Breast Cancer Research

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SMYD4 promotes MYH9 ubiquitination through lysine monomethylation modification to inhibit breast cancer progression

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Abstract

Background Breast cancer is the leading cause of female mortality worldwide. (SET And MYND Domain Containing 4) SMYD4 has been reported to be a tumour suppressor. However, the molecular mechanism of SMYD4 remains unclear.

Methods The expression level of SMYD4 in breast cancer cells was detected by qRT-PCR and western blot. The effect of SMYD4 was verified in vitro and in vivo. The interaction between SMYD4 and MYH9 was investigated by co-IP assay. The regulation of SMYD4 on WNT signaling pathway was detected by luciferase reporter assay and ChIP analysis.

Results This study found that SMYD4 downregulation was associated with poor prognosis. SMYD4 was performed as a tumor suppressor both in vitro and in vivo. SMYD4 was found to interact with the downstream protein MYH9 and impede WNT signaling pathway. Further studies revealed that SMYD4 impeded the binding of MYH9 to the CTNNB1 promoter region by promoting lysine monomethylation and ubiquitination degradation of MYH9.

Conclusions These findings reveal the emerging character of SMYD4 in Wnt/β-catenin signaling and bring new sights of gene interaction. The discovery of this SMYD4/MYH9/CTNNB1/WNT/β-Catenin signalling pathway axis suggests that SMYD4 is a potential therapeutic target for breast cancer.

Keywords Breast cancer, SMYD4, MYH9, Wnt/βcatenin signaling, Methylation, Ubiquitin

Introduction

Breast cancer remains the the leading cause of cancer death in females worldwide, and the declines in breast cancer mortality have slowed in recent years [1]. Advances in cancer treatment have dramatically improved the prognosis for breast cancer patients, but it is still unsatisfactory [2–5]. Breast cancer is a heterogeneous disease influenced by multiple factors such as genetics, environment and hormone levels [6]. Emerging evidence suggests that, in addition to genetic changes, epigenetic reprogramming of cancer cells is critical for progression, metastasis and thrapy resistance in breast cancer [7, 8]. In recent years, research on the

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pathogenesis of breast cancer has made great achievements, shedding light on the process of its occurrence and development [9–13]. However, the carcinogenic pathways and novel pathogenic genes need to be further investigated.

The SMYD (SET and MYND domain-containing proteins) family is a subgroup of protein methyltransferases that consists of five members (SMYD1-5). These proteins contain SET domains responsible for lysine methylation [14, 15]. SMYD4, located at human 17p13.3, is a potential tumour suppressor in multi cancer types [14, 16].Han et al. identify SMYD4 as a target of miR-1307-3p action to inhibit breast cancer progression [17]. Zhou et al. found that SMYD4 monomethylates PRMT5 and forms a positive feedback loop to promote hepatocellular carcinoma progression [18]. Besides, Liu et al. showed that miR-135a combined with SMYD4 activated Nanog expression and induced the conversion of non-CSCs to CSCs, significantly affecting the proportion of CSCs and tumour progression [19]. These findings provide compelling evidence that SMYD4 plays a key role in cancer. However, the potential mechanism of SMYD4's role in breast cancer remains to be further investigated.

Abnormal Wnt signalling regulation is an important issue in the cancer process.Wnt signals are subdivided into three well-established pathways [20]. Canonical Wnt signaling is a β-Catenin dependent and T cell factor (TCF)/lymphoid enhancer factor (LEF) involved pathway that is responsible mainly for breast cancer cell proliferation and 'stemness' maintenance [20, 21]. While Wnt-PCP (planar cell polarity) signalling, which is a non-canonical Wnt signalling, does not require β-catenin or TCF molecules. In addition, Wnt-Ca2 + signalling is another non-canonical Wnt signalling with less research, but plays crucial roles in several biological processes [20]. Recent studies have revealed that Wnt signalling is involved in breast cancer proliferation [22], metastasis [23], stemness maintenance [24], phenotype shaping [25], immune microenvironment regulation [26] and drug resistance [27].Therefore, the study of the molecular mechanism of Wnt signalling is crucial for the treatment of breast cancer.

The aim of this study was to reveal that SMYD4 can interact with MYH9 to inhibit the nuclear localisation of CTNNB1, thereby down-regulating Wnt signalling and its target genes. This effect is caused by lysine monomethylation of MYH9 by SMYD4 and prevents transcriptional activation of CTNNB1 by MYH9. The SMYD4/MYH9/CTNNB1 axis may play a key role in breast cancer.

Materials and methods

Immunohistochemistry (IHC)

Heat-induced antigen retrieval was performed on deparaffinised, dehydrated tissue sections in 0.01 M sodium citrate buffer at 98 °C for 18 min. 3% H2O2 was used to block endogenous peroxidase activity. The sections were then treated with 5% bovine serum albumin (BSA) for 1 h at room temperature (RT) and primary antibodies overnight at 4 °C. The sections were washed with phosphate buffer saline (PBS), treated with HRP-conjugated secondary antibodies for 1 h at RT and then visualised with 3,3′-diaminobenzidine (DAB) reagent. Finally, after hematoxylin staining and hydrochloric acid alcohol differentiation, the sections were coverslipped with neutral gum. All sections were photographed and evaluated by two experts under a light microscope.

Cell culture, plasmids, and transfection

The normal mammary epithelial cell line MCF-10 A and the breast cancer cell lines MDA-MB-231, MCF-7, SK-BR3, T47D and Cal51 were obtained from ATCC (American Type Culture Collection, Manassas, USA). MCF-10 A was cultured in MCF-10 A cell specific medium (Procell, China). MCF-7, T47D, Cal51 and MDA-MB-231 were cultured in DMEM (Gibco, Grand Island, USA). All medium included 10% fetal bovine serum (FBS, NEWZERUM, Australia) and 1% penicillin/streptomycin (Gibco, Grand Island, USA). All media contained 10% fetal bovine serum (FBS, NEWZERUM, Australia) and 1% penicillin/streptomycin (Gibco, Grand Island, USA). Cells were cultured at 37 °C in a humidified cell incubator with a 5% CO2 atmosphere. The SMYD4 plasmids and corresponding vectors as well as the SMYD4 siRNA kits were purchased from RiboBio (Guangzhou, China). MYH9 plasmids and corresponding vectors were purchased from GeneCopoeia (Guangzhou, China). The siRNA kits of MYH9 were obtained from Keyybio (Shandong, China). Transfection was performed using Lipofectamine 3000 (Invitrogen, California, USA) according to the manufacturer's recommendations. Lentiviruses (RiboBio, China) were used to infect CAL-51 cells and generate stable SMYD4-overexpressed cells according to the manufacturer's protocol. The siRNAs oligonucleotides are listed in Supplemetary information: Table S1.

Antibodies

Antibodies against SMYD4, Flag, MYH9, IgG, Vimentin, Survivin were purchased from Proteintech. Antibody against HA was produced by Immunoway. Antibodies against N-cadherin, E-cadherin, cyclinD1, c-myc, β-catenin, Histone H3, α-tubulin, HIS, ubiquitin and β-actin were from Cell Signaling Technology, pan-lysine methylation antibodies were from Abclonal.All antibodies used for western blot or IHC are listed in Supplemetary information: Table S2.

RNA isolation and quantitative reverse transcription PCR (RT qPCR)

Total RNA was isolated from cells using Rapid cellular RNA extraction kit (Sparkjade, China) according to the manufacturer's recommendations.RNA quality and concentration were measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Massachusetts, USA). For mRNA, the All-in-One First Strand cDNA Synthesis SuperMix for qPCR (Transgene, China) was used for RT-qPCR. SMYD4 and GAPDH primers were synthesized by Genewiz (Tianjin, China). All specific sequences are listed in Supplemetary information: Table S3.

Proliferation assay

For CCK8 assay, cells were plated in 96-well plates at a density of 2,000 cells per well and 10 μL CCK8 solution was added on days 1–5. After 1 h of incubation in the dark, absorbance values were recorded and analysed using a microplate reader (Bio-Rad, California, USA) at 450 nm. For colony formation, 500 cells were plated in six-well plates and incubated until the colonies reached the appropriate size. Colonies were then fixed with 4% paraformaldehyde and stained with 1% crystal violet, followed by imaging and counting. The EdU assay was performed using the EdU Assay Kit (RiboBio, China) according to the manufacturer's instructions. The ratio of EdU-positive cells was counted and analysed using a fluorescence microscope (Leica, Germany).

Migration and invasion assay

The Matrigel-coated Transwell (BD Biosciences, New Jersey, USA) was used to determine the invasive capacity of breast cancer cells. Briefly, 50,000 cells were seeded in the upper chambers with FBS-free medium, while medium containing 20% FBS was added in the lower chambers. After 16–24 h of incubation, the migrating cells were fixed and stained using a three-step kit (Thermo Scientific, Massachusetts, USA). The images were then examined and counted under a light microscope (Olympus, Japan) at 100× magnification. In the cell scratch assay, cells were treated and incubated in 6-well plates for 48 h to allow the cells to reach confluence. A single scratch was made in each well using a sterile 10 μL pipette tip. All cells were then incubated in FBS-free medium to exclude the effect of FBS on migration. Images of the scratches were taken at 0 and 24 h under a light microscope (Olympus, Japan). The distances (relative to 0 h) were measured and analysed.

Flow cytometry assay

For apoptosis assay, cells were stained with Annexin V Apoptosis Detection Kit (BD Pharmingen, New Jersey, USA) according to the official instructions after digestion and washing. A flow cytometer (BD Biosciences, New Jersey, USA) was used for analysis.

Western blotting and immunofluorescence (IF)

SDS-PAGE electrophoresis was performed to separate cell lysates. The target proteins were immunoblotted with appropriate antibodies and visualised using an ECL reagent (Millipore, Bedford, MA, USA). The scaling values of each individual stripe were measured using ImageJ software and normalised to β-actin (Supplemetary information: Fig.S1). For the immunofluorescence assay, cells were plated on glass coverslips at 50,000 cells per well, followed by washing, fixation and permeabilization. Primary antibodies were incubated on the coverslips overnight at 4 °C. The coverslips were then treated with FITC/TRITC conjugated secondary antibodies for 1 h at RT. After staining with 4′,6-diamidino-2-phenylindole (DAPI), the coverslips were captured and analysed using a fluorescence microscope (Zeiss).

Co-immunoprecipitation (Co-IP) and protein identification

Breast cancer cells were washed with pre-cooled PBS and treated with IP lysis buffer (Thermo, Massachusetts, USA). The lysates were then immunoprecipitated with specific antibodies against SMYD4, MYH9, Flag, HIS, HA or IgG (negative control) at 4 °C overnight. The next day, 40 μL of pre-cleaned Protein A/G agarose beads (Santa Cruz, California, USA) were added to the system and incubated for 2 h at 4 °C. The agarose beads were then washed 3 times with PBS and the immunoprecipitation complexes were isolated. For the Co-IP assay, the lysates were subjected to immunoblotting as described above. For protein identification, the lysates were stained with Coomassie brilliant blue solution (Solarbio, China) after separation on SDS-PAGE gels. The proteins were identified by mass spectrometry, which was performed and analysed by Novogene Company (China). IP-MS was performed using FLAG antibody and IGG antibody (negative control) in SMYD4-FLAG overexpressing CAL51 cells. The proteins detected in the negative control group were excluded to eliminate the effect of false-positive results. The proteins detected were then checked for reliability using the q-value.

Chromatin immunoprecipitation (ChIP) analysis

The ChIP assay was performed as previously described according to the manufacturer's recommendations of Upstate Biotechnology [26]. The primers used are listed in Supplemetary information: Table S3.

TOP/FOP assay

TOP/FOP-Flash and pRL-TK plasmids (Kyybio, China) were co-transfected into different treated cells. The Dual-Luciferase Reporter Assay Kit (Transgene) was used to

detect firefly and Renilla luciferase activities according to the instructions. Firefly fluorescence values for TOP/FOP-Flash were calculated normalised to the renal fluorescence values for pRL-TK.

Xenograft

Stable SMYD4-overexpressed CAL-51 cells and control cells (8 × 106 cells) were injected with 5% Matrigel (BD Biosciences, New Jersey, USA) into the mammary fat pads of 5-week-old female NSG mice and reared for 6 weeks, during which time tumour growth was recorded once a week. After sacrifice, final volume and weight were determined. Paraffin-embedded tumour from mice were sectioned for haematoxylin and eosin (H&E) staining and IHC analysis. Each group contained 7 mice, and all protocols were approved by the Ethics Committee of Tianjin Medical University Cancer Institute and Hospital.

Statistics and analysis

Prism 9(Graphpad Software, CA) was used for data visualisation and analysis. Student's t-test was used for comparisons between two groups, and Pearson correlation analysis was used for correlation analysis. Data were presented as mean ± standard deviation (SD), and $p < 0.05$ was considered significant.

Data collection

The Cancer Genome Atlas [28], The Genotype-Tissue Expression Project [29] and METABRIC [30] databases were used to obtain clinical data and gene expression profiles.

Results

SMYD4 was downregulated in breast cancer

Firstly, the preliminary analysis of the expression of SMYD family members in different cancer types was carried out in Gepia online analysis website(http://gepia.cancer-pku.cn/), and the results showed that the expression of SMYD1 and SMYD4 among SMYD family was lower than control group in breast cancer tissues, whereas the expression of SMYD2, SMYD3, SMYD5 was higher than control group(Supplemetary information: Fig.S1).

Combined with the current state of research, we found that SMYD4 may have a correlation on the progression of breast cancer, but there are fewer existing studies, so SMYD4 was used as a target gene for subsequent research.

The expression of SMYD4 gene in different cancer types was further validated by the TCGA pan-cancer database(Fig. 1A). The result showed that SMYD4 expression was significantly down-regulated in TCGA and GTEx database(T = 1085,*N* = 291)(Fig. 1B).

Then the prognostic impact of SMYD4 in breast cancer was analyzed via the KM-plotter website, which showed that high SMYD4 expression levels were associated with better prognosis, and this correlation differed significantly in DMFS and RFS, but not in OS(Fig. 1C-E).

Finally, RT-qPCR and western blot were conducted to detect SMYD4 mRNA and protein levels in breast cell lines, including normal breast cell line MCF10A and breast cancer cell lines MDA-MB-231, CAL-51, SKBR3, MCF-7, and T47D, indicating that SMYD4 was decreased in all breast cancer cell lines as compared to MCF10A cells(Fig. 1F).

Interestingly, the expression level of SMYD4 was much lower in triple-negative breast cancer cells and CAL-51 and lumial A type cancer cells MCF-7, compared with SKBR3, MDA-MB-231 and T47D cells(Fig. 1G). In addition, the METABRIC database was used to analyse the expression of SMYD4 in different subtypes of breast cancer, but the variation between each subtype was not significant. Indicating that SMYD4 expression in breast cancer cell lines is not following with conventional Luminal type.

Taken together, these results suggest that SMYD4 is downregulated in breast cancer tissues and breast cancer cell lines, and low SMYD4 expression is associated with poor prognosis.

SMYD4 inhibits cell proliferation and invasion

To evaluate the effect of SMYD4 on breast cancer proliferation, SMYD4-FLAG overexpressed CAL-51 cells were constructed and verified by western blot(Fig. 2A).

SMYD4 overexpressed cells exhibited lower rates of cell clone formation, less cell proliferation rate and lower EDU positiv ratio compared to control cells, suggesting that SMYD4 could inhibit breast cancer proliferation(Fig. 2B, D,F).

Meanwhilie SMYD4 overexpressed cells showed less aggressive in transwell and scratch assays(Fig. 2C, E).

Cell flow assay shows SMYD4 promotes apoptosis in breast cancer cells(Fig. 2G).

Furthermore, western blot showed that SMYD4 overexpressed CAL-51 cells expressed more E-cadherin, less N-cadherin, and less Vimentin, while SMYD4 knockdown MDA-MB-231 cells were associated with less E-cadherin, more N- cadherin, and more Vimentin, indicating that SMYD4 is negatively associated with EMT(Fig. 2H).

To assess the role of SMYD4 in vivo, SMYD4 overexpressed CAL51 cells were injected in situ in SCID mice, which showed that SMYD4 inhibited tumor proliferation(Fig. 2I, J,K).

These cell function assays showed the same results in SMYD4 overexpressed MCF-7 cells as well as SMYD4 knockdown MDA-MB-231 cells(Supplemetary information: Fig.S2-S3).

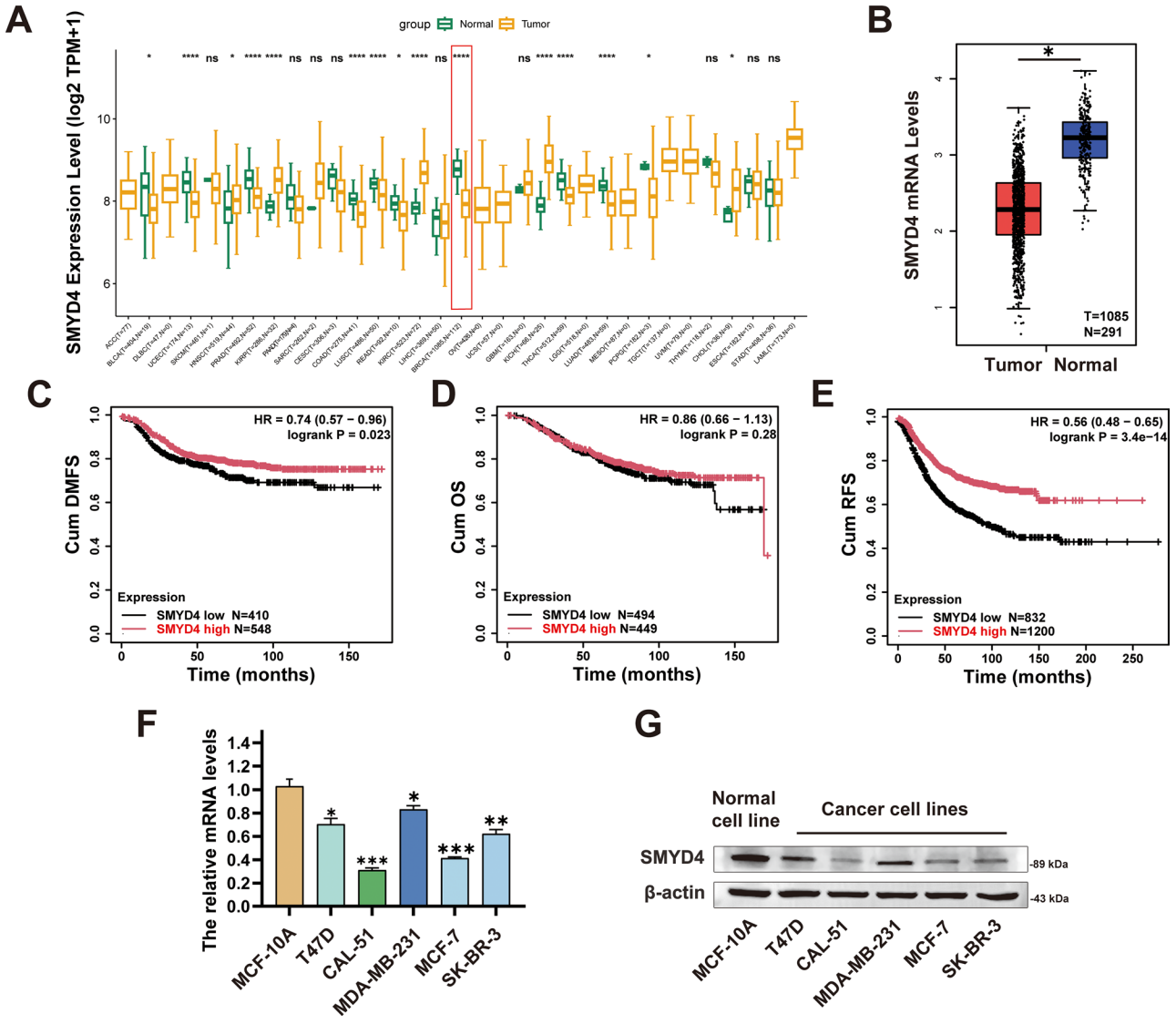

Fig. 1 SMYD4 was downregulated in breast cancer. (**A**)The SMYD4 expression level in multiple cancer types. (**B**)The SMYD4 expression level in breast cancer tissues compared with normal tissues. The survival analysis of breast cancer patients with high or lowSMYD4 expression levels, including cumulative DMFS (**C**), OS (**D**), and RFS (**E**), predicted by the KM plotter. The SMYD4 expression level in breast cancer cell lines detected by RTqPCR (**F**) and western blot (**G**). All experiments were repeat three times ($n=3$), compared variances using F test and analyzed using an unpaired t-test, error bars represent SD.*$p<0.05$; **$p<0.01$;***$p<0.001$

These results suggest that SMYD4 can inhibit breast cancer proliferation and invasion both in vivo and in vitro.

SMYD4 interacts with MYH9

Immunoprecipitation was performed and followed by mass spectrometry to further determine the mechanism of SMYD4 function. A total number of 349 SMYD4 binding proteins were identified, among which, MYH9 ranked at the leading and was selected as a cancer related target gene(Fig. 3A).

The protein binding of SMYD4 to MYH9 was then predicted, analysed and visualised by GRAMM, PDBePISA and Pymol2 software, and the binding model of SMYD4 and MYH9 was presented as a cartoon structure(Fig. 3B).

In addition, the co-localisation levels of SMYD4 and MYH9 in breast cancer cells were analysed by immunofluorescence and fluorescence co-localisation analysis(Fig. 3C).

In addition, the interaction between SMYD4 and MYH9 was verified by endogenous and exogenous immunoprecipitation experiments(Fig. 3D-F). These results suggest that SMYD4 can directly interact with MYH9 at the protein level.

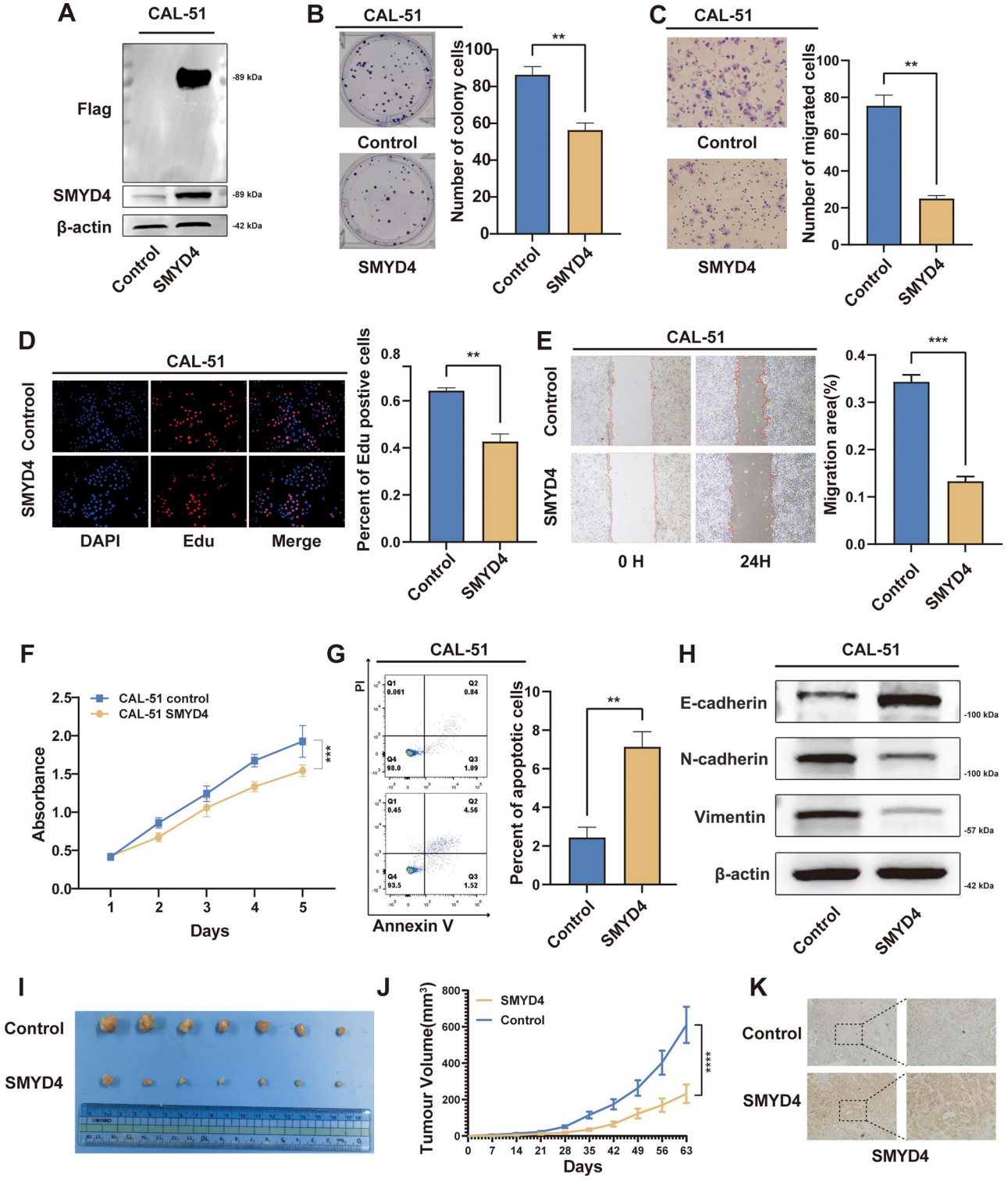

Fig. 2 SMYD4 inhibits cell proliferation and invasion. (**A**) The construction of SMYD4overexpressed CAL-51 cells validated by western blot. The cell proliferation assays and invasion assays were conducted, including, colony formation assay (**B**), transwell assay (**C**), EdU assay (**D**), scratch assay (**E**) and CCK-8 (**F**). The apoptotic cells were analyzed through cell cytometry(**G**). (**H**)The expression levels of EMTrelated biomarkers, including Ecadherin, Ncadherin, and Vimentin in SMYD4overexpressed CAL-51 cells detected by western blot. (**I**) The image of tumor volume after in situ injection in SCID mice. (**J**) The data of tumor volume growth after in situ injection in SCID mice. (**K**) The protein expression level of SMYD4 in xenograft by IHC. All experiments were repeat three times ($n = 3$), compared variances using F test and analyzed using an unpaired t-test, error bars represent SD. $^{**}p < 0.01$;$^{***}p < 0.001$

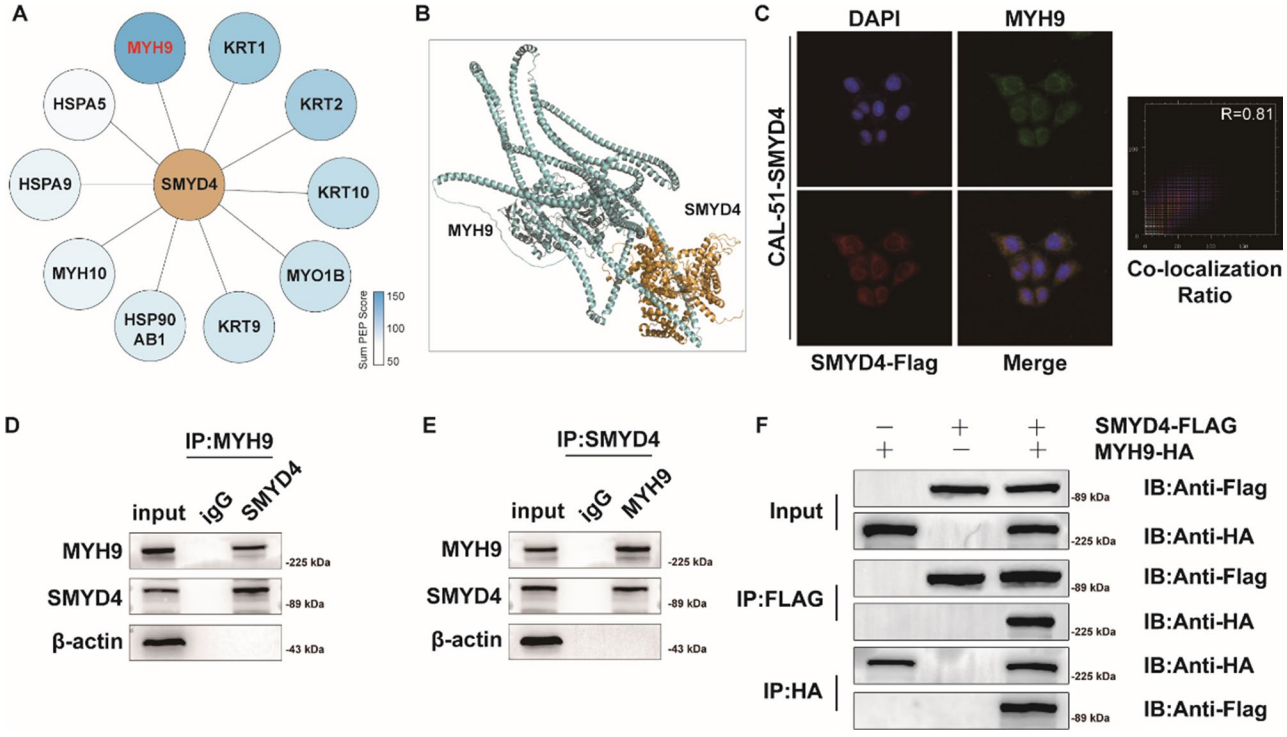

Fig. 3 SMYD4 interacted with MYH9. (**A**) The top 10 interacting genes identified by mass spectrometry. (**B**) The predictive protein binding model of SMYD4 and MYH9. (**C**) The intracellular colocalization of SMYD4 and MYH9 visualized by IF and analysis by co-localizasion ratio. The interaction of SMYD4 and MYH9 was verified by coIP assay, including endogenous (**D**, **E**) and exogenous (**F**). All experiments were repeat three times ($n=3$)

SMYD4 acts by inhibiting the wnt signalling pathway

Three groups of SMYD4 overexpressed CAL51 cells and control cells were subjected to RNAseq analysis. The analysis showed 277 up-regulated genes and 408 down-regulated genes(Fig. 4A).

GO analysis of these differential genes showed that up-regulated genes were enriched in endoplasmic reticulum lumen, and down-regulated genes were mainly enriched in blood vessel morphogenesis an morphogenesis of an epithelium(Fig. 4B, C).

All the sequenced genes were then subjected to GSEA analysis and the enrichment results showed that the WNT signalling pathway was significantly inhibited in the SMYD4 overexpressed group(Fig. 4D, E). Therefore, the Wnt signalling pathway is considered to be a potential major pathway through which SMYD4 exerts its function.

As extensively studied, Wnt/β-catenin signalling is the classical pathway of Wnt signalling, starting with the nuclear localization of β-catenin.

Nucleoplasmic separation assay showed that in SMYD4-overexpressing CAL-51 cells, β-catenin expression was decreased in the nucleus and increased in the cytoplasm(Fig. 4F).

To assess the regulatory effect of SMYD4 on Wnt signalling, TOP/FOP luciferase assay was performed. The results showed that SMYD4 overexpression decreased TOP/FOP luciferase activity, whereas SMYD4 down-regulation increased TOP/FOP luciferase activity(Fig. 4G).

In addition, the effect of SMYD4 on the localisation of β-catenin was explored using immunofluorescence assays. The results showed that in SMYD4 overexpressed CAL-51 cells, nuclear localisation of β-catenin was significantly reduced, whereas β-catenin was abundantly present on the cell membrane(Fig. 4H).

Moreover, the expression of Wnt/β-catenin downstream target genes was detected by western blot, and it was found that SMYD4 down-regulated the expression of MYH9 protein and WNT pathway-related proteins, N-cadherin, Vimentin, C-myc, CyclinD1, and Survivin proteins(Fig. 4I).

In contrast, the expression of MYH9 and WNT pathway-related proteins was upregulated in SMYD4 knockdown MDA-MB-231 cells(Fig. 4I).

Besides, altered WNT pathway proteins due to SMYD4 overexpression were also detected in xenografts(Supplemetary information: Fig.S4).

The above results suggest that SMYD4 exerts cancer inhibition by suppressing the expression of MYH9 and inhibiting the WNT signalling pathway.

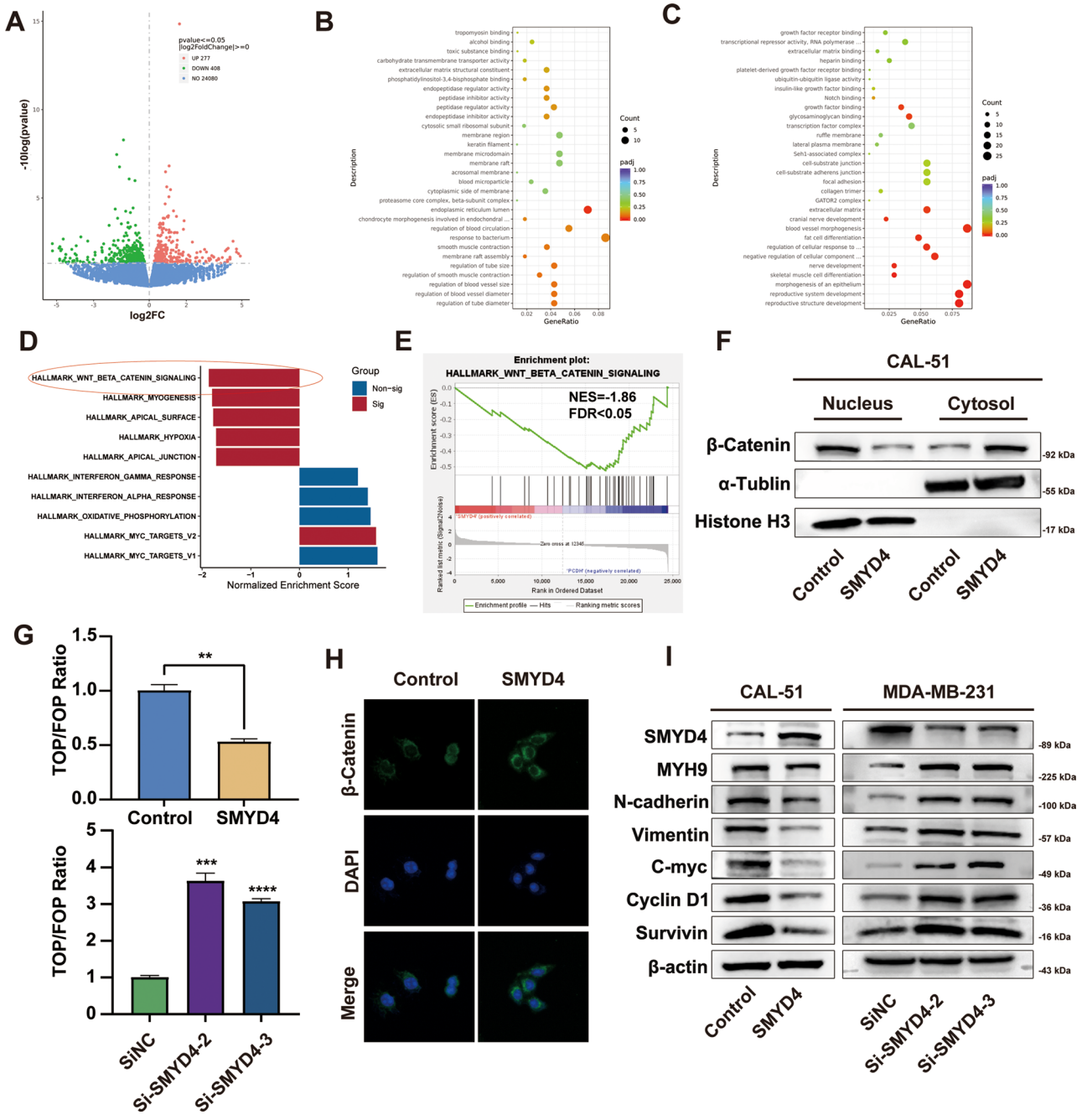

Fig. 4 SMYD4 suppressed Wnt signaling by inhibiting the nuclear localization of βcatenin. (**A**) RNAseq differential gene volcano map between SMYD4 overexpressed and control group. The GO analysis was performed among up-regulated genes (**B**)and down-regulated genes(**C**). (**D**)TOP5 up- and down-regulated gene sets after GSEA analysis and the enrichment results of WNT signalling pathway(**E**). (**F**)The βcatenin expression level in the nucleus and cytoplasm detected by nucleocytoplasmic separation western blot. (**G**)The TOP/FOP luciferase reporter assay in SMYD4overexpressed CAL-51 cells and in SMYD4 knock-down MDAMB-231 cells. (**H**) The localization transformation of βcatenin visualized by IF. (**I**)The expression levels of downstream target genes of Wnt/βcatenin signaling, including MYH9,N-cadherin, Vimentin, C-myc, Cyclin D1 and Survivin detected by western blot. All experiments were repeat three times ($n=3$), compared variances using F test and analyzed using an unpaired t-test, error bars represent SD. **$p<0.01$;***$p<0.001$; ****$p<0.0001$

MYH9 rescues SMYD4 function in breast cancer

To further validate the role of SMYD4 in relation to MYH9, rescue experiments were performed to identify MYH9 as a key protein for SMYD4 to exert its function.

Cell clone formation assays illustrated that overexpression of MYH9 reversed the inhibition of clone formation rate, and EDU positivity in SMYD4 overexpressed cells, whereas knockdown of MYH9 rescued the facilitation

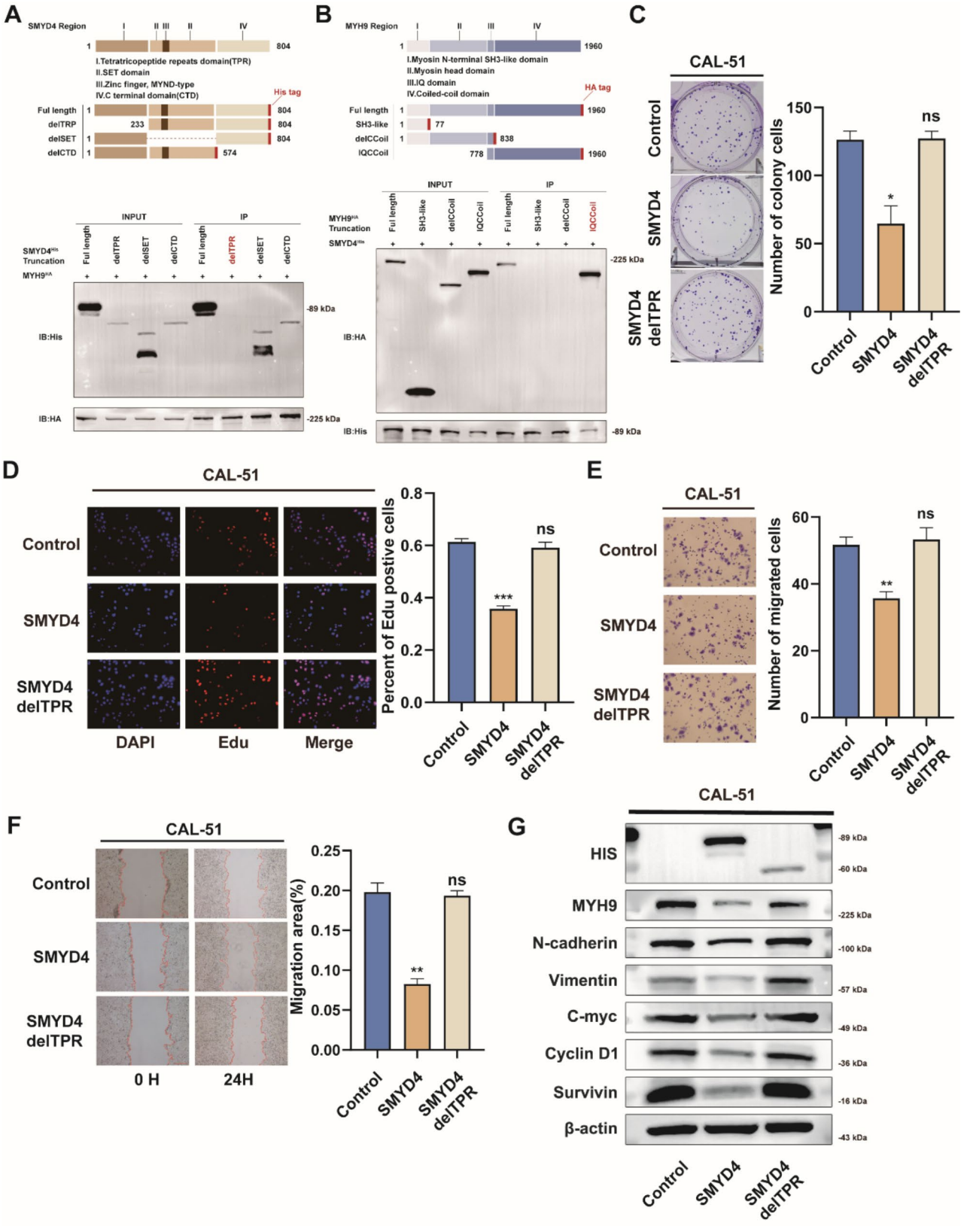

Fig. 5 (See legend on next page.)

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Fig. 5 SMYD4 functions through the TPR domain combined with the Ccoil domain of MYH9. (**A**)Diagrammatic representation of SMYD4 and its truncated forms. 293T cells were transfected with the indicated constructs subjected to immunoprecipitation with anti-HA (against MYH9). The red boxes represents the pull-down bands. (**B**) Diagrammatic representation of MYH9 and its truncated forms. 293T cells were transfected with the indicated constructs subjected to immunoprecipitation with anti-HIS (against SMYD4). The red boxes represents the pull-down bands. SMYD4 delTPR rescues SMYD4-inhibited cell proliferation and invasion including colony formation assay (**C**), EdU assay (**D**), transwell assay (**E**), scratch assay (**F**). (**G**)The expression levels of downstream target genes of Wnt/βcatenin signaling, including MYH9, N-cadherin, Vimentin, C-myc, Cyclin D1 and Survivin detected by western blot. All experiments were repeat three times ($n=3$), compared variances using F test and analyzed using an unpaired t-test, error bars represent SD.*$p<0.05$; **$p<0.01$; ***$p<0.001$

funcation in SMYD4 knockdown cells(Supplemetary information: Fig.S5 A, B,F, H).

Similarly, MYH9 overexpressed cells showed function rescue in transwell and scratch assays(Fig.S5 C, E). Knockdown of MYH9 restored the increase in cell invasiveness after SMYD4 knockdown(Fig.S5 D, G).

In addition, MYH9 rescued SMYD4-induced changes in the WNT signalling pathway.

Nuclear localisation of β-catenin was restored after both MYH9 overexpression and knockdown(Fig.S5 I, J), and the addition of MYH9 also reversed the changes in TOP/FOP luciferase activity after SMYD4 overexpression and knockdown treatments(Fig.S5 K, L). The WNT pathway-related proteins N-cadherin, Vimentin, C-myc, Cyclin D1, and survivin, which were detected to be altered in the SMYD4 overexpression as well as knockdown groups, were also rescued after MYH9 overexpression and knockdown, respectively(Fig.S5 M).

SMYD4 functions through the TPR domain combined with the Ccoil domain of MYH9

To further investigate the mechanism of direct interaction between SMYD4 and MYH9, truncation mutants with different domains were constructed for SMYD4 and MYH9 for subsequent studies.

Taking into account the literature and protein domain prediction websites, the 5'TPR domain, SET domain and 3'CTD domain of SMYD4 were truncated and fused to express the HIS tag. SMYD4 full-length plasmid and three truncations of SMYD4 were co-transfected with MYH9-HA into 293T tool cells for CO-IP assays.

By CO-IP assay, SMYD4 full-length, delSET and delCTD could immunoprecipitate the MYH9-HA protein, however delTPR could not. It suggests that the 5'TPR domain of SMYD4 the domain directly interacts with the MYH9 protein(Fig. 5A).

After that, MYH9 was designed as SH3-like, delCoil, and IQCcoil truncations and fused to express HA tags, MYH9 full-length plasmid and three truncations were co-transfected with SMYD4-HIS plasmid into 293T tool cells for CO-IP, and the results showed that only IQCcoil truncation successfully immunoprecipitated the SMYD4-HIS protein, showing that the Ccoil structural domain of MYH9 is the direct binding site for SMYD4(Fig. 5B).

To verify that the TPR domain of SMYD4 is the core structural domain exerts protein function, we designed rescue experiments on the truncated protein of TPR. SMYD4 full-length and SMYD4 delTPR were transfected into breast cancer cells to verify the function.

Consistent with the hypothesised results, truncated TPR SMYD4 was unable to exert an inhibitory effect on the rate of clone formation, cell proliferation and EDU positivity, as well as an impediment to invasiveness in transwell and scratch assays(Fig. 5C-F).

Similarly, SMYD4 delTPR no longer inhibited MYH9 expression as well as the expression of WNT pathway-related proteins(Fig.S5 G). In addition, SMYD4 delTPR also rescued the effects on β-catenin nuclear localisation and the inhibitory function of TOP/FOP luciferase activity(Fig. 6A, B).

The above results indicate that SMYD4 binds the Ccoil domain of MYH9 through the TPR domain and affects the expression of MYH9 protein to inhibit the WNT signalling pathway.

SMYD4 acts by methylating MYH9 protein to promote ubiquitination degradation of MYH9 and affects its binding to the CTNNB1 promoter

SMYD4 is a histone and non-histone methylation transfer protein, so it is presumed that the mechanism of SMYD4 regulation of downstream MYH9 proteins is through non-histone methylation. Pan-lysine methylation antibodies purchased from Abclonal(China) were used to examine methylation of MYH9 by SMYD4.

Immunoprecipitation of MYH9 protein from CAL-51 cells transfected with full-length SMYD4 and SMYD4 delTPR was performed to detect the level of panmethylation at the location of MYH9, which showed that SMYD4 overexpression markedly increased the level of lysine mono-methylation of MYH9 but did not alter the level of lysine di-methylation and tri-methylation of MYH9, and SMYD4 delTPR did not affect any methylation level of MYH9(Fig. 6C).Similarly, the lysine monomethylation level of MYH9 was significantly reduced in the SMYD4 knockdown group(Fig. 6D).

Protein stability assays were then performed in SMYD4-overexpressed CAL51 cells, and MYH9 degradation was faster in the SMYD4-overexpressed group compared to the control group after the addition of CHX(Fig. 6E). To investigate the ubiquitination pathway through which SMYD4 promotes the degradation of MYH9, MG132 and CQ addition treatments were

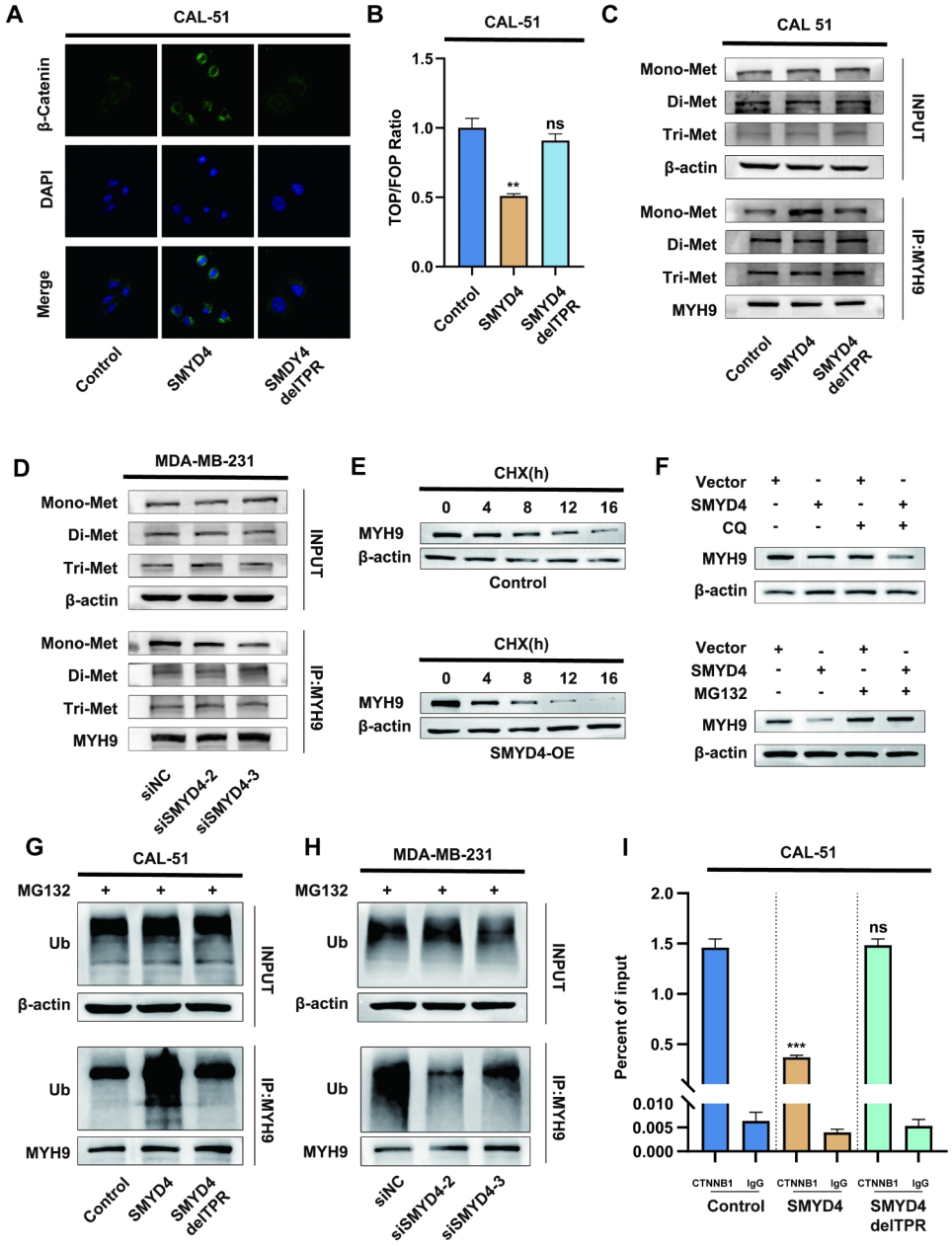

Fig. 6 SMYD4 acts by methylating MYH9 protein to promote ubiquitination degradation of MYH9. (**A**)The localization transformation of βcatenin visualized by IF after rescue treatment by SMYD4 delTPR (**B**)The TOP/FOP luciferase reporter assay after rescue treatment by SMYD4 delTPR. (**C**) MYH9 lysine methylation levels after SMYD4 and SMYD4delTPR overexpression detected by immunoprecipitation and western blot. (**D**) MYH9 lysine methylation levels after SMYD4 knockdown detected by immunoprecipitation and western blot. (**E**) Changes in MYH9 protein expression levels over time after CHX treatment detected by westernblot between SMYD4 overexpression and control groups. (**F**) After CQ and MG132 treatments, MYH9 protein expression levels in SMYD4 overexpression and control groups were detected by westernblot. Ubiquitination levels of MG132-treated MYH9 proteins detected by immunoprecipitation and western blot in SMYD4 overexpression group(**G**) and SMYD4 knockdown group (**H**).(**I**) Number of MYH9-bounded CTNNB promoter regions detected by CHIP and real-time qPCR. All experiments were repeat three times ($n = 3$), compared variances using F test and analyzed using an unpaired t-test, error bars represent SD.*$p < 0.05$; **$p < 0.01$;***$p < 0.001$; ****$p < 0.001$

performed respectively, The results showed that the degradation rate of MYH9 was significantly altered by the addition of MG132(Fig. 6F), suggesting that MYH9 is degraded via the proteasome pathway.

MYH9 immunoprecipitation assay was performed on different groups of cells after 8 h of MG132-treated culture.The results showed that SMYD4 overexpression significantly increased the level of ubiquitination of MYH9 and was rescued by SMYD4 delTPR(Fig. 6G). Meanwhile knockdown of SMYD4 significantly reduced the ubiquitination level of MYH9(Fig. 6H).

In addition we verified by CHIP-qPCR that overexpression of SMYD4 significantly reduced the binding of MYH9 to the CTNNB1 promoter region, and the effect could be similarly reversed by SMYD4 delTPR(Fig. 6I).

Discussion

This study reveals the regulatory role of the SMYD4/MYH9/CTNNB1/WNT axis in breast cancer progression. In breast cancer, the expression of SMYD4, a cancer suppressor gene, is abnormally reduced. This alteration results in reduced binding of the TPR domain of the SMYD4 to the MYH9 Ccoil domain and reduces the level of MYH9 lysine monomethylation, weakening the inhibitory effect on MYH9 protein function.Thus MYH9 enhances the transcriptional activity of CTNNB1 and activates the WNT signalling pathway, which increases the proliferation and invasiveness of breast cancer. These results were further validated by rescue assays. The mechanism of the SMYD4/MYH9/CTNNB1/WNT axis in breast cancer was elucidated and provided a promising strategy for breast cancer treatment.

The SMYD family of lysine methyltransferases contains a functional SET structural domain that functions by mono-, di- or trimethylation modifications of histones and non-histone proteins [31–33]. However, the SET structural domain is highly conserved among the different family members [34]. Several previous studies have demonstrated the promoting role of SMYD family members SMYD2, SMYD3 in a wide range of cancers [35]. Some bioinformatics analyses showed that SMYD5 is also a potential oncogene for gastric and breast cancers [36, 37]. Whereas studies on SMYD1 have focused on the regulation of cardiac function.In contrast, SMYD4 was first identified as a potential cancer suppressor gene in breast cancer [16]. The different roles of SMYD family members may be related to the unique structure of each protein and the degree of openness of the different protein conformations.SMYD4 has a unique N-terminal TPR structural domain compared to other members, which may influence the mechanism of protein-protein interactions [38]. Surprisingly, our findings are consistent with this structural feature. The N-terminal TPR domain of SMYD4 is precisely the domain that interacts with downstream MYH9.Unfortunately, X-ray structures of SMYD1, SMYD2, and SMYD3 have been analysed by scholars [38], and there are ongoing studies on the structural analysis of SMYD5 [39]. However no analyses have been reported for the structure of SMYD4.So the function of the unique N-terminal TPR domain of SMYD4 still needs further study.

The myosin heavy chain 9 (MYH9) gene encodes the heavy chain of non-muscle myosin IIA (NMIIA), which belongs to the myosin II subfamily of actin-based molecular motors [40]. Adhesion junctions (consist of E-cadherin/catenin complexes) and tight junctions are key components of epithelial cell-cell adhesion. The cytoskeleton containing NMII and actin filaments provides them with the necessary mechanical traction [40, 41]. MYH9 was initially identified as a tumour suppressor gene [42]. But an increasing number of studies have identified its oncogenic role [43–46]. It causes proliferation, epithelial mesenchymal transition (EMT), invasion, metastasis, radiotherapy resistance, stem cell preservation and metabolic regulation in a variety of tumours [40]. Several studies have found that MYH9 also acts by activating the WNT pathway in a variety of ways, including promoting the transcription of CTNNB1 [47], inhibiting ubiquitination of GSK3β [48], and inhibiting β-catenin degradation [49]. In this study, we found that SMYD4 interacts with MYH9. It prevents MYH9 from binding to the CTNNB1 promoter and inhibits the nuclear localisation of β-catenin to inhibit the WNT pathway, thereby preventing breast cancer progression.

Post-translational modification (PTM) is an important way of regulating biological processes. The important role of non-histone lysine methylation modifications is gradually being discovered [50]. Typically, lysine can receive up to three methyl groups to form mono-, dimethyl- or trimethyl-lysine, and different methylation states of lysine have different functions [51]. Non-histone methylation has many regulatory functions, such as regulating protein stability, altering subcellular localisation, affecting DNA binding capacity, tuning protein interactions, or crosstalking with other PTMs [52]. SMYD family member SMYD2 was found to have multiple non-histone substrates such as p53, Rb, ERα, PARP1 and the chaperone protein HSP90 [50]. Our study revealed for the first time that SMYD4 can lysine monomethylate modified MYH9 and affect the binding of MYH9 to the CTNNB1 promoter.However, whether other non-histone substrates of SMYD4 as well as methylation modifications of MYH9 have additional regulatory functions requires further investigation.

Conclusion

In our study, we identified SMYD4 as a tumour suppressor. It modifies MYH9 through lysine monomethylation and hinders the binding of MYH9 to the CTNNB1 promoter. This in turn leads to decreased nuclear localisation of β-catenin, which is involved in the inhibition of the expression of proteins downstream of the Wnt/β-catenin signalling pathway. In summary, our study identified the existence of the SMYD4/MYH9/CTNNB1/Wnt signalling pathway axis. This may provide new targets and promising strategies for breast cancer treatment.

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
This study and included experimental procedures were approved by the institutional animal care and use committee of Tianjin Medical university (approval no.2023026). All animal housing and experiments were conducted in strict accordance with the institutional guidelines for care and use of laboratory animals.

Consent for publication
Written informed consent for publication was obtained from all participants.

Competing interests
The authors declare no competing interests.

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