

Analysis of phosphatases in ER-negative breast cancers identifies DUSP4 as a critical regulator of growth and invasion

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Abstract Estrogen receptor (ER)-negative cancers have a poor prognosis, and few targeted therapies are available for their treatment. Our previous analyses have identified potential kinase targets critical for the growth of ER-negative, progesterone receptor (PR)-negative and HER2-negative, or “triple-negative” breast cancer (TNBC). Because phosphatases regulate the function of kinase signaling pathways, in this study, we investigated whether phosphatases are also differentially expressed in ER-negative compared to those in ER-positive breast cancers. We compared RNA expression in 98 human breast cancers (56 ER-positive and 42 ER-negative) to identify phosphatases differentially expressed in ER-negative compared to those in ER-positive breast cancers. We then examined the effects of one selected phosphatase, dual specificity phosphatase 4 (DUSP4), on proliferation, cell growth, migration and invasion, and on signaling pathways using protein microarray analyses of 172 proteins, including

phosphoproteins. We identified 48 phosphatase genes are significantly differentially expressed in ER-negative compared to those in ER-positive breast tumors. We discovered that 31 phosphatases were more highly expressed, while 11 were underexpressed specifically in ER-negative breast cancers. The DUSP4 gene is underexpressed in ER-negative breast cancer and is deleted in approximately 50 % of breast cancers. Induced DUSP4 expression suppresses both in vitro and in vivo growths of breast cancer cells. Our studies show that induced DUSP4 expression blocks the cell cycle at the G1/S checkpoint; inhibits ERK1/2, p38, JNK1, RB, and NFκB p65 phosphorylation; and inhibits invasiveness of TNBC cells. These results suggest that DUSP4 is a critical regulator of the growth and invasion of triple-negative breast cancer cells.

Keywords TNBC · Phosphatase · Mouse xenograft · MAPK pathways

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Introduction

Breast cancer is the most frequently diagnosed cancer in women and the second highest cause of cancer-related death in United States women in 2016 [1]. Selective estrogen receptor modulators (SERMs) and aromatase inhibitors are currently used to treat ER-positive breast cancers [2–6], while anti-HER2 drugs are used to treat ER-negative HER2-positive breast cancers [7–9]. However, there are few treatment options available for breast cancers that do not express ER, progesterone receptor (PR), or the HER2 protein, otherwise known as “triple-negative” breast cancers (TNBCs). As no targeted therapy is available for most TNBC tumors, cytotoxic chemotherapy is the current treatment strategy.

We previously conducted cDNA microarray studies to identify potential therapeutic targets of ER-negative breast cancers [10]. These studies identified several kinases critical for the growth of TNBC. In this study, we sought to identify phosphatases differentially expressed in ER-negative tumors that also regulate breast cancer growth. We identified 11 underexpressed and 31 overexpressed phosphatases in ER-negative compared to those in ER-positive breast cancers. We initially focused on those 11 phosphatases underexpressed exhibiting decreased expression in ER-negative tumors and found that of these 11 underexpressed phosphatases, *DUSP4* is the most frequently deleted phosphatase in ER-negative breast cancer.

DUSP4 specifically regulates extracellular regulated kinase (ERK), and the phosphatase activity of *DUSP4* is enhanced upon interaction with ERK and p38 [11–13]. *DUSP4* expression has been shown to play an important role in senescence [14] and emerging evidence suggests *DUSP4* is involved in the growth and progression of cancer [15, 16]. Our results demonstrate that *DUSP4* is frequently deleted in breast cancer and underexpressed in TNBCs, and that *DUSP4* overexpression halts TNBC tumor growth and invasion. Our results also demonstrate that all three MAPK proteins (ERK1/2, p38, and JNK1) are negatively regulated by *DUSP4*. Moreover, re-expression of *DUSP4* also inhibits other growth inducing pathways, including NF κ B and Rb. Overall, this study demonstrates that *DUSP4* is frequently deleted in breast cancers and is a critical regulator of growth and invasion of TNBCs, suggesting *DUSP4* is an important tumor suppressor gene in these aggressive breast cancers.

Materials and methods

Breast tumor and microarray datasets

All tumors were collected by Dr. Jenny Chang and approved by the institutional review board of Baylor College of Medicine. This tumor set, including tumor stage, size, and patient race and menopausal status, has been previously described [10]. In this study, four samples were removed from the analysis. These four samples had an unconfirmed ER status or appeared as outliers on the principal component analysis (PCA) plot and thus removed from the dataset. Therefore, 98 samples, 56 ER-positive and 42 ER-negative invasive breast cancers, were used for this analysis. Gene expression was estimated using the Robust Multi-array Average (RMA) procedure. We limited our data and clustering analysis to 262 genes (454 probe-sets on Affymetrix U133A chip), which encode known phosphatases and proteins with phosphatase in their name. We used the most variable probe-set for each of the 262

genes. Statistical analysis was done using Partek software (<http://partek.com>). The following criteria were used to find genes differentially expressed between ER-negative and ER-positive tumors: Benjamini–Hochberg false discovery rate (FDR) p value ≤ 0.1 , fold change ≥ 1.2 or ≤ 0.8 .

Cell lines and cell culture

The MCF-7 (HTB-22), MDA-MB-231 (HTB-26), and MDA-MB-468 (HTB-132) cell lines (American Type Culture Collection (ATCC), Manassas, VA, USA) were cultured in DMEM (Cellgro by Mediatech, Inc., Manassas, VA, USA) supplemented with 10 % fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin. These were grown and maintained as described in Supplementary Methods. STR profiles were compared to (1) known ATCC fingerprints (ATCC.org); (2) the Cell Line Integrated Molecular Authentication database (CLIMA) version 0.1.200808 (<http://bioinformatics.istge.it/clima/>) [17]; and (3) the MD Anderson fingerprint database.

Reagents and antibodies

The *DUSP4* antibody was purchased from BD Transduction Laboratories (Lexington, KY, USA). Phospho-ERK1/2 (#4370) and total ERK1/2 (#4372) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antiactin antibody was purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Antimouse (#NA931 V) and antirabbit (NA934 V) secondary antibodies were obtained from GE Healthcare Bio-sciences Corp. (Piscataway, NJ, USA).

Viral vectors and modification of cell lines

The *DUSP4* ORF clone was obtained from Open Biosystems (Huntsville, AL, USA) and cloned into tetracycline (Tet)-inducible vector (pTIPZ) through a gateway LR clonase reaction. Final constructs were verified through restriction digestion and sequencing. Lentiviral vectors were prepared as described previously [18]. Stable cell lines expressing inducible cDNAs were generated by lentiviral infection using a pTIPZ lentiviral expression system in the presence of 4 μ g/ml polybrene, followed by puromycin selection at 48 h post infection. All pTIPZ stable cell lines were maintained in media with Tet-safe serum (Clontech Laboratories Inc., Mountain View, CA, USA).

Quantitative RT-PCR (qRT-PCR) assessments

Quantitative PCR assays of reverse-transcribed transcripts (4 replicates per transcript) were carried out using an ABI

PRISM 7900 Sequence Detection System (Life Technologies (formerly Applied Biosystems Inc., Foster City, CA, USA), as previously described [10].

Cell growth assays

Cellular proliferation and anchorage-independent growth were measured as described previously [19].

Cell cycle assays

To measure cell cycle distribution, Tet-inducible cells were treated for 4 days with or without doxycycline (2 µg/ml) to induce DUSP4 gene expression. Cells were then harvested and fixed overnight in 70 % ethanol at −20 °C. Cells were then stained with propidium iodide (PI) (1 µg/ml) in 0.1 % Triton X-100 and RNase in PBS and analyzed using a FACSCalibur Flow Cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Western blot analysis

Western blot analyses were performed as described previously [19]. Antibodies used include anti-DUSP4 antibody (BD Biosciences, San Jose, CA, 1:1000), anti-phospho-ERK1/2 antibody (1:1000), and anti-ERK1/2 (1:1000) (both from Cell Signaling Technology Inc., Danvers, MA, USA). Anti-Vinculin antibody (Sigma-Aldrich Corp., St. Louis, MO, USA) was used at a 1:2000 dilution.

Boyden chamber invasion assay

Matrigel-coated transwell cell culture chambers (BD Transduction Laboratory, Lexington, KY, USA) and inserts of 8 µm pore size (Corning Inc. Life Sciences, Lowell, MA, USA) were used to measure invasion of TIPZ-DUSP4 in MDA-MB-231 and SUM 159 cells. 3×10^5 cells/well were placed in the upper chamber of the transwell inserts in serum-free media. Media containing 10 % FBS was placed in the lower chambers. All samples were incubated for 20 h at 37 °C in a humidified atmosphere with 5 % CO₂. Non-invasive cells in the upper chambers were removed by cotton swab. Invasive cells were then fixed and stained with HEMA3 (Fisher Scientific Company, LLC, Kalamazoo, MI, USA). Invasive cells on the lower surface of the filters which penetrated through the Matrigel were then mounted on glass slides, counted, and photographed using a light microscope at $\times 40$ magnification. Migration assays were performed using transwell inserts without Matrigel. Percent invasion was calculated based on number of cells invaded divided by number of cells migrated, and the results were multiplied by 100. All assays were performed in triplicate and the results shown as average \pm standard deviation.

Reverse phase protein array (RPPA)

RPPA assays were performed in the MD Anderson Core facility. Experimental details are provided in Supplementary Materials.

Mouse experiments

Experiments using nude mice (The Jackson Laboratory, Bar Harbor, ME, USA) were performed in accordance with M.D. Anderson Institutional Animal Care and Use Committee (IACUC)-approved protocols. Experimental details of our mouse experiments are provided in Supplementary Materials.

Results

Identification of phosphatases differentially expressed in ER-negative breast cancer

To identify phosphatases differentially expressed in ER-negative breast cancers, we compared RNA levels in ER-positive and ER-negative human breast cancer samples using a strategy analogous to our previous exploration of kinase expression [10]. Tumor samples from 98 invasive breast cancer patients (56 ER-positive, 42 ER-negative) were previously classified as ER positive or ER negative using IHC staining for ER α [10]. RNA expression in ER-positive and ER-negative breast tumors was then analyzed by Affymetrix gene expression profiling to identify molecules differentially expressed in ER-negative breast cancer compared to those in ER-positive breast cancers. We identified 31 overexpressed (fold ≥ 1.2 , FDR-adjusted $p \leq 0.1$) and 11 underexpressed phosphatases (fold ≤ 0.80 , adjusted $p \leq 0.1$) in ER-negative tumors compared to those in ER-positive tumors (Table 1; Fig. 1a). Figure 1b shows the hierarchical clustering seen when only ER-negative tumors are clustered using the differentially expressed genes identified in Fig. 1a. Through these analyses, we identified several phosphatases critical to cell signaling and metabolic pathways in both the over- and underexpressed phosphatase groups, including overexpressed phosphatases that regulate kinases and cell cycle progression (CDC25A, CDC25B, and CDKN3), and underexpressed phosphatases that regulate the MAPK and PI3 K pathways (INPP4B, DUSP4, and DUSP11).

Focusing on phosphatases underexpressed in ER-negative breast cancer, we next investigated whether these differentially expressed genes were homogeneously expressed in ER-negative tumors. As shown in Fig. 1b, these genes are differentially expressed in ER-negative tumors. The entire ER-negative group is divided into three

Table 1 Differential expression of overexpressed phosphatases in ER-negative versus ER-positive breast cancer

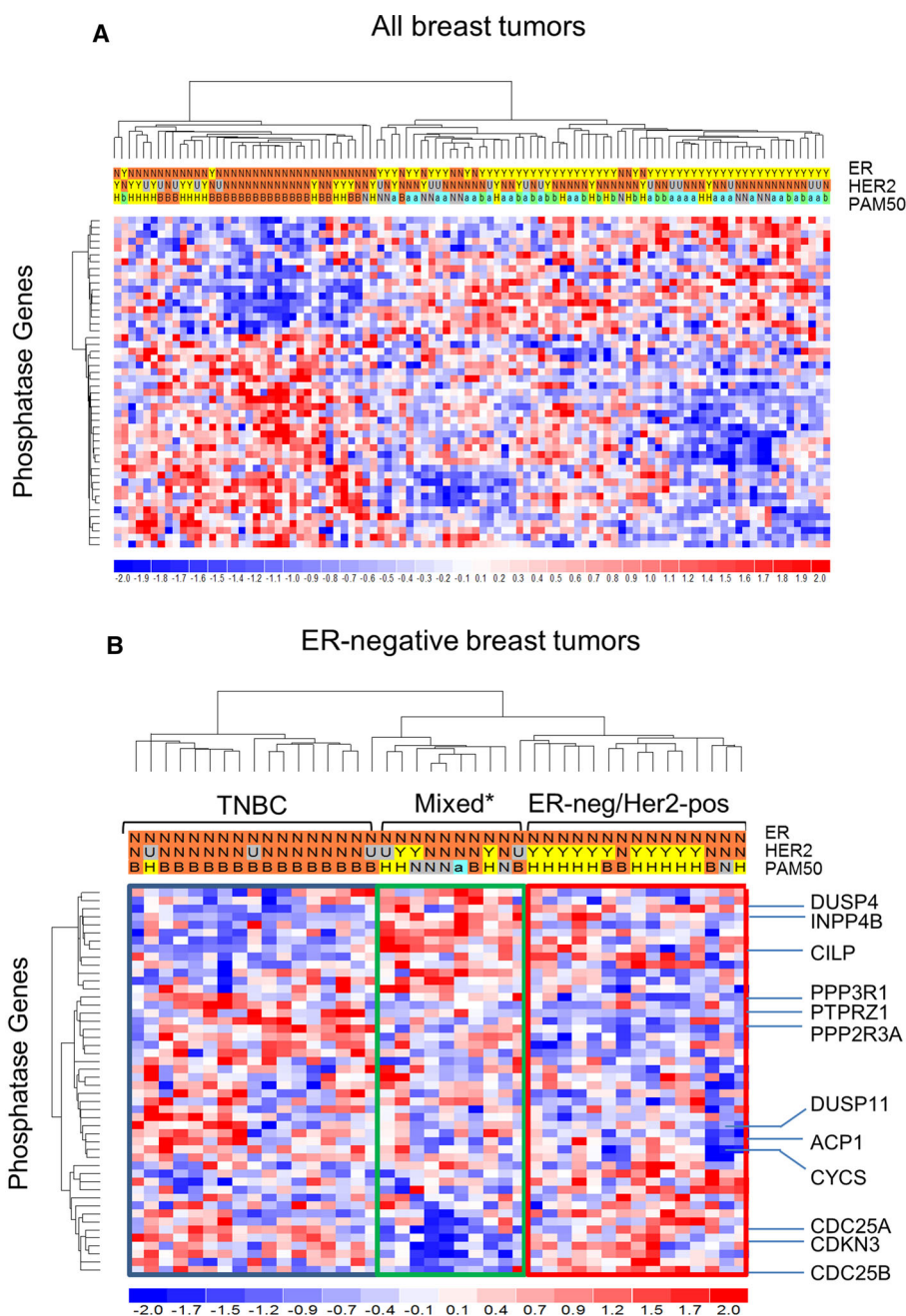
Gene name	Symbol	Fold	Adjusted <i>p</i> value ^a
<i>Overexpressed phosphatases (≥ 1.2-fold, p < 0.1)</i>			
Inositol(myo)-1(or 4)-monophosphatase 2	IMPA2	1.87	0.0006
Lipin 1	LPIN1	1.85	0.0000
Protein tyrosine phosphatase-like (proline instead of catalytic arginine), member A	PTPLA	1.84	0.0002
Discs, large (Drosophila) homolog-associated protein 5	DLGAP5	1.84	0.0004
Pyruvate dehydrogenase phosphatase catalytic subunit 1	PDP1	1.62	0.0009
Acid phosphatase 1, soluble	ACP1	1.56	0.0002
Protein tyrosine phosphatase, receptor-type, Z polypeptide 1	PTPRZ1	1.51	0.0073
Protein tyrosine phosphatase, receptor type, K	PTPRK	1.48	0.0239
Protein tyrosine phosphatase, receptor type, C	PTPRC	1.46	0.0956
Chromosome 12 open reading frame 5	C12orf5	1.43	0.0038
Protein phosphatase 3, regulatory subunit B, alpha	PPP3R1	1.36	0.0000
Pyrophosphatase (inorganic) 1	PPA1	1.36	0.0105
Protein phosphatase 1, catalytic subunit, beta isozyme	PPP1CB	1.34	0.0003
Cyclin-dependent kinase inhibitor 3	CDKN3	1.32	0.0508
Protein tyrosine phosphatase-like (proline instead of catalytic arginine), member B	PTPLB	1.28	0.0652
Cell division cycle 25B	CDC25B	1.27	0.0723
Protein tyrosine phosphatase, nonreceptor type 2	PTPN2	1.26	0.0067
Cell division cycle 14B	CDC14B	1.25	0.0531
Signal-regulatory protein alpha	SIRPA	1.25	0.0195
Protein phosphatase 2, regulatory subunit B'', alpha	PPP2R3A	1.24	0.0227
Protein tyrosine phosphatase, receptor type, F	PTPRF	1.24	0.0723
Myotubularin-related protein 2	MTMR2	1.23	0.0337
Nudix (nucleoside diphosphate linked moiety X)-type motif 15	NUDT15	1.23	0.0358
Cell division cycle 25A	CDC25A	1.23	0.0227
Cytochrome c, somatic	CYCS	1.22	0.0837
Dual specificity phosphatase 11 (RNA/RNP complex 1-interacting)	DUSP11	1.21	0.0358
Nudix (nucleoside diphosphate linked moiety X)-type motif 1	NUDT1	1.21	0.0780
Inositol monophosphatase domain containing 1	IMPAD1	1.20	0.0027
Protein tyrosine phosphatase, nonreceptor type 4 (megakaryocyte)	PTPN4	1.20	0.0227
Protein tyrosine phosphatase, nonreceptor type 22 (lymphoid)	PTPN22	1.20	0.0024
<i>Underexpressed phosphatases (≤0.80-fold, p < 0.1)</i>			
Protein phosphatase 1, regulatory subunit 3C	PPP1R3C	0.46	0.0001
Dual specificity phosphatase 4	DUSP4	0.47	0.0052
Ectonucleotide pyrophosphatase/phosphodiesterase 1	ENPP1	0.47	0.0000
Inositol polyphosphate-4-phosphatase, type II, 105 kDa	INPP4B	0.48	0.0001
Cartilage intermediate layer protein, nucleotide pyrophosphohydrolase	CILP	0.52	0.0001
Fructose-1,6-bisphosphatase 1	FBP1	0.58	0.0001
Protein tyrosine phosphatase, receptor type, T	PTPRT	0.71	0.0001
Myotubularin-related protein 9	MTMR9	0.75	0.0064
Protein tyrosine phosphatase, receptor type, G	PTPRG	0.75	0.0011
Calcium activated nucleotidase 1	CANT1	0.76	0.0931
Inositol polyphosphate-5-phosphatase J	INPP5 J	0.80	0.0006

^a FDR adjustment based on 262 features analyzed

major subgroups that express different sets of phosphatases: TNBC, mixed TNBC+Her2, and predominantly Her2-positive tumors. We identified six sets of

phosphatases differentially expressed in ER-negative tumors, including one set of underexpressed and one set of overexpressed phosphatases for each of the ER-negative

Fig. 1 Phosphatases differentially expressed in ER-negative and ER-positive breast cancers. **a** Supervised hierarchical clustering of breast cancers based on phosphatase expression that distinguishes ER-positive from ER-negative breast tumors. Clinical parameters (HER2, ER, Pam50) are annotated for the samples (*x*-axis), and gene signature is annotated for the genes (*y*-axis). **b** Hierarchical clustering analysis of only ER-negative breast tumors using the phosphatases genes (as shown) selected in **a**



Footnotes: *Mixed = TNBC; ER-neg/Her2-pos.

Abbreviations: ER & Her2 Banner: Y = Yes; N = No; U = unknown. **PAM50 Banner:** a = Luminal A; b = Luminal B; B = Basal; H = Her2; N = Normal.

subgroups (TNBC, mixed TNBC+Her2, and Her2-enriched) (Supplementary Table 1). One set of phosphatases, which includes INPP4B, DUSP4, and CILP, is underexpressed in the TNBC subgroup compared to that in the other 2 ER-negative subgroups, while another set, which includes PPP3R1, PTPLA, and PTPRZ1, is overexpressed in the TNBC subgroup compared to that in the other two

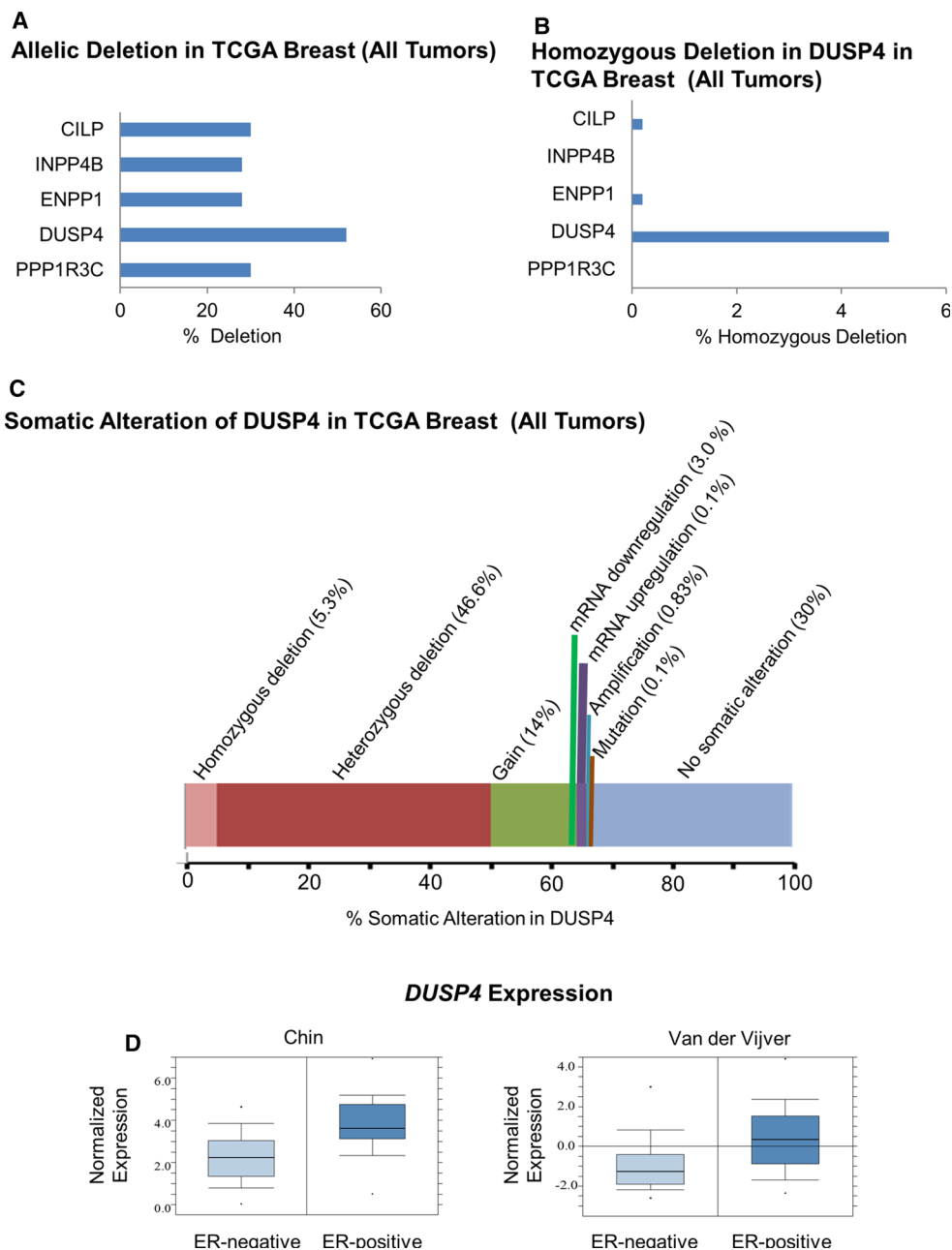
subgroups. A third set of phosphatases, which includes CDC25B, NUDT1, and DLGAP5, is overexpressed in the Her2-enriched subgroup compared to that in the other subgroups (Supplementary Table 1).

We next examined the Breast Invasive Carcinoma TCGA dataset (accessed through the cBioPortal for Cancer Genomics on 01/15/2014) with the genetic alteration

parameters to include both homozygous deletion and heterozygous loss [20, 21]. We found that among the 5 phosphatases most underexpressed in ER-negative tumors, *DUSP4* was the only phosphatase with high-frequency allele loss (Fig. 2a) and exhibited the highest degree of homozygous deletion (Fig. 2b). *DUSP4* is frequently somatically altered (66.6 %) in breast cancers in the TCGA dataset with high proportions of hetero (46.6 %)- and homozygous deletion (5.3 %) (Fig. 2c). In addition to gene loss, copy number gain (14 %), mRMA upregulation (3.0 %), and amplification (0.83 %) also occur. We also

analyzed *DUSP4* somatic alterations in both ER-negative and TNBC populations (Supplementary Figure 1). For this reason, we selected *DUSP4* for further study. We next conducted an examination of *DUSP4* expression across 10 independent publically available breast cancer microarray datasets (Supplementary Table 3) [22–31]. We selected datasets with >100 patients which displayed differential *DUSP4* expression in ER-positive versus ER-negative breast cancers ($p < 0.001$). Differential expression of *DUSP4* was observed in all 10 datasets (two are shown in Fig. 2d).

Fig. 2 Genomic alteration and expression of phosphatases. **a** Percent of all tumors with any deletion among the top five underexpressed genes in the TCGA dataset. **b** Homozygous deletion of selected phosphatases in TCGA. **c** All somatic alterations of *DUSP4* gene represented by the cBio Oncoprint. **d** Differential expression of *DUSP4* in two breast cancer datasets



Inducible expression of DUSP4 inhibits ER-negative but not ER-positive breast cancer cell growth in vitro

To determine the role of DUSP4 in regulating growth of ER-negative breast cancer cells, we cloned the *DUSP4* into a tetracycline (Tet)-inducible vector (pTIPZ). Lentiviral particles containing pTIPZ-DUSP4 or pTIPZ-vector were infected and stable pools of ER-negative (SUM 159, MDA-MB-231) and ER-positive (MCF-7) cell lines were generated by puromycin selection for doxycycline-inducible DUSP4 expression. After 4 days of doxycycline induction, DUSP4 expression was determined by Western blot using anti-DUSP4 antibody. Our results show that after 4 days of doxycycline treatment, DUSP4 expression was significantly induced in all three cell lines (Fig. 3a).

We then determined the effect of DUSP4 on breast cancer cell growth with and without doxycycline using the trypan blue exclusion assay. Our results show that induced DUSP4 expression strongly suppresses cell growth in DUSP4-transfected ER-negative cell lines (SUM159: 89 % repression; MDA-MB-231: 83 % repression), but not in DUSP4-transfected MCF7 ER-positive breast cancer cells (Fig. 3b–d, respectively). Conversely, no detectable change in cell growth rate is associated with doxycycline treatment in any of the vector-transfected cells. Collectively, these results demonstrate induction of DUSP4 inhibits proliferation of ER-negative but not ER-positive breast cancer cells.

Induction of DUSP4 inhibits anchorage-independent growth of ER-negative but not ER-positive breast cancer cells

We next investigated whether DUSP4 regulates anchorage-independent growth of ER-negative and ER-positive breast cancer cells using the doxycycline-inducible cell lines described above. Our results show induced DUSP4 expression significantly suppresses anchorage-independent growth of DUSP4-transfected ER-negative cells (SUM 159: 95 % suppression; MDA-MB-231: 85 % suppression), but not DUSP4-transfected MCF7 ER-positive cells (Fig. 3e–g, respectively). Vector clones show no change in anchorage-independent growth when treated with doxycycline. These results further demonstrate that DUSP4 induction inhibits growth of ER-negative but not ER-positive breast cancer cells.

Induction of DUSP4 inhibits ER-negative breast cancer growth in vivo

We next investigated whether DUSP4 expression inhibits in vivo growth of TNBCs using nude and SCID mouse

models. After injected cell lines grew into tumors approximately 50 mm³ in volume, mice were randomized to receive either doxycycline-treated or nondoxycycline-treated water. Growth rate of MDA-MB-231 vector clones was not affected by doxycycline treatment (Fig. 4a). However, strong suppression of tumor growth was associated with doxycycline-induced DUSP4 expression in MDA-MB-231 xenografts compared to nondoxycycline treatment (Fig. 4c). Furthermore, while no difference in growth rate (slope) was observed in MDA-MB-231-vector control clones (Fig. 4b), there was a significant difference in MDA-MB-231-DUSP4 clone growth rates depending on the presence and absence of doxycycline ($p = 0.03$) (Fig. 4d). Significant differences in tumor growth were also observed in inducible SUM159–DUSP4 clones implanted into the mammary fat pad of SCID mice in the presence or absence of doxycycline, but not in SUM159-vector clones (Fig. 4e, g). Similarly, while no difference was apparent in the growth rate of SUM159-vector control clones, there was a significant difference in the SUM 159-DUSP4 clone growth rates in the presence and absence of doxycycline ($p = 0.0002$) (Fig. 4f, h). As shown in Fig. 5a, b, Ki67 expression in MDA-MB-231 tumors (measured by immunohistochemistry) was significantly reduced in the DUSP4-transfected group treated with doxycycline ($p = 0.004$). These results are consistent with our in vitro studies and demonstrate that DUSP4 induction inhibits the growth of ER-negative breast tumors in vivo.

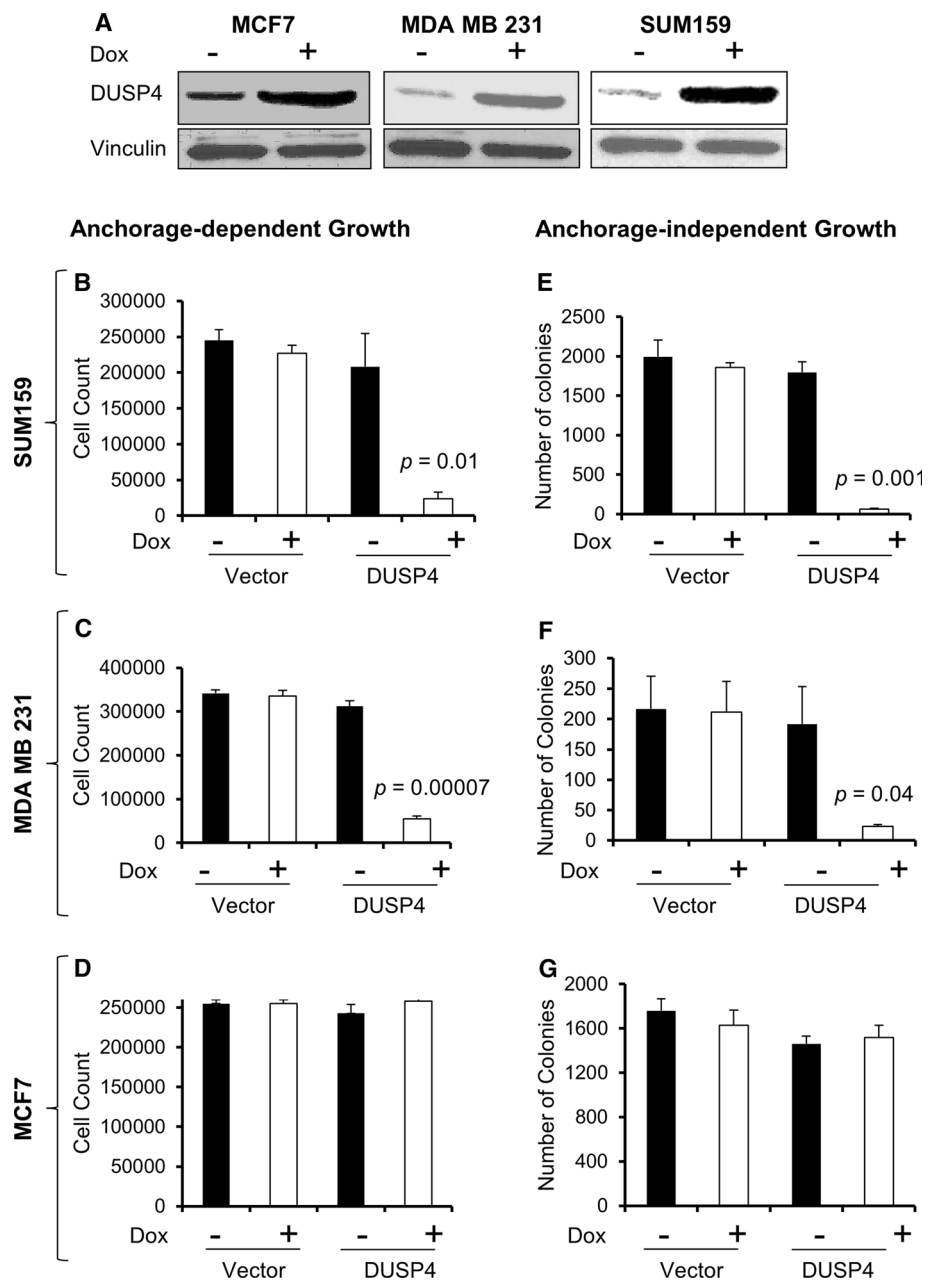
Induction of DUSP4 inhibits invasion and causes a cell cycle block

We next investigated the invasive potential of ER-negative breast cancers before and after DUSP4 induction, and the effect of DUSP4 overexpression on cell motility. Our data show that DUSP4 induction slightly reduces migration of ER-negative breast cancer cells ($p = 0.002$) (Fig. 5c, d) and inhibits the invasiveness of MDA-MB-231 breast cancer cells (Fig. 5e, f) ($p = 0.00001$). We then studied the effect of DUSP4 on the cell cycle using flow cytometry analysis and PI staining. Our results show that DUSP4 expression increases the proportion of cells in G0/G1 ($p = 0.01$) and reduces the G2/M population (Fig. 5g), suggesting that a DUSP4-induced cell cycle blockade is one of the mechanisms preventing growth of ER-negative breast cancer cells.

Induced expression of DUSP4 inhibits the activation of multiple signaling pathways

We next conducted RPPA protein microarray analyses of 172 proteins before and after DUSP4 overexpression to determine the effects of DUSP4 on these proteins in ER-

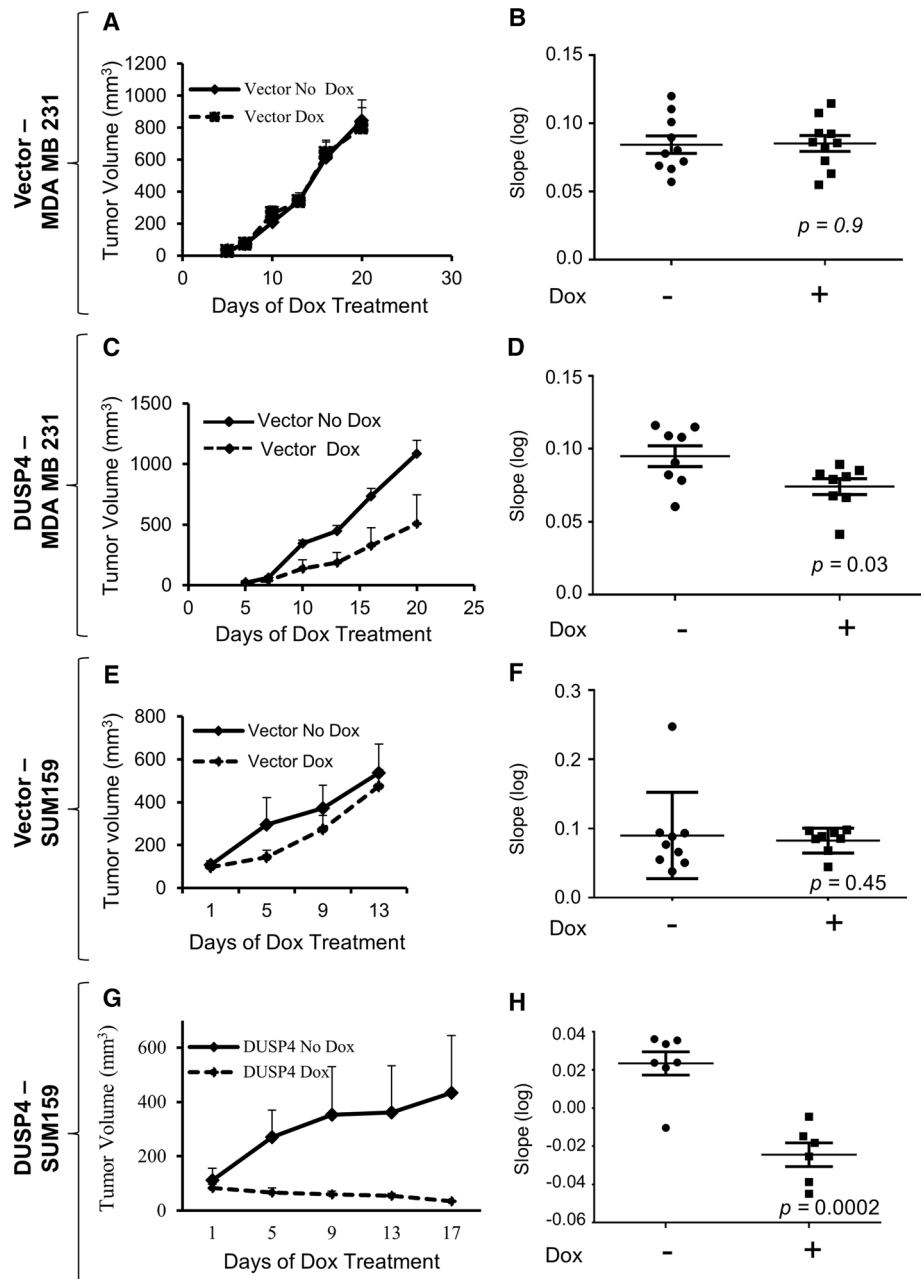
Fig. 3 Induced expression of DUSP4 inhibits ER-negative but not ER-positive growth in vitro. **a** Western blot analysis of doxycycline-induced expression of DUSP4 in SUM159, MDA-MB-231, and MCF7 breast cancer cells. **b–d** Proliferation analysis of breast cancer cell lines upon expression of *DUSP4*. **e–g** Anchorage-independent colony formation assay of breast cancer cell lines upon expression of *DUSP4*



negative cells (Fig. 6a). DUSP4 overexpression significantly affects a number of differentially expressed proteins (Fig. 6a). The most commonly up-regulated and down-regulated proteins are shown in Supplementary Table 2. Induced DUSP4 expression significantly alters expression of a number of signaling proteins, including several MAPK family members. Our RPPA results indicate DUSP4 expression significantly reduces phosphorylation of ERK1/2 at T202/204, p38 at T180/Y182, and JNK1 at T183/T185 (Fig. 6b). To confirm our RPPA results, we examined the effect of DUSP4 expression on phospho-ERK1/2, pJNK1, and phospho-p38 status by Western blot analysis. Our

results indicate DUSP4 down-regulates ERK1/2, JNK1, and p38 signaling in both MDA-MB-231 and SUM 159 cells (Supplementary Figure 2). In addition, induced DUSP4 overexpression also affected other signaling proteins, resulting in significant inhibition of the phosphorylation of Rb (at S807/S811) and p53 (at S536), and increases AMPK phosphorylation (Fig. 6c). Collectively, these RPPA results suggest that DUSP4 induction significantly impacts growth regulatory kinases (ERK1/2, JNK1, and p38) and downstream regulators of proliferation (p53 and Rb), which directly or indirectly reduce tumor growth in ER-negative breast cancer cells.

Fig. 4 Induction of DUSP4 expression inhibits the growth of ER-negative breast cancer cells in vivo. **a** Induced expression of DUSP4 does not inhibit in vivo xenograft growth of MDA-MB-231 vector clone \pm Dox; **b** calculated slope from MDA-MB-231 vector clone xenograft. **c** MDA-MB-231-DUSP4 clone \pm Dox growth curves; **d** calculated slope from MDA-MB-231-DUSP4 xenograft. **e** Induced expression of DUSP4 does not inhibit in vivo xenograft growth of SUM 159-Vector clone \pm Dox growth curves; **f** calculated slope from SUM 159-vector \pm Dox, **g** SUM 159-DUSP4 clone \pm Dox growth curves; **h** calculated slope from SUM 159-DUSP4 clone xenograft. *t*-test *p* values are indicated



Discussion

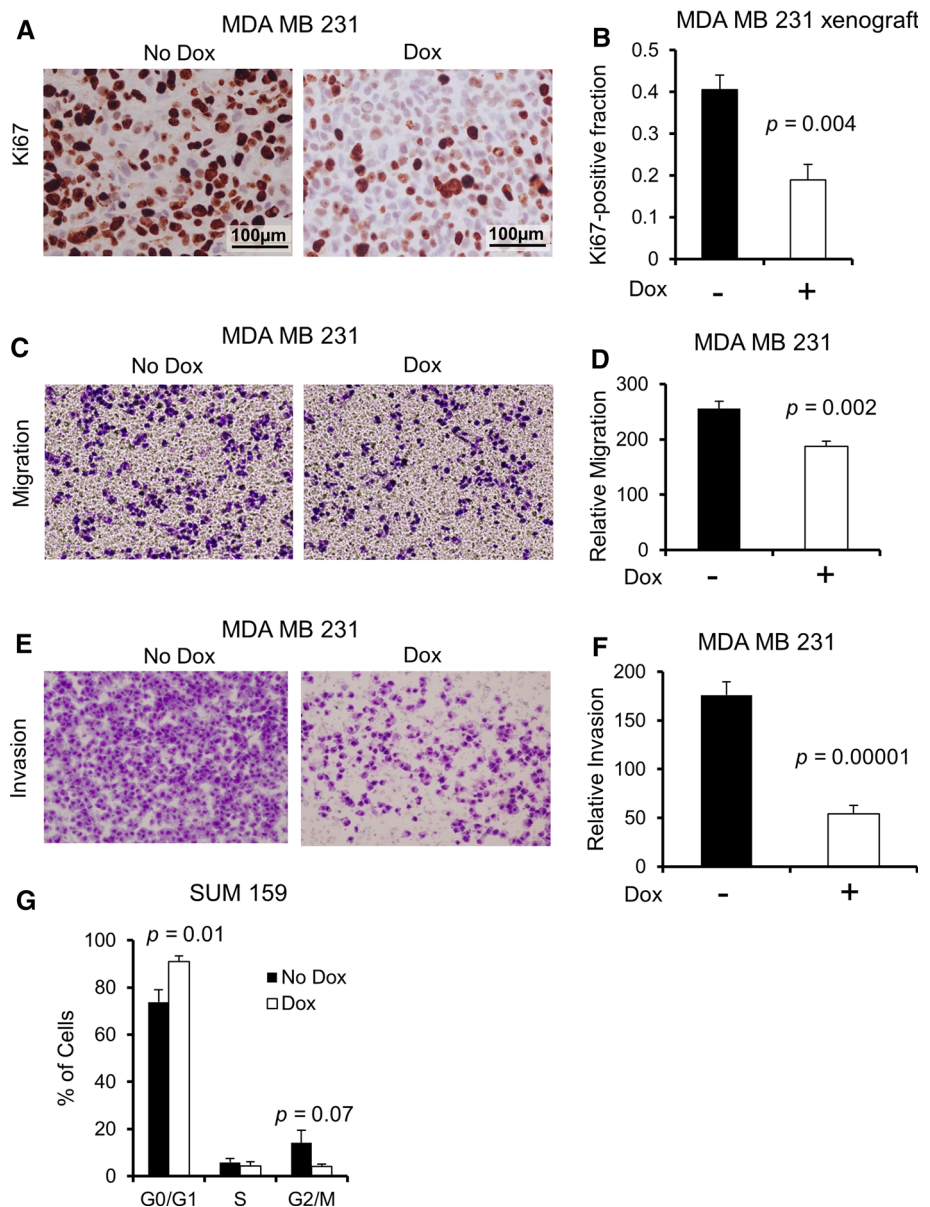
In this study, we identified phosphatases differentially expressed in ER-negative compared to those in ER-positive breast cancers. We found 31 phosphatases significantly overexpressed and 11 phosphatases significantly underexpressed in ER-negative versus ER-positive breast cancers. Included in the set of underexpressed phosphatases are phosphatases regulating growth factor signaling pathways (INPP4B, PTPRT, and DUSP4), as well as phosphatases involved in gluconeogenesis (FPB1), nucleotide cleavage (ENPP1), and metabolism (CANT1). Of these 11

underexpressed phosphatases, DUSP4 is the most commonly deleted phosphatase, deleted in approximately 50 % of human breast cancers. Our results show that DUSP4 overexpression in TNBCs suppresses breast cancer cell growth by suppression of MAPKs (ERK1/2, JNK1, and p38), NF κ B, and Rb signaling pathways, ultimately causing a cell cycle block.

DUSP4 is an early response gene synthesized after growth factor stimulation [11, 32, 33] and has also been mapped to a gene locus that is frequently lost in breast and prostate cancer [34]. *DUSP4* is localized in chromosome 8p, part or all of which is commonly lost in multiple

Fig. 5 Increased DUSP4 expression causes reduced proliferation, invasion, and a G1 cell cycle block.

a Representative Ki67 staining of MDA-MB-231 xenograft sections. **b** Quantitation of Ki67; **c** representative pictures of Boyden chamber assay for migration for the cells before and after DUSP4 induction. **d** Relative migration in MDA-MB-231; **e** representative pictures of Boyden chamber assay for invasion for the cells before and after DUSP4 induction. **f** Relative invasion in MDA-MB-231. **g** Cell cycle changes after DUSP4 induction. *t*-test *p* values are indicated



cancers including breast cancer [35–38]. Armes et al. [34] have shown loss of the DUSP4 gene and protein in early-onset and high-grade breast cancer. DUSP4 loss or epigenetic silencing has been described in lung cancer and glioblastomas [15, 39, 40]. Re-expression of DUSP4 in lung cancer cells with 8p loss and low endogenous DUSP4 reduces growth, while knockdown of DUSP4 in cell lines with high DUSP4 expression enhances cell growth [15]. Our results are in agreement with these previously published studies [34, 40, 41]; together, our results and those of others suggest that DUSP4 is an important tumor suppressor gene in breast cancer.

Balko et al. have demonstrated that *DUSP4* mRNA expression levels correlate inversely with MEK inhibitor

sensitivity, suggesting that DUSP4 expression is a biomarker for MEK inhibitor sensitivity in PTEN-positive tumors [16, 42]. Our results also show that DUSP4 inhibits ERK1/2 phosphorylation in TNBC cells, as well as p38 and JNK1/2 phosphorylation. Since termination of MAPK signaling is maintained by MAP phosphatases, lower expression of DUSP phosphatases results in increased MAPK activity [32, 43]. p38 has been shown to be involved in cell proliferation and tumorigenesis [44], and high levels of p38 in breast cancer patients correlates with invasiveness and poor prognosis [45]. Previously, we have shown that inhibition of the p38 kinase suppresses proliferation of ER-negative breast cancer cells [19]. Our present study suggests that inhibition of all three MAPKs by DUSP

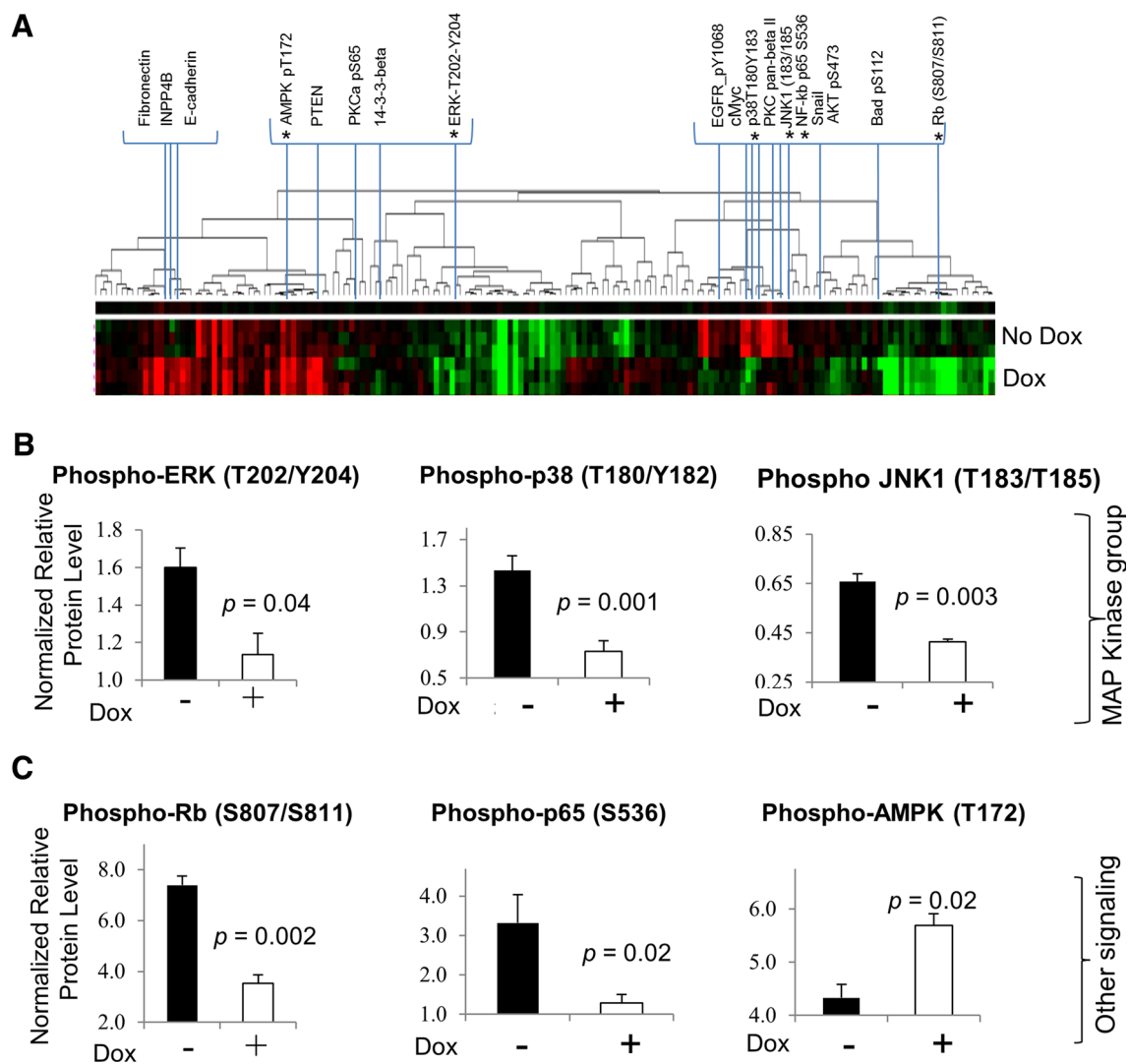


Fig. 6 RPPA analysis identifies signaling pathways altered upon induced expression of DUSP4. **a** RPPA analysis was performed on SUM159 cells with and without induction of DUSP4 (–Dox = No DUSP4 over expression and +Dox = DUSP4 overexpression). Selective markedly altered proteins are indicated after DUSP4 expression. The median expression values for each protein treated with

Dox was subtracted from the protein expression value in cells treated with vehicle to obtain a difference value for each protein studied. Those proteins that showed the greatest change (increased or decreased) are shown in Supplementary Table 2. **b** Changes in MAP Kinase group: p-ERK1/2, p-p38, and p-JNK1/2. **c** Changes in the other signaling pathways: p-Rb, p-p65 (S536), and p-AMPK (T172)

expression has a stronger growth inhibitory effect than inhibition of only ERK1/2 alone. Creighton et al. [46] previously demonstrated that increased MAPK activity causes loss of ER α expression and plays a role in the generation of the ER α phenotype. Our results suggest reduced expression of DUSP4 activates phosphorylation of all three MAPKs. Therefore, regulation of DUSP4 expression may influence the generation of ER α -negative breast cancer via MAPK activation. Expression of DUSP4 also inhibits NF κ B and Rb signaling, in addition to MAPK signaling, ultimately resulting in profound suppression of growth.

In this study, we demonstrated that several other phosphatases (PPP1R3C, INPP4B, FBP1, PTPRT, MTMR9,

and CANT1) are underexpressed in ER-negative compared to those in ER-positive breast cancers. The PPP1R3C gene is hypermethylated in colorectal cancer (CRC) [47], and is a candidate tumor suppressor gene in melanoma and is inactivated through promoter methylation [48]. Gewinner et al. have shown that INPP4B regulates the PI3 K pathway and that its gene is located in a region frequently deleted in both breast cancer cell lines and high-grade breast tumors [49–51]. Deletion of INPP4B has been shown to increase growth of breast cancer cells in vitro, and overexpression of INPP4B reduces growth in vivo [49]. Another phosphatase underexpressed in ER-negative breast cancer is FBP1. The FBP1 protein regulates glycolysis and epithelial-to-mesenchymal transition in breast cells, and

overexpression of FBP1 reduces growth of breast cancer cells [52]. The other phosphatases we identified as under-expressed in ER-negative breast cancer (PTPRT, MTMR9, CANT1, ENPP1, and CILP) may play important roles in signal transduction and tumorigenesis in breast as well as other cancers. Thus, this approach of examining the differential expression of phosphatase genes in ER-positive and ER-negative breast cancer has identified many important phosphatases that regulate tumor growth and tumorigenesis.

Through this genomic study of RNA from human breast cancers, we identified specific phosphatases differentially expressed in ER-negative breast cancers compared to those in ER-positive breast cancers. For this study, we focused on those phosphatases underexpressed in ER-negative versus ER-positive breast cancer. Such phosphatases may be important tumor suppressor genes in ER-negative breast cancer. We also demonstrated that DUSP4 controls the growth and invasiveness of ER-negative breast cancer, and alters the phosphorylation of several growth-promoting signaling proteins, including three MAPKs (ERK, p38, and JNK) and NF κ B. These results suggest that targeting this pathway by targeting the downstream genes ERK, p38, JNK, and NF κ B, or by reactivating DUSP4 (possibly by using demethylation agents), provides a novel approach for the treatment of ER-negative, and particularly triple-negative, breast cancer.

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Compliance with ethical standards

Conflict of interest PH Brown is on the Scientific Advisory Board of Susan G. Komen for the Cure. All the remaining authors declare no actual, potential, or perceived conflict of interest that would prejudice the impartiality of this article.

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References

- Siegel R, Ma J, Zou Z, Jemal A (2014) Cancer statistics. *CA Cancer J Clin* 64(1):9–29
- Dowsett M, Cuzick J, Ingle J, Coates A, Forbes J, Bliss J, Buyse M, Baum M, Buzdar A, Colleoni M, Coombes C, Snowdon C, Gnant M, Jakesz R, Kaufmann M, Boccardo F, Godwin J, Davies C, Peto R (2010) Meta-analysis of breast cancer outcomes in adjuvant trials of aromatase inhibitors versus tamoxifen. *J Clin Oncol* 28(3):509–518
- Jakesz R, Jonat W, Gnant M, Mittlboeck M, Greil R, Tausch C, Hilfrich J, Kwasny W, Menzel C, Samonigg H, Seifert M, Gademann G, Kaufmann M, Wolfgang J (2005) Switching of postmenopausal women with endocrine-responsive early breast cancer to anastrozole after 2 years' adjuvant tamoxifen: combined results of ABCSG trial 8 and ARNO 95 trial. *Lancet* 366(9484):455–462
- Mouridsen H, Giobbie-Hurder A, Goldhirsch A, Thurlimann B, Paridaens R, Smith I, Mauriac L, Forbes J, Price KN, Regan MM, Gelber RD, Coates AS (2009) Letrozole therapy alone or in sequence with tamoxifen in women with breast cancer. *N Engl J Med* 361(8):766–776
- Cuzick J, Sestak I, Baum M, Buzdar A, Howell A, Dowsett M, Forbes JF (2010) Effect of anastrozole and tamoxifen as adjuvant treatment for early-stage breast cancer: 10-year analysis of the ATAC trial. *Lancet Oncol* 11(12):1135–1141
- Cuzick J, Sestak I, Forbes JF, Dowsett M, Knox J, Cawthorn S, Saunders C, Roche N, Mansel RE, von Minckwitz G, Bonanni B, Palva T, Howell A (2014) Anastrozole for prevention of breast cancer in high-risk postmenopausal women (IBIS-II): an international, double-blind, randomised placebo-controlled trial. *Lancet* 383(9922):1040
- Tan AR, Swain SM (2001) Adjuvant chemotherapy for breast cancer: an update. *Semin Oncol* 28(4):359–376
- Baselga J, Bradbury I, Eidtmann H, Di Cosimo S, de Azambuja E, Aura C, Gomez H, Dinh P, Fauria K, Van Dooren V, Aktan G, Goldhirsch A, Chang TW, Horvath Z, Coccia-Portugal M, Domont J, Tseng LM, Kunz G, Sohn JH, Semiglazov V, Lerzo G, Palacova M, Probachai V, Puztai L, Untch M, Gelber RD, Piccart-Gebhart M, Neo AST (2012) Lapatinib with trastuzumab for HER2-positive early breast cancer (NeoALTTO): a randomised, open-label, multicentre, phase 3 trial. *Lancet* 379(9816):633–640
- Geyer CE, Forster J, Lindquist D, Chan S, Romieu CG, Pienkowski T, Jagiello-Gruszfeld A, Crown J, Chan A, Kaufman B, Skarlos D, Campone M, Davidson N, Berger M, Oliva C, Rubin SD, Stein S, Cameron D (2006) Lapatinib plus capecitabine for HER2-positive advanced breast cancer. *N Engl J Med* 355(26):2733–2743
- Speers C, Tsimelzon A, Sexton K, Herrick AM, Gutierrez C, Culhane A, Quackenbush J, Hilsenbeck S, Chang J, Brown P (2009) Identification of novel kinase targets for the treatment of estrogen receptor-negative breast cancer. *Clin Cancer Res* 15(20):6327–6340
- Robinson CJ, Sloss CM, Plevin R (2001) Inactivation of JNK activity by mitogen-activated protein kinase phosphatase-2 in EAhy926 endothelial cells is dependent upon agonist-specific JNK translocation to the nucleus. *Cell Signal* 13(1):29–41
- Chu Y, Solski PA, Khosravi-Far R, Der CJ, Kelly K (1996) The mitogen-activated protein kinase phosphatases PAC1, MKP-1, and MKP-2 have unique substrate specificities and reduced activity in vivo toward the ERK2 sevenmaker mutation. *J Biol Chem* 271(11):6497–6501

13. Chen P, Hutter D, Yang X, Gorospe M, Davis RJ, Liu Y (2001) Discordance between the binding affinity of mitogen-activated protein kinase subfamily members for MAP kinase phosphatase-2 and their ability to activate the phosphatase catalytically. *J Biol Chem* 276(31):29440–29449
14. Tresini M, Lorenzini A, Torres C, Cristofalo VJ (2007) Modulation of replicative senescence of diploid human cells by nuclear ERK signaling. *J Biol Chem* 282(6):4136–4151
15. Chitale D, Gong Y, Taylor BS, Broderick S, Brennan C, Somwar R, Golas B, Wang L, Motoi N, Szoke J, Reinersman JM, Major J, Sander C, Seshan VE, Zakowski MF, Rusch V, Pao W, Gerald W, Ladanyi M (2009) An integrated genomic analysis of lung cancer reveals loss of DUSP4 in EGFR-mutant tumors. *Oncogene* 28(31):2773–2783
16. Balko JM, Cook RS, Vaught DB, Kuba MG, Miller TW, Bhola NE, Sanders ME, Granja-Ingram NM, Smith JJ, Meszoely IM, Salter J, Dowsett M, Stemke-Hale K, Gonzalez-Angulo AM, Mills GB, Pinto JA, Gomez HL, Arteaga CL (2012) Profiling of residual breast cancers after neoadjuvant chemotherapy identifies DUSP4 deficiency as a mechanism of drug resistance. *Nat Med* 18(7):1052–1059
17. Romano P, Manniello A, Aresu O, Armento M, Cesaro M, Parodi B (2009) Cell line data base: structure and recent improvements towards molecular authentication of human cell lines. *Nucleic Acids Res* 37:D925–D932
18. Hartman ZC, Poage GM, den Hollander P, Tsimelzon A, Hill J, Panupinthu N, Zhang Y, Mazumdar A, Hilsenbeck SG, Mills GB, Brown PH (2013) Growth of triple-negative breast cancer cells relies upon coordinate autocrine expression of the proinflammatory cytokines IL-6 and IL-8. *Cancer Res* 73(11):3470–3480
19. Chen L, Mayer JA, Krisko TI, Speers CW, Wang T, Hilsenbeck SG, Brown PH (2009) Inhibition of the p38 kinase suppresses the proliferation of human ER-negative breast cancer cells. *Cancer Res* 69(23):8853–8861
20. Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, Sun Y, Jacobsen A, Sinha R, Larsson E, Cerami E, Sander C, Schultz N (2013) Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Sci Signal* 6(269):p11
21. 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 (2012) The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov* 2(5):401–404
22. VandeVijver MJ, He YD, van't Veer LJ, Dai H, Hart AA, Voskuil DW, Schreiber GJ, Peterse JL, Roberts C, Marton MJ, Parrish M, Atsma D, Witteveen A, Glas A, Delahaye L, vander Velde T, Bartelink H, Rodenhuis S, Rutgers ET, Friend SH, Bernards R (2002) A gene-expression signature as a predictor of survival in breast cancer. *N Engl J Med* 347(25):1999–2009
23. Desmedt C, Piette F, Loi S, Wang Y, Lallemand F, Haibe-Kains B, Viale G, Delorenzi M, Zhang Y, d'Assignies MS, Bergh J, Lidereau R, Ellis P, Harris AL, Klijn JG, Foekens JA, Cardoso F, Piccart MJ, Buyse M, Sotiriou C (2007) Strong time dependence of the 76-gene prognostic signature for node-negative breast cancer patients in the TRANSBIG multicenter independent validation series. *Clin Cancer Res* 13(11):3207–3214
24. Kao KJ, Chang KM, Hsu HC, Huang AT (2011) Correlation of microarray-based breast cancer molecular subtypes and clinical outcomes: implications for treatment optimization. *BMC Cancer* 11:143
25. Chin K, DeVries S, Fridlyand J, Spellman PT, Roydasgupta R, Kuo WL, Lapuk A, Neve RM, Qian Z, Ryder T, Chen F, Feiler H, Tokuyasu T, Kingsley C, Dairkee S, Meng Z, Chew K, Pinkel D, Jain A, Ljung BM, Esserman L, Albertson DG, Waldman FM, Gray JW (2006) Genomic and transcriptional aberrations linked to breast cancer pathophysiology. *Cancer Cell* 10(6):529–541
26. Wang Y, Klijn JG, Zhang Y, Sieuwerts AM, Look MP, Yang F, Talantov D, Timmermans M, Meijer-van Gelder ME, Yu J, Jatkoe T, Berns EM, Atkins D, Foekens JA (2005) Gene-expression profiles to predict distant metastasis of lymph-node-negative primary breast cancer. *Lancet* 365(9460):671–679
27. Minn AJ, Gupta GP, Siegel PM, Bos PD, Shu W, Giri DD, Viale A, Olshen AB, Gerald WL, Massague J (2005) Genes that mediate breast cancer metastasis to lung. *Nature* 436(7050):518–524
28. Saal LH, Johansson P, Holm K, Gruvberger-Saal SK, She QB, Maurer M, Koujak S, Ferrando AA, Malmstrom P, Memeo L, Isola J, Bendahl PO, Rosen N, Hibshoosh H, Ringner M, Borg A, Parsons R (2007) Poor prognosis in carcinoma is associated with a gene expression signature of aberrant PTEN tumor suppressor pathway activity. *Proc Natl Acad Sci USA* 104(18):7564–7569
29. Curtis C, Shah SP, Chin SF, Turashvili G, Rueda OM, Dunning MJ, Speed D, Lynch AG, Samarajiwa S, Yuan Y, Graf S, Ha G, Haffari G, Bashashati A, Russell R, McKinney S, Group M, Langerod A, Green A, Provenzano E, Wishart G, Pinder S, Watson P, Markowitz F, Murphy L, Ellis I, Purushotham A, Borresen-Dale AL, Brenton JD, Tavare S, Caldas C, Caldas C, Aparicio S (2012) The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. *Nature* 486(7403):346–352
30. Lu X, Lu X, Wang ZC, Iglehart JD, Zhang X, Richardson AL (2008) Predicting features of breast cancer with gene expression patterns. *Breast Cancer Res Treat* 108(2):191–201
31. Hatzis C, Pusztai L, Valero V, Booser DJ, Esserman L, Lluch A, Vidaurre T, Holmes F, Souchon E, Wang H, Martin M, Cotrina J, Gomez H, Hubbard R, Chacon JI, Ferrer-Lozano J, Dyer R, Buxton M, Gong Y, Wu Y, Ibrahim N, Andreopoulou E, Ueno NT, Hunt K, Yang W, Nazario A, DeMichele A, O'Shaughnessy J, Hortobagyi GN, Symmans WF (2011) A genomic predictor of response and survival following taxane-anthracycline chemotherapy for invasive breast cancer. *JAMA* 305(18):1873–1881
32. Amit I, Citri A, Shay T, Lu Y, Katz M, Zhang F, Tarcic G, Siwak D, Lahad J, Jacob-Hirsch J, Amariglio N, Vaisman N, Segal E, Rechavi G, Alon U, Mills GB, Domany E, Yarden Y (2007) A module of negative feedback regulators defines growth factor signaling. *Nat Genet* 39(4):503–512
33. Hirsch DD, Stork PJ (1997) Mitogen-activated protein kinase phosphatases inactivate stress-activated protein kinase pathways in vivo. *J Biol Chem* 272(7):4568–4575
34. Armes JE, Hammet F, de Silva M, Ciciulla J, Ramus SJ, Soo WK, Mahoney A, Yarovaya N, Henderson MA, Gish K, Hutchins AM, Price GR, Venter DJ (2004) Candidate tumor-suppressor genes on chromosome arm 8p in early-onset and high-grade breast cancers. *Oncogene* 23(33):5697–5702
35. Radford DM, Fair KL, Phillips NJ, Ritter JH, Steinbrueck T, Holt MS, Donis-Keller H (1995) Allelotyping of ductal carcinoma in situ of the breast: deletion of loci on 8p, 13q, 16q, 17p and 17q. *Cancer Res* 55(15):3399–3405
36. Thor AD, Eng C, Devries S, Paterakos M, Watkin WG, Edgerton S, Moore DH 2nd, Etzell J, Waldman FM (2002) Invasive micropapillary carcinoma of the breast is associated with chromosome 8 abnormalities detected by comparative genomic hybridization. *Hum Pathol* 33(6):628–631
37. Yokota T, Yoshimoto M, Akiyama F, Sakamoto G, Kasumi F, Nakamura Y, Emi M (1999) Localization of a tumor suppressor gene associated with the progression of human breast carcinoma within a 1-cM interval of 8p22-p23.1. *Cancer* 85(2):447–452
38. Adams J, Cuthbert-Heavens D, Bass S, Knowles MA (2005) Infrequent mutation of TRAIL receptor 2 (TRAIL-R2/DR5) in

- transitional cell carcinoma of the bladder with 8p21 loss of heterozygosity. *Cancer Lett* 220(2):137–144
39. Britson JS, Barton F, Balko JM, Black EP (2009) Deregulation of DUSP activity in EGFR-mutant lung cancer cell lines contributes to sustained ERK1/2 signaling. *Biochem Biophys Res Commun* 390(3):849–854
 40. Waha A, Felsberg J, Hartmann W, von dem Knesebeck A, Mikeska T, Joos S, Wolter M, Koch A, Yan PS, Endl E, Wiestler OD, Reifenberger G, Pietsch T (2010) Epigenetic downregulation of mitogen-activated protein kinase phosphatase MKP-2 relieves its growth suppressive activity in glioma cells. *Cancer Res* 70(4):1689–1699
 41. Venter DJ, Ramus SJ, Hammet FM, de Silva M, Hutchins AM, Petrovic V, Price G, Armes JE (2005) Complex CGH alterations on chromosome arm 8p at candidate tumor suppressor gene loci in breast cancer cell lines. *Cancer Genet Cytogenet* 160(2):134–140
 42. Balko JM, Schwarz LJ, Bholra NE, Kurupi R, Owens P, Miller TW, Gomez H, Cook RS, Arteaga CL (2013) Activation of MAPK pathways due to DUSP4 loss promotes cancer stem cell-like phenotypes in basal-like breast cancer. *Cancer Res* 73(20):6346–6358
 43. Camps M, Nichols A, Arkinstall S (2000) Dual specificity phosphatases: a gene family for control of MAP kinase function. *FASEB J* 14(1):6–16
 44. Bulavin DV, Higashimoto Y, Popoff IJ, Gaarde WA, Basrur V, Potapova O, Appella E, Fornace AJ Jr (2001) Initiation of a G2/M checkpoint after ultraviolet radiation requires p38 kinase. *Nature* 411(6833):102–107
 45. Gutierrez MC, Detre S, Johnston S, Mohsin SK, Shou J, Allred DC, Schiff R, Osborne CK, Dowsett M (2005) Molecular changes in tamoxifen-resistant breast cancer: relationship between estrogen receptor, HER-2, and p38 mitogen-activated protein kinase. *J Clin Oncol* 23(11):2469–2476
 46. Creighton CJ, Hilger AM, Murthy S, Rae JM, Chinnaiyan AM, El-Ashry D (2006) Activation of mitogen-activated protein kinase in estrogen receptor alpha-positive breast cancer cells in vitro induces an in vivo molecular phenotype of estrogen receptor alpha-negative human breast tumors. *Cancer Res* 66(7):3903–3911
 47. Takane K, Midorikawa Y, Yagi K, Sakai A, Aburatani H, Takayama T, Kaneda A (2014) Aberrant promoter methylation of PPP1R3C and EFHD1 in plasma of colorectal cancer patients. *Cancer Med* 3(5):1235–1245
 48. Bonazzi VF, Irwin D, Hayward NK (2009) Identification of candidate tumor suppressor genes inactivated by promoter methylation in melanoma. *Genes Chromosomes Cancer* 48(1):10–21
 49. Gewinner C, Wang ZC, Richardson A, Teruya-Feldstein J, Etemadmoghadam D, Bowtell D, Barretina J, Lin WM, Rameh L, Salmena L, Pandolfi PP, Cantley LC (2009) Evidence that inositol polyphosphate 4-phosphatase type II is a tumor suppressor that inhibits PI3 K signaling. *Cancer Cell* 16(2):115–125
 50. Chin SF, Wang Y, Thorne NP, Teschendorff AE, Pinder SE, Vias M, Naderi A, Roberts I, Barbosa-Morais NL, Garcia MJ, Iyer NG, Kranjac T, Robertson JF, Aparicio S, Tavare S, Ellis I, Brenton JD, Caldas C (2007) Using array-comparative genomic hybridization to define molecular portraits of primary breast cancers. *Oncogene* 26(13):1959–1970
 51. Naylor TL, Greshock J, Wang Y, Colligon T, Yu QC, Clemmer V, Zaks TZ, Weber BL (2005) High resolution genomic analysis of sporadic breast cancer using array-based comparative genomic hybridization. *Breast Cancer Res* 7(6):R1186–R1198
 52. Dong C, Yuan T, Wu Y, Wang Y, Fan TW, Miriyala S, Lin Y, Yao J, Shi J, Kang T, Lorkiewicz P, St Clair D, Hung MC, Evers BM, Zhou BP (2013) Loss of FBPI by snail-mediated repression provides metabolic advantages in basal-like breast cancer. *Cancer Cell* 23(3):316–331