# Decitabine Reverses CSC-Induced Docetaxel Resistance via Epigenetic Regulation of DAB2IP in TNBC.

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# Abstract

**Background:** Although docetaxel (DOC)-based chemotherapy, the standard of care for triple-negative breast cancer (TNBC), has greatly improved cancer survival, chemoresistance invariably evolves.

**Methods:** Using TNBC and docetaxel-resistant TNBC cell lines, we established murine breast cancer xenograft models to evaluate the impact of decitabine on DOC resistance. The methylation status of DAB2IP was evaluated in 226 TNBC patients and TNBC cell lines by BSP analysis. A limiting dilution assay was performed to evaluate the tumorigenic capacity in vivo. Cellular and molecular mechanisms were demonstrated using in vivo and in vitro biochemical methods.

**Results:** Here, we observed that a low dose of DAC significantly prevented the evolution of DOC resistance via inhibition of cancer stem cell (CSC) enrichment in TNBC. The bisulfite sequencing PCR analysis showed that DAC treatment increased DAB2IP expression via inhibition of DNA methylation in both DOC-resistant TNBC cells and tumorspheres. Loss of DAB2IP boosted the enrichment of CSCs through activation of Erk/β-catenin signaling in vitro and facilitated tumor initiation in vivo. In mouse model, TNBC tumors with inhibition of DAB2IP exhibited poor response to DOC and DOC-resistant tumors were resensitized to DOC treatment by low-dose DAC treatment in vivo. Finally, hypermethylation of DAB2IP was correlated with low expression of DAB2IP and poor outcome of TNBC patients treated with DOC-based chemotherapy.

**Conclusions:** Collectively, we discovered that epigenetic silencing of DAB2IP is a driver of CSC enrichment, which results in the evolution of DOC resistance, and a low dose of DAC reverses DOC resistance through epigenetic re-expression of DAB2IP.


# 1. Background

Triple-negative breast cancer (TNBC) accounts for 12%-18% of breast cancer (BC) cases and lacks expression of the estrogen and progesterone receptors and overexpression/amplification of the Her2 receptor[1]. As TNBC exhibits poor response to endocrine/anti-Her2 therapy, chemotherapy is the only established systemic-therapy for TNBC treatment. Docetaxel (DOC), which inhibits cell division and causes cell death, is one of the most widely used cytotoxic drugs in TNBC. Although DOC-based chemotherapy has greatly improved the outcome of TNBC patients, many of them develop resistance to DOC (acquired chemoresistance), resulting in cancer relapse in 3-5 years [2]. The search for effective treatments that might reverse resistance to DOC is an important unmet medical need.

Previous study revealed that genomic aberrations associated with chemoresistance pre-exist before chemotherapy, while chemoresistance phenotypes are commonly caused by nongenetic mechanisms (including epigenetic silencing, transcriptional repression and proteasome-mediated degradation) [3]. DNA methylation, an epigenetic modification that connects a methyl group to CpG dinucleotides in the genome mediated by DNA methyltransferases (DNMTs), often causes gene silencing. In BC, hypermethylation was extensively observed at promoters of tumor suppressors that regulate tumor

survival, cell apoptosis and CSC capacities [4, 5]. Demethylating agents, represented by decitabine (5-aza-2'-deoxycytidine, DAC) and azacitidine, can reactivate epigenetically silenced tumor suppressors and become a potential treatment for drug-resistance[6]. DAC, a chemical analogue of cytidine, regulates DNA methylation by inhibiting DNA methyltransferases at low doses. In hematopoietic malignancies, DAC and azacitidine are the only demethylation regimens approved by the FDA [7]. In solid tumors (including breast, bladder and prostate cancers), studies have shown that a low dose of DAC produces an antitumor effect via sustained inhibition of DNA methylation [7, 8]. Whether DAC is a potential strategy for the treatment of DOC resistance in TNBC worth further investigation.

Cancer stem cells (CSCs) are a subset of cancer cells with self-renewal potential, multidirectional differentiation capacity and intrinsic chemoresistance. Accumulating evidence showed that CSCs are enriched in the residual population of chemoresistant tumor cells, leading to the evolution of chemoresistance in TNBC [9, 10]. Elimination of CSCs becomes necessary for the treatment of chemoresistance. Recent study reported that DAC treatment at low dose suppresses CSC capacity in BC, raising the question of whether DAC is capable of impacting the evolution of chemoresistance[8].

DAB2IP, a Ras GTPase-activating protein (RAS GAP), inactivates several oncogenic signaling pathways (such as the RAS, NF-κB and PI3K-AKT pathways) and inhibits cancer cell growth, migration and survival. In previous studies, aberrant promoter methylation was detected in the DAB2IP gene in several cancers (including breast, prostate and lung cancers), which caused DAB2IP silencing [11, 12]. Our study discovered that low expression of DAB2IP caused by hypermethylation at the DAB2IP promoter region was detected in both DOC-resistant TNBC cells and cancer stem-like cells. Loss of DAB2IP boosted the enrichment of CSCs through activation of Erk/β-catenin signaling and induced DOC-resistance. A low dose of DAC restored the expression of DAB2IP and subsequently reversed CSC-induced chemoresistance. Collectively, we discovered that low-dose DAC treatment reverses DOC resistance through epigenetic re-expression of DAB2IP in TNBC.

# 2. Materials And Methods

### *2.1. Patients and tissue specimens*

Formalin-fixed paraffin-embedded (FFPE) specimens from 304 TNBC patients were collected for evaluation of DAB2IP expression at Sun Yat-sen University Cancer Center from 2000 to 2015. All cases were pathologically diagnosed as breast cancer with negative expression of ER, PR and Her2 receptor. The clinical and pathological characteristics are listed in Table S1. Follow-up data were determined by clinical review or a telephone interview. Our study was in accordance with the Declaration of Helsinki and was ethically approved by the institutional review board.

### *2.2. Promoter methylation analysis*

Genomic DNA was isolated from breast cell lines (Omega, Tissue DNA Kit; cat. D3396-02) or FFPE specimens with thicknesses less than 10 mm (Qiagen Kit; cat. # 1071592) and bisulfite converted

(Qiagen Kit; cat. # 59824) according to the manufacturers' instructions. Primers used in the bisulfite sequencing PCR (BSP) experiments were listed in supplemental table 3. The PCR products were cloned into the pGEM-T Easy Vector System and sequenced using the M13F universal primer. Three clones for each cell line or each tissue were analyzed, and the average methylation frequency was counted. The DNA methylation level was assessed as methylated-loci / (methylated + unmethylated loci). The methylation level of DAB2IP was detected in 304 TNBC cases, and promoter analysis in 78 cases were failed because of insufficient tissue volume or tissue DNA degradation.

### *2.3. Xenograft tumor model and limiting dilution assay*

Female BALB/c-nude mice (4–5 weeks old, 18–20 g) were supplied by the Beijing Vital 17 River Laboratory Animal Technology Company Limited, China. Mice were subcutaneously injected with luciferase-tagged cells ($1\times10^6$) into the right mammary fat pads, and tumors were measured every 4 days beginning on the $8^{th}$ day after inoculation. Decitabine/docetaxel (DOC) treatment began when the volume of tumors reached approximately 200 $mm^3$. For DAC treatment, mice were treated with a low dose of DAC (2.5 mg/kg, i.p. 4 times weekly), which was estimated based on the low dose for humans (7.5 $mg/m^2$) (the dose conversion was based on the FDA recommendation: https://www.fda.gov/downloads/drugs/guidances/ucm078932.pdf); the 7.5 $mg/m^2$ dose can reach a plasma concentration of up to 100 nM in humans [13]. For DOC treatment, mice were treated with DOC (15 mg/kg, i.p. weekly). For the combined treatment of DAC and DOC, mice were treated with DAC on days 1-4 and treated with DOC on day 5 weekly (Fig. 1C). The tumor burden was evaluated by luciferase activity using the Xenogen IVIS spectrum imaging system (Caliper) or the tumor volume ($(length\times width^2)/2$).

The limiting dilution assay was performed to evaluate the tumorigenic capacity in vivo (Fig. 3H). The indicated cells were mixed with Matrigel (Corning) and subcutaneously injected into 4 mammary fat pads of female NOD/SCID mice (Beijing Vital 17 River Laboratory Animal Technology Company Limited, China). The influence of tumor formation was assessed by extreme limiting dilution analysis (http://bioinf.wehi.edu.au/software/elda/) and luciferase activity using the Xenogen IVIS spectrum imaging system (Caliper). All experimental procedures were approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University.

### *2.4. Statistical analysis*

The SPSS 20.0 statistical software package was used for statistical analyses. The clinicopathological characteristics of groups of patients with high/low expression were analyzed using the $\chi^2$ test. Survival analysis was performed with the Kaplan-Meier method and the log-rank test. Univariate and multivariate Cox regression models were used to assess the survival data. $P < 0.05$ was considered statistically significant.

## 3. Results

### *3.1 Decitabine enhances the TNBC response to docetaxel.*

The anticancer effect of DAC was shown in several studies, most of which used a high dose [14]. As DAC at high dose rapidly induced DNA damage and cell-apoptosis, it inevitably caused substantial toxicity. To identify a suitable dose of DAC for our study, TNBC cells were treated with various concentrations of DAC. DAC treatment did not significantly decrease cell viability until the concentration exceeded 100 nM (Fig. S1A). Thus, a low dose of DAC (100 nM) was used to further study.

TNBC cells were pretreated with DAC or vehicle (veh) before DOC treatment to investigate whether DAC improves the DOC response of TNBC. Pretreatment of DAC improved the response to DOC in TNBC cells (Fig. 1A). Using flow cytometry, we observed a higher proportion of cell apoptosis in the DAC-pretreatment groups than in the control groups (Fig. 1B). Further, xenograft tumor model was used to evaluate the anticancer effect of DAC in vivo (Fig. 1C). The volume of tumors pretreated with DAC before DOC treatment barely increased during chemotherapeutic treatment, and these tumors grew much slower and smaller than the DOC group (Fig. 1D-F). These data suggested that DAC enhanced the TNBC response to DOC. Although DAC treatment moderately suppressed tumor growth (Fig. 1D), we did not observe a significantly increased proportion of cell apoptosis compared to that in the veh group (Fig. 1G), indicating that the anticancer effect of DAC treatment is independent of cell apoptosis.

### *3.2 Decitabine inhibits the CSC enrichment induced by DAB2IP hyper-methylation.*

Several studies revealed that cells surviving after chemotherapy exhibited high CSC capacities (self-renewal ability and CD44 expression) [9, 15]. To analyze the changes of CSC population under DOC or DAC+DOC treatment, we labeled CSCs and dead cells with anti-CD44 antibodies and PI, respectively. Cells treated with DOC exhibited a higher surface expression of CD44 than the veh group, while DAC-pretreatment decreased the CD44 expression in the DOC-treated cells (Fig. 2A and Fig. S2A). Consistently, the mRNA expression of CD44 was higher in DOC-treated cells than in vehicle-treated cells, and pretreatment with DAC decreased the expression of CD44 (Fig. 2B). Although we did not observe significant changes in the expression of CD24 (Fig. S2A), another CSC-specific marker, previous studies have proven that CD44 expression is adequate for CSC population selection [15]. These data indicated that DOC treatment induced the enrichment of $CD44^{hi}$ population which could be significantly inhibited by DAC treatment. Further, we established DOC-resistant (DOC-R) cell lines (Fig. S2B-C). Compared with DOC-naive (DOC-N) cells, DOC-R cells exhibited higher proportion of $CD44^{hi}$ cells and increased CD44 expression (Fig. 2C-D and Fig. S2D). The DAC treatment attenuated the $CD44^{hi}$ population enrichment and the elevated CD44 expression in DOC-R cells (Fig. 2C-D and Fig. S2D). Using tumor-sphere assay, we observed a higher self-renewal potential in DOC-R cells than in DOC-N cells (Fig. 2E). Importantly, low-dose DAC treatment significantly suppressed self-renewal in DOC-R cells (Fig. 2E).

We further investigated what drives CSC enrichment and how DAC inhibits CSC enrichment. Previous studies suggested that Ras, a driver gene for human cancers, plays a causal role in CSC enrichment, and RAS GAPs are negative regulators for Ras activity [9, 16, 17]. Thus, we examined whether loss of RAS

GAPs caused CSC enrichment. We compared the mRNA expression of 13 RAS GAP genes in suspension-cultured TNBC cells with that in attachment-cultured cells. IQGAP2, IQGAP3 and DAB2IP were consistently silenced in suspension-cultured cells (Fig. 2F). DAB2IP was aberrantly silenced in several cancers which caused activation of Ras, and IQGAP 2/3, although do not exhibit RAS GAP activity, were shown to affect the Ras signaling through direct association with Rac1[18]. Further, we collected 10 paired normal mammary and breast cancer tissues and observed that the mRNA level of DAB2IP was significantly decreased in 9/10 tumor tissues Compared to the respective expression levels in normal tissues (Fig. S2E-G). The above findings were also supported by TCGA BRCA dataset (Fig. S2H). Furthermore, we observed a higher DAB2IP protein expression in attachment-cultured cells than in suspension-cultured spheroids (Fig. 2G-H). The expression of DAB2IP negatively correlated with the size of spheroids indicating that the expression of DAB2IP might be negatively correlated with self-renewal capacity in TNBC (Fig. 2H). Further, GSEA showed that DAB2IP expression was positively correlated with a methylated gene signature (Fig. S2I). Using the CCLE database, we observed that the DAB2IP-expression negatively correlated with the DNA-methylation level in breast cancer cell lines (Fig. S2J).

DAB2IP is encoded by 4 most commonly seen transcripts (Supplementary table 3). Although transcription of the four transcripts is regulated by different promoters, 4 functional domains (including C2, RAS GAP, Pleckstrin homology (PH) and DUF3498 Domain) are similarly contained in 4 protein isoforms indicating that all these protein isoforms have the potential to regulate the activity of RAS signaling. Primers were designed to detect methylation status according to the distribution of CpG islands in the promoter region of 4 transcripts (Supplemental table 3). Comparing to attachment-cultured cells, we observed a significantly increased methylation level at the promoter region of the ENST00000259371.6 transcript in suspension-cultured cells (Fig. 2I and Fig. S2K). In TNBC cells, DNA methylation level of DAB2IP (ENST00000259371.6) negatively correlated with mRNA expression that MDA-MB-231 exhibited the lowest level of DNA methylation and highest mRNA level of DAB2IP while HCC1806 exhibited the highest level of DNA methylation and lowest mRNA level of DAB2IP (Fig. S2L). By inhibition of DNA methylation, DAC increased the expression of DAB2IP in TNBC cells (Fig. S2M). Thus, the transcript ENST00000259371.6 is selected to further study. Further, DAC treatment significantly inhibited DNA methylation at DAB2IP (ENST00000259371.6) promoter and increased the expression of DAB2IP in suspension-cultured cells (Fig. 2J-K).

### *3.3 Loss of DAB2IP enhances cancer stem cell capacity through activation of Erk/β-catenin signaling in TNBC.*

DAB2IP is a tumor suppressor in several cancers (breast, liver, prostate and lung cancers) and has been implicated in the regulation of tumor growth, cancer metastasis and drug resistance [11, 19]. GSEA showed that DAB2IP expression was negatively correlated with CSC-related gene signatures (Fig. S3A). To investigate the function of DAB2IP, we established stable cell lines overexpressing/silencing DAB2IP (Fig. S3B). Tumor-sphere culture showed that self-renewal potential was enhanced by DAB2IP-inhibition and inhibited by DAB2IP-overexpression in TNBC cells (Fig. 3A and Fig. S3C). Enrichment of $CD44^{hi}$ population was enhanced in DAB2IP-inhibition cells and attenuated in DAB2IP-overexpression cells (Fig.

3B and Fig. S3D). As key regulators of self-renewal in CSCs, the mRNA levels of Nanog and Sox2 were significantly increased in DAB2IP-silenced cells but decreased in DAB2IP-overexpressing cells (Fig. 3C and Fig. S3E).

To understand the mechanism underlying inhibition of CSC enrichment by DAB2IP, we performed GSEA and observed that DAB2IP expression was associated with repression of the Wnt-target gene signature (Fig. S3A right panel). β-Catenin, the downstream effector of canonical Wnt signaling, is necessary for CSC capacities in BC. Previous study has revealed that phosphorylation of Erk1/2, the main downstream effector of Ras, maintains the expression of β-catenin and subsequently facilitates the nuclear translocation of β-catenin [20]. In TNBC spheroids, cells with DAB2IP-inhibition increased the expression of p-Erk1/2 and activated β-catenin (Fig. 3D). To examine whether DAB2IP-inhibition promoted the nuclear translocation of β-catenin, we stimulated TNBC spheroids with Wnt3a (250 ng/ml), which is a stimulator of β-catenin signaling. At the early phase of Wnt3a treatment (15 min), active β-catenin staining was readily detected in the cytoplasm and nucleus of cells silencing DAB2IP. In control cells, active β-catenin staining was mostly detected at the cell membrane and hardly detected in the cytoplasm and nucleus (Fig. 3D). As we prolonged the interval of Wnt3a treatment to 30 min, confocal analysis showed that active β-catenin staining could only be detected in the nucleus in cells with silenced DAB2IP, indicating the nuclear translocation of β-catenin. In contrast, active β-catenin staining was mostly detected at the cell membrane rather than in the cytoplasm and nucleus in control cells (Fig. 3D). Conversely, DAB2IP overexpression significantly weakened the staining of p-Erk1/2 and active β-catenin and further inhibited the nuclear accumulation of β-catenin in TNBC spheroids (Fig. S3F).

Next, we performed a limiting dilution assay to explore the effect of DAB2IP on the tumor-initiation capacity of TNBC cells. A higher frequency of tumor initiation was observed in cells with DAB2IP-inhibition than in the control cells (Fig. 3E). On the contrary, we observed a decreased incidence of tumor initiation in cells overexpressing DAB2IP compared to that in the control group (Fig. S3G). DAB2IP significantly suppressed the tumor initiation capacity in TNBC (Table 1).

### *3.4 Loss of DAB2IP induces docetaxel resistance in TNBC.*

As loss of DAB2IP induced CSC enrichment, we further investigated whether loss of DAB2IP induces DOC resistance in TNBC. Compared to control cells, DAB2IP-silenced cells exhibited a higher viability accompanied by a lower proportion of cell apoptosis when treated with DOC (Fig. 4A-B). When treated with DOC, DAB2IP-silenced cells exhibited a decreased proportion of cells at the peak of the G2/M phase (Fig. 4C). In contrast, DAB2IP-overexpression significantly improved the cytotoxic effect of DOC in TNBC cells (Fig. S4A-C). Using Western blotting, we observed that DAB2IP expression was negatively correlated with the level of p-Erk1/2, while there were no significant changes in Erk1/2 expression in DAB2IP-silenced or DAB2IP-overexpressing cells (Fig. 4D and Fig. S4D). As downstream targets of Erk1/2, Akt, CCNB1 and CDK1 are key regulators of cell apoptosis and cell cycle (G2/M) transitions. In cells with DAB2IP-inhibition, we detected an increased phosphorylation level of Akt and CDK1 with no significant changes in the protein level (Fig. 4D). In addition, the protein level of CCNB1 was increased in DAB2IP-

inhibition cells (Fig. 4D). In contrast, the levels of phosphorylated-Akt, phosphorylated-CDK1 and CCNB1 were decreased in DAB2IP-overexpressing cells (Fig. S4D).

In animal models, tumors with DAB2IP-inhibition grew much faster and larger than those in controls under DOC treatment, while the growth of tumors overexpressing DAB2IP was significantly suppressed by DOC treatment. (Fig. 4E-F and Fig. S4E-G). We also observed that the proportion of cell apoptosis was decreased in tumors with DAB2IP-inhibition, while an increased proportion of cell apoptosis was shown in DAB2IP-overexpression tumors (Fig. 4G and Fig. S4H).

### *3.5 Low dose of decitabine reverses docetaxel resistance by restoring DAB2IP expression.*

We further examined whether DAC overcomes DOC resistance through re-expression of DAB2IP. In Doc-R cells, we observed that the DNA-methylation level of DAB2IP was higher and the expression of DAB2IP was lower than those in DOC-N cells (Fig. 5A and Fig. S5A). When treated with DAC, the DNA-methylation level was decreased in Doc-R cells, and DAB2IP expression was increased (Fig. 5B and Fig. S5B). In Doc-R cells, DAC+DOC treatment induced a higher proportion of cell apoptosis than DOC treatment alone (Fig. 5C). We also observed that DAC treatment could not restore the DAB2IP expression in DAB2IP-silenced cells and DAC pretreatment could not improve the response to DOC in DAB2IP-silenced cells (Fig. S5C-D). These data indicated that DAB2IP is essential for DAC to improve the DOC response in TNBC cells.

In vivo, tumors in the DAC+DOC group grew slower and smaller than the tumors in the DOC group (Fig. 5D and Fig. S5E). The weights of tumors sequentially treated with DAC and DOC were less than those of tumors treated with DOC alone (Fig. 5E). Moreover, we observed stronger DAB2IP staining in tumors treated with DAC than that in tumors without DAC treatment (Fig. 5F). Combination of DAC and DOC increased the proportion of apoptotic cells in DOC-R tumors (Fig. 5G).

### *3.6 Epigenetic silencing of DAB2IP predicts poor outcomes in TNBC.*

Further, we investigated whether the expression or methylation status of DAB2IP acts as a prognostic marker for TNBC. We collected 304 TNBC patient tissues and detected the expression and methylation level of DAB2IP (Fig. 6A-B). Clinical and pathological features of the TNBC patients are listed in Table S1. Low expression of DAB2IP was significantly correlated with a hyper-methylation status in TNBC (Fig. 6B-C). Survival analysis showed that low expression and hypermethylation of DAB2IP correlated with poor outcome in TNBC patients (Fig. 6D). Among patients treated with DOC-based chemotherapy, both low DAB2IP expression and hypermethylation correlated with worse outcomes (Fig. 6E).

## 4. Discussion

Although DOC-based chemotherapy has greatly decreased the risk of cancer relapse in TNBC, 30% of patients would encounter chemoresistance soon after chemotherapy [21]. How to prevent chemoresistance evolution and treat patients with chemoresistance become major challenges for BC treatment. Previous studies have revealed the mechanisms of chemoresistance, including the efflux of

membrane "drug pump" on drugs (ABC transporter), the cellular abnormal metabolism of drugs, the dysregulation of DNA damage repair and cell cycle transition, the activation of anti-apoptosis or pro-survival signaling pathways and the enrichment of cancer stem cells (CSCs). In our study, we discovered that a low dose of decitabine improved DOC response via inhibition of CSC enrichment. Thus, pretreating TNBC patients with low dose of DAC before DOC treatment might prevent the evolution of chemoresistance. Because CSC was enriched in chemoresistant TNBC cell population, combination of DAC and DOC could also be a rescue measures for patients who were resistant to DOC.

DAC is an effective demethylating agent that it induces rapid cell apoptosis and causes intolerable cytotoxicity at high dose while causes DNA demethylation and exhibits improved safety at low dose[22, 23]. Recently, Hsing-Chen Tsai et al found that a low dose of DAC exhibited a sustained demethylating effect and suppressed CSC capacity in breast cancer [8]. Thus, the improvement of DOC response by DAC was not simply caused by increased cell cytotoxicity. As low dose of DAC treatment rarely causes cell apoptosis, the addition of DAC at low dose to DOC treatment could be well tolerated. Combination of DAC and DOC can improve the therapeutic effect and avoid increasing the damage of chemotherapy to normal tissues.

Due to the overexpression of ATP-binding cassette (ABC) transporter proteins and the high activity of aldehyde dehydrogenase (ALDH), CSCs are resistant to chemotherapy. Taking advantage of its drug-resistance and self-renewal potential, CSCs are enriched by chemotherapeutic pressure and regenerate the tumor after cancer-therapy. Identification the mechanism of CSC enrichment becomes the key to discovering the treatment strategy for DOC resistance in TNBC. In human cancer, Ras signaling is one of the major pathways involved in the self-renewal of CSCs, and its aberrant activation is a driving factor for CSC enrichment [24]. Activated Ras proteins are turned off by RAS GTPase-activating proteins (RAS GAPs). However, several RAS GAPs are silenced in multiple human cancers, commonly caused by nongenetic mechanisms [25, 26]. In our study, we discovered that silence of DAB2IP, caused by promoter hyper-methylation, induced DOC-resistance in TNBC. As a member of RAS GAPs, loss of DAB2IP significantly activated the Erk/β-catenin signaling, downstream of RAS signaling, and enhanced the CSC capacities in TNBC.

Accumulating evidence indicates that aberrant DNA methylation contributes to the development of drug resistance in solid tumors [27, 28]. Hyper-methylation at the promoter region epigenetically silences tumor suppressors, which enables tumor cells to evade chemotherapy. In our study, we discovered that low-dose DAC treatment re-expressed DAB2IP in CSCs through the inhibition of DNA methylation, which provides a theoretical basis for the treatment of chemotherapy resistance by low-dose DAC. However, the lack of biomarkers that predict the sensitivity to DAC has prohibited the clinical application of DAC [7, 29]. As a low dose of DAC reversed DOC resistance through epigenetic re-expression of DAB2IP, hyper-methylation of DAB2IP could serve as a biomarker to identify patients with DOC-resistant TNBC who will benefit from DAC treatment.

In addition to DOC-resistant cells, we also observed DNA methylation at the DAB2IP promoter region in TNBC cells naïve to DOC treatment. Moreover, hyper-methylation of DAB2IP predicted a higher risk of cancer mortality in TNBC patients receiving DOC treatment than a hypo-methylated status. Since our specimens were all collected before chemotherapy, epigenetic silencing of DAB2IP, which caused DOC resistance, might exist long before DOC treatment. As TNBC cells with epigenetic silence of DAB2IP exhibited worse DOC response than the general TNBC cells, these cells might be more likely to survive during chemotherapy. The above observation was supported by a recent study of single-cell sequencing in which gene profiles causing chemoresistance were found to be pre-existing and adaptively selected by chemotherapy[30]. Thus, a hyper-methylation status of DAB2IP could be a marker for DOC resistance in TNBC, and DAC-treatment before DOC-based chemotherapy might prevent the development of DOC resistance in TNBC patients with DAB2IP-hypermethylation. Although clinical data supporting the above viewpoint are lacking, our data from an animal model showed that the combined treatment (DAC-pretreatment followed by DOC treatment) exhibited a better tumor-suppressive effect than DOC treatment alone in DOC-naïve TNBC. Whether the DAB2IP methylation status can identify those patients who will benefit from combined treatment with DAC and DOC warrants further studies.

# 5. Conclusion

Collectively, our study discovered that loss of DAB2IP expression caused by DNA methylation at the promoter region was critical for CSC enrichment and DOC resistance evolution in TNBC. A low dose of DAC effectively treats DOC resistance through epigenetic regulation of DAB2IP, while the methylation status of DAB2IP could be a potential marker for identifying those TNBC patients who will benefit from DAC treatment (Fig. 8F).

# Abbreviations

TNBC, triple-negative breast cancer; BC, breast cancer; ER, estrogen receptor; PR, progesterone receptor; CSC, cancer stem cell; DOC, docetaxel; DAC, decitabine; DNMT, DNA methyltransferase; RAS GAP, Ras GTPase-activating protein; BSP, bisulfite sequencing PCR; qRT-PCR, quantitative real-time polymerase chain reaction; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; ICC, immunocytochemistry; IF, immunofluorescence; IHC, immunohistochemistry; veh, vehicle; TCGA, The Cancer Genome Atlas; GSEA, gene set enrichment analysis; OS, overall survival.

# Declarations

### Ethics approval and consent to participate

Ethical approval was obtained from the respective institutional review boards of the Ethics Committee of Sun Yat-sen University Cancer Center. All patients provided written informed consent to participate in this study.

### Consent for publication

Not applicable.

### Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its additional files.

### Competing interest

All the authors declare that they have no conflicts of interest.

### Author Contributions

Zhenchong Xiong: Conception, methodology and Data curation.

Lin Yang: Methodology design and Data curation.

Lu Yang: Investigation and Visualization.

Jianchang Fu: Methodology and Software.

Peng Liu: Formal analysis.

Peng Sun: Validation.

Hailin Tang: Writing - original draft and project administration ZC.

Xiaoming Xie: Supervision and funding acquisition.

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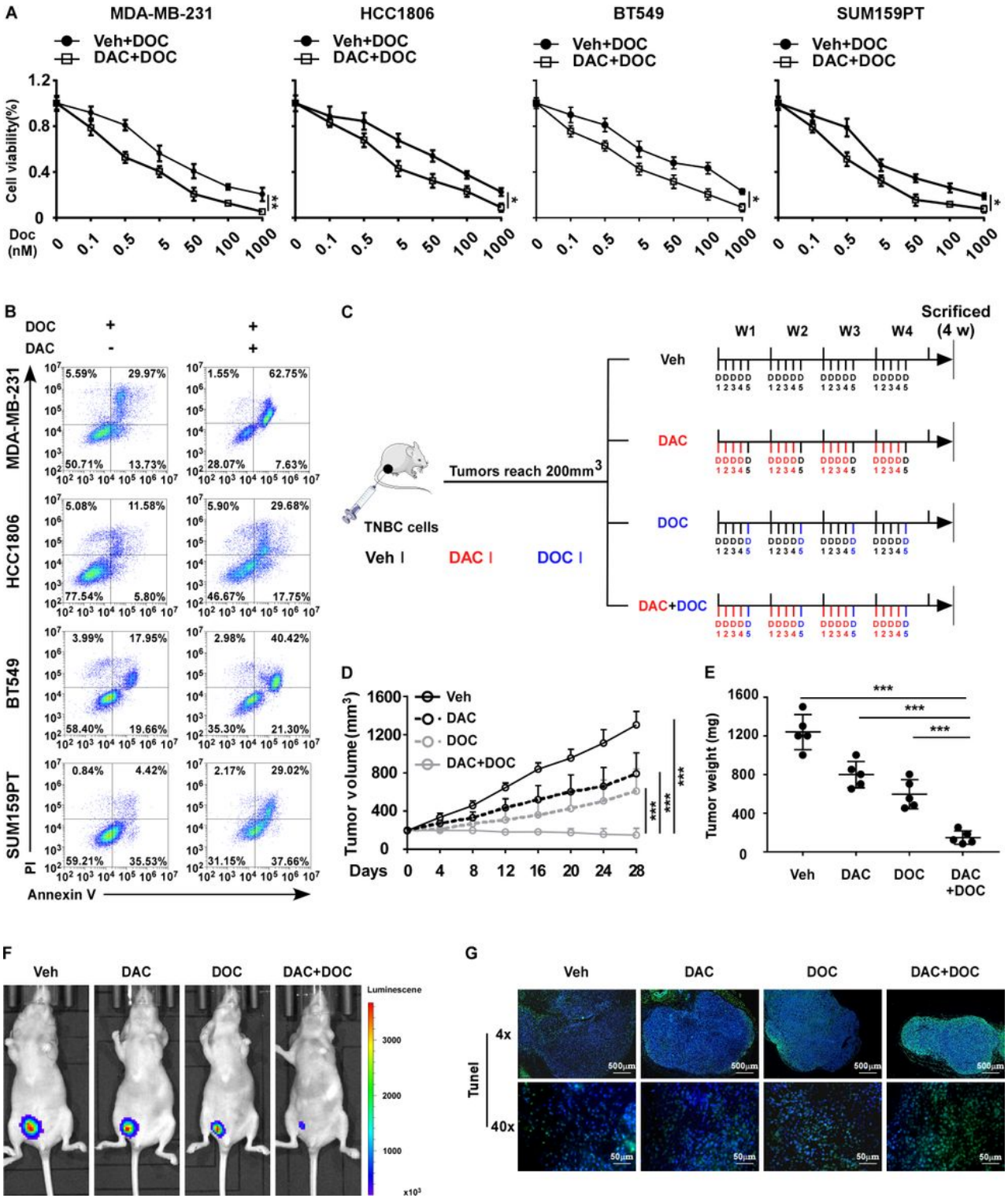
# Tables

**Table 1. Incidence of Tumors in NOD/SCID Mice**

| Number of cells | Number of outgrowths | | | |
|---|---|---|---|---|
| | Vector | DAB2IP | Control | RNAi#1 |
| $2\times10^5$ | 6/6 | 5/6 | 6/6 | 6/6 |
| $2\times10^4$ | 5/6 | 2/6 | 4/6 | 6/6 |
| $2\times10^3$ | 1/6 | 0/6 | 1/6 | 3/6 |
| $2\times10^2$ | 1/6 | 0/6 | 1/6 | 2/6 |
| *P*-value | Stem cell frequency | | *P*-value | Stem cell frequency | |
| <0.005 | 1/9,353 | 1/92,028 | 0.002 | 1/13,788 | 1/1,895 |

# Figures

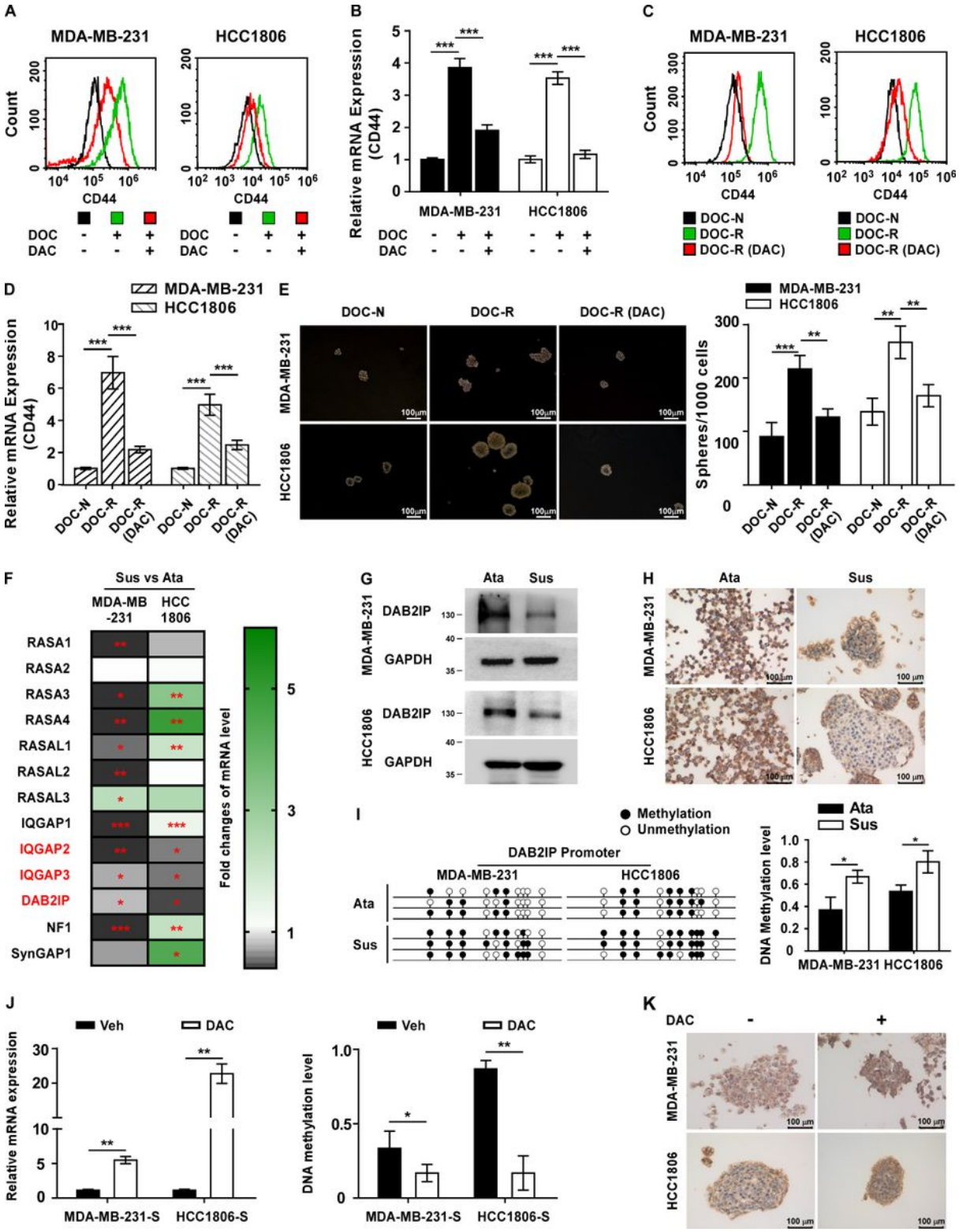
Figure 1




## Figure 1

Decitabine enhances TNBC response to docetaxel. A. Cell viability was assessed in cells with the indicated treatments. TNBC cells pretreated with a low dose of decitabine (DAC, 100 nM) or vehicle (veh) for 72 h were treated with the indicated concentrations of docetaxel (DOC, 72 h), and cell viability was evaluated by CCK-8 assay. B. Cell apoptosis (Annexin V-positive) was evaluated by an Annexin V/PI assay using flow cytometry. TNBC cells pretreated with a low dose of DAC (100 nM, 72 h) or veh (72 h)

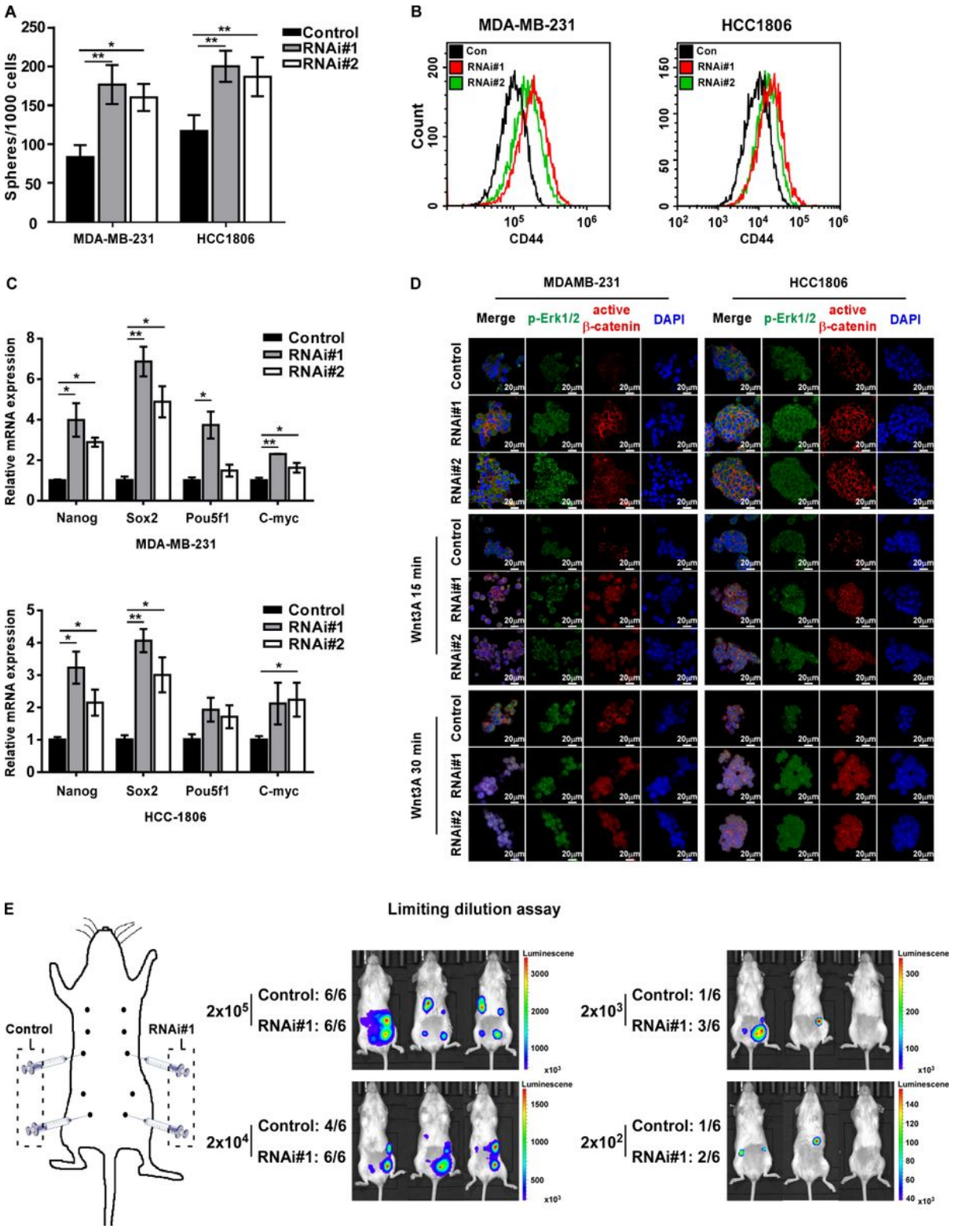
were treated with DOC (5 nM) for 72 h. C. Schematic diagram of the in vivo experimental strategies. MDA-MB-231 cells (1×106) were subcutaneously injected into the right mammary fat pads of mice. Mice were randomly assigned to the veh group, DAC group, DOC group or DAC+DOC group when tumors reached a volume of 200 mm3. Mice were sacrificed on day 28. D. The volumes of tumors in the indicated groups (n = 5/group) were measured every 4 days and are shown by tumor growth curves. E. Tumor weights of tumors in the indicated groups. F. Representative images of tumor-bearing mice in the indicated groups are presented with luciferase activity. G. Representative images of TUNEL staining of apoptotic cells in tumors treated with veh, DAC, DOC or DOC+DAC. The cell nucleus was labeled with DAPI (in blue), and the apoptotic cell fraction was labeled with TUNEL (in green). Scale bars: 50 μm and 500 μm. In A and D, Data are presented as the mean ± SD. P-values were determined by two-way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001. In E, data are presented as the mean ± SD. P-values were determined by one-way ANOVA. ***P < 0.001.




## Figure 2

Decitabine inhibits the CSC enrichment induced by DAB2IP hyper-methylation. A. Flow cytometry for CD44 cell surface expression in TNBC cells with the indicated treatment. TNBC cells and TNBC cells pretreated with DAC were treated with DOC, and TNBC cells treated with vehicle were selected as blank control. B. qRT-PCR analysis of CD44 expression in the indicated cells. GAPDH was used as an internal control. C. Flow cytometry for CD44 cell surface expression in TNBC cells treated with the indicated

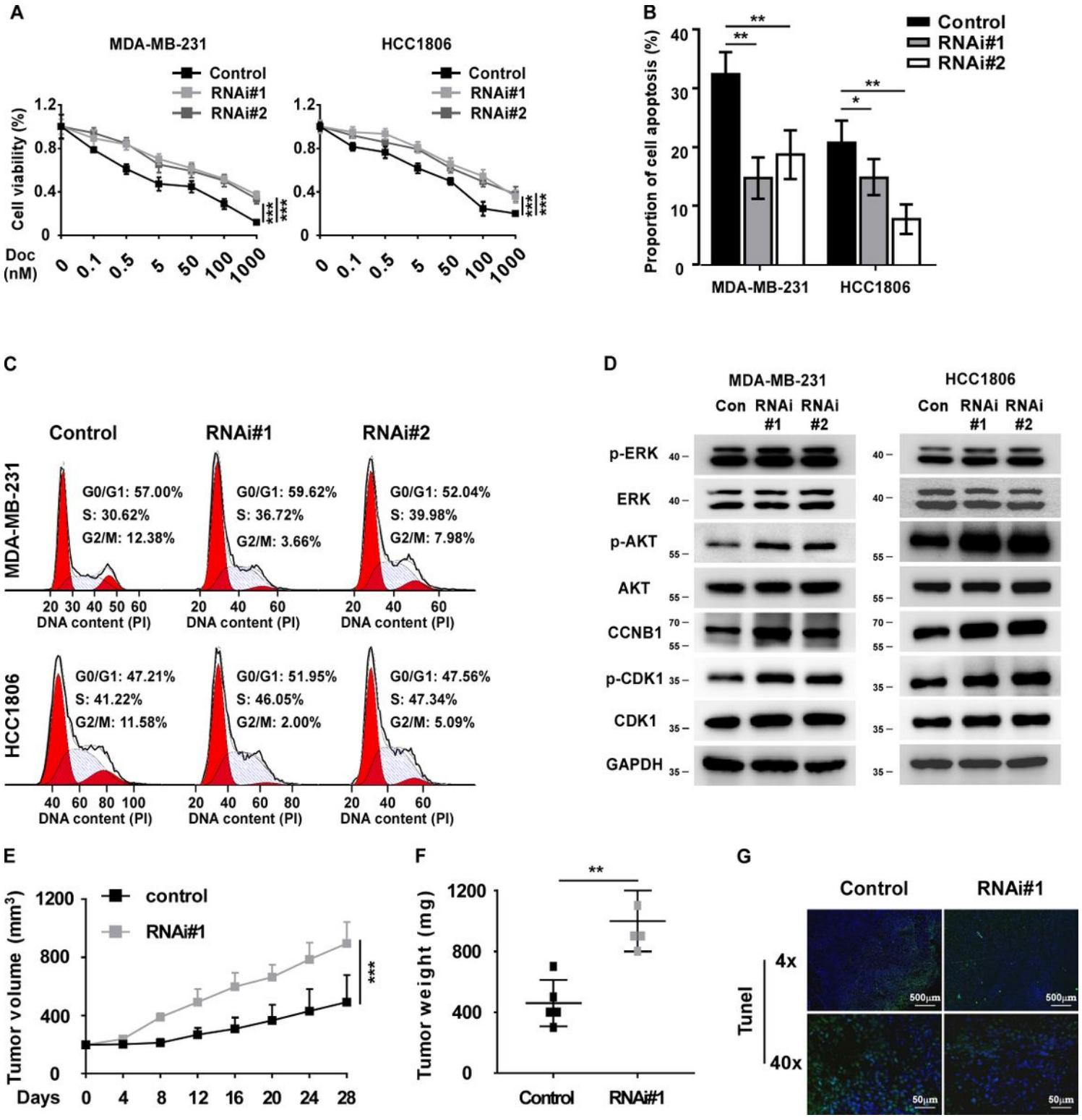
treatment. DOC-N, DOC-R cells and DOC-R cells pretreated with DAC were stained with anti-CD44 antibodies to assess the CD44hi population. D. qRT-PCR analysis of CD44 expression. GAPDH was used as an internal control. E. Tumor-sphere assay of DOC-N, DOC-R cells and DOC-R cells pretreated with DAC. Scale bars: 100 μm. F. Heatmap depicting the relative expression of genes between suspension-cultured and attachment-cultured cells. GAPDH was used as an internal control. P-values were determined by a two-tailed Student's t test. *P < 0.05, **P < 0.01, ***P < 0.001. G. The protein expression of DAB2IP in attachment-cultured or suspension-cultured cells. GAPDH was used as an internal control. H. Representative images of DAB2IP staining using immunocytochemistry (ICC). Scale bar: 100 μm. I. DNA methylation analysis (left panel) and quantification (right panel) of DAB2IP. J. qRT-PCR analysis (left panel) and DNA methylation analysis (right panel) of DAB2IP in the indicated suspension-cultured cells. K. Representative images of DAB2IP staining using ICC in tumor spheres. Scale bar, 100 μm. In I and J, primers corresponding to the ENST00000259371.6 transcript were used. In B,D and E, Data are presented as the mean ± SD. P-values were determined by one-way ANOVA. ***P < 0.001. In I-J, Data are presented as the mean ± SD, P-values were determined by a two-tailed Student's t test. *P < 0.05, **P < 0.01.




## Figure 3

Loss of DAB2IP caused by promoter methylation enhances cancer stem cell capacity in TNBC. A. Quantification of tumor spheres formed by the indicated TNBC cells. Data are presented as the mean ± SD, and P-values were determined by one-way ANOVA. *P < 0.05, **P < 0.01. B. Flow cytometry for CD44 cell surface expression in the indicated cell population. C. qRT-PCR analysis of Nanog, Sox2, Pou5f1 and C-myc in the indicated cells. Data are presented as the mean ± SD, P-values were determined by a two-

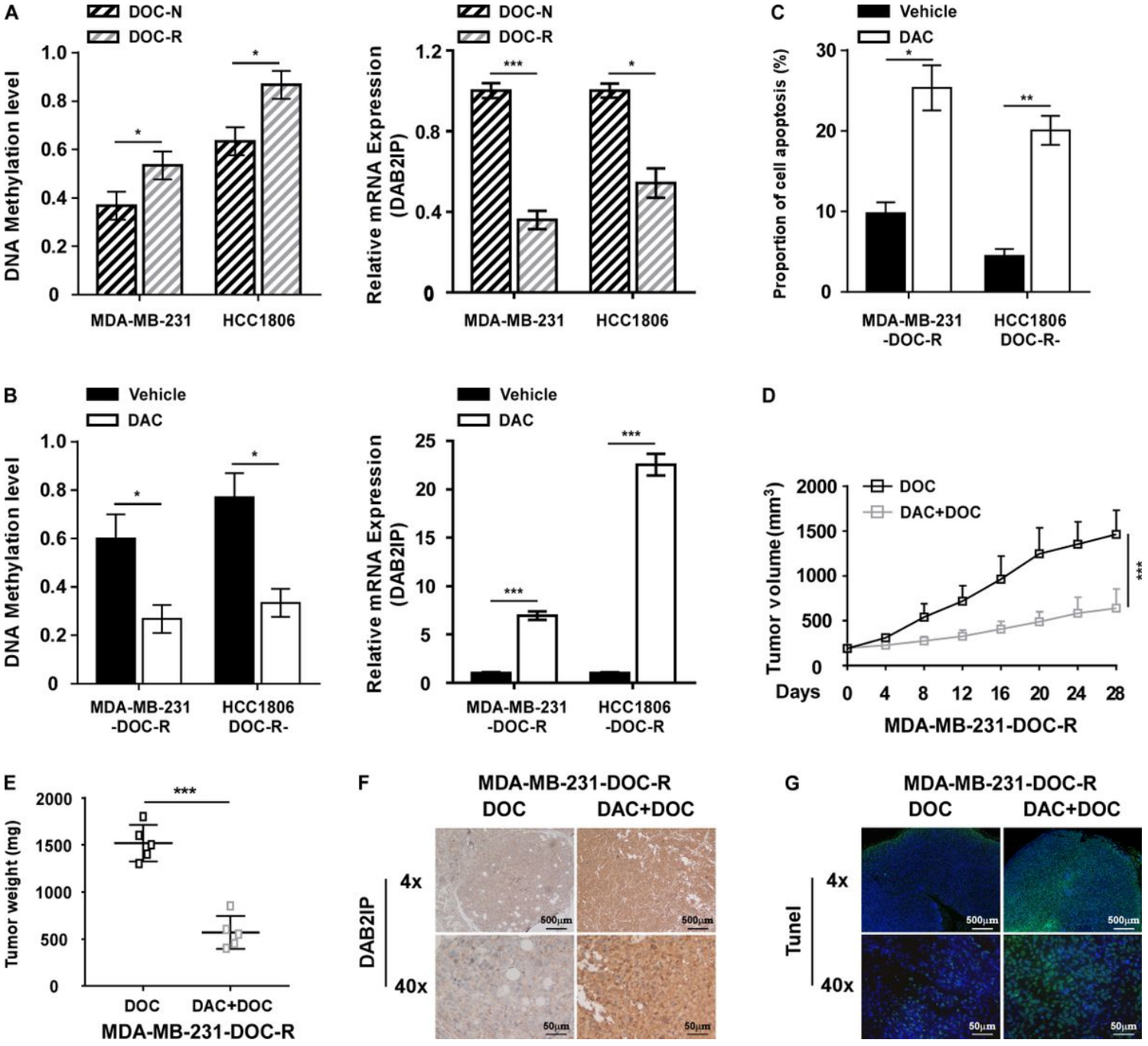
tailed Student's t test. *P < 0.05, **P < 0.01. D. Representative images of phosphorylated-Erk1/2 (phosphorylated levels represented as p-, in green) and non-phosphorylated (active) β-catenin (in red) stained using immunofluorescence (IF) in tumor spheres formed by the indicated cells. Tumor spheres were stimulated by Wnt3a (upper panel: 0 min; middele panel: 15 min; lower panel: 30 min). Slides of tumor spheres were stained for p-Erk1/2 and active β-catenin, and cell nuclei were labeled with DAPI (in blue). Images were captured using confocal microscopy. Scale bar: 20 μm. E. A limiting dilution assay was used to evaluate the tumor initiation capacity. The indicated number of MDA-MB-231-control cells or MDA-MB-231-RNAi#1 (with DAB2IP inhibition) cells were mixed with Matrigel and subcutaneously injected into right (MDA-MB-231-control) and left (MDA-MB-231-RNAi#1) mammary fat pads of female NOD/SCID mice. A schematic diagram of the limiting dilution assay is shown (left panel). The tumor initiation frequency was assessed by luciferase activity (middle and right panel).




## Figure 4

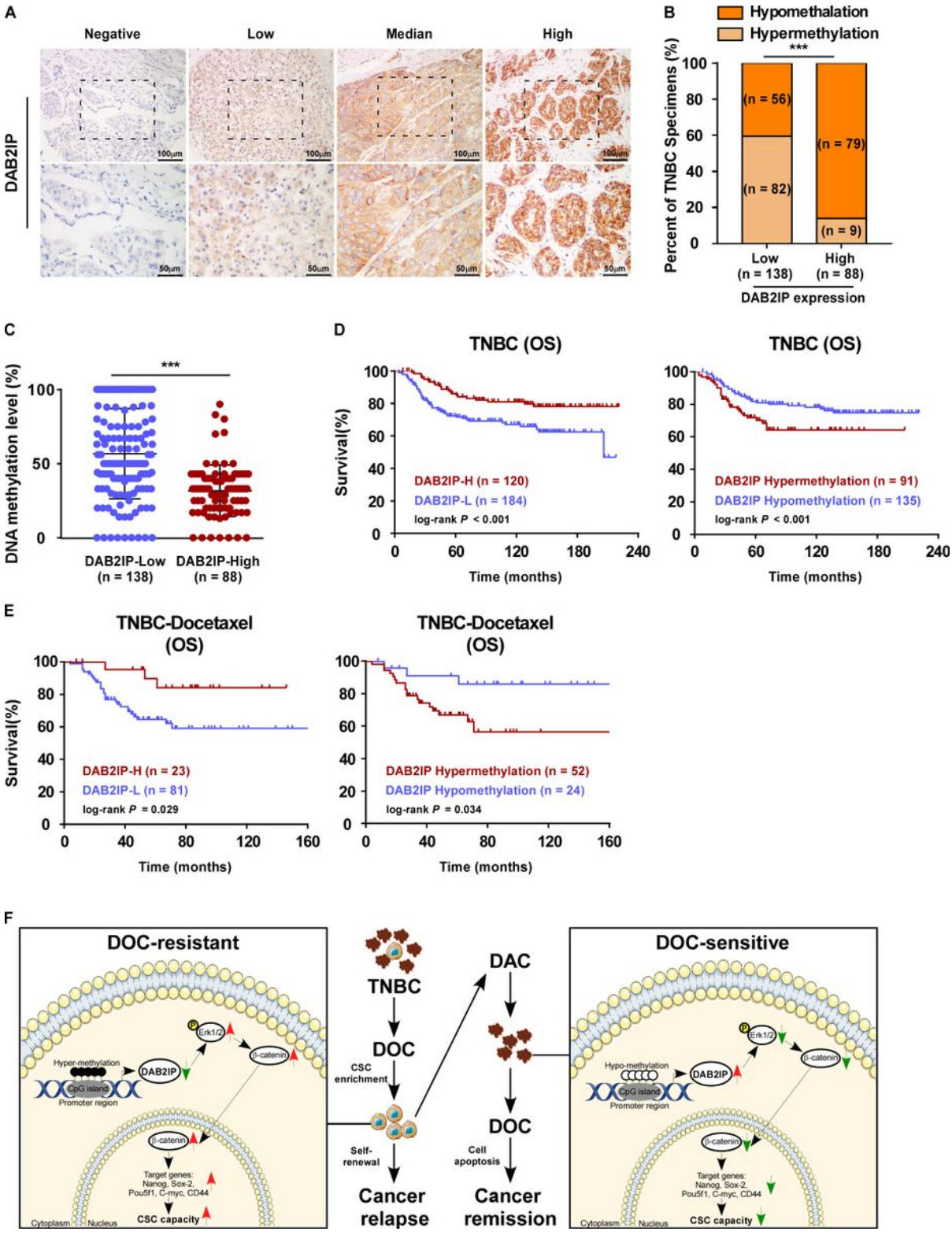
Loss of DAB2IP induces docetaxel resistance in TNBC. A. Cell viability was assessed in the indicated cells treated with DOC. TNBC cells were pretreated with various concentrations of docetaxel, and cell viability was evaluated by CCK-8 assay. B. Cell apoptosis was evaluated by an Annexin V/PI assay using flow cytometry. The indicated cells were treated with DOC. C. Cell cycle analysis of the indicated cells by flow cytometry. A total of 2×104 cells were stained with propidium iodide (PI), and the cell cycle distribution

was evaluated. D. Protein expression of p-Erk1/2, Erk1/2, p-Akt, Akt, CCNB1, p-CDK1 and CDK1 in the indicated cells. GAPDH was used as an internal control. E. Volumes of tumors in the indicated groups (n = 5/group). Mice were treated with DOC (15 mg/kg, i.p. weekly) when tumors reached a volume of 200 mm3. Tumor volumes were measured every 4 days and are shown by tumor growth curves. F. Tumor weights of tumors in the indicated groups. G. Representative images of TUNEL staining of apoptotic cells in the indicated tumors treated with DOC. Cell nuclei were labeled with DAPI (in blue), and the apoptotic cell fraction was labeled with TUNEL (in green). Scale bars: 50 μm and 500 μm. In A and E, Data are presented as the mean ± SD, P-values were determined by two-way ANOVA. ***P < 0.001. In B, Data are presented as the mean ± SD, P-values were determined by one-way ANOVA. *P < 0.05, **P < 0.01. In F, data are presented as the mean ± SD, P-values were determined by a two-tailed Student's t test. **P < 0.01.



## Figure 5

Decitabine reverses docetaxel resistance by restoring DAB2IP expression. A. Quantification of the methylation status (left panel) and mRNA expression (right panel) of DAB2IP in the indicated cells. B. Quantification of the methylation status (left panel) and mRNA expression (right panel) of DAB2IP in DOC-resistant cells treated with vehicle or DAC. C. Cell apoptosis was evaluated by an Annexin V/PI assay. The indicated cells pretreated with vehicle or DAC were treated with DOC. D. Volumes of tumors in the indicated groups (n = 5/group). Mice were treated with DOC (15 mg/kg, i.p. weekly) or DAC (2.5 mg/kg, i.p. 4 times weekly) and DOC (15 mg/kg, i.p. weekly) when tumors reached a volume of 200 mm3. E. Tumor weights of tumors in the indicated groups. F. Representative images of DAB2IP staining using immunohistochemistry (IHC) in the indicated tumors. Scale bars: 50 μm and 500 μm. G. Representative images of TUNEL staining of apoptotic cells in the indicated tumors. Cell nuclei were labeled with DAPI (in blue), and the apoptotic cell fraction was labeled with TUNEL (in green). Scale bars: 50 μm and 500 μm. In A and B, primers corresponding to the ENST00000259371.6 transcript were used. In A, B and E, Data are presented as the mean ± SD, P-values were determined by a two-tailed Student's t test. *P < 0.05. ***P < 0.001. In C-D, Data are presented as the mean ± SD, P-values were determined by two-way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001.




## Figure 6

Epigenetic silencing of DAB2IP predicts poor outcomes in TNBC. A. Representative images of DAB2IP staining using immunohistochemistry (IHC) in TNBC tissues. Scale bars: 50 μm and 100 μm. B. Statistical quantification of DAB2IP staining and DNA methylation levels in TNBC tissues (n = 226). P-values were determined by the χ2 test. ***P < 0.001. C. Quantification of DAB2IP methylation levels in specimens with high DAB2IP expression and specimens with low DAB2IP expression. Data are presented

as the mean ± SD (right panel). P-values were determined by a two-tailed Student's t test. *P < 0.05. ***P < 0.001. D. Survival analysis of the expression (left panel) and methylation status (right panel) of DAB2IP using Kaplan–Meier curves in TNBC patients. E. Survival analysis of the expression (left panel) and methylation status (right panel) of DAB2IP using Kaplan–Meier curves in TNBC patients treated with docetaxel-based chemotherapy. F. Model of DAC reverses CSC-induced DOC resistance via epigenetic regulation of DAB2IP in TNBC. In TNBC, epigenetic silencing of DAB2IP enhanced CSC capacity through activation of Erk/β-catenin signaling (left panel). Cells with DAB2IP silencing exhibiting poor response to DOC were selectively enriched by chemotherapeutic pressure and contributed to DOC resistance evolution/cancer relapse in TNBC. In DOC-resistant TNBC, a low dose of DAC re-expressed DAB2IP via demethylation and suppressed CSC capacity (right panel). DOC-resistant TNBC regained the response to DOC via pretreatment of DAC. In B-E, primers corresponding to the ENST00000259371.6 transcript were used.

## Supplementary Files

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