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Original Paper

SUPT5H Post-Transcriptional Silencing Modulates PIN1 Expression, Inhibits Tumorigenicity, and Induces Apoptosis of Human Breast Cancer Cells

Bilal Ahmad Lone Faiz Ahmad Shibendra Kumar Lal Karna Yuba Raj Pokharel

Faculty of Life Science and Biotechnology, South Asian University, Akbar Bhawan, Chanakyapuri, New Delhi, India

Key Words
SUPT5H • Breast cancer • Apoptosis • Cell proliferation

Abstract
Background/Aims: Breast cancer (BrCa) is one of the most common cancers and a highly heterogenous disease, both at the pathological and molecular levels. A common element for the progression of cancer is the presence of aberrant transcription. Targeting the misregulation of transcription may serve as a tool for cancer therapeutics. SUPT5H (Suppressor of Ty 5 homolog) is a highly conserved RNA polymerase II-associated transcription elongation and processivity factor. However, few studies have examined the relationship between SUPT5H and cancer. ***Methods:*** Yeast two-hybrid and colocalization by immunofluorescence were performed to investigate protein-protein interaction. Colony formation assay, CTG assay, and crystal violet assays were performed for cell viability, clonogenicity, and cell proliferation study. Data mining was performed for expression analysis of SUPT5H in breast cancers. Flow cytometry was performed for the assessment of cell cycle and apoptosis. The Transwell chambers were employed for the migration and invasion assays. Quantitative real-time polymerase chain reaction (qRT PCR) and Western blotting were performed to measure the mRNA and protein levels of SUPT5H and other markers related to viability, migration, cell cycle, and apoptosis. Silent mutations were generated for rescue experiments. The biological function of SUPT5H was investigated through siRNA depletion of SUPT5H mRNA *in vitro*. ***Results:*** We showed that SUPT5H is upregulated in breast cancer tissue as compared with the adjacent normal tissue in breast cancer patients. In human breast cancer cells, the levels of SUPT5H and PIN1 are positively correlated with each other. Our biochemical analysis showed that PIN1 interacts with SUPT5H through WW domain, that was required to promote SUPT5H protein stability. Depletion of SUPT5H by siRNA technology reduced the tumorigenic and metastatic

Yuba Raj Pokharel
Faculty of Life Science and Biotechnology, South Asian University, Akbar Bhawan, Chanakyapuri, New Delhi-110021 (India)
E-Mail yrp@sau.ac.in

properties, promoted s-phase cell cycle arrest and apoptosis of MDA-MB-231 cells. Moreover, depletion of SUPT5H abrogated MAPK molecules thereby regulates the oncogenic behavior of breast cancer cells. ***Conclusion:*** Our findings demonstrated an essential role of SUPT5H in BrCa tumorigenicity by regulating the expression levels of genes that control proliferation, migration, cell cycle, and apoptosis of breast cancer MDA-MB-231 cells.

Introduction

Breast cancer is the most frequently diagnosed cancer with a high mortality rate in the female population worldwide. Among the breast cancers, the triple-negative breast cancer(TNBC); lacking the expression of estrogen receptor (ER), progesterone receptor (PR) as well as human epidermal growth receptor 2(HER2) has been challenging due to heterogeneity of the disease and the absence of well-defined molecular targets [1]. TNBC accounts for 10 ~ 20% of invasive breast cancer [2] and displays aggressive clinical behaviour with a high rate of proliferation and has a poor prognosis [3]. Compared to other breast cancers, TNBC frequently affects younger women and is more prevalent in African-American women [4].

Cancer progression is strongly associated with the dysregulated versions of transcriptional programs [5]. Alteration of genes involved in chromatin structure, transcriptional stimulation and elongation, and post-transcriptional RNA processing has a profound effect on the development and progression of cancer [6]. Transcription elongation by RNA polymerase II involves the coordinated assembly of multiple transcription elongation factors, including SUPT5H, to the core promoter of coding transcripts. SUPT5H in association with second protein-Spt4 forms the complex, DRB (5,6-dichloro-1bata-D-ribofuranosyl-benzimidazole) Sensitivity Inducing Factor (DSIF) that together binds with RNA polII thereby regulates transcription cycle, coordinates in chromatin remodelling, transcription elongation, pre-mRNA processing and mRNA capping in eukaryotes [7–9]. SUPT5H (NusG in bacteria) is conserved in all domains of life; it is a crucial accessory factor required for regulating and maintaining the processivity of RNA pol II [10]. SUPT5H interacts with MYC causes an increase in the speed and processivity of polymerase II, and is implicated in cancer progression [11]. Moreover, SUPT5H was identified to be overexpressed in colorectal cancer and is involved in colorectal cancer progression [12].

Among the multiple oncoproteins, a peptidyl-prolyl cis-trans isomerase(PPIase)-PIN1, which is stimulated by activated E2F [13], works as a molecular switch in multiple pathways to determine the fate of proline-directed phosphoproteins [14]. Aberrant expression of PIN1 is associated with the distortions of the wider signaling network that fuels cancer progression. In cancers, overactivation of PIN1 is correlated with poor patient survival. Because silencing of PIN1 is associated with the inhibition of breast cancer cell proliferation and tumorigenesis of breast cancer stem cells, the molecular therapy for targeting PIN1 is growing [15–17].

In the present study, we dissected the role and molecular mechanism of SUPT5H in triple-negative breast cancer. We used the siRNA approach to address the effects of SUPT5H silencing on breast cancer cell lines. We showed that SUPT5H contributes to breast cancer cell proliferation, migration, invasion, cell cycle, and apoptosis. Our study revealed the oncogenic function of SUPT5H and could be a potential therapeutic target of interest to counter breast cancer.

Materials and Methods

Cell Culture, antibodies, and reagents

MDA-MB-231 cells were purchased from NCCS Cell Repository (Pune, India) and maintained in L-15 medium supplemented with 10% FBS, 100 units/ml penicillin (all from Gibco, ThermoFisher Scientific, USA) at 37°C and 5% CO2. Anti-SUPT5, Anti-PIN-1, Anti- β-catenin, Anti-E2F1, Anti-Bax, Anti- Bcl-xl, Anti-

Caspase-3, Anti-P38, Anti- pP38, Anti-Cyclin E1, Anti-Slug, and Anti-p-J antibodies were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA); Anti-PCNA, Anti-cyclinA1 and Anti-PARP antibodies from Cloud-Clone Corp (Houston, USA); Anti-Vimentin and Anti-MMP-2 from Thermo Fisher; Anti-cJun and anti-p-c-Jun antibodies from Cell Signaling Technology, anti-β-actin antibody and MG132 from SigmaAldrich. Matrigel was purchased from Corning Incorprated Lifescience, (Tewksbury, MA, USA). CellTiter-Glo® luminescent cell viability assay kit was purchased from Promega Corp (Madison, Wisconsin, USA).

Table 1. Sequence of siRNAs

siRNAs	Scramble	SUPT5H
Target (5'-3')	AATTCTCCGAACGTGTCACGT	CCGGAGGGACAACGAACTCAT
Sense (5'-3')	UUCUCCGAACGUGUCACGUdTdT	GGAGGGACAACGAACUCAUdTdT
Antisense (5'-3')	ACGUGAGACACGUUCGGAGAAdTdT	AUGAGUUCGUUGUCCCUCCdGdG

Transfection of siRNAs

Cells were transiently transfected with oligo siRNAs (FlexiTube siRNA, QIAGEN) using Lipofectamine RNAiMAX (ThermoFisher Scientific) according to the manufacturer's instructions. The sequences of the siRNAs used in this study are listed in Table 1.

Generation of silent mutations

First, the wild-type SUPT5H expression plasmid was constructed by fusing the full-length human SUPT5H ORF to the pcDNA3.1(+) vector (Invitrogen). Then, two silent mutations were introduced into the siRNA targeted region of wild-type SUPT5H expression plasmid (shown in red below) using QuickChange II site-Directed kit (Agilent) to generate the mutated SUPT5H expression construct (Δ2) for rescue experiment. A double nucleotide mismatch has been found sufficient to abolish the siRNA knockdown effect on target mRNA [18, 19]. The sequencing analysis of the siRNA-resistant SUPT5H expression construct confirmed the mutations.

SUPT5H
Wt: C CGG AGG GAC AAC GA**A** CT**C** AT
Δ2: C CGG AGG GAC AAC GA**G** CT**A** AT
Arg Arg Asp Asn Glu Leu

Knockdown-rescue experiment

For the knockdown-rescue experiment, the MDA-MB-231 cells were transfected with siRNA, as already described. After 24 hours of siRNA transfection, the siRNA resistant SUPT5H overexpression plasmid was transfected using Lipofectamine LTX (Thermo), according to the manufacturer's instruction. After 6 hours post-transfection, the serum-free media was replaced with complete media, and the cells were incubated for the next 48 hours to assay the SUPT5H knockdown rescue with western blotting, cell cycle, and apoptosis.

Cell cycle

siRNA scramble and Supt5h transfected MDA-MB-231 cells were analyzed for the distribution of the cell population in the different phases of the cell cycle. Following a 72 hr transfection, cells were harvested and washed with PBS. Then cells were fixed with 70% chilled ethanol and stored at 4°C overnight. Next day cells were centrifuged, and ethanol was removed, followed by two PBS wash. Next, cells were stained with RNase (10 µg /mL) and Propidium iodide (20 µg/mL) and kept at 37°C for half an hour. Samples were analyzed by cytometry using FACS verse. A total of 30,000 events were analyzed for each sample, and data were analyzed using ModFit LT software.

Colony Formation assay

MDA-MB-231 cells were assessed to form colonies following transfection with siRNA scramble and siRNA SUPT5H. Cells, transfected for 72 h, were harvested, and 5 X 10^2 cells from the scramble and SUPT5H knockdown cells were seeded in each well. Every 48 h media was replenished with fresh media, and the culture was kept for 12 days. Then cells were fixed with methanol and stained with 0.4% crystal violet. Colonies were counted using Image J software.

Wound healing assay

4 x10^5 MDA-MB-231 cells were seeded in each well of six-well and transfected with siRNA SUPT5H. Once the monolayer formed, a scratch was given with a 200 µl pipette tip. Cells were washed, and detached cells were removed. Then images of wound area were taken at two-time intervals (0 and 24 h). Then the percentage wound healed area was calculated after normalizing the wound area.

Table 2. Real time RT-PCR Primers used for the study

Gene	Forward Primer Sequence	Reverse Primer Sequence
SUPT5H	5'-TACCAGTTCACAGACACGCC-3'	5'-GGCCTCCACGTAGATGTAGC-3'
Noxa	5'-AAGAACGCTCAACCGAGCC-3'	5'-CTGCCGGAAGTTCAGTTTGTC-3'
p21	5'-CTGCCCAAGCTCTACCTTCC-3'	5'-CGAGGCACAAAGGGTACAAGA-3'
CIAP	5'-AAGGAGTCTTGCTCGTGCTG-3'	5'-AGCATCAGGCCACAACAGAA-3'
GAPDH	5'-GTCAAGGCTGAGAACGGGAA-3'	5'-AGTGGCTCCATTCACCGC-3'
XIAP	5'-CGAGGAACCCTGCCATGTAT-3'	5'-TGACCAGGCACGATCACAAG- 3'

Migration and invasion assay

Transwell chambers (Corning), 8 µm pore size, were inserted in 24 well plate to determine the migration and invasion potential of breast cancer cells. For migration assay, the 24-hour post-transfection cells (4 X10^4) were seeded on the upper chamber in 200 µL of serum-free media, whereas the bottom chamber was filled with 700µl of 10% serum-supplemented media as a source of chemoattractant. The same procedure was carried out for invasion assay, however, the upper chamber was first uniformly coated with 80 µg Matrigel (Corning) diluted in 100 µl DMEM and incubated at 37°C for 2 hours. The cells were suspended in chambers and further incubated at 37˚C, 5% CO_2 for 48 hours. Thereafter, non-migrated and non-invaded cells were removed with a cotton swab, and the cells migrated and invaded to the lower surface of upper chamber were fixed in 70% ethanol and stained in 0.4% crystal violet followed by washing to remove excess dye and air drying. Images of migrated and invaded cells were taken in ten randomly selected fields using NIKON ECLIPSE Ti computerized image analysing system.

RNA isolation and quantitative PCR

Total RNA was isolated from the scramble and SUPT5H knockdown MDA-MB-231 cells using TRIzol reagent. Next, cDNA was synthesized from 2µg of total RNA using a cDNA synthesis kit (Thermo) as per manufacturer's instruction. Primers used against genes under the study are mentioned in Table 2. PCR cycling program was as follows- 10 min at 95°C and 40 cycles of 15 sec at 95°C, 30 sec at 60°C and the melt curve with single reaction cycle with the following conditions: 95°C for 15 sec, 60°C for 1 min and dissociation at 95°C for 15 sec. Ct values thus obtained were normalized to the housekeeping gene β actin. $2^{-\Delta\Delta Ct}$ method was used to determine the relative expression of genes.

Crystal violet assay

Cell viability of SUPT5H depleted cells was assessed by crystal violet assay. Briefly, MDA-MB-231 cells cultured in a six-well plate were transfected with a scramble and SUPT5H siRNA and incubated for 48 h. Then cells were harvested and counted. Approx 5X10^3 cells from each sample were seeded in each well of 96 wells plate and further incubated for 48 h. Following this, the media was discarded, and cells were stained with 0.4% crystal violet (prepared in 50% methanol) and incubated for 30 min. Then wells were washed with water to remove excess stain followed by an air dry for 12 h. Next day, the dye was dissolved in 100 µL of methanol, followed by taking absorbance of dissolved dye at 570 nm. Viability was calculated as fold change in absorbance value of siRNA SUPTH w.r.t siRNA scramble.

CellTiter-Glo® luminescent cell viability assay

The CellTiter-Glo® luminescent cell viability assay determines the viability of cells based on the quantification of ATP. Briefly, freshly passaged cells are added to pre-plated si-RNA transfection complex in 96 well white culture plates at a density of 5000 cells/well. Seventy-two hours after transfection, 100 µl CellTiter-Glo® reagent was added to each well that contains 100 µl of cell culture medium. Cells were then lysed by shaking on a shaker for 2 minutes, and luminescence was measured after 10 minutes of incubation at room temperature using a microtiter plate reader (BioTek, Winooski, USA).

JC-1 Staining and Flow cytometry

The effect of silencing SUPT5H on mitochondrial membrane potential was measured by JC-1 fluorescent dye using a flow cytometer. MDA-MB-231 cells transfected with scramble and siRNA SUPT5H were incubated for 48 h and stained with JC-1 dye for 30 min in the dark. Then cells were harvested and washed with PBS and analyzed for fluorescence by flow cytometry (FACS Verse, BD).

Fluorescent microscopy for assessing intracellular ROS

We measured intracellular reactive oxygen species (ROS) levels following knockdown of SUPT5H. SiRNA Scramble and siRNA-SUPT5H transfected cells were incubated for 48 h, and the level of intracellular ROS was assessed by probe CM-H2DCFDA incubated for 30 min in the dark at 37°C. Then cells were washed once with PBS, and images were taken using a fluorescent microscope. Moreover, we also checked the level of ROS with pre-treatment with N-acetyl cysteine followed by knockdown of SUPT5H. This was done to observe the role of SUPT5H in maintaining the redox state of MDA-MB-231 cells.

Western blotting

Cells were lysed with SDS lysis buffer containing protease inhibitors, and the protein concentration was measured with the Pierce™ BCA assay kit (ThermoFisher). The proteins extracted were subjected to SDS-polyacrylamide gel electrophoresis. The proteins separated on the gel were transferred to the PVDF membrane and exposed to a blocking buffer for 2 hours. The membranes were then probed with specific primary antibody (1:500) with appropriate dilution at 4°C overnight. The blots were rinsed three times for 5 minutes with Tris buffer solution with tween-20 (TBST) and incubated in HRP-conjugated secondary antibody solution for 1 hour at room temperature followed by three washes with 1 x TBST. The immunoreactive bands were visualized by ECL Elistar ETA C ultra 2.0 (Cyanagen).

Apoptosis assessment by flow cytometry

To assess the effect of silencing of SUPT5H on apoptosis, Annexin V-FITC and Propidium iodide staining were done, and samples were analyzed by FACS verse (BD). Briefly, SUPT5H+/-siRNA treated cells were incubated for 72 h. Then cells were harvested and incubated with 1 X binding buffer followed by incubation with Annexin and Propidium iodide solution for 30 min. Next, samples were acquired for analysis using FACS verse (BD).

Yeast two-hybrid (Y2H) assays

Yeast two-hybrid (Y2H) assays were performed using Matchmaker™ Gold Yeast two-hybrid system (Clonetech, Mountain View, CA, USA) as per manufacturer's protocol. The human PIN1 ORF and segments of PIN1, WW, and PPI domains were fused with Gal 4 DNA binding domain into bait vector pGBKT7, and human SUPT5H ORF was fused with Gal 4 activation domain into prey vector pGADT7 using In-Fusion HD cloning kit (Takara Bio USA). The bait and prey vectors were transformed into yeast strains Y2H Gold and Y187 respectively and mated together in 2X yeast peptone dextrose adenine (YPDA) media. The diploid cells were selected on synthetically defined (SD) media dropped out for leucine and tryptophan (SD/-Leu/-Try). For the interaction of bait and prey proteins, the colonies from double dropout media were examined and selected on minimal media with the restrictiveness of Leucine(L), Tryptophan(W), Histidine(H) and Adenine(A) (SD/-Leu/-Trp/-His/-Ade). Only the diploids that contain both bait and prey plasmids and which express the reporter genes in response to two-hybrid interaction will grow on QDO media.

Immunohistochemistry

The excised tumors were fixed, dehydrated, and embedded in paraffin. Paraffin sections of 4 μm thickness are dried and baked. Before immunostaining, the sections were deparaffinized, hydrated, and washed. For antigen retrieval, the sections were immersed in Tris-EDTA buffer (pH 9.0) for 6 min at boiling temperature. After retrieval, the slides were placed in 3% hydrogen peroxide in methanol for 20 minutes to quench the endogenous peroxidase activity. Then non-specific antigens were blocked with 10% goat serum for 20 minutes followed by overnight incubation with anti SUPT5H antibody (1:200 in 0.5% BSA) at 4°C. Next day, the specimen was washed and incubated with appropriately diluted biotinylated secondary antibody followed by the incubation with 100 μl appropriately diluted SAv-HRP conjugates (BD Bioscience) for 30 minutes at room temperature, protected from light and the signals were visualized with 3,3'-Diaminobenzi-

dine (DAB) substrate solution (0.05% DAB, 0.015% H2O2) as the chromagen and counter-stained by hematoxylin. Images were captured using NIKON ECLIPSE Ti computerized image analysing system.

Bioinformatic analysis

RNA-seq data from The Cancer Genome Atlas (TCGA: http://cancergenome.nih.gov/) of tumors were compared with their respective normal tissues using BioXpress (https://hive.biochemistry.gwu.edu/bioxpress). UALCAN database (http://ualcan.path.uab. edu) was used to perform an in-depth analysis of protein expression data from Clinical Proteomic Tumor Analysis Consortium (CPTAC) confirmatory/discovery dataset. mRNA expression status in different types of breast cancer and in breast cancer cell lines across available data sets was evaluated using the KM-express tool (http://ec2-52-201-246-161.compute-1.amazonaws.com/kmexpress/ index.php). The co-expression correlation between SUPT5H and PIN1 in breast cancer were retrieved from TCGA using cBioPortal (https://www.cbioportal.org/).

Statistical analysis

In this study, the experimental data were statistically analyzed using Graphpad Prism 6 software and presented as mean ± s.e.m. A comparison between different groups was analyzed by unpaired two-tailed t-test or ANOVA test. Spearman,s rank correlation was applied to determine the correlation between SUPT5H and PIN1. A P-value of less than 0.05 was regarded as statistically significant.

Results

Elevated expression of SUPT5H is observed in breast cancers

To investigate the differential expression of SUPT5H in breast cancers, we retrieved the RNA-sequencing datasets from TCGA using BioXpress [20]. During the paired analysis of TCGA data, the SUPT5H was found overexpressed in breast and other cancers with respect to their normal tissues (Fig. 1A). The BioXpress database indicated that SUPT5H is significantly overexpressed in 57.02% (65/114, $p=0.002$) of breast cancer tissues compared with the paired normal tissues. Next, we determined the overexpression of SUPT5H in breast tumors, their subtypes, and pathological stages using proteomic CPTAC datasets [21]. Statistical analysis of CPTAC data retrieved from UALCAN database [22] indicated the significant overexpressed of SUPT5H protein in breast cancer tissues (Fig. 1B), and in subclasses (Luminal, HER2 positive and TNBC) of breast cancers compared to normal breast tissues, with more significant in TNBC followed by HER2 positive and luminal($p<0.05$, Fig. 1C). CPTAC dataset further revealed the increased expression levels of SUPT5H in advanced stages of breast cancer compared to normal, with more significant in stage 2, followed by stage 3 (Fig. 1D, $p<0.05$). Moreover, analysis of TCGA SUPT5H datasets using UALCANS also exhibited the consistency with CPTAC datasets (data not shown). In datasets from KM-Express [23] (E-MTAB-4993), the transcript profile of SUPT5H is upregulated in triple-negative breast cancer (TNBC) patients compared to ER+ patients (Fig. 1E), and among the breast cancer cell line (GSE48213) the triple-negative type or basal-like cells have elevated expression levels of SUPT5H RNA compared to non-malignant or other types of breast cancer (Fig. 1F). Furthermore, our investigation of protein expression of SUPT5H in breast cancer tissues by immunohistochemistry has confirmed the increased expression of SUPT5H protein in 6 of 20 (30%) breast cancer tissues compared to their respective normal tissues (Fig. 1G). Taken together, these data indicated the up-regulation of SUPT5H expression in breast cancers.

SUPT5H specifically interacts with PIN1, and this interaction depends on the N-terminal (WW) domain of PIN1

SUPT5H is ranked among the top-ranked protein with similar functions to peptidyl-prolyl cis/trans isomerase (PIN1) [24]. Using cBioportal [25], we identified a positive correlation between SUPT5H and PIN (Pearson=0.36) from the TCGA breast invasive carcinoma dataset (Fig. 2A). Western blotting was performed to verify the expression correlation between SUPT5H and PIN1. The result showed that the silencing of SUPT5H was efficient in

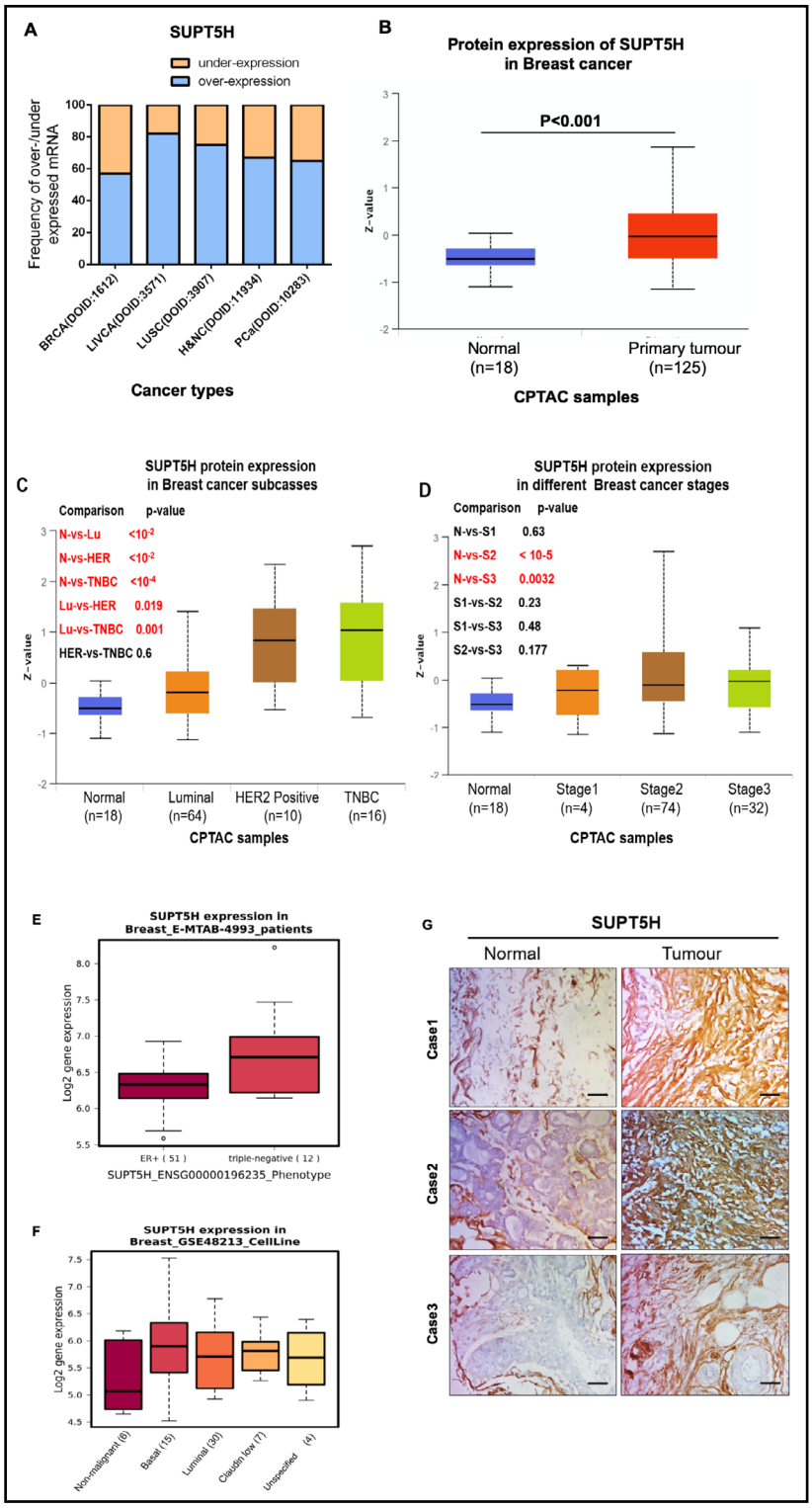

Fig. 1. SUPT5H is highly expressed in breast cancer (A) Expression status of SUPT5H in different cancers and their respective normal tissues obtained from BioXpress database. Log2 fold change (Log2FC) between the mRNA expression in cancerous and adjacent tissues of greater than zero for SUPT5H is considered to be over-expression and less than zero to be under-expression. Abbreviations: Breast cancer [BRCA], Liver cancer [LIVCA], Lung squamous cell carcinoma [LUSC], Head and neck cancer [H&NC], Prostate cancer [PCa]. (B) CPTAC data from UALCAN indicated that expression of SUPT5H was significantly up-regulated in breast cancer tissues (n=125) compared to normal breast tissues (n=18), $P<10^{-7}$. (C) Based on CPTAC from UALCAN, the expression of SUPT5H in normal breast tissues and breast cancer tissue with different subclasses (D) The expression of SUPT5H protein in normal tissues and breast cancer tissues with different grades of tumor. Significant up-regulation was observed in stage 2 and stage 3 of breast cancers. (E) SUPT5H transcript profiling of two breast cancers in a cohort of 63 patients (51 ER+, 12 triple-negative), with increased expression in triple-negative according to E-MTAB-4993 dataset from KM-express database. (F) Expression status of SUPT5H in breast cancer subtype cell lines according to GSE48213 dataset from KM-express database. (G) Representative immunohistochemistry images of SUPT5H protein expression in breast cancer and normal tissues. Scale bar, 100 µm.

MDA-MB-231 and led to inhibition of the expression of PIN1. Further, Rescue experiment demonstrated that siRNA-SUPT5H transfection silences the expression of endogenous SUPT5H protein and the SUPT5H protein that is expressed from native (Supplementary Fig. S1) but not from mutated SUPT5H ORF (Fig. 2B) (for all supplementary material see www.cellphysiolbiochem.com). The rescue of observed knockdown phenotypes with an exogenously expressed siRNA-resistant SUPT5H expression construct (Δ2) confirmed the efficiency of double nucleotide mismatch to abolish the siRNA knockdown effect and validated the specificity as well as the selectivity of the siRNA for the endogenous SUPT5H gene. The rescue of SUPT5H and PIN1 proteins with exogenously expressed engineered SUPT5H ORF in SUPT5H depleted cells confirmed the positive correlation between SUPT5H and PIN1.

PIN1 is 163 residue protein, containing the N-terminal WW domain and C-terminal PPIase domain [26] (Fig. 2C). Earlier, *in vitro* studies have shown the interaction between PIN1 and SUPT5H[27]. We validated the interaction between SUPT5H and PIN1 using an independent yeast-two-hybrid assay, and we identified the functional domain of PIN1 required for direct interaction (Fig. 2D). To determine the functional region of PIN1 that is required for direct interaction with SUPT5H, we employed two truncated forms of PIN1. We showed the gene encoding N-terminal region (1-138, WW) but not the C-terminal region (118-492, PPIase) interacts with SUPT5H, suggesting the N-terminal area which includes the WW domain may be involved in PIN1-SUPT5H interaction.

Using Immunofluorescence (IF) confocal microscopy, we visualized the colocalization pattern of PIN1 and split PIN1 domains with SUPT5H by co-transfection of pmTurquoiseC1-PIN1 and mVenusC1-SUPT5H or pmTurquoise-$PIN1_{WW}$ and mVenusC1-SUPT5H or pmTurquoiseC1-$PIN1_{PPIase}$ and mVenusC1-SUPT5H plasmids, and cells were allowed to express fusion protein for 48 hours after transfection. Finally, we detected prominent overlapping with Pearson's value of 0.70 and 0.72 between PIN1 and SUPT5H, and PIN1-WW and SUPT5H, respectively, whereas the less overlapping, was observed between PIN1-PPIase and SUPT5H with Pearson's value of 0.3 (Fig. 2E). Together, these results demonstrate the physical interaction and high affinity of the WW domain for SUPT5H.

SUPT5H abundance is regulated by PIN1

Interestingly, the silencing of PIN1 in MDA-MB-231 cells resulted in a down-regulation of endogenous SUPT5H proteins (Fig. 2F). Because SUPT5H directly binds to PIN1, we then asked whether the protein-protein interaction increased the stability of SUPT5H. For this purpose, we transfected siRNA-scr and siRNA-PIN1 in MDA-MB-231 cells and treated each well with proteasome inhibitor MG1322, all other wells with DMSO. Our results revealed that treatment of MG132 completely blocked the PIN1 knockdown-induced loss of SUPT5H, suggested that PIN1 binding to SUPT5H promotes stability of SUPT5H.

Silencing of SUPT5H decreases cell viability, cell proliferation, and colony formation of breast cancer cells

To explore whether knockdown of SUPT5H gene expression affects the oncogenic behavior of breast cancer cells, crystal violet, cell-Titer-Glo (CTG), and colony formation were assayed. Crystal violet assay demonstrated a significant decrease in cell viability in siRNA-SUPT5H transfected MDA-MB-231 cells compared with scramble siRNA after 72 hours of transfection (Fig. 3A). Next, we examined the growth of breast cancer cells after silencing SUPT5H with siRNA using CTG assay. Compared with cells transfected with siRNA-scr, cells transfected with siRNA-SUPT5H demonstrated reduced growth and displayed lower viability (Fig. 3B). In consistent with the results of crystal violet and CTG assays, colony formation assay demonstrated that the silencing of SUPT5H decreased the colony-formation in MDA-MB-231 cells (Fig. 3C). Essentially identical results were observed when one more independent SUPT5H siRNA (siRNA-SUPT5H#) was used (the sequence is shown in Supplementary Table S1). The knockdown with siRNA-SUPT5H# further validated that phenotype is the result of target mRNA knockdown rather than an off-target event (Supplementary Fig. S2, S3). Moreover, SUPT5H knockdown with siRNA-SUPT5H also decreased the viability and

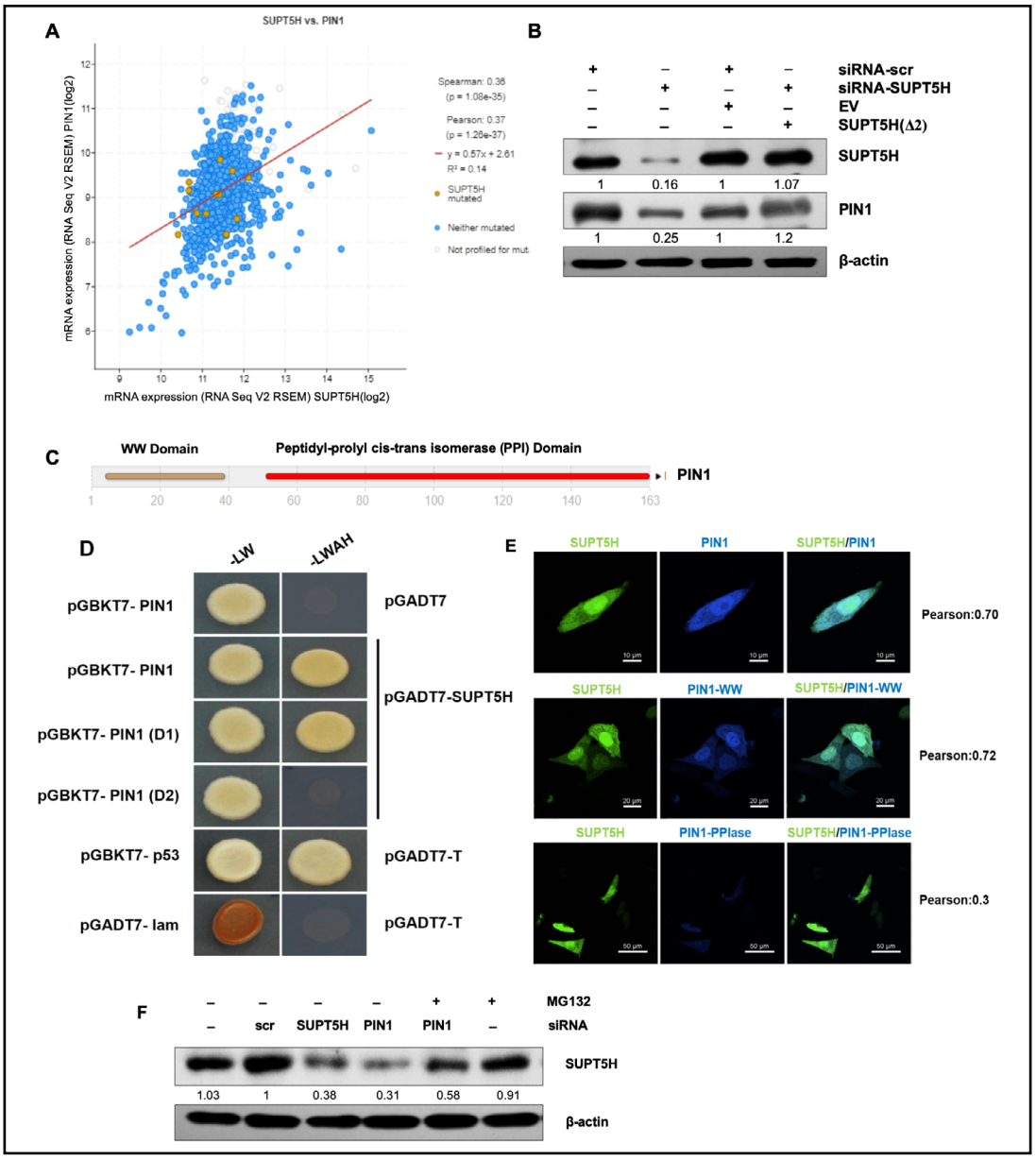

Fig. 2. Co-expression correlation and stability landscape of interaction between SUPT5H and PIN1. (A) Dataset from cBioportal showed a positive correlation between SUPT5H and PIN expression in breast cancer. (B) Western blot of SUPT5H and PIN1 proteins in MDA-MB-231 cells transfected with siRNA-SUPT5H only, siRNA-SUPT5H plus the siRNA-SUPT5H resistant ORF construct SUPT5H(Δ2), non-silencing siRNA-scr or siRNA-scr plus empty vector (EV) (representative blots are shown from two independent experiments). The quantitative comparison of siRNA-scr v/s siRNA-SUPT5H and siRNA-scr+EV v/s siRNA-SUPT5H+SUPT5H(Δ2) was performed. (C) A 163 residue protein PIN1 with two domains: WW domain and PPI domain (D) Interaction of pGBKT7-PIN1 with pGADT7-SUPT5H and pGBKT7-PIN1$_{WW}$ domain with pGADT7-SUPT5H were detected by DDO (-LW) and QDO (-LWAH) selection using yeast two-hybrid screening. Vectors pGBKT7-p53 with pGADT7-T and pGBKT7-Lam with pGADT7-T were used as a positive and negative control, respectively. The possibility of false-positive was eliminated by confirming that the activation domain in the empty vector could not interact with the pGBKT7-PIN1 bait vector. (E) MDA-MB-231 cells were co-transfected with mtorquois-PIN1 and mVenus-SUPT5H, mtorquois-PIN1$_{WW}$ and mVenus-SUPT5H, and mtorquois-PIN1$_{PPIase}$ and mVenus-SUPT5H plasmids. The colocalization of signals were visualized and analyzed using confocal microscopy. (F) MDA-MB-231 cells were transfected with siRNA-scr, siRNA-SUPT5H, or siRNA-PIN1. About 48h later, cells were further treated with MG132 or DMSO for 8h. Western blotting examination indicated the role of proteasome on PIN1 regulation of SUPT5H abundance (representative blots are shown from two independent experiments).

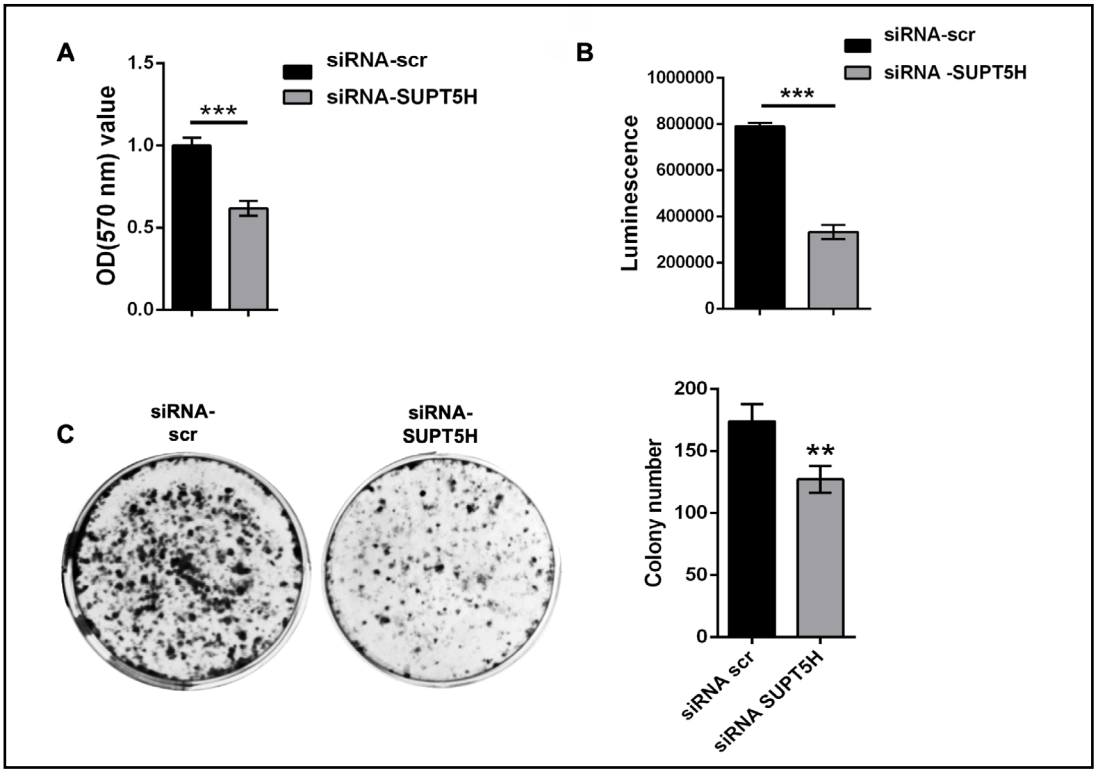

Fig. 3. SUPT5H depletion suppresses the tumorigenesis and colony formation ability of breast cancer MDA-MB-231 cells. (A) Crystal violet assay was performed to determine the proliferation ability of breast cancer cells transfected with siRNA-SUPT5H (B) Cell viability of siRNA SUPT5H transfected cells was determined by CellTiter-Glo® luminescent cell viability assay. (C) The Clonogenicity of SUPT5H depleted cells was determined by colony formation assay. Bars show the mean ± SD of at least triplicates. **p<0.01, ***p<0.001.

clonogenicity of MCF-7 and MDA-MB-453 cells (Supplementary Fig. S2, S3). These results implicated the involvement of SUPT5H in regulating the tumorigenic properties of breast cancer cells.

SUPT5H silencing in MDA-MB-231 reduces their migratory and invasive capacities

We first determined the effect of SUPT5H silencing on cell migration by wound healing assay. As shown in Fig. 4A, the artificial wound gap of scramble siRNA-transfected cells was significantly decreased compared with SUPT5H siRNA-transfected cells at 24 hours. To study the invasiveness and migration potentials of siRNA SUPT5H knockdown cells, we performed migration and invasion assays using transwell coated with/ without matrigel, respectively. We found that depletion of SUPT5H significantly reduced the cell migration and invasion capacity of breast cancer cells (Fig. 4B). The effects of SUPT5H knockdown were likely specific, as we obtained identical results with siRNA-SUPT5H# (Supplementary Fig. S4). Consistent with our data in MDA-MB-231, SUPT5H knockdown with siRNA-SUPT5H also significantly decreased the migration and invasion potential in MCF-7 and MDA-MB-453 cells (Supplementary Fig. S4). Furthermore, western blot analysis revealed a reduction in the expression level of migration-related proteins (MMP-2, integrin, slug, vimentin, and β-catenin) in the SUPT5H-siRNA transfected breast cancer cells compared with scramble-siRNA transfected (Fig. 4C). These results suggest the involvement of SUPT5H in regulating the metastatic properties of breast cancer.

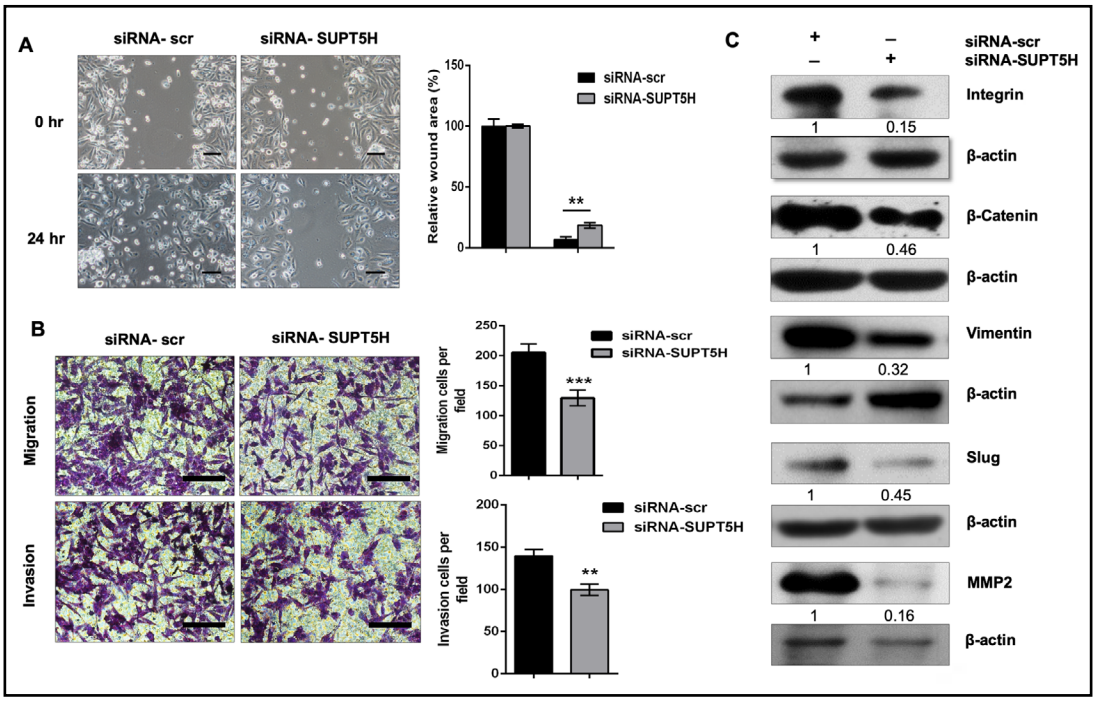

Fig. 4. SUPT5H depletion inhibits the migratory and invasive behavior of MDA-MB-231 cells. (A) A wound-healing assay was performed to measure the migration potential of siRNA-scr and siRNA-SUPT5H transfected cells. The wound area was quantified by ImageJ 64 software. Scale bar, 100µm, and magnification, 10x. (B) Transwell assay was used to assess the migration and invasion potentials of MDA-MB-231 after transfection with siRNA-scr or siRNA-SUPT5H. Scale bar, 100µm, and magnification, 20x. (A, B) data is the representation of three independent experiments. **p<0.01, ***p<0.001. (C) Western blot analysis of the expression of migration-related proteins of MDA-MB-231 cells following transfection of siRNA-scr and siRNA-SUPT5H (representative blots are shown from two independent experiments).

Silencing of SUPT5H induces cell cycle arrest in breast cancer cells

To determine if SUPT5H plays a functional role in MDA-MB-231 cells, we explored its function in cell cycle regulation using RNAi approach. The siRNA transfected cells were stained with propidium iodide and analyzed using Fluorescence-activated cell sorter. Silencing of SUPT5H led to the accumulation of cells in the S phase with a concomitant decrease in G1, and this loss-of-function phenotype was rescued upon the exogenous expression of siRNA resistant ORF that further confirmed the specific silencing effect of siRNA (Fig. 5A). Consistent with our data, the S-phase arrest was also observed in MCF-7 and MDA-MB-453 upon the silencing of SUPT5H and with siRNA-SUPT5H# in MDA-MB-231 cells. (Supplementary Fig. S5). In agreement with the cell-cycle distribution analysis, silencing of SUPT5H induced the expression of p21 (Fig. 5B) at mRNA level and inhibited the expression of proteins such as PCNA, E2F, cyclinE, which are involved in cell cycle process, along with induction of cyclin A1 protein (Fig. 5C).

Silencing of SUPT5H promotes apoptosis, induces mitochondrial membrane potential, and reactive oxygen species (ROS) changes in breast cancer cells

The annexin V/propidium iodide apoptosis assay was performed to investigate whether silencing of the SUPT5H gene induces apoptosis. The apoptotic index of MDA-MB-231 cells transfected with siRNA-SUPT5H was significantly higher than that of cells transfected with siRNA-scr and the exogenous SUPT5H engineered construct successfully rescued MDA-MB-231 cells from apoptosis obtained with siRNA-SUPT5H transfection, demonstrated that this phenotype is directly linked to an anti-apoptotic SUPT5H function (Fig. 6A). Besides,

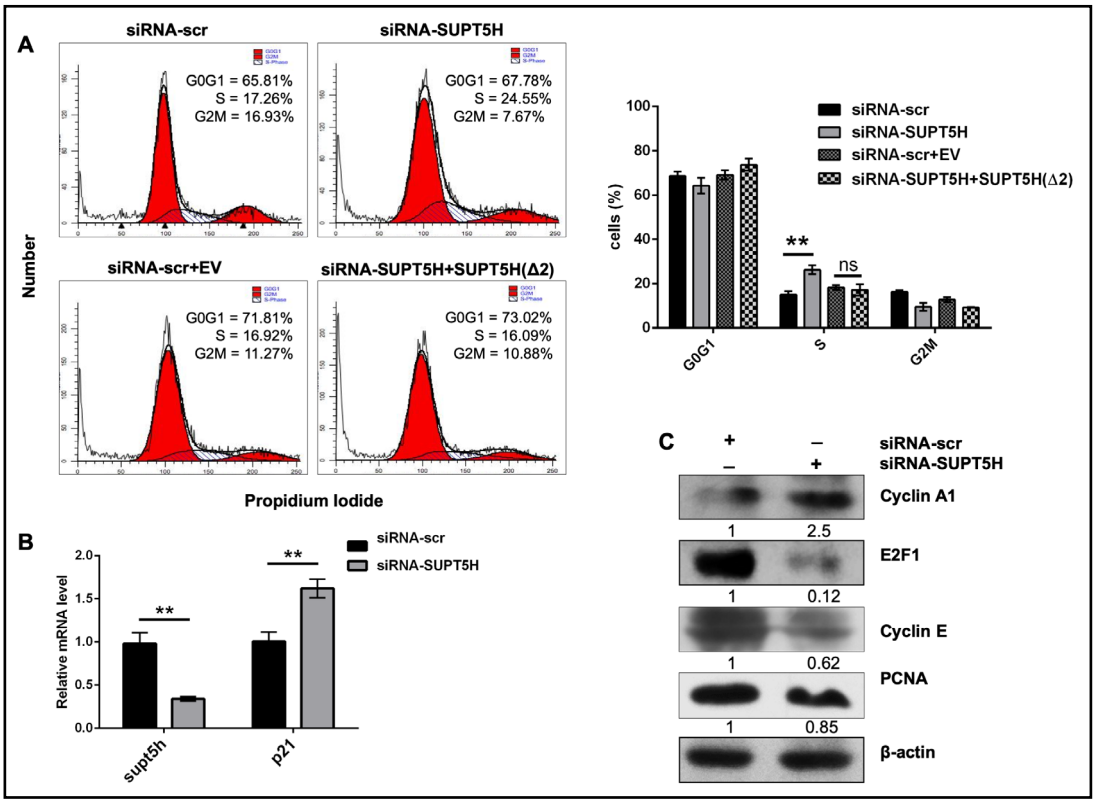

Fig. 5. The depletion of SUPT5H gene expression induces cell cycle arrest in MDA-MB-231 cells. (A) Cells transfected with siRNA-SUPT5H only (knockdown), siRNA-SUPT5H plus the siRNA resistant SUPT5H ORF (Δ2), non-silencing siRNA-scr or siRNA-scr plus empty vector (EV) were stained with propidium iodide after 72 hours of transfection and subjected to FACS analysis to determine cell-cycle distribution. Depletion of SUPT5H by siRNA-SUPT5H induced the S-phase cell cycle arrest, and the effect of siRNA-SUPT5H was rescued by engineered SUPT5H gene construct. (B) Quantification of p21 gene expression in SUPT5H silenced MDA-MB-231 cells using real-time qPCR. (A, B) data represented as means ± SD (n=3). *p<0.05, **p<0.01, not significant, ns (C) Western blot analysis of cell-cycle regulatory proteins in MDA-MB-231 cells transfected with siRNA-SUPT5H or siRNA-scr (representative images are shown from two independent experiments).

significant apoptosis was further observed in MDA-MB-231 cells with siRNA-SUPT5H# (Supplementary Fig. S6). Furthermore, compared with control cells, SUPT5H knockdown significantly induces apoptosis in MCF-7 and MDA-MB-231 cells (Supplementary Fig. S6).

Further, we examined the effect of silencing of SUPT5H on the expression of molecules that regulates apoptosis; western blotting analysis revealed that silencing of SUPT5H effectively increased the level of pro-apoptotic Bax and decreased the levels of anti-apoptotic Bcl-xl, Bcl-2, pro-caspase 3, and pro-PARP proteins (Fig. 6B). Compared with siRNA-scr transfected, RT-qPCR analysis demonstrated that depletion of SUPT5H increased expression of pro-apoptotic marker Noxa and decreased the expression of anti-apoptotic markers Xiap and Ciap in siRNA-SUPT5H transfected cells (Fig. 6C). These results indicated that the silencing of SUPT5H promotes apoptosis via the activation of the intrinsic pathway.

To investigate whether reactive oxygen species (ROS) is involved in siRNA-SUPT5H mediated apoptosis, we first observed the intracellular ROS by H2DCFDA staining dye using fluorescence microscopy. As shown in Fig. 6D, the silencing of SUPT5H promotes the fluorescence intensity (ROS production), and the ROS strength in SUPT5H silenced cells was diminished by free radicle scavenger *N*-acetyl-L-cysteine (NAC). The increase of ROS production is associated with loss of mitochondrial membrane potential that reflects the apoptotic state of cells by the mitochondrial pathway. We tested the change of mitochondrial membrane

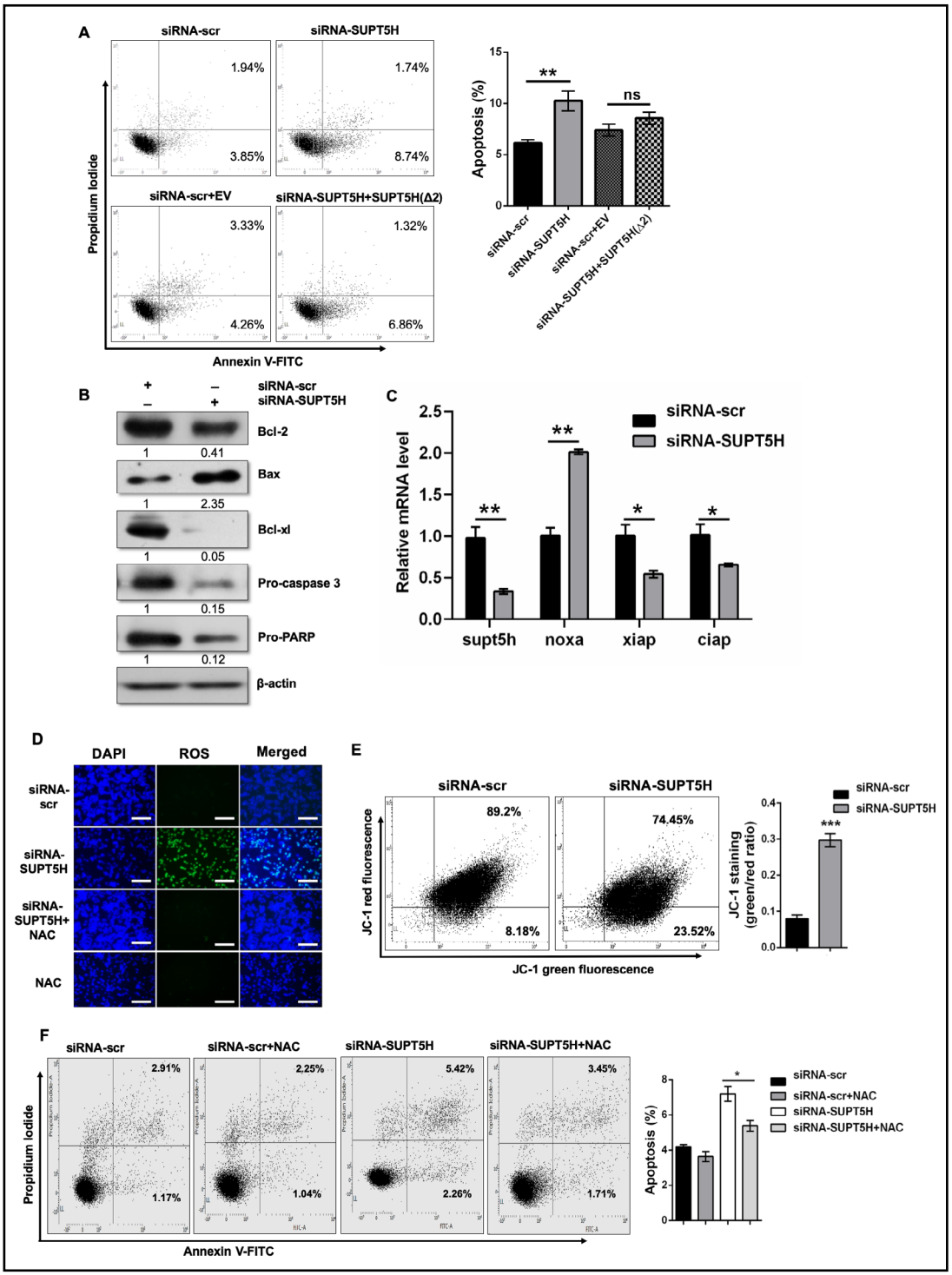

Fig. 6. SUPT5H depletion promotes apoptosis, generates ROS, and change mitochondrial membrane potential (MMP) in MDA-MB-231. (A) Flow cytometric analysis demonstrated the levels of cell apoptosis in MDA-MB-231 cells. (B) Western blot analysis of apoptosis regulatory proteins in MDA-MB-231 treated cells with siRNA-SUPT5H or siRNA-scr (representative blots are shown from two independent experiments). (C) qRT-PCR analysis of the expression of genes related to apoptosis. (D) SUPT5H depletion promotes ROS production and the levels of ROS were diminished in presence of N-acetyl-L-cysteine (NAC). Scale bar, 100μm and magnification, 20X. (E) Flowcytometric analysis of the change of MMP in SUPT5H silenced MDA-MB-231 cells using JC-1 dye. (F) Treatment of NAC reagent to SUPT5H depleted MDA-MB-231 cells rescues the cells from apoptosis as indicated by flow cytometric analysis. The mean ± was shown in bar plots (n=3). *p<0.05, **p<0.01, ***p<0.001, not significant, ns.

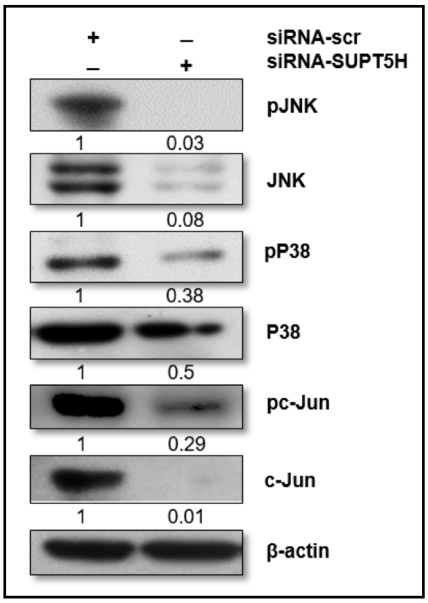

Fig. 7. SUPT5H depletion in MDA-MB-231 inhibits the expression of MAPK pathway proteins. Western blot showing the effects of silencing of SUPT5H on the levels of total and phosphorylated JNK, P38, and c-Jun. An equal amount of cellular protein (50μg) was separated, and β-actin was applied as a control for equal loading (representative blots are shown from two independent experiments).

potential using the JC-1 dye by flow cytometry. We demonstrated that compared to scramble, the silencing of SUPT5H promotes the loss of mitochondrial membrane potential as revealed by the increase of green-to-red (monomer/aggregate) ratio (Fig. 6E). As shown in Fig. 6E, the JC-1 green/red ratio was 0.31 in SUPT5H silenced group, while it was 0.09 in the scramble group, suggested that the silencing of SUPT5H promotes mitochondrial dysfunction by changing the membrane potential ($\Delta\Psi m$).

To explore the mechanism that apoptosis by silencing of SUPT5H was ROS dependent, we performed the AnnexinV/PI apoptosis assay in siRNA-scr and siRNA-SUPT5H transfected cells, treated with/without NAC. Our results demonstrated that the treatment of NAC partially rescues the SUPT5H silenced cells from apoptosis, indicating that apoptosis in SUPT5H silenced cells is partially ROS dependent (Fig. 6F).

Silencing of SUPT5H suppresses the MAPK signaling pathway in breast cancer cells

To explain the effects of silencing of SUPT5H in reducing the cell viability and migration of MDA-MB-231 cells, the expression and activation of MAPK molecules were analyzed by western blot. Our results showed that silencing of SUPT5H was associated with deactivation of active MAPK molecules with a change in the levels of total and activated MAPK proteins (Jnk, P38, c-Jun) (Fig. 7), thus accounting for the involvement of SUPT5H in activation of MAPK pathway or influence other pathway(s) relating to cell proliferation/migration/viability.

Discussion

TNBC is considered the most aggressive form of breast cancer and has poor prognosis due to a lack of effective classification markers, molecular signatures, and targeted therapies. Gaining better insights into the pathological mechanism of breast cancer would play an important role in identifying target sites.

Our study provides evidences of the increased expression of SUPT5H in breast cancers as determined by database mining and immunohistochemical studies, suggesting it acts as a proto-oncogene. We then showed the targeting effects of the silencing of SUPT5H on MDA-MB-231 breast cancer cells *in vitro.* The observations obtained predicted the contribution of SUPT5H toward breast cancer progression and demonstrated a rationale for using SUPT5H as a targeting candidate in breast cancer.

RNAi technology was used toward the functional study of SUPT5H loss. SUPT5H specific siRNA was transfected into MDA-MB-231 breast cancer cell line to screen the effects. It was first proved that transfection of siRNA-SUPT5H efficiently down-regulated the expression of SUPT5H at mRNA and protein levels, as demonstrated by quantitative real-time PCR and Western blot analysis, respectively. Rescue of siRNA knockdown phenotype by ectopic expression of the protein of interest with an engineered construct can be used to confirm the target specificity of siRNA [28]. The knockdown-rescue approach was applied to determine the target specificity of siRNA-SUPT5H. Further, our results clearly showed for the first time in breast cancer MDA-MB-231 cells that SUPT5H silencing attenuates cell growth, inhibits migration and invasion, induces apoptosis, and changes the cell cycle. The MAPK signaling molecules were markedly inhibited by SUPT5H silencing and revealed the association of SUPT5H with the MAPK pathway.

In the previous study, PIN1, a *cis/trans* isomerase that activates oncoproteins and inactivates tumor supressors [14], has been found to interact with Cdk9-phosphorylated SUPT5H. We validated the interaction between PIN1 and SUPT5H and discovered that the functional WW domain of PIN1 is critical for direct interaction with SUPT5H. We further demonstrated that SUPT5H interaction with PIN1 increased the stability of SUPT5H. Moreover, we showed that SUPT5H positively regulates the expression of PIN1 at translation levels, which determines the correlation between SUPT5H and PIN1. The interaction between SUPT5H and PIN1 implies that that SUPT5H might be involved in regulating the different pathways of cancer cells.

The invasive/metastatic potential is an essential parameter for the progression of cancer cells. MDA-MB-231 is already known to have high metastatic/invasive potential [29]. Patients with TNBC have higher incidences of lymph node invasion and distant metastasis than other breast cancers [30]. We have demonstrated that the silencing of SUPT5H significantly suppressed the migration and invasion abilities of MDA-MB-231 breast cancer cells. Previously, a positive correlation has been observed between the expression of vimentin, MMP2, integrin, β-catenin, and human melagnancies [31–33]. To further confirm that SUPT5H is involved in migration and invasion of breast cancer cells, gene silencing of SUPT5H was done. As a result of gene silencing of SUPT5H, the expression of vimentin, MMP2, integrin, and β-catenin was inhibited as determined by western blot analysis. These results suggested that SUPT5H is involved in migration and invasion by regulating the expression of metastasis-associated proteins in breast cancer MDA-MB-231 cells.

In this study, we have further demonstrated that the silencing of SUPT5H promotes cell cycle arrest at the S-phase by inhibiting the proliferation of MDA-MB-231 breast cancer cells. This effect may have been mediated with increased expression of CCNA1 and CKI p21 genes and decreased expression of E2F1, PCNA and cyclin E. Cyclin A1 is an important regulator that contributes to G1 to S cell cycle progression [34, 35]. p21 has the unique ability to interacts with PCNA and blocks PCNA activity [36]. The loss of PCNA blocks DNA synthesis and arrest the cells in the S phase of cell cycle [37]. During the S phase, Cyclin A-kinase has been found to inactivate E2F1 [38]. Cyclin E, which accumulates in late G1 by the E2F-mediated gene transcription program, accelerates S-phase entry, but it is rapidly degraded. Thus, the regulation of these critical molecules by SUPT5H is of vital importance in cell cycle progression.

Evasion of apoptosis is a hallmark of various cancers that may contribute to uncontrolled proliferation, carcinogenesis, and resistance to therapy [39, 40]. Apoptotic cells display a series of characteristic morphological changes such as cell shrinkage, membrane blebbing, chromatin condensation, and nuclear fragmentation [41]. Mitochondria is an essential organelle that contributes to the progression of cancer via the evasion of apoptosis. Targeting mitochondria is a crucial strategy for cancer therapy [42]. Previous reports showed that excessive ROS generation is associated with the destruction of the integrity of the mitochondrial membrane that results in the loss of mitochondrial membrane potential, eventually cell death by activating apoptotic pathways. Our study demonstrated that mitochondrial membrane potential showed severe collapse, and apoptosis occurs in siRNA-SUPT5H transfected

cells as compared to siRNA-scr transfected cells. We examined the expression of different pro-apoptotic and anti-apoptotic markers upon the silencing of SUPT5H. Bcl-2 family members are the regulators of the molecular mechanism that organizes caspase-dependent intrinsic apoptosis. Overexpression Bcl-2 anti-apoptotic proteins evade the tumor cells from programmed cell death. Targeted inhibition of Bcl-2 is an attractive strategy that sensitizes certain cancers. NOXA is a pro-apoptotic BH3 only protein molecule that potently antagonizes Bcl-2 and triggers the activation of pro-apoptotic BAX protein. Bcl-xl is another potent apoptosis suppressor that is upregulated in certain cancer. Conversely, Bax is an apoptosis promoter that is inactivated in certain cancers. Ratios of Bax/Bcl-2 and Bax/Bcl-xl determines cell susceptibility to cancer [43, 44]. Our results showed that silencing of SUPT5H increased the expression of NOXA and BAX and decreased the expressions of Bcl-2 and Bcl-xl, that support the existence of a specific apoptotic mechanism that cells with elevated NOXA, Bax/Bcl-2, and Bax/Bcl-xl ratios are more sensitive to lose cell viability and displays enhanced apoptosis. For efficient apoptosis, inhibition of inhibitor of apoptosis (IAP) family members X-linked IAP (XIAP) and cellular IAP1 (CIAP1) are required to promote the activation of caspase-3 that are essential for the induction of PARP cleavage [45–47]. Cleavage of PARP prevents DNA repair, and executes caspase-mediated programmed cell death [48]. Considering the TNBC, the most dangerous form of breast cancer, the silencing of SUPT5H may be an encouraging approach for targeting breast cancer.

To identify the mechanism affected by SUPT5H silencing, we examined the MAPK signaling pathway that controls a variety of cellular events, including cell proliferation, cell survival, migration, and invasion. Dysregulation of MAPK cascade promotes the development and progression of cancer [49]. We found that SUPT5H silencing has a strong inhibitory effect on MAPK pathway molecules, JNK, P38 and c-jun. Phosphorylation of MAPK molecules is required for their full activation. We observed that phosphorylation of key MAPK molecules was effectively suppressed in SUPT5H silenced cells compared to scrambled cells. Activated JNK and p38 can translocate into the nucleus and phosphorylate an important transcription factor c-Jun. Phosphorylated c-Jun promotes the expression of numerous genes and can induce oncogenic transformation [50].

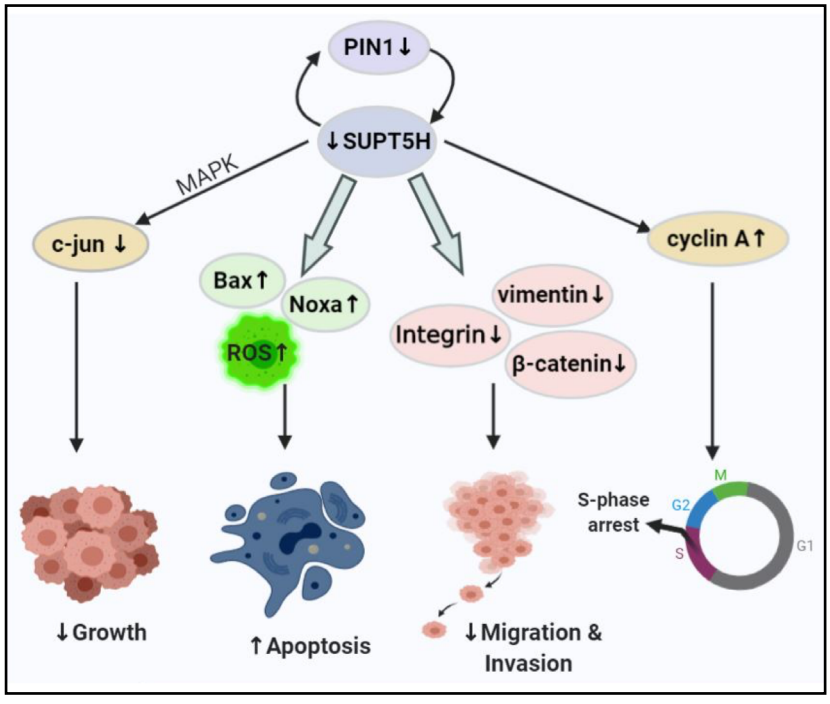

Fig. 8. Schematic diagram depicting the role of SUPT5H in breast cancer progression. The silencing of SUPT5H regulates a number of genes involved in cell proliferation, migration, and apoptosis.

Conclusion

This study illuminated the role of SUPT5H as a molecular signature of breast cancer that regulates tumorigenic potential; proliferation, migration, invasion, and apoptosis (Fig. 8), and its higher expression might be correlated with patient's poor prognosis. Our study displayed promising results on the silencing of SUPT5H in the most aggressive breast cancer cell and may serve as a new target for the treatment of breast cancers. However, the study was performed *in-vitro;* in this context, further extrapolation of research *in vivo,* exploring the regulatory mechanism of SUPT5H, and role in more extensive ranges of tumors will be necessary.

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Author contributions

Y.R.P and B.A.L designed the experiments. B.A.L., F.A and S.K.L.K performed the experiments. B.A.L and Y.R.P wrote the manuscript with help from F.A.

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Disclosure Statement

The authors declare no competing interests.

References

1 Tofigh A, Suderman M, Paquet ER, Livingstone J, Bertos N, Saleh SM, Zhao H, Souleimanova M, Cory S, Lesurf R, Shahalizadeh S, Lopez NG, Riazalhosseini Y, Omeroglu A, Ursini-Siegel J, Park M, Dumeaux V, Hallett M: The Prognostic Ease and Difficulty of Invasive Breast Carcinoma. Cell Rep 2014;9:129–142.

2 Perou CM: Molecular stratification of triple-negative breast cancers. Oncologist 2010;15:39–48.

3 Darrel W Cleere: Triple-negative breast cancer: a clinical update. Community Oncol 2010;7:203–211.

4 Morris GJ, Naidu S, Topham AK, Guiles F, Xu Y, McCue P, Schwartz GF, Park PK, Rosenberg AL, Brill K, Mitchell EP: Differences in breast carcinoma characteristics in newly diagnosed African-American and Caucasian patients: a single-institution compilation compared with the National Cancer Institute's Surveillance, Epidemiology, and End Results database. Cancer 2007;110:876–884.

5 Bradner JE, Hnisz D, Young RA:Transcriptional Addiction in Cancer. Cell 2017;168:629–643.

6 David CJ, Manley JL: Alternative pre-mRNA splicing regulation in cancer: pathways and programs unhinged. Genes Dev 2010;24:2343–2364.

7 Hartzog GA, Fu J: The Spt4 – Spt5 complex : A multi-faceted regulator of transcription elongation. Biochim Biophys Acta 2013;1829:105–115.

8 Wen Y, Shatkin AJ: Transcription elongation factor hSPT5 stimulates mRNA capping. Genes Dev 1999;13:1774–1779.

9 Mandal SS, Chu C, Wada T, Handa H, Shatkin AJ, Reinberg D:Functional interactions of RNA-capping enzyme with factors that positively and negatively regulate promoter escape by RNA polymerase II. Proc Natl Acad Sci 2004;101:7572-7577.

10 Fitz J, Neumann T, Pavri R: Regulation of RNA polymerase II processivity by Spt5 is restricted to a narrow window during elongation. EMBO J 2018;37:e97965.
11 Baluapuri A, Hofstetter J, Dudvarski Stankovic N, Endres T, Bhandare P, Vos SM, Vos SM, Adhikari B, Schwarz JD, Narain A, Vogt M, Wang SY, Düster R, Jung LA, Vanselow JT, Wiegering A, Geyer M, Maric HM, et al.: MYC Recruits SPT5 to RNA Polymerase II to Promote Processive Transcription Elongation. Mol Cell 2019;74:674-687.
12 Chen R, Zhu J, Dong Y, He C, Hu X: Suppressor of Ty homolog-5 , a novel tumor-specific human telomerase reverse transcriptase promoter-binding protein and activator in colon cancer cells. Oncotarget 2015;6:32841-32855.
13 Ryo A, Liou YC, Wulf G, Nakamura M, Lee SW, Lu KP: PIN1 is an E2F target gene essential for Neu/Ras-induced transformation of mammary epithelial cells. Mol Cell Biol 2002;22:5281–5295.
14 Zhou XZ, Lu KP: The isomerase PIN1 controls numerous cancer-driving pathways and is a unique drug target. Nat Rev Cancer 2016;16:463–478.
15 Karna SKL, Ahmad F, Lone BA, Pokharel YR: Knockdown of PTOV1 and PIN1 exhibit common phenotypic anti-cancer effects in MDA-MB-231 cells. PloS One 2019;14:e0211658.
16 Luo M-L, Gong C, Chen C-H, Lee DY, Hu H, Huang P, Yao Y, Guo W, Reinhardt F, Wulf G, Lieberman J, Zhou XZ, Song E, Lu KP: Prolyl isomerase Pin1 acts downstream of miR200c to promote cancer stem-like cell traits in breast cancer. Cancer Res 2014;74:3603–3616.
17 Rustighi A, Zannini A, Tiberi L, Sommaggio R, Piazza S, Sorrentino G, Nuzzo S, Tuscano A, Eterno V, Benvenuti F, Santarpia L, Aifantis I, Rosato A, Bicciato S, Zambelli A, Sal GD: Prolyl-isomerase Pin1 controls normal and cancer stem cells of the breast. EMBO Mol Med 2014;6:99–119.
18 Dahlgren C, Zhang HY, Du Q, Grahn M, Norstedt G, Wahlestedt C, Liang Z: Analysis of siRNA specificity on targets with double-nucleotide mismatches. Nucleic Acids Res 2008;36:e53.
19 Wu W, Hodges E, Höög C: Thorough validation of siRNA-induced cell death phenotypes defines new anti-apoptotic protein. Nucleic Acids Res 2006;34:e13.
20 Wan Q, Dingerdissen H, Fan Y, Gulzar N, Pan Y, Wu TJ, Yan C, Zhang H, Mazumder R: BioXpress: an integrated RNA-seq-derived gene expression database for pan-cancer analysis. Database (Oxford) 2015;2015:bav019.
21 Chen F, Chandrashekar DS, Varambally S, Creighton CJ: Pan-cancer molecular subtypes revealed by mass-spectrometry-based proteomic characterization of more than 500 human cancers. Nat Commun 2019;10:5679.
22 Chandrashekar DS, Bashel B, Balasubramanya SAH, Creighton CJ, Ponce-Rodriguez I, Chakravarthi BVSK, Varambally S: UALCAN: A Portal for Facilitating Tumor Subgroup Gene Expression and Survival Analyses. Neoplasia 2017;19:649–658.
23 Chen X, Miao Z, Divate M, Zhao Z, Cheung E. KM-express: an integrated online patient survival and gene expression analysis tool for the identification and functional characterization of prognostic markers in breast and prostate cancers. Database (Oxford) 2018;2018:bay069.
24 Pokharel YR, Saarela J, Szwajda A, Rupp C, Rokka A, Karna SLK, Teittinen K, Corthals G, Kallioniemi O, Wennerberg K, Aittokallio T, Westermarck J: Relevance Rank Platform (RRP) for Functional Filtering of High Content Protein-Protein Interaction Data. Mol Cell Proteomics 2015;14:3274–3283.
25 Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA,Jacobsen A, Byrne CJ, Heuer ML, Larsson E, Antipin Y, Reva B, Goldberg AP, Sander C, Schultz N: The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discov 2012;2:401–404.
26 Bayer E, Goettsch S, Mueller JW, Griewel B, Guiberman E, Mayr LM, Bayer P: Structural analysis of the mitotic regulator hPin1 in solution: insights into domain architecture and substrate binding. J Biol Chem 2003;278:26183–26193.
27 Lavoie SB, Albert AL, Handa H, Vincent M, Bensaude O: The peptidyl-prolyl isomerase Pin1 interacts with hSpt5 phosphorylated by Cdk9. J Mol Biol. 2001;312:675–685.
28 Lassus P, Rodriguez J, Lazebnik Y: Confirming specificity of RNAi in mammalian cells. Sci STKE 2002;2002:pl13.
29 Li WM, Zhou LL, Zheng M, Fang J: Selection of Metastatic Breast Cancer Cell-Specific Aptamers for the Capture of CTCs with a Metastatic Phenotype by Cell-SELEX. Mol Ther Nucleic Acids 2018;12:707–717.
30 Wahba HA, El-Hadaad HA: Current approaches in treatment of triple-negative breast cancer. Cancer Biol Med 2015;12:106–116.

31 Yin HL, Wu CC, Lin CH, Chai CY, Hou MF, Chang SJ, Tsai HP, Hung WC, Pan MR, Luo CW: β1 Integrin as a Prognostic and Predictive Marker in Triple-Negative Breast Cancer. Int J Mol Sci 2016;17:1432.
32 Satelli A, Li S: Vimentin in cancer and its potential as a molecular target for cancer therapy. Cell Mol Life Sci 2011;68:3033-3046.
33 Xu J, Prosperi JR, Choudhury N, Olopade OI, Goss KH: β-Catenin is required for the tumorigenic behavior of triple-negative breast cancer cells. PloS One 2015;10:e0117097.
34 Henglein B, Chenivesse X, Wang J, Eick D, Brechot C: Structure and cell cycle-regulated transcription of the human cyclin A gene. Proc Natl Acad Sci 1994;91:5490–5494.
35 Yin H, Subrata S, Yang W, Yang G: Overexpression of cyclin A leads to S-phase arrested apoptosis. Hua Xi Yi Ke Xue Xue Bao 2001;32:188–190.
36 Chen J, Peters R, Saha P, Lee P, Theodoras A, Pagano M, Wagner G, Dutta A: A 39 amino acid fragment of the cell cycle regulator p21 is sufficient to bind PCNA and partially inhibit DNA replication *in vivo*. Nucleic Acids Res 1996;24:1727–1733.
37 Gehen SC, Vitiello PF, Bambara RA, Keng PC, O'Reilly MA: Downregulation of PCNA potentiates p21-mediated growth inhibition in response to hyperoxia. Am J Physiol Lung Cell Mol Physiol 2007;292:L716-L724.
38 Bertoli C, Skotheim JM, de Bruin RAM: Control of cell cycle transcription during G1 and S phases. Nat Rev Mol Cell Biol 2013;14:518–528.
39 Fulda S: Tumor resistance to apoptosis. Int J Cancer 2009;124:511–515.
40 Arbiser JL, Bonner MY, Gilbert LC: Targeting the duality of cancer. NPJ Precis Oncol. NPJ Precis Oncol 2017;1:1-7.
41 Lowe SW, Lin AW: Apoptosis in cancer. Carcinogenesis 2000;21:485–495.
42 Fulda S, Galluzzi L, Kroemer G: Targeting mitochondria for cancer therapy. Nat Rev Drug Discov 2010;9:447–464.
43 Perlman H, Zhang X, Chen MW, Walsh K, Buttyan R: An elevated bax/bcl-2 ratio corresponds with the onset of prostate epithelial cell apoptosis. Cell Death Differ 1999;6:48–54.
44 Gonzalez MS, De Brasi CD, Bianchini M, Gargallo P, Moiraghi B, Bengio R, Larripa IB: BAX/BCL-XL gene expression ratio inversely correlates with disease progression in chronic myeloid leukemia. Blood Cells Mol Dis 2010;45:192–196.
45 Bratton SB, Lewis J, Butterworth M, Duckett CS, Cohen GM: XIAP inhibition of caspase-3 preserves its association with the Apaf-1 apoptosome and prevents CD95- and Bax-induced apoptosis. Cell Death Differ 2002;9:881–892.
46 Roy N, Deveraux QL, Takahashi R, Salvesen GS, Reed JC:The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. EMBO J 1997;16:6914–6925.
47 Deveraux QL, Roy N, Stennicke HR, Van Arsdale T, Zhou Q, Srinivasula SM, Alnermi ES, Salvesen GS, Reed JC: IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. EMBO J 1998;17:2215–2223.
48 Soldani C, Scovassi AI: Poly(ADP-ribose) polymerase-1 cleavage during apoptosis: an update. Apoptosis 2002;7:321–328.
49 Dhillon AS, Hagan S, Rath O, Kolch W: MAP kinase signalling pathways in cancer. Oncogene 2007;26:3279–3290.
50 Shaulian E: AP-1--The Jun proteins: Oncogenes or tumor suppressors in disguise? Cell Signal 2010;22:894–899.