**SP3 is associated with migration, invasion and Akt/PKB signalling in MDA-MB-231 breast cancer cells**

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Running title: SP3 promotes progression of breast cancer

**Data availability statement:** the data supporting the findings of this study are available upon request.

**Abstract**

Specificity proteins (SP) exert pro-oncogenic functions in cancer cells ranging from cancer cell proliferation, migration, invasion and angiogenesis. There is a strong evidence that several antineoplastic drugs target depletion of SP proteins via different pathways. However, the mode of action of SP3 and the underlying consequences of its depletion are not well understood. Here, we demonstrate that SP3 is overexpressed in invasive breast cancer cells vs normal counterparts. Gene expression analysis from TCGA datasets indicated that SP3 is strongly correlated with Akt-signalling related proteins, GNA13 (G Protein Subunit Alpha 13) and RAB33B (RAB33B, Member RAS Oncogene Family). RNAi of SP3 decreased active phosphorylation of Akt at serine and threonine sites. These findings indicate that SP3 exhibits pro-oncogenic function which clearly fits the description of a NOA (non-oncogene addiction) gene. Future analyses are prompted to uncover SP3 gene regulation function and to reveal downstream targets of SP3 in breast cancer.

**Keywords**: Breast Cancer; SP3; transcription factor; Akt signalling; GNA13

1. **Introduction**

Breast carcinoma is a heterogeneous disease in which there are remarkable differences in cell types contributing to drug resistance and tumour recurrence [1]. Stages of breast cancer development have been linked to temporal-dependent mutations and chromosomal translocations which lead to oncogenes activation and tumour suppressor genes deactivation [2]. Gene expression profiling and immunohistochemical expression proved this heterogeneity and achieved a huge progress in our understanding of this heterogeneity [3]. These advances lead to a more precise classification including luminal A, luminal B, basal-like, HER2+ and normal subtypes [4]. The basal subtype is characterized by the absence of oestrogen receptor (ERα), Progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2), hence it is known as triple negative. However, basal and triple-negative are not completely interchangeable [5]. Regarding sensitivity to cancer therapies, luminal breast cancer cells are inhibited by the phosphoinositide 3-kinase inhibitor GSK1069615 and the Akt inhibitor GSK690693. However, basal cells preferentially respond to the MEK protein kinase inhibitor GSK1120212 [6].

Specificity proteins (SP) are 25 transcription factors (TFs) playing crucial roles in early development and maintaining cellular homeostasis [7]. SP1 was the first member identified and characterized, and SP1 knockout in mouse embryos exhibited retarded development and embryo lethality [8]. SP1-9 share structural homology with C2H2 zinc finger DNA binding domain, which binds to GC-boxes more than CT and GT boxes. Also, SP1-4 contain an N-terminus transactivation domain (TAD), whereas SP5-9 lack this domain [7]. These fine structural differences confer the different functions of these TFs in normal development and diseases including cancer. Numerous studies report that SP proteins especially SP1 and SP3 are highly expressed in tumour vs. non-tumour tissues and are poor prognostic indicators for cancer patients including breast cancers [9-12].

SP3 acts as an activator or repressor depending on isoform and/or post-translational modifications (PTMs) [11]. SP3 binds to GT and GC boxes promoter elements competing with SP1 for the GC-box promoters [13]. SP3 activates a number of genes related to crucial biological processes such as cell-cycle regulation, hormone-induction and housekeeping. Mechanistically, SP3 regulates transcription by binding to consensus GC- and GT-box regulatory elements in regulated genes. The protein contains a zinc finger DNA-binding domain and several transactivation domains with four different transcript variants encoding different isoforms [14].

Most cancer studies have focused on the pro-oncogenic role of SP1 in cancer cells as a promoter of cell growth, survival and migration/invasion [11,15]. However, there is a lack of cancer studies investigating the functional role of SP3 in cancer cells especially breast cancer. In this study, we demonstrated that SP3 is overexpressed in invasive breast carcinoma (mainly ER+ PR+ HER2-) and invasive breast cancer cells vs normal or non-invasive counterparts. SP3 is associated with breast tumour stages 1-3 and invasion of MDA-MB-231 breast cancer cells. Gene expression analysis from human datasets indicated that SP3 is strongly correlated with Akt-signalling related protein GNA13 (G Protein Subunit Alpha 13) and the tumour suppressor protein CTCF (CCCTC-Binding Factor). Interestingly, knockdown of SP3 decreased active phosphorylation of Akt at serine and threonine sites. These findings indicate that SP3 exhibits pro-oncogenic function which clearly fits the description of a NOA (non-oncogene addiction) gene in breast carcinoma.

1. **Materials and Methods**
	1. ***Culture cells and antibodies***

Breast epithelial (ZenBio MBE, MCF10A) and cancer cells (MCF7, T47D, MDA-MB-453, BT20, Hs578T and MDA-MB-231), were obtained from the American Type Culture Collection (ATCC). The antibodies used were purchased from the following manufacturers: Anti-SP3 antibody, Santa cruz, CA, USA; anti-SATB2 antibody, Abcam, Cambridge, UK; anti-SATB1, anti-phospho-Akt (Ser473), anti-phospho-Akt (Thr308) and anti-Akt antibodies, cell signalling technology®, Danvers, MA, USA; anti-α-tubulin and anti-β-actin antibodies, Sigma-Aldrich, St. Louis, MO, USA.

* 1. ***siRNA transfection***

Knockdown of SP3 gene in MDA-MB-231 was performed by RNAi technology using siRNAs. siRNA or short-interfering RNA is the most commonly knockdown tool for short-term silencing of genes of interest. siRNA targeting SP3 was obtained from Sigma-Aldrich (St. Louis, MO, USA). The sequence of the control siRNA is 5′-CUUACGCUGAGUACUUCGATT-3′. Using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA), MDA-MB-231 cells were transfected with 50 nM of SP3 siRNA according to the manufacturer's instructions and as described before [16].

* 1. ***Oncomine database analysis***

In order to analyse the expression pattern of SP3 in cancer gene datasets especially breast cancer, we used the cancer microarray database Oncomine (https://www.oncomine.org). In brief, SP3 gene was searched in metastatic breast cancer dataset and the results were filtered by selecting breast carcinoma vs. normal analysis (https://www.oncomine.org/resource) and as described before [17].

* 1. ***Immunoblot (IB) analysis***

Western blotting or immunoblot analysis was performed as described previously [18]. In brief, protein lysates were prepared by 2X sample buffer (1M Tris-HCl (pH 6.8), SDS, Glycerol, BPB (bromophenol blue, 2-Mercaptoethanol) for 5 min at 100°C. The protein concentration was measured in each sample using Lowry protein assay (Thermal Scientific). 15-20 µg protein from each sample were loaded on SDS-polyacrylamide gel electrophoresis (PAGE) which is transferred to a polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were incubated in phosphate buffered saline (PBS) containing 1% non-fat skim milk for 1 h at room temperature for blocking nonspecific binding to proteins. Primary antibody was diluted in blocking buffer and incubated with the membrane at 4˚C overnight. The membranes were washed with TBS-T buffer 3 times 10 min each and incubated with HRP-labelled secondary antibodies for 1 h at room temperature. The membranes were then washed, and protein bands' signals were visualized using ECL system (Nacalai tesque, Kyoto, Japan). The signals' intensities were detected and measured using Light Capture II equipped with CS analyser and ImageJ software package.

* 1. ***Migration assay***

In order to measure migration of cancer cells, the lower surface of the filter of 24-well Boyden chambers was coated with fibronectin overnight. Then, 5 × 104 cancer cells were loaded onto the upper chamber of Boyden chambers with starved medium (DMEM and 0.1% BSA), whereas the lower chamber was filled with nutrients and 10% chemoattractant FBS. Six hours after loading, the cells were fixed, stained with 0.5% crystal violet and washed 3 times in PBS for counting. Cells that migrated to the lower chamber were monitored under a microscope (40X magnification) and counted in 5 randomly selected fields as described previously [19]. Three independent experiments were performed independently.

* 1. ***Matrigel invasion assay***

In order to measure invasion of cancer cells, the filter of 24-well Boyden chambers was coated with Matrigel (BD Biosciences, San Jose, CA, USA) overnight. 1 x 105 cells were loaded onto the upper chamber with starved medium (DMEM and 0.1% BSA), whereas the lower chamber was filled with nutrients and 10% chemoattractant FBS. After 18 h incubation at 37°C, the cells were fixed using, stained with 0.5% crystal violet and washed 3 times in PBS for counting. Cells invading the lower chamber were monitored under a microscope (40x magnification) and counted in 5 randomly selected fields as described previously [20]. Three independent experiments were performed independently.

* 1. ***SP3 homology modelling***

Swiss-model server queries for different protein sequences with Blast and HHBlits against template library. Models are built based on SP3-5wjq.1.c alignment using ProMod3. Coordinates conserved between SP3 and 5wjq.1.c are copied from the 5wjq.1.c to SP3 model. The geometry of the resulting SP3 model is regularized using a force field. Homo-oligomeric structure of the SP3 protein is predicted based on the analysis of pairwise interfaces of the identified 5wjq.1.c structure. Further details of model building and other parameters including oligomeric state conservation, quality estimation and ligand modelling can be found on the server (https://swissmodel.expasy.org).

* 1. ***Statistical analysis***

All presented data are in the format of mean ± Standard Error (SE). Statistical analyses for cancer cell experiments were performed using unpaired t-test by Prism (Graphpad). *P values (\*P < 0.05, \*\*P < 0.01, and \*\*\*P < 0.001)* were considered statistically significant. Fiji (Fiji Is Just ImageJ) is used for western blot band intensity analysis relative to control marker.

1. **Results**
	1. ***Genetic alterations of SP3 in multiple types of cancers***

In order to reveal SP3 function in cancer, we first examined SP3 genetic mutations in several types of cancers using phosphosite database (https://www.phosphosite.org). SP3 gene mutations occur in malignancies including endometrial, bladder, colorectal, stomach, prostate and breast cancers (Fig. 1A). Percentage (%) of tumour samples showing mutations of SP3 range from 0.5-4%. Since SP3 is highly associated with malignant phenotype of breast carcinoma, we investigated SP3 expression in normal epithelial breast vs. invasive breast carcinoma using independent datasets from Oncomine database. Importantly, Fig. 1B exhibits up to 3-fold (Log2 median-centred ratio) increase of SP3 expression in breast cancer patients as compared to normal breast tissues. For further analysis of SP3 association with ER, PR and HER2 status, and tumour stage, we analysed The Cancer Genome Atlas (TCGA) using cBioPortal breast cancer (METABRIC, Nature 2012 & Nat Comm 2016). SP3 mutation spectrum in breast cancer includes gene amplification and high mRNA expression (Fig. 1C). Most of cancer patients with high SP3 expression have ER+ PR+ HER2- status. This finding indicates the strong association between SP3 expression and the presence of ER & PR but not HER2. Moreover, high SP3 expression are found in tumour stages 1-4 giving importance of SP3 in development and progression of the disease.

We further examined the level of expression of SP3 protein in multiple breast cancer cells vs. normal cells. To measure SP3 expression as compared to normal breast cells, we used FiJi analysis software to compare SP3 band intensity in different samples relative to Actin (control). SP3 expression (79 & 108 KDa) is higher in most of breast cancer cells as compared to normal breast cells (ZenBio MBE & MCF10A) (Fig. 1D & 1E). Interestingly, SP3 expression was even higher than the expression of transcription factor SATB1 which is mediating strong oncogenic phenotype in different cancers including breast cancer as previously reported [16].

* 1. ***TCGA gene expression analysis for SP3***

To dissect the molecular pathways related to SP3 in breast cancer, we performed The Cancer Genome Atlas (TCGA, Provisional) profiling of SP3 in human breast cancer patient’s dataset (METABRIC, Nature 2012 & Nat Comm 2016). Both SP3 and SP1 are closely related transcription factors. Both proteins contain a highly conserved DNA-binding domain (C terminus) and two Gln-rich domains (N terminus). We first measured correlation analysis between SP1 & SP3 in breast cancer patients’ samples. As shown in Fig. 2A, the two proteins are not significantly correlated which suggest that each gene has distinct functions. However, this does not completely exclude the possibility of redundancy of the two proteins as reported in previous studies [13]. Then, we analysed the top positively and inversely correlated genes with SP3 to dissect molecular mechanisms of SP3 in breast cancer.

Among top ten genes positively correlated with SP3, GNA13 (G Protein Subunit Alpha 13) and RAB33B (RAB33B, Member RAS Oncogene Family) have been identified (Table 1). GNA13 regulates several oncogenic pathways including signalling by Rho GTPases and Akt/Protein Kinase B (PKB). Also, GNA13 exerts a pro-oncogenci function in several human cancers [21,22]. On the other hand, among inversely correlated genes with SP3, several tumour suppressor genes were identified such as CTCF (CCCTC Binding Factor) (Table 2). CTCF acts as a transcriptional repressor which binds to promoters of MYC & BAG1 genes [23,24]. To define correlation between SP3, RAB33B, GNA13 and CTCF at mRNA level in clinical invasive breast cancer specimens, we used cBioPortal platform. As shown in Fig. 2B & 2C, there is a strong positive correlation between SP3 and RAB33B (Pearson: 0.81; Spearman: 0.77; P<0.001) & GNA13 (Pearson: 0.79; Spearman: 0.75; P<0.001). Conversely, SP3 inversely correlated with CTCF (Pearson: -0.36; Spearman: -0.35; P<0.001) (Fig. 2D). To confirm the strong association between SP3, SP1, RAB33B, GNA13 and CTCF, we checked the expression of the five genes in patients with breast cancer (Fig. 2E). Consistently, SP3, RAB33B and GNA13 are highly expressed, but CTCF is under expressed in majority of breast cancer patients. Moreover, expressions of SP3 & SP1 are not correlated in clinical samples as shown in Fig. 2E.

* 1. ***Knockdown of SP3 inhibits Akt/PKB signalling, migration and invasion***

To investigate the mechanistic insights of SP3 in breast cancer cells *in vitro*, we chose MDA-MB-231 as a model for breast cancer given its strong metastatic and aggressive characteristics. By using short interfering RNA (siRNA) technology, we depleted the expression of SP3 in MDA-MB-231 cells as shown in Fig. 3A. We further measured band intensity using FiJi software. As shown in Fig. 3B, SP3 expression in SP3 siRNA is much lower than control siRNA relative to Actin as a loading control. Since SP3 is strongly correlated with GNA13 which activates Akt/Protein Kinase B (PKB) signalling [21], we checked Akt activity upon SP3 depletion. Surprisingly, silencing SP3 inhibited the active phosphorylation of Akt at Ser473 and Thr308 (Fig. 3A) which mediates the activity of Akt signalling in breast cancer [25]. Then, we investigated whether SP3 is driving MDA-MB-231 migration and invasion. As shown in Fig. 3C, silencing SP3 using siRNA in MDA-MB-231 drives mesenchymal epithelial transition (MET) using phase contrast images. Also, using Boyden chambers, we assessed the impact of SP3 depletion on migration and invasion with the absence or presence of Matrigel in extracellular matrix (ECM) respectively. By counting the number of migrated or invaded cells using 5 independent fields, we found a significant decrease in migration (*p<0.01* & *p<0.001*) and invasion (*p<0.05* & *p<0.01*) giving strong association of SP3 activity and malignant phenotype of MDA-MB-231 cells (Fig. 3D and 3E).

* 1. ***Homology Modelling of SP3***

Due to the lack of pure 3-dimensional (3D) structure of SP3 in Protein data bank (https://www.rcsb.org), we used SWISS-MODEL to predict structure of SP3. The SWISS-MODEL template library was queried for SP3 with Blast and HHBlits for similar structures. The template protein was found to be 5wjq.1.C which is a zinc finger protein with 31.82 sequence identity and 0.36 sequence similarity (Fig. 4A and 4B). SP3 shares residues spanning 556-714 amino acids with the template. The software predicted formation of four helices after comparing amino acids residues with the template protein (5wjq.1.C) (Fig. 4C and 4D). These findings confirm the previously reported data about SP3 structural analysis [26]. Importantly, this includes the presence of highly conserved DNA binding domain (3 adjacent Cys2His2- type zinc fingers) and serine/threonine-rich subregions as sites of post-translational modifications.

1. **Discussion**

Our findings highlight the crucial role of SP3 expression in breast cancer cells’ migration and invasion. Although members of SP transcription factors are critical for development, there are accumulating evidences that these proteins are overexpressed in various types of cancers [11,27-29]. The major structural difference between SP1 and SP3 is the location of the inhibitory domain. Whilst Sp1 has the inhibitory domain at the N-terminus, the Sp3 inhibitory domain is located immediately in front of the DNA-binding domain. This structural difference confers the functional distinction of SP1 and SP3 [30]. Members of the miR-17-92 (miR-20a/miR-17-50) and miR-239∼27a∼24-2 (miR-27a) clusters are highly expressed in cancer cells and are upstream regulators of SP proteins to maintain their high expressions in cancer cells [31,32].

RNAi studies showed that individual depletion of one of SP proteins suppresses tumourigenesis [11]. RNAi of SP1, SP3 or SP4 in MDA-MB-231 breast, 786-O kidney, SW480 colon, A549 lung, and pancreatic cancer cells results in cell growth inhibition, survival decrease, and inhibition of migration and invasion. SP knockdown decreases several SP-regulated genes which has pro-oncogenic functions such as survivin, bcl-2, VEGF, and EGFR [33]. Given the oncogenic-like activity of SP proteins and their overexpression in cancer cells, SP genes are defined as NOA genes which are “attractive drug targets” [34].

Four isoforms of SP3 are expressed in vivo and retain different parts of the N terminus. Whilst these isoforms are derived from alternative translational start sites (1, 37, 856 & 907), an upstream ORF (position -47 to -18) regulates expression of the 2 long isoforms [13]. Although SP1 and SP3 share structural similarity, unlike SP1, none of SP3 isoforms is glycosylated. Alternatively, SP3 isoforms are SUMO-modified specifically and exclusively at Lys 551 [13]. Our results demonstrate that Sp3 has many unique features and is not simply a functional equivalent of Sp1 since SP1 & SP3 are not correlated in clinical samples analysed using TCGA. However, this hypothesis doesn’t completely exclude the possibility of redundancy in function between the two proteins which requires further studies. Gene expression analysis studies showed that SP transcription factors regulate unique and common sets of genes associated with carcinogenesis with the maximum gene overlap between SP3 and SP4 [11]. Also, Ingenuity Pathway Analysis (IPA) confirmed that three SP TFs regulate both cancer-promoting and cancer-inhibiting genes. However, the analysis strongly predicted that SP1, SP3, and SP4 are associated with cancer cell growth, survival and migration/invasion and not the reverse pathways [11].

Consistently, our results demonstrated that SP3 correlates with RAB33B and the signal transducing activator GNA13. GNA13 has an important role in promoting tumourigenicity of several tumours and represent a prognostic biomarker and therapeutic target [21]. GNA13 promotes G1/S cell cycle transition by upregulating c-Myc and cyclin-dependent kinase (CDK) regulator cyclin D1, however suppressing FOXO1 activity and CDK inhibitors p21Cip1 and p27Kip1 [21]. Also, GNA13 activates Akt/PKB and ERK signalling in cancer cells [22]. This function is consistent with our finding showing inhibition of Akt/PKB signalling upon SP3 depletion. In summary, we propose SP3 to function as a bifunctional transcription factor that either stimulates or represses the transcription of numerous genes. SP3 confers migration and invasion of breast cancer cells via activation of Akt/PKB signalling. The pro-oncogenic function of SP3 in breast cancer prompts further research to assess its clinical efficacy as a therapeutic target.

**Compliance with Ethical Standards
Funding:**No funding received for this study.
**Disclosure of potential conflicts of interest:**The author declares that there is no conflict of interest
**Informed consent:** Not applicable

**Figure Legends**

**Figure 1.** SP3 is upregulated in clinical breast cancer tissues and cell lines. (A) The percentage (%) of tumour samples showing mutations of SP3 in different cancers was calculated using phosphosite database. (B) Oncomine database analysis using invasive breast cancer vs. normal breast datasets showing the relative expression of SP3 in clinical breast cancer tissues as compared to the normal breast tissue. (C) Analysis of The Cancer Genome Atlas (TCGA) cBioPortal for SP3 mutation spectrum and its association with ER, PR & HER2 states in addition to tumour stage. (D) The expression levels of SATB1, SATB2 & SP3 were checked in breast cancer cells (Non-invasive & invasive) by western blotting. Β-actin was used as a loading control for the western blot analysis. (E) Band signal intensity is analysed by FiJi software for SP3 expression in different samples relative to Actin as loading control (1 replicate).

**Figure 2.** TCGA correlation analysis of SP3, SP1, RAB33B, GNA13 & CTCF (A) Analysis of TCGA metastatic breast cancer database (TCGA, Provisional) using cBioPortal showing the correlation between SP3 and SP1 mRNA levels. (B) The correlation analysis performed by TCGA to compare between SP3 and RAB33B mRNA levels. (C) The correlation analysis performed by TCGA to compare between SP3 and GNA13 mRNA levels. (D) The correlation analysis performed by TCGA to compare between SP3 and CTCF mRNA levels. (E) The TCGA expression heatmap of SP3, SP1, RAB33B, GNA13 & CTCF in breast cancer patients.

**Figure 3.** Silencing SP3 inhibits Akt signalling and MDA-MB-231 cells migration & invasion. (A) The expression levels of the indicated proteins (SP3, pAkt (Ser473), pAkt (Thr308) & Akt) in MDA-MB-231 cells were examined by immunoblotting with β-actin as the loading control. (B) Band signal intensity is analysed by FiJi software for SP3 expression in SP3 and control siRNAs relative to Actin as loading control (1 replicate). (C) Representative images of the cell morphology (Phase contrast; scale bar = 50 µm), migration and invasion of MDA-MB-231 cells with either control or SP3 siRNAs. (D) Migration graph indicates the average number (mean ± SE) of migrated cells per field (*\*P<0.05; \*\*P<0.01; \*\*\*P<0.001*). (E) Invasion graph indicates the average number (mean ± SE) of invaded cells per field *(\*P<0.05; \*\*P<0.01; \*\*\*P<0.001*).

**Figure 4.** Homology modelling of SP3. (A) Homology modelling analysis results indicating 5wjq.1.c as the most similar template for SP3 with 31.82 sequence identity. (B) SP3-template alignment by server Swiss-model. (C) The ribbon structure of SP3 protein is shown with coil, helix and strand. (D) Hydrophobicity of SP3 protein is shown with colours (orange red for the most hydrophobic to white at 0.0 to dodger blue for the most hydrophilic).

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