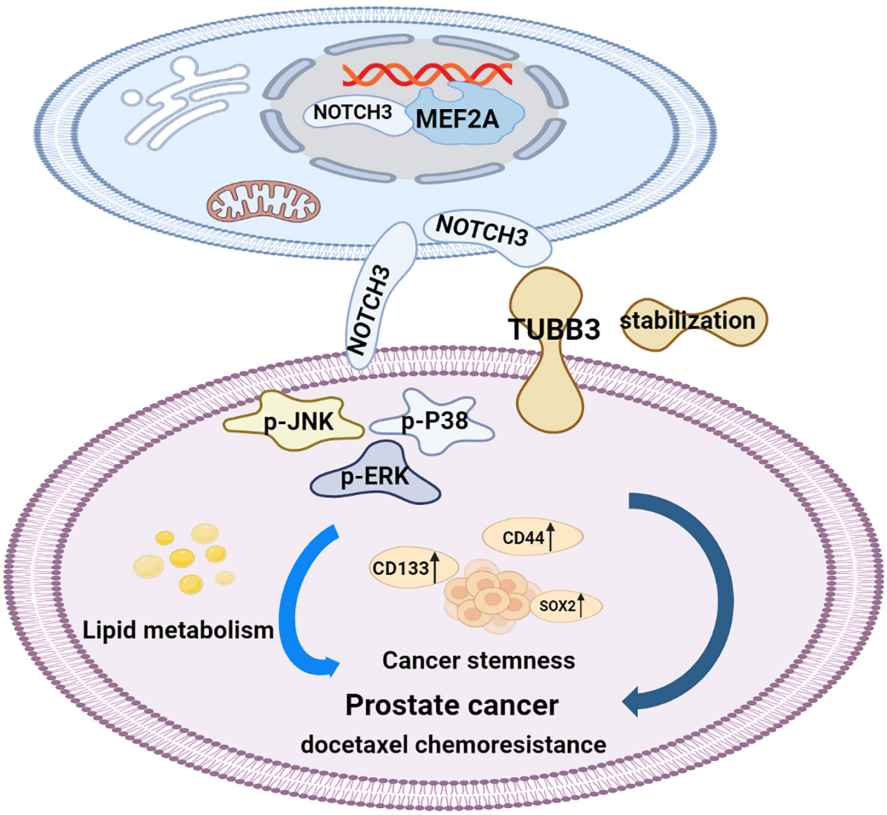

FIGURE 9 A hypothetical schematic diagram explaining the involvement of NOTCH3 on docetaxel resistance in PCa.

cells. Activation of NOTCH3 signaling is crucial for tumor resistance to platinum and taxanes. Together in clinical investigation of ovarian cancer patients, increased NOTCH3 expression was related to poor survival in stage III and stage IV patients treated with taxane chemotherapy.[30] NOTCH3 signaling initiation activates the stem cell reprogramming factor PBX1, which promotes CSC activity and contributes to platinum chemoresistance.[31] Overexpression of NOTCH3 is associated with a decrease in tumor-suppressor miR-136 and miR-150 expression that is resistant to DTX.[32] In addition, a range of pre-clinical studies has demonstrated that the combination of DTX and NOTCH3-specific inhibitors, such as γ-secretase inhibitor (GSI), small interfering RNA or antibody medicines, can boost the efficacy of DTX treatment in several tumors.[33,34] The combination of EGFR-TKIs and β-catenin inhibitor limits the NOTCH3-dependent activation of β-catenin, thereby significantly suppressing tumor initiation and enhancing the survival time of xenograft mice.[35] In pancreatic adenocarcinoma cells, the presence of NOTCH3 is associated with an increase in the function of PI3K/Akt signaling in response to gemcitabine administration.[36]

Implications of MEF2A in human cancer were revealed in PCa, and MEF2A was demonstrated to engage in stress-induced advancement of PCa.[37] MEF2A promotes tumor proliferation and metastasis in gastric cancer.[38] Through increasing the expression of MMP10, TGF-β mediates the effects of the growth factor on breast cancer metastasis by activating the MEF2A.[39] In addition, MEF2A is regarded as one of the transcription factors reacting to norepinephrine-involved carcinogenesis in ovarian cancer.[40,41]

Mechanically, we demonstrated that NOTCH3 was controlled by MEF2A, bound to TUBB3, and activated the MAPK signaling pathway. Most cancers utilize lipids and cholesterol to satisfy their insatiable energy needs. The FAO pathway is responsible for lipid catabolism and involves both exogenous and endogenous fatty acids.[42] Cancer-associated adipocytes (CAAs) in the tumor microenvironment facilitate tumor growth and drug response in a complex and dynamic manner.[43] Numerous intricate processes are implicated in the adipocyte- and lipid metabolism-mediated chemoresistance of diverse solid tumors.[44] Targeting CAAs might have a profoundly positive effect on chemotherapy efficacy. As its role in cancer development and treatment resistance, the MAPK signaling pathway has attracted considerable attention in the cancer research community.[45] Additionally, it has been linked to lipid metabolism in a variety of malignancies, including lung and colorectal cancers.[46,47] In our study, we demonstrated that NOTCH3 is capable of activating the MAPK signaling pathway in PCa cells, and that this activation may be associated with cancer stemness and lipid metabolism. Of course, additional research is required to validate these findings and elucidate the underlying mechanisms of NOTCH3 regulating the MAPK pathway.

AUTHOR CONTRIBUTIONS

Xianchao Sun: Investigation; methodology; project administration; software; writing – original draft; writing – review and editing. **Ying Zhang:** Data curation; methodology; software. **Shiyong Xin:** Formal analysis; software; supervision. **Liang Jin:** Resources; software; validation. **Qiong Cao:** Data curation; validation. **Hong Wang:** Data curation; project administration. **Keyi Wang:** Methodology; project administration. **Xiang Liu:** Project administration; software; supervision. **Chaozhi Tang:** Data curation; formal analysis; visualization. **Weiyi Li:** Formal analysis; software. **Ziyao Li:** Methodology; project administration. **Xiaofei Wen:** Project administration; supervision. **Guosheng Yang:** Project administration; supervision. **Changcheng Guo:** Resources; software; validation; writing – original draft. **Zhiyu Liu:** Project administration; supervision. **Lin Ye:** Formal analysis; funding acquisition; investigation; resources; software; writing – original draft; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

Approval of the research protocol by an Institutional Review Board: This study was approved by the Ethical Review Committee of Shanghai Tenth People's Hospital.

Informed consent: Informed consent was signed before collecting samples.

Registry and the Registration No. of the study/trial: N/A.

Animal studies: Animal experiments were approved by the Ethics Committee of the Shanghai Tenth People's Hospital.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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