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 NFkB p65 phosphorylation; and inhibits invasiveness of TNBC cells. These results suggest that that DUSP4 is a critical regulator of the growth and invasion of triple-negative breast cancer cells.

Keywords TNBC · Phosphatase · Mouse xenograft · MAPK pathways

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 NFκB and Rb. Overall, this study demonstrates that DUSP4 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 probesets on Affymetrix U133A chip), which encode known phosphatases and proteins with phosphatase in their name. We used the most variable probeset 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 \( \leq 0.1 \), fold change \( \geq 1.2 \) or \( \leq 0.8 \).

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 µg/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 × 10⁵ 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 ×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 ± standard deviation.

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 ERα [10]. RNA expression in 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 ≤ 0.1) and 11 underexpressed phosphatases (fold ≤ 0.80, adjusted p ≤ 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

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

| Gene name                                                                 | Symbol   | Fold  | Adjusted p value* |
|----------------------------------------------------------------------------|----------|-------|-------------------|
| **Overexpressed phosphatases (≥ 1.2-fold, p < 0.1)**                      |          |       |                   |
| 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                                               | ACPI     | 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            |
| **Underexpressed phosphatases (≤0.80-fold, p < 0.1)**                    |          |       |                   |
| 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            |

* 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
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, \textit{DUSP4} was the only phosphatase with high-frequency allele loss (Fig. 2a) and exhibited the highest degree of homozygous deletion (Fig. 2b). \textit{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 %), mRNA 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 \textit{DUSP4} for further study. We next conducted an examination of \textit{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 \textit{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).

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

\textbf{A} Allelic Deletion in TCGA Breast (All Tumors)

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{figureA}
\caption{Allelic Deletion in TCGA Breast (All Tumors)}
\end{figure}

\textbf{B} Homozygous Deletion in DUSP4 in TCGA Breast (All Tumors)

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{figureB}
\caption{Homozygous Deletion in DUSP4 in TCGA Breast (All Tumors)}
\end{figure}

\textbf{C} Somatic Alteration of DUSP4 in TCGA Breast (All Tumors)

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{figureC}
\caption{Somatic Alteration of DUSP4 in TCGA Breast (All Tumors)}
\end{figure}

\textbf{D} DUSP4 Expression

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{figureD}
\caption{DUSP4 Expression}
\end{figure}
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-
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 p65 (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 (p65 and Rb), which directly or indirectly reduce tumor growth in ER-negative breast cancer cells.
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), NFkB, 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...
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. 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.
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\(\alpha\) expression and plays a role in the generation of the ER\(\alpha\) 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\(\alpha\)-negative breast cancer via MAPK activation. Expression of DUSP4 also inhibits NFkB 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

**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 overexpression and +Dox = DUSP4 overexpression). Selective markedly altered proteins are indicated after DUSP4 expression. The median expression values for each protein in cells 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).
overexpression of FBP1 reduces growth of breast cancer cells [52]. The other phosphatases that we identified as underexpressed 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 NFκB. These results suggest that targeting this pathway by targeting the downstream genes ERK, p38, JNK, and NFκB, 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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