Title:

Defining the Na^+/H^+ Exchanger NHE1 interactome in triple-negative breast cancer cells

Authors and Affiliations:

Schammim Ray Amith[1,2], Krista Marie Vincent[3,4], Jodi Marie Wilkinson[1], Lynne Marie Postovit[3], and Larry Fliegel[1]

[1]Department of Biochemistry, University of Alberta, Edmonton, Alberta, T6G 2H7, Canada.

[2]Present address: School of Life Sciences, Keele University, Keele, Staffordshire, ST5 5BG, United Kingdom.

[3]Department of Oncology, University of Alberta, Edmonton, Alberta, T6G 2H7, Canada.

[4]Department of Anatomy and Cell Biology, University of Western Ontario, London, Ontario, N6A 3K7, Canada.

Authors' email addresses:

s.r.amith@keele.ac.uk

kvincent@ualberta.ca

jmwilkin@ualberta.ca

postovit@ualberta.ca

lfliegel@ualberta.ca

Corresponding Author:

Larry Fliegel, Professor and Associate Chair

345 Medical Sciences Building

Dept. of Biochemistry

University of Alberta

Edmonton, Alberta, Canada, T6G 2H7

Tel: 780-492-1848; Fax: 780-492-0886; Email: lfliegel@ualberta.ca

Abstract:

Mounting evidence supports a major role for the Na^+/H^+ exchanger NHE1 in cancer progression and metastasis. NHE1 is hyperactive at the onset of oncogenic transformation, resulting in intracellular alkalinization and extracellular microenvironmental acidification. These conditions promote invasion and facilitate metastasis. However, the signal pathways governing the regulation of exchanger activity are still unclear. This is especially important in the aggressively metastatic, triple-negative basal breast cancer subtype. We used affinity chromatography followed by mass spectrometry to identify novel and putative interaction partners of NHE1 in MDA-MB-231 triple-negative breast cancer cells. NHE1 associated with several types of proteins including cytoskeletal proteins and chaperones. We validated protein interactions by co-immunoprecipitation for: 14-3-3, AKT, α-enolase, CHP1, HSP70 and HSP90. Additionally, we used The Cancer Genome Atlas (TCGA) to study *NHE1* gene expression in primary patient breast tumours *versus* adjacent normal tissue. *NHE1* expression was elevated in breast tumour samples and, when broken down by breast cancer subtype, *NHE1* gene expression was significantly lower in tumours of the basal subtype compared to luminal and HER2+ subtypes. Reverse phase protein array (RPPA) analysis showed that NHE1 expression positively correlated with $p90^{RSK}$ expression in basal, but not luminal, primary tumours. Other proteins were negatively correlated with *NHE1* expression in basal breast cancer tumours. Taken together, our data provides the first insight into the signalling molecules that form the NHE1 interactome in triple-negative breast cancer cells. These results will focus our search for novel targeted therapies.

Keywords:

NHE1

Triple-negative breast cancer

Signal scaffold

14-3-3

$p90^{RSK}$

Highlights:

- Na^+/H^+ exchanger NHE1 is a signal scaffold in triple-negative basal breast cancer.
- NHE1 forms a complex with 14-3-3, AKT, α-enolase, CHP1, HSP70 and HSP90.
- NHE1 positively correlates with $p90^{RSK}$ expression in primary breast tumours.
- NHE1 associated with cytoskeletal proteins and chaperones
- NHE1 associated with many proteins that promote breast cancer

Abbreviations:

ACC1 (acetyl-CoA carboxylase); AKT (protein kinase B, PKB); CAII (carbonic anhydrase II); CHP1 (calcineurin B homologous protein 1); ER (estrogen receptor); ERK1/2 (extracellular signal-regulated kinase 1/2); ERM (ezrin-radixin-moesin); FOXO3a (forkhead box O3a); GSK (glycogen synthase kinase); HA, hemagglutinin; HER2 (human epidermal growth factor receptor 2); HSP70/HSP90; heat shock protein70/90; p38MAPK (p38 mitogen-activated protein kinases); NF2 (neurofibromin 2); NHE1, Na^+/H^+ exchanger isoform 1; PDGF (platelet-derived growth factor); PI3K (phosphoinositide-3-kinase); PIP_2 (phosphatidylinositol 4,5-biphosphate); PR

(progesterone receptor); $p90^{RSK}$ (p90 ribosomal S6 kinase); p160ROCK (rho-associated coiled coil-containing protein kinase 1); TNBC (triple-negative breast cancer).

1. Introduction

The Na^+/H^+ exchanger isoform 1 (NHE1 [*SLC9A1*]) is the major protein involved in the regulation of pH homeostasis and is ubiquitously expressed in mammalian cells. Human NHE1 is an integral plasma membrane protein comprised of 815 amino acids. The N-terminal transmembrane domain consisting of amino acids 1 to 500 is responsible for ion exchange, and the C-terminal cytosolic domain of amino acids 501 to 815 regulates the exchanger's activity *via* phosphorylation or interactions with intracellular signalling molecules [1, 2]. In normal cells, NHE1 is activated either by growth factors and hormones in serum, which leads to direct or indirect phosphorylation of the exchanger by intracellular signal kinases (e.g. PI3K, ERK1/2, p38MAPK), or by binding to a variety of cytosolic proteins and lipids (e.g. ezrin-radixin-moesin, 14-3-3, calmodulin, phosphatidylinositol 4,5-biphosphate (PIP_2), carbonic anhydrase; reviewed in [3, 4]). This activation is due to an increased affinity of NHE1's allosteric proton-binding site for intracellular H^+ ions [4]. In cancer cells, however, exchanger activity is dysregulated and NHE1 becomes overactive. The increased activity of NHE1 is pivotal to tumourigenesis. It results in increased sodium uptake and elevated proton extrusion that leads to intracellular alkalinization of cancer cells and extracellular acidification of the tumour microenvironment [5, 6].

In breast cancer, the dysregulation of pH homeostasis and altered dynamics of proton transport are critical determinants of metastasis. Elevated intracellular pH with decreased extracellular pH is thought to lead to enhanced cellular proliferation and metastasis [6]. Breast cancer is a heterogeneous disease with three major molecular profiles: estrogen and/or progesterone receptor (ER/PR)-positive; human epidermal growth factor receptor 2 (HER2)-positive; and triple-negative, which lacks the expression of estrogen, progesterone, or HER2 receptors [7]. In breast cancer cells, NHE1 becomes hyperactive early in neoplastic transformation. This hyper-activation has been shown to be particularly important in triple

negative breast cancer [8] where it is primarily responsible for the acidic pH of the tumour microenvironment that facilitates metastasis [5]. Inhibiting NHE1 activity, or preventing the dysregulation of NHE1, may thus provide an attractive avenue for preventing metastasis of breast cancer [3, 8]. This is particularly important for aggressive metastatic triple-negative breast cancer for which hormone and HER2-targeted therapies do not work [9].

NHE1 regulation is mediated through its cytosolic C-terminal tail by both phosphorylation and protein-protein interactions. Phosphorylation of NHE1 directly increases exchanger activity in response to hormonal stimulation; however, protein-protein interactions are thought to account for approximately 50% of NHE1 activation by growth factors. These protein interactions are increasingly considered to be important in NHE1 regulation [3, 10-13]. NHE1 hyper-activation could be due to an altered regulation of the exchanger's activity by signal kinases and its intracellular binding partners. We recently demonstrated that NHE1 regulation by $p90^{RSK}$ *via* the serine 703 residue can influence the metastatic potential of triple-negative breast cancer cells. Additionally, the metastatic potential of breast cancer cells was enhanced by mutation of the calmodulin-binding domain to make an artificially hyperactive NHE1 protein [14].

There has been increasing attention on the role of NHE1 as a signal scaffold in recent years. NHE1 binds signalling complexes including ERK2 in Chinese hamster ovary cells [15, 16]. It acts as an anchor for actin filaments [13] and binds several regulatory proteins including calmodulin [1], HSP70 [17] and others (reviewed in [4]). As noted above, the enhanced activity of NHE1 is critical in metastasis and the protein-mediated regulation of NHE1 is likewise crucial in its activity. However, there is no information available on protein-mediated regulation of NHE1 in breast cancer cells, particularly in the triple-negative breast cancer (TNBC) subtype.

In the present study, affinity chromatography followed by mass spectrometry was used to identify intracellular protein binding partners of NHE1 in a triple-negative breast cancer model, MDA-MB-231 cells. We further validated key intracellular molecular associations that make up the NHE1 interactome in these cells by co-immunoprecipitation. Our data was supplemented by an analysis of The Cancer Genome Atlas (TCGA; https://tcga-data.nci.nih.gov/tcga/) database, where we compared NHE1 gene expression in primary patient breast tumours and normal breast tissue. Reverse phase protein array (RPPA) clinical data from TCGA was used to examine correlations in NHE1 mRNA expression with that of signal proteins of primary breast tumours by subtype *versus* normal tissue. We also looked for correlations between NHE1 expression and other interacting proteins or signal kinases. Our data, for the first time, provide insight into the NHE1 signal scaffold that regulates metastasis in triple-negative basal-like breast cancer and could provide critical insights for the development of novel chemotherapeutics targeted towards TNBC.

2. Materials and Methods

2.1. Cell lines and culture conditions

MDA-MB-231 breast cancer cells were cultured in high-glucose modified DMEM (HyClone) supplemented with 10% fetal calf serum (HyClone), 10 mM HEPES, and 1000 units/ml penicillin/streptomycin (Gibco) under standard culture conditions (5% CO_2, 37°C and high humidity). Low serum media, to activate NHE1, contained 0.2% serum but was otherwise identical in composition. We used a previously generated stable MDA-MB-231 cell line where the endogenous NHE1 was knocked out [8] and replaced with a hemagglutinin (HA) tagged

NHE1 protein that does not interfere with NHE1 activity [18]. Cells expressing wild-type HA-tagged NHE1 protein were grown in the presence of 400 μg/ml G418 (Geneticin Sulfate, Santa Cruz). DNA analysis (DDC Medical, Ohio) was used to authenticate parental MDA-MB-231 cells, which showed >95% homology to the ATCC STR profile.

2.2. Preparation of Cell Lysates

Seventy-five 150 mm plates of MDA-MB-231 cells were grown in standard medium and lysis buffer [20 mM HEPES, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1% Triton X-100, and protease inhibitors] was added to generate whole cell lysates. Lysates were centrifuged at 10,000 rpm for 15 min. at 4°C and the supernatant was used for affinity chromatography.

2.3. Protein Expression and Purification

Affinity chromatography was used to identify the interacting protein partners of NHE1 in MDA-MB-231 breast cancer cell lysates as previously described [19]. The C terminus of the rabbit NHE1 spanning amino acids 545 to 816 was produced as a glutathione S-transferase (GST) fusion protein [17]. The primer pair used for amplification of the sequence was 5′-acggatccattggaaagacaagctcaaccggttta-3′ and 5′-aagaattcactgccctttggggatgaaaggct-3′. For cloning and expression, the pGEX-3X plasmid in the BamHI-EcoRI sites was utilized. GST was produced using the same plasmid without an exogenous insert (control). Proteins were expressed in TOP2 *Escherichia coli* and a single colony was selected for overnight culture. After induction for 5 hours at 30°C with isopropyl β-D-thiogalactopyranoside, bacterial cultures were centrifuged

in lysis buffer at 7,000 rpm at 4°C prior to sonication of the pellet to induce greater cell disruption and protein extraction. Debris was removed by further centrifugation at 20,000 rpm at 4°C and the supernatant collected and filtered before transferring to column. Purification from the supernatant was essentially as described earlier [19] using a glutathionine-agarose column. The supernatant was loaded onto the column and passed through it twice. The column was washed with 20 volumes of phosphate buffered saline and eluates with the purified protein were collected and centrifuged at 3,000 × g. Purified proteins were resuspended in 0.1 M MOPS buffer with 80 mM $CaCl_2$ (pH 7.5) and concentrated. Five mg of either GST or NHE1-GST proteins were bound to Affi-Gel 10 beads (Bio-Rad) as described by the manufacturer. After blockage of any remaining reactive esters and washing, MDA-MB-231 cell lysates were then passed through the Affi-Gel 10 column 10 times and the column was washed. Bound proteins were eluted with buffer [20 mM HEPES, pH 7.2, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1% SDS and protease inhibitors], and precipitated with trichloroacetic acid. Precipitates were separated by SDS-PAGE and separated proteins were cut from the gel for further analysis by mass spectrometry.

2.4. Sample Preparation and Mass Spectrometry

The samples were digested with trypsin in-gel. Briefly, the excised gel bands were de-stained twice in 100 mM ammonium bicarbonate/acetonitrile (50:50). The samples were then reduced with 10 mM BME in 100 mM bicarbonate and the alkylated with 55 mM iodoacetamide in 100 mm bicarbonate. After dehydration, gel segments were digested with trypsin (6 ng/μL) overnight at room temperature. Tryptic peptides were extracted from the gel with a 97%

water/2% acetonitrile/1% formic acid solution, followed by a second extraction using 50% of the first extraction buffer and 50% acetonitrile.

Fractions containing tryptic peptides dissolved in aqueous 25% v/v acetonitrile and 1% v/v formic acid were resolved and ionized by using nanoflow HPLC (Easy-nLC II, Thermo Scientific) coupled to the LTQ XL-Orbitrap hybrid mass spectrometer (Thermo Scientific). Nanoflow chromatography and electrospray ionization were accomplished by using a PicoFrit fused silica capillary column (ProteoPepII, C18) with 100 μm inner diameter (300Å, 5μm, New Objective). Peptide mixtures were next injected onto the column with a flow rate of 3,000 nL/min and were resolved at 500 nL/min using 70 min linear gradients from 0 to 45% v/v aqueous ACN in 0.2% v/v formic acid. The mass spectrometer was operated in a data-dependent acquisition mode, recording high-accuracy and high-resolution survey Orbitrap spectra using external mass calibration, with a resolution of 60,000 and m/z range of 400 to 2,000. The fourteen most intense multiply charged ions were sequentially fragmented using collision-induced dissociation, and the spectra of their fragments were recorded in the linear ion trap. After two fragmentations, all precursors selected for dissociation were dynamically excluded for 60 sec.

Data were processed using Proteome Discoverer 1.4 (Thermo Scientific) and SEQUEST was used to search the Uniprot Human protein database (Thermo Scientific). Search parameters included a fragment mass tolerance of 0.8 Da and a precursor mass tolerance of 10 ppm. Peptides were searched with the dynamic modifications of carbamidomethyl cysteine as a static modification and oxidized methionine and deamidated glutamine and asparagine. Data shown are representative of three independent experiments.

2.5. Co-immunoprecipitation and Immunoblotting

Cells were stimulated (+, 0.2% serum) or not (−, 10% serum) overnight prior to adding 2.5 mM DSP (dithiobis[succinimidyl propionate]), an amine-reactive cleavable cross-linker (Thermo Scientific). Incubation with DSP was for 2 hours on ice in the dark. The reaction was stopped with 30 mM Tris (Fisher BioReagents) for 15 min. prior to lysis with RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 80 mM NaF, 5 mM EDTA, 1 mM EGTA, 1 mM Na-orthovanadate, 1 % NP-40, 0.05 % deoxycholate and protease inhibitor cocktail). After centrifugation, 25 µL of pre-washed HA-tagged beads (Santa Cruz Biotechnology) was added to the supernatant and incubated overnight at 4°C. Beads were removed by centrifugation and washed with phosphate buffered saline (PBS, 150 mM NaCl, 5 mM sodium phosphate, pH 7.4). Samples were boiled in SDS before separation on SDS-PAGE gels. Western blotting was onto nitrocellulose membranes. Co-immunoprecipitation of putative protein interaction partners of NHE1 was confirmed by immunoblot analysis. Blots were incubated overnight with the following antibodies against targeted proteins: AKT (Cell Signalling Technologies); α-Enolase (ProteinTech); HA-probe, CHP1 and 14-3-3 (Santa Cruz Biotechnologies); and HSP70 and HSP90 (Enzo Life Sciences). Chemiluminescence was used to detect immunoreactive co-precipitating proteins.

2.6. Bioinformatics

An analysis of primary patient clinical samples in The Cancer Genome Atlas (TCGA; https://tcga-data.nci.nih.gov/tcga/) database was used to evaluate NHE1 gene expression in normal breast tissue *versus* primary breast tumours. Level 3 TCGA RNAseqV2 gene expression, reverse phase protein array (RPPA), and clinical data from 1062 breast cancer and 113 associated normal patient samples were obtained from the TCGA Data Portal (https://tcga-

data.nci.nih.gov/tcga/) in August 2014. Subtype data for tumours was accessed from the UCSC cancer genome browser (RNASeqV2 defined). All statistical analyses and visualizations for bioinformatics datasets were conducted in the RStudio programming environment (v0.98.501). R/Bioconductor packages ggplot2 and plyr were used where appropriate.

3. Results

3.1. Determination of NHE1 interacting partners by mass spectrometry

We used mass spectrometry analysis in order to determine potential novel intracellular interaction partners of NHE1 in triple-negative MDA-MB-231 breast cancer cells (Table 1). An affinity chromatography-based protein purification method using a glutathione S-transferase (GST) fusion protein of the C terminus of the rabbit NHE1 spanning amino acids 545 to 816 (NHE1-GST) was used to detect protein-protein interactions in MDA-MB-231 cell lysates. Interacting proteins unique to the NHE1-GST sample were selected based on the number of detected peptide spectral matches (PSM). The highest number of PSMs was attributed to NHE1 (*SLC9A1*), indicating the presence of NHE1 homodimers, which has been reported earlier [20]. Several proteins involved in cellular biosynthesis, metabolism, signal transduction, and cytoskeletal organization were found to uniquely interact with NHE1 (over the control) as listed in Table 1. For interacting proteins present in both the GST control and the NHE1-GST sample, the binary logarithm of the ratio of the number of PSMs in the sample *versus* control was calculated ($\log2[PSM_{NHE1\text{-}GST}/PSM_{GST}]$). A two-fold or higher detection of interacting proteins in the sample over the control was considered significant (Table 2).

3.2. Validation of NHE1 interaction partners by co-immunoprecipitation

We validated several putative intracellular protein binding partners of NHE1, both as determined by the above MS analysis and previous studies (reviewed in [3]), by co-immunoprecipitation of HA-tagged NHE1 with target proteins. In breast cancer cells, NHE1 becomes hyper-active (stimulated) with serum deprivation [14, 21, 22]. We cultured MDA-MB-231 cells in stimulated (+, serum-deprived, 0.2% serum) and unstimulated (−, serum-supplemented, 10% serum) conditions prior to lysis and co-immunoprecipitation. This was followed by immunoblotting with antibodies against target proteins. NHE1 was found to co-immunoprecipitate with: AKT (protein kinase B, PKB), α-enolase, CHP1 (calcineurin homologous protein 1), 14-3-3, and heat shock proteins HSP70 and HSP90 [Fig. 1; N=3]. No significant differences in the interaction of NHE1 with AKT, CHP1, HSP70 or HSP90 were observed between stimulated and unstimulated cells. However, in stimulated MDA-MB-231 cells where basal NHE1 exchange activity is significantly higher [14, 21], the dimeric form of 14-3-3 was associated with NHE1 in greater amounts than the monomeric form. Also, α-enolase is mainly associated with NHE1 in stimulated cells.

3.3. Na^+/H^+ exchanger NHE1 mRNA expression in primary patient clinical breast cancer subtypes

The Cancer Genome Atlas (TCGA; www.cancergenome.nih.gov), an initiative of the National Cancer Institute and the National Human Genome Research Institute, is a cancer genomic database containing tumour tissue and normal tissue samples from hundreds of patients. We used TCGA to analyze the expression of *NHE1* mRNA in primary breast tumours and

associated normal mammary tissue from patient samples (n=1062 and 113, respectively). Our analysis revealed that *NHE1* mRNA expression significantly increased in breast tumour samples over their matched normal counterparts (Fig. 2a, *P<0.001). When expression levels were examined by breast cancer subtype, however, we found significantly decreased *NHE1* expression in the basal subtype compared to luminal and HER2+ subtypes [23] (Fig. 2b, *P<0.001). Though *NHE1* expression levels tended to be higher than normal in HER+ tumours, this effect was not significant.

3.4. Reverse phase protein array (RPPA) analysis of NHE1 interacting partners from primary patient tumours

Reverse phase protein arrays (RPPA) are a high-throughput, antibody-based determination of protein expression levels in primary patient tumour tissue samples from TCGA. We analyzed correlations of *NHE1* mRNA expression with that of proteins in primary tumours from patients with the basal (triple-negative) clinical subtype of breast cancer using the RPPA-TCGA database (N=220). As seen in Table 3, NHE1 expression levels positively and significantly correlated with the expression of: phospho-p90^{RSK} (pT359/pS363; $r = 0.31$), phospho-GSK3 (α(pS21), β(pS9); $r = 0.31$), β-catenin ($r = 0.27$), AKT (pS473; $r = 0.26$), NF2 (neurofibromin 2; $r = 0.24$), and Src (pY416; $r = 0.23$). A further analysis of *NHE1* mRNA expression levels in basal *versus* luminal breast cancer samples revealed a weakly positive but significant correlation of NHE1 with phospho-p90^{RSK} (T359/S363) protein expression in only basal-type cancers ($r = -0.05$ in luminal samples *versus* $r = 0.31$ in basal samples; Fig. 3). Additionally, *NHE1* expression in basal breast cancer tumours negatively correlated with the expression of: Notch1 ($r = -0.22$), FOXO3a (forkhead box O3a; $r = -0.23$), ACC1 (acetyl-CoA

carboxylase; $r = -0.23$), p27 (pT157; $r = -0.24$), Chk1 ($r = -0.25$), Smad4 ($r = -0.25$), and cyclin B1 ($r = -0.26$) (Table 3).

4. Discussion

NHE1 is regulated by both kinase-mediated phosphorylation and by interactions with intracellular proteins and lipids that modulate its activity. These regulators primarily affect NHE1 activity by shifting its pH *versus* activity curve to make it more active at more alkaline intracellular pH [3, 11-13]. In transformed cells, cytoplasmic alkalinization brought about by increased Na^+ uptake is largely driven by NHE1 and is a critical step in tumourigenesis. The resultant increased H^+ efflux also mediates metastasis by providing a more acidic extracellular microenvironment that not only promotes the development of a more invasive cell phenotype [6], but is also optimal for the activity of secreted serine proteases and matrix metalloproteinases. These proteolytic enzymes digest a path through the extracellular matrix allowing for cells to leave the primary tumour and enter the bloodstream [24, 25]. It is also of note that elevated NHE1 activity contributes of sodium loading of cells. Elevated sodium loading by NHE1 and other sodium loading proteins such as sodium channels and Na^+/HCO_3^- co-transport, may promote mitogenesis and/or oncognesis [26-30] and elevated tissue sodium is reported in malignant breast cancer [31] and other cancer types [32, 33]. Thus elevated NHE1 activity may contribute to oncogenesis by more than one mechanism.

Since the elevated activity of NHE1 contributes to metastasis, there is great interest in deciphering how this activity is regulated in breast cancer cells. In other tissues, the cytosolic C-terminal tail of NHE1 is a binding site for signal kinases, regulatory proteins, and linkages to the cell cytoskeleton [4, 13, 15, 16] suggesting that it is acting as a scaffolding rather than an adaptor

protein. In this study, we used different approaches to identify individual molecular players associated with the NHE1 signal scaffold in breast cancer. We looked at direct interactions determined by affinity chromatography and identified by mass spectrometry and we also analyzed hundreds of primary patient tumour samples characterized by The Cancer Genome Atlas.

4.1. NHE1 and cytoskeletal proteins

Our affinity chromatography experiments identified alpha-actinin-4, moesin, ezrin, F-actin-capping protein, talin-1, profilin and alpha-actinin-1 (Table 1, 2) as cytoskeletal proteins from MDA-MB-231 cells that were associated with the cytosolic tail of NHE1. Our study is the first identification of the association of most of these proteins with NHE1 in this cell type, which is representative of basal triple-negative breast cancer. However, many of these and related proteins have been suggested to be involved with NHE1 in other cell types. Ezrin and moesin (and radxin) are known to bind to NHE1 [34]; in fact, ezrin co-localizes with NHE1 in MDA-MB-231 cells [35]. We also found talin-1 associated with NHE1. Talin is a large protein that links integrins to the actin cytoskeleton, binds moesin, and recruits a moesin-NHE1 complex to invadopodia, stabilizing them and promoting matrix degradation [36].

Our results also revealed novel interactions of NHE1 with the actin-binding proteins alpha-actinin 1 and 4, and profilin-1 in MDA-MB-231 cells. Alpha-actinin-1 and 4 are the non-muscle isoforms of the protein generally believed to be structural components of F-actin cohesion in cells [37]. Alpha-actinin-4 is upregulated in some types of cancer and promotes proliferation of luminal MCF-7 breast cancer cells [37, 38]. Profilin is a ubiquitously expressed actin-binding protein that can regulate invadopodia maturation [39] and is overexpressed in breast cancer cells

[40]. To our knowledge, profilin-1 has not been previously shown to be associated with NHE1, and this interaction is intriguing considering its role in invadopodia maturation [39]. However, we were unable to confirm these interactions further by co-immunoprecipitation.

4.2. NHE1 and chaperone proteins

Various chaperone proteins were also identified as associating with NHE1 by affinity chromatography, including heat shock proteins. We confirmed the interaction of HSP70 and HSP90 with NHE1 by co-immunoprecipitation. NHE1 and HSP70 were previously shown to interact in PS127A Chinese hamster fibroblasts [17]. More recently [41], HSP70 was found to be dynamically associated with NHE1 in macrophages. Heat shock proteins are inducible molecular chaperones that facilitate the folding of proteins and their transport across membranes, and prevent protein aggregation in response to cellular stress. Tumour cells express higher levels of HSP70 and HSP90, which play a role in metastasis, proliferation and protection against apoptosis [42]. This leaves open the possibility that some of their actions are mediated through NHE1, which also facilitates tumour cell metastasis [8, 14].

Other chaperones found associated with NHE1 included protein disulfide isomerase, peroxiredoxin-2, thioredoxin, calnexin and calreticulin. These proteins are likely involved in the folding of NHE1. Several laboratories [43, 44] have shown that elevated NHE1 expression is associated with increased calreticulin and protein disulfide isomerase expression, implicating an association of some of these proteins with NHE1 in other tissues. Glucose-regulated proteins were also increased with elevated NHE1 expression in the myocardium [43]. Here, we found an association of 78 kDa glucose-regulated protein with NHE1 (Table 2). Endoplasmin, which was

also found associated with the NHE1 tail (Table 1), is known to be elevated in breast cancer with distant metastasis and is associated with decreased patient survival [45].

4.3. NHE1 and biosynthetic proteins

We identified several proteins of biosynthetic pathways associated with NHE1 including glutamate dehydrogenase, fructose-bisphosphate aldolase, transketolase, glyceraldehyde-3-phosphate dehydrogenase and α-enolase (Table 1, 2). α-enolase is a cytosolic glycolytic enzyme and oxidative stress protein. Its expression is elevated in many tumours, including those of the breast, where it is also found on the cell surface [46, 47]. The NHE1-α-enolase complex was confirmed by both affinity chromatography and co-immunoprecipitation. Interestingly, α-enolase was found to be more strongly associated with NHE1 from cells cultured in stimulated, serum-depleted conditions where the exchanger is hyperactive. The physiological significance of such associations are not clear; however, glycolytic enzymes are known to bind to other membrane proteins, such as band 3, and the binding process assists in regulation of metabolism [48]. Whether NHE1 plays a similar role is not known. The only kinase found to be directly associated with the NHE1 cytosolic tail in affinity chromatography experiments was phosphoglycerate kinase, which plays a role in regulating the glycolytic pathway.

4.4. NHE1 and signal transduction proteins

Calcineurin homologous protein 1 (CHP1) has been shown to form a complex with the cytosolic domain of NHE1, helping to stabilize it at the cell surface in some cell types [4, 49]. Surprisingly, we did not find CHP1 associated with NHE1 by affinity chromatography

experiments but were able to demonstrate an association with NHE1 through co-immunoprecipitation from MDA-MB-231 cells. Other proteins found associated with NHE1 by affinity chromatography experiments included: cathepsin D, galectin 1, voltage-dependent anion selective channel protein 1, a chloride channel, a 40s ribosomal protein SA, nucleolin and heterogeneous nuclear ribonucleoprotein A1. Some of these proteins have an association with cancer progression. For example, cathepsin D is a lysosomal protease overexpressed in many metastatic breast cancer cell lines and its presence in the cytosol of primary breast tumours is indicative of an increased risk of metastatic disease [50]. Galectin-1 is a β-galactosidase-binding protein thought to be involved in cell-cell interactions. It is abnormally expressed in cancer cells and increased expression in many cancers, including breast cancer, is generally associated with poor prognosis [51]. Nucleolin is also overexpressed in breast cancer cell populations [52]. Our attempts to validate the interaction of cathepsin D with NHE1 by co-IP experiments were unsuccessful; validation of the other protein-protein interactions were not attempted at this time.

Significantly, we identified several isoforms of 14-3-3 proteins that bind to the NHE1 tail region by affinity chromatography (Table 1, 2) and this was confirmed by co-immunoprecipitation experiments. 14-3-3 proteins bind intracellular ligands which include signal kinases, phosphatases and other proteins. The 14-3-3 proteins have been demonstrated to interact with the NHE1 tail. They regulate phosphorylation of serine 703 of NHE1 in the muscle tissues [53, 54]. 14-3-3 binding to NHE1 in breast cancer cells has not been reported. We have, however, previously demonstrated that serine 703, the binding site of 14-3-3 on the NHE1 C-terminal, plays an important role in the regulation of epithelial-mesenchymal transition of triple-negative MDA-MB-231 breast cancer cells [14]. These results support the suggestion that 14-3-3 binding to NHE1 is important in the regulation of the exchanger in TNBC cells. Furthermore, this data also supports the idea that NHE1 is acting more as a scaffold protein, as opposed to just an

adaptor protein, with definitive functional consequences to many of these interactions as suggested earlier [15].

4.5. NHE1 expression and protein associations in primary patient breast tumours

To supplement the above findings, we examined correlations in the expression of NHE1 and other signal proteins in primary patient tumours. We previously used the ONCOMINE™ database [21] to show that, in a comparison of multiple cancers *versus* matched normal tissue, *NHE1* mRNA expression is elevated in some types of cancer including breast cancer. In this study, we used The Cancer Genome Atlas (TCGA) to analyse *NHE1* mRNA expression in primary patient breast tumours of various clinical subtypes. The TCGA is a more robust RNA-sequencing database with a much larger patient breast tumour and normal tissue cohort. The TCGA also has an additional analytic capability linked to a reverse phase protein array (RPPA) database that enables the comparison of the expression of multiple proteins with a particular gene of interest. A limitation is that NHE1 expression can only be determined at the mRNA level in currently available tumour genomics databases. Despite this limitation, useful information can still be collected from this analysis that will lead to further investigations.

Our initial TCGA analysis showed that *NHE1* mRNA expression was significantly higher in breast tumours than in normal mammary tissue (Fig. 2a). Interestingly, a more in-depth analysis classified by specific breast cancer subtypes (luminal A and B, HER2-amplified and basal) revealed that *NHE1* mRNA expression is mostly unchanged in luminal and HER2-amplified tumours and actually lower in basal (triple-negative) tumours compared to normal mammary tissue (Fig. 2b). The reason for this discrepancy is unclear. However, it is possible that, based on the sample sizes for the two analyses (see legends for Fig. 2a and 2b), the subtype-

specific tumour tissues in Fig. 2b may only account for a percentage of the sample pool used to generate Fig. 2a.

The lower *NHE1* mRNA expression in basal breast tumours may be indicative of a likewise lower expression of NHE1 protein. At first glance, this result appears to contradict the suggestion that NHE1 plays an important role in breast tumour progression. Particularly since the basal subtype represents the most aggressively metastatic cancers. However, NHE1 is a tightly regulated protein that is modulated by both phosphorylation and protein binding, and the regulation of its activity is critical in metastatic basal-like breast cancer cells. More NHE1 protein, if inactive, would not affect intracellular pH. Several laboratories, including ours, have shown that NHE1 is hyper-activated in breast cancer cells in the acidic, hypoxic, and serum-depleted tumour microenvironment [5, 8, 14]; in our studies, this increased activity was not accompanied by a similar increase in NHE1 protein expression. In heart disease an analogous situation exists. The activation of NHE1, and not increased expression of the protein per se, is the critical factor determining NHE1-mediated disease progression [55]. Further to this end, we recently demonstrated that replacement of endogenous NHE1 in MDA-MB-231 cells with a hyperactive NHE1 protein resulted in an increased metastatic behaviour of this cell line [14]. This also supports the hypothesis that activation and regulation of NHE1 are critical determinants.

We used the TCGA-RPPA database to identify correlations between *NHE1* mRNA expression and a number of intracellular signalling molecules. We found several novel, as well as previously identified, potential regulators of the protein. $p90^{RSK}$ and AKT (protein kinase B) are known to regulate NHE1 activity. $p90^{RSK}$ phosphorylates NHE1 at Ser703 and stimulates its activity, mediating growth factor activation of the protein in some cell types [4, 56]. We recently showed that a mutation at serine 703 to non-phosphorylatable alanine markedly changed the morphology of MDA-MB-231 breast cancer cells from a mesenchymal to a more epithelial-like

phenotype with a concomitant loss of metastatic potential [14]. In this study, we found a weak but significant positive correlation between *NHE1* mRNA and the expression of phosphorylated $p90^{RSK}$ at the protein level in primary metastatic basal (triple-negative), but not luminal, breast tumours (Fig. 3). This leaves open the possibility that $p90^{RSK}$-mediated phosphorylation of NHE1 is at least partially responsible for the aggressive metastatic behaviour of TNBC cells. Indeed, we have previously demonstrated that BI-D1870, a specific inhibitor of $p90^{RSK}$, inhibits the metastatic potential of several TNBC cell types, supporting this hypothesis [14]. Though these results are significant, the pattern of the positive correlation is dispersed. The reason for this is not immediately clear but could be due to the influence of other NHE1 regulators.

Another kinase known to regulate NHE1 is AKT. The exact role of AKT-mediated phosphorylation of NHE1 is dependent on cell type [4]. In the myocardium, NHE1 activity is inhibited by AKT-mediated phosphorylation of serine 648 [57, 58]. However, in fibroblasts, AKT phosphorylation of NHE1 is stimulatory, induces cytoskeletal remodeling, and is necessary for the activation of NHE1 by insulin and PDGF [59]. We confirmed that AKT interacts with NHE1 in MDA-MB-231 cells by co-immunoprecipitation experiments (Fig. 1). TCGA-RPPA analysis showed that AKT protein expression in patient tumours of the triple-negative basal clinical subtype positively, correlated with NHE1 expression though weakly (Table 3).

Other proteins that were found to weakly, positively correlate with NHE1 in RPPA were pGSK3, NF2, Src and β-catenin. To our knowledge, the direct demonstration of these correlations with NHE1 in triple-negative breast cancer would be novel and could be the subject of future investigations. Potential phosphorylation sites for GSK3 exist [4]. The expression of some proteins negatively correlated with *NHE1* mRNA expression (Table 3). The significance of this is not yet clear.

Conclusion

In breast cancer, the dysregulation of pH homeostasis chiefly caused by hyper-activation of NHE1 plays a critical role in triggering and sustaining metastasis, where altered dynamics of Na^+/H^+ exchange are a key determinant of metastatic potential. This hyper-activation of NHE1 is particularly important in triple-negative breast cancer where it is primarily responsible for the acidic pH of the tumour microenvironment that facilitates metastasis [5, 6, 8]. NHE1 is regulated by protein-protein interactions and by phosphorylation/dephosphorylation. Here, we examined proteins binding to NHE1 in triple-negative breast cancer cells and those correlating with NHE1 gene expression in primary patient breast tumour samples. We found many categories of proteins binding to the NHE1 regulatory cytosolic domain including several cytoskeletal, chaperone, biosynthesis, and signalling proteins. It is noteworthy that many NHE1-binding proteins have elevated expression in breast and other cancer types; this raises the possibility that their function could impact NHE1 activity (or *vice versa*) and contribute to its role in metastasis. Our study is the first definition of the interactome of NHE1 in triple-negative breast cancer cells.

Contributors:

SRA, KMV, LMP and LF designed the study and methodology. SRA, KMV, LMP and LF wrote the manuscript, and LF and SRA made final corrections prior to submission. JMW completed the affinity chromatography and immunoprecipitation experiments, and KMV did the acquisition and analysis of TCGA and RPPA data. All authors read and approved the final article.

Disclosure:

The authors declare no potential conflicts of interest.

Acknowledgements:

We thank Jack Moore and Dr. Richard Fahlman from the Alberta Proteomics and Mass Spectrometry Facility at the University of Alberta for their assistance with the mass spectrometry data acquisition and analysis respectively. SRA was supported by a Postdoctoral Fellowship Grant from the Canadian Breast Cancer Foundation (CBCF), Prairies-Northwest Territories Division and by CIHR funding to LF. JMW was supported by Women and Children's Health Research Institute funding to LF. Research support for this project was to LF from CIHR and from the Women and Children's Health Research Institute through the Breast Cancer Society of Canada. This work was supported by an Alberta Innovates Health Solutions Translational Health Chair in cancer and a Canadian Breast Cancer Foundation operating grant awarded to LMP. LMP was the recipient of the Peter-Lougheed Premier New Investigator Award from the Canadian Institutes of Health Research. KMV is a Vanier Scholar. Funding sources had no involvement in study design.

References

[1] S. Wakabayashi, T. Ikeda, T. Iwamoto, J. Pouyssegur, M. Shigekawa, Calmodulin-Binding autoinhibitory domain controls "pH-Sensing" in the Na^+/H^+ exchanger NHE1 through sequence specific interaction, Biochem. 36 (1997) 12854-12861.

[2] B.L. Lee, B.D. Sykes, L. Fliegel, Structural and functional insights into the cardiac Na(+)/H(+) exchanger, J Mol Cell Cardiol 61 (2013) 60-7.

[3] S.R. Amith, L. Fliegel, Regulation of the Na^+/H^+ Exchanger (NHE1) in Breast Cancer Metastasis, Cancer Res 73(4) (2013) 1259-64.

[4] R. Hendus-Altenburger, B.B. Kragelund, S.F. Pedersen, Structural dynamics and regulation of the mammalian SLC9A family of Na(+)/H(+) exchangers, Curr Top Membr 73 (2014) 69-148.
[5] S.J. Reshkin, A. Bellizzi, S. Caldeira, V. Albarani, I. Malanchi, M. Poignee, M. Alunni-Fabbroni, V. Casavola, M. Tommasino, Na^+/H^+ exchanger-dependent intracellular alkalinization is an early event in malignant transformation and plays an essential role in the development of subsequent transformation-associated phenotypes, FASEB J 14(14) (2000) 2185-2197.
[6] R.A. Cardone, V. Casavola, S.J. Reshkin, The role of disturbed pH dynamics and the Na^+/H^+ exchanger in metastasis, Nat Rev Cancer 5(10) (2005) 786-95.
[7] S. Badve, D.J. Dabbs, S.J. Schnitt, F.L. Baehner, T. Decker, V. Eusebi, S.B. Fox, S. Ichihara, J. Jacquemier, S.R. Lakhani, J. Palacios, E.A. Rakha, A.L. Richardson, F.C. Schmitt, P.H. Tan, G.M. Tse, B. Weigelt, I.O. Ellis, J.S. Reis-Filho, Basal-like and triple-negative breast cancers: a critical review with an emphasis on the implications for pathologists and oncologists, Mod Pathol 24(2) (2011) 157-67.
[8] S.R. Amith, J.M. Wilkinson, S. Baksh, L. Fliegel, Na^+/H^+ exchanger (NHE1) as a novel co-adjuvant target in paclitaxel therapy of triple-negative breast cancer cells, Oncotarget 6((2)) (2015) 1262-75.
[9] S.A. O'Toole, J.M. Beith, E.K. Millar, R. West, A. McLean, A. Cazet, A. Swarbrick, S.R. Oakes, Therapeutic targets in triple negative breast cancer, J Clin Pathol 66(6) (2013) 530-42.
[10] S. Wakabayashi, B. Bertrand, M. Shigekawa, P. Fafournoux, J. Pouyssegur, Growth factor activation and "H^+-sensing" of the Na^+/H^+ exchanger isoform 1 (NHE1), J. Biol. Chem. 269 (1994) 5583-5588.
[11] L. Fliegel, Regulation of the Na^+/H^+ exchanger in the healthy and diseased myocardium, Expert Opin Ther Targets 13(1) (2009) 55-68.
[12] S. Bandyopadhyay, C.Y. Chiang, J. Srivastava, M. Gersten, S. White, R. Bell, C. Kurschner, C.H. Martin, M. Smoot, S. Sahasrabudhe, D.L. Barber, S.K. Chanda, T. Ideker, A human MAP kinase interactome, Nat Methods 7(10) (2010) 801-5.
[13] M.E. Meima, J.R. Mackley, D.L. Barber, Beyond ion translocation: structural functions of the sodium-hydrogen exchanger isoform-1, Curr Opin Nephrol Hypertens 16(4) (2007) 365-72.
[14] S.R. Amith, J.M. Wilkinson, L. Fliegel, Na^+/H^+ exchanger NHE1 regulation modulates metastatic potential and epithelial-mesenchymal transition of triple-negative breast cancer cells, Oncotarget 7(16) (2016) 21091-113.

[15] R. Hendus-Altenburger, E. Pedraz-Cuesta, C.W. Olesen, E. Papaleo, J.A. Schnell, J.T. Hopper, C.V. Robinson, S.F. Pedersen, B.B. Kragelund, The human Na(+)/H(+) exchanger 1 is a membrane scaffold protein for extracellular signal-regulated kinase 2, BMC Biol 14 (2016) 31.
[16] M. Baumgartner, Patel H, Barber DL, Na(+)/H(+) exchanger NHE1 as plasma membrane scaffold in the assembly of signaling complexes, Am J Physiol Cell Physiol 287 (2004) C844-C850.
[17] N.L.C.L. Silva, R.S. Haworth, D. Singh, L. Fliegel, The Carboxyl-terminal region of the Na^+/H^+ exchanger interacts with mammalian heat shock protein., Biochemistry 34 (1995) 10412-10420.
[18] B.L. Bullis, X. Li, D.N. Singh, L.G. Berthiaume, L. Fliegel, Properties of the Na^+/H^+ exchanger protein. Detergent-resistant aggregation and membrane microdistribution, Eur J Biochem 269(19) (2002) 4887-4895.
[19] P. Karki, X. Li, D. Schrama, L. Fliegel, B-RAF is associated with and activates the NHE1 isoform of the Na^+/H^+ exchanger, J Biol Chem 286(15) (2011) 13096-105.
[20] P. Fafournoux, J. Noel, J. Pouysségur., Evidence that Na^+/H^+ exchanger isoforms NHE1 and NHE3 exist as stable dimers in membranes with a high degree of specificity for homodimers, J. Biol. Chem. 269 (1994) 2589-2596.
[21] S.R. Amith, S. Fong, S. Baksh, L. Fliegel, Na (+)/H (+)exchange in the tumour microenvironment: does NHE1 drive breast cancer carcinogenesis?, Int J Dev Biol 59(7-9) (2015) 367-77.
[22] S.J. Reshkin, A. Bellizzi, V. Albarani, L. Guerra, M. Tommasino, A. Paradiso, V. Casavola, Phosphoinositide 3-kinase is involved in the tumor-specific activation of human breast cancer cell Na(+)/H(+) exchange, motility, and invasion induced by serum deprivation, J. Biol. Chem. 275(8) (2000) 5361-5369.
[23] X. Dai, T. Li, Z. Bai, Y. Yang, X. Liu, J. Zhan, B. Shi, Breast cancer intrinsic subtype classification, clinical use and future trends, Am J Cancer Res 5(10) (2015) 2929-43.
[24] M.R. Greco, E. Antelmi, G. Busco, L. Guerra, R. Rubino, V. Casavola, S.J. Reshkin, R.A. Cardone, Protease activity at invadopodial focal digestive areas is dependent on NHE1-driven acidic pHe, Oncol Rep 31(2) (2014) 940-6.

[25] G. Busco, R.A. Cardone, M.R. Greco, A. Bellizzi, M. Colella, E. Antelmi, M.T. Mancini, M.E. Dell'Aquila, V. Casavola, A. Paradiso, S.J. Reshkin, NHE1 promotes invadopodial ECM proteolysis through acidification of the peri-invadopodial space, FASEB J 24(10) (2010) 3903-15.
[26] R. Onkal, M.B. Djamgoz, Molecular pharmacology of voltage-gated sodium channel expression in metastatic disease: clinical potential of neonatal Nav1.5 in breast cancer, Eur J Pharmacol 625(1-3) (2009) 206-19.
[27] P. Wong, H.W. Kleemann, I.F. Tannock, Cytostatic potential of novel agents that inhibit the regulation of intracellular pH, British J Cancer 87(2) (2002) 238-45.
[28] I.L. Cameron, N.K. Smith, T.B. Pool, R.L. Sparks, Intracellular concentration of sodium and other elements as related to mitogenesis and oncogenesis in vivo, Cancer Res 40(5) (1980) 1493-500.
[29] R.L. Sparks, T.B. Pool, N.K. Smith, I.L. Cameron, Effects of amiloride on tumor growth and intracellular element content of tumor cells in vivo, Cancer Res 43(1) (1983) 73-7.
[30] M. Lobikin, B. Chernet, D. Lobo, M. Levin, Resting potential, oncogene-induced tumorigenesis, and metastasis: the bioelectric basis of cancer in vivo, Phys Biol 9(6) (2012) 065002.
[31] R. Ouwerkerk, M.A. Jacobs, K.J. Macura, A.C. Wolff, V. Stearns, S.D. Mezban, N.F. Khouri, D.A. Bluemke, P.A. Bottomley, Elevated tissue sodium concentration in malignant breast lesions detected with non-invasive 23Na MRI, Breast Cancer Res Treat 106(2) (2007) 151-60.
[32] K.R. Thulborn, D. Davis, H. Adams, T. Gindin, J. Zhou, Quantitative tissue sodium concentration mapping of the growth of focal cerebral tumors with sodium magnetic resonance imaging, Magn Reson Med 41(2) (1999) 351-9.
[33] M. Stubbs, L. Rodrigues, F.A. Howe, J. Wang, K.S. Jeong, R.L. Veech, J.R. Griffiths, Metabolic consequences of a reversed pH gradient in rat tumors, Cancer Res 54(15) (1994) 4011-6.
[34] S.P. Denker, D.C. Huang, J. Orlowski, H. Furthmayr, D.L. Barber, Direct binding of the Na--H exchanger NHE1 to ERM proteins regulates the cortical cytoskeleton and cell shape independently of H(+) translocation, Mol Cell 6(6) (2000) 1425-1436.
[35] E. Antelmi, R.A. Cardone, M.R. Greco, R. Rubino, F. Di Sole, N.A. Martino, V. Casavola, M. Carcangiu, L. Moro, S.J. Reshkin, ss1 integrin binding phosphorylates ezrin at T567 to

activate a lipid raft signalsome driving invadopodia activity and invasion, PLoS One 8(9) (2013) e75113.
[36] B.T. Beaty, Y. Wang, J.J. Bravo-Cordero, V.P. Sharma, V. Miskolci, L. Hodgson, J. Condeelis, Talin regulates moesin-NHE-1 recruitment to invadopodia and promotes mammary tumor metastasis, J Cell Biol 205(5) (2014) 737-51.
[37] K.G. Oikonomou, K. Zachou, G.N. Dalekos, Alpha-actinin: a multidisciplinary protein with important role in B-cell driven autoimmunity, Autoimmun Rev 10(7) (2011) 389-96.
[38] S. Khurana, S. Chakraborty, X. Cheng, Y.T. Su, H.Y. Kao, The actin-binding protein, actinin alpha 4 (ACTN4), is a nuclear receptor coactivator that promotes proliferation of MCF-7 breast cancer cells, J Biol Chem 286(3) (2011) 1850-9.
[39] A. Valenzuela-Iglesias, V.P. Sharma, B.T. Beaty, Z. Ding, L.E. Gutierrez-Millan, P. Roy, J.S. Condeelis, J.J. Bravo-Cordero, Profilin1 regulates invadopodium maturation in human breast cancer cells, Eur J Cell Biol 94(2) (2015) 78-89.
[40] J.V. Coumans, D. Gau, A. Poljak, V. Wasinger, P. Roy, P.D. Moens, Profilin-1 overexpression in MDA-MB-231 breast cancer cells is associated with alterations in proteomics biomarkers of cell proliferation, survival, and motility as revealed by global proteomics analyses, OMICS 18(12) (2014) 778-91.
[41] C. Huang, J. Wang, Z. Chen, Y. Wang, W. Zhang, 2-phenylethynesulfonamide Prevents Induction of Pro-inflammatory Factors and Attenuates LPS-induced Liver Injury by Targeting NHE1-Hsp70 Complex in Mice, PLoS One 8(6) (2013) e67582.
[42] M. Shevtsov, G. Multhoff, Heat Shock Protein-Peptide and HSP-Based Immunotherapies for the Treatment of Cancer, Frontiers in immunology 7 (2016) 171.
[43] A.R. Cook, S.C. Bardswell, S. Pretheshan, K. Dighe, G.S. Kanaganayagam, R.I. Jabr, S. Merkle, M.S. Marber, S. Engelhardt, M. Avkiran, Paradoxical resistance to myocardial ischemia and age-related cardiomyopathy in NHE1 transgenic mice: a role for ER stress?, J Mol Cell Cardiol 46(2) (2009) 225-33.
[44] P. Karki, L. Fliegel, Overexpression of the NHE1 isoform of the Na(+)/H (+) exchanger causes elevated apoptosis in isolated cardiomyocytes after hypoxia/reoxygenation challenge, Mol Cell Biochem 338(1-2) (2010) 47-57.
[45] T.R. Cawthorn, J.C. Moreno, M. Dharsee, D. Tran-Thanh, S. Ackloo, P.H. Zhu, G. Sardana, J. Chen, P. Kupchak, L.M. Jacks, N.A. Miller, B.J. Youngson, V. Iakovlev, C.J. Guidos, K.A.

Vallis, K.R. Evans, D. McCready, W.L. Leong, S.J. Done, Proteomic Analyses Reveal High Expression of Decorin and Endoplasmin (HSP90B1) Are Associated with Breast Cancer Metastasis and Decreased Survival, Plos One 7(2) (2012) e30992.
[46] K.C. Hsiao, N.Y. Shih, H.L. Fang, T.S. Huang, C.C. Kuo, P.Y. Chu, Y.M. Hung, S.W. Chou, Y.Y. Yang, G.C. Chang, K.J. Liu, Surface alpha-enolase promotes extracellular matrix degradation and tumor metastasis and represents a new therapeutic target, PloS one 8(7) (2013) e69354.
[47] J. Gao, R. Zhao, Y. Xue, Z. Niu, K. Cui, F. Yu, B. Zhang, S. Li, Role of enolase-1 in response to hypoxia in breast cancer: exploring the mechanisms of action, Oncol Rep 29(4) (2013) 1322-32.
[48] I.A. Lewis, M.E. Campanella, J.L. Markley, P.S. Low, Role of band 3 in regulating metabolic flux of red blood cells, Proc Natl Acad Sci U S A 106(44) (2009) 18515-20.
[49] M. Matsushita, H. Tanaka, K. Mitsui, H. Kanazawa, Dual functional significance of calcineurin homologous protein 1 binding to Na(+)/H(+) exchanger isoform 1, Am J Physiol (Cell Physiol) 301(2) (2011) C280-8.
[50] M. Garcia, N. Platet, E. Liaudet, V. Laurent, D. Derocq, J.P. Brouillet, H. Rochefort, Biological and clinical significance of cathepsin D in breast cancer metastasis, Stem Cells 14(6) (1996) 642-50.
[51] V.L. Thijssen, R. Heusschen, J. Caers, A.W. Griffioen, Galectin expression in cancer diagnosis and prognosis: A systematic review, Biochim Biophys Acta 1855(2) (2015) 235-47.
[52] N.A. Fonseca, A.S. Rodrigues, P. Rodrigues-Santos, V. Alves, A.C. Gregorio, A. Valerio-Fernandes, L.C. Gomes-da-Silva, M.S. Rosa, V. Moura, J. Ramalho-Santos, S. Simoes, J.N. Moreira, Nucleolin overexpression in breast cancer cell sub-populations with different stem-like phenotype enables targeted intracellular delivery of synergistic drug combination, Biomaterials 69 (2015) 76-88.
[53] M.E. Cavet, S. Lehoux, B.C. Berk, 14-3-3beta is a p90 ribosomal S6 kinase (RSK) isoform 1-binding protein that negatively regulates RSK kinase activity, J Biol Chem 278(20) (2003) 18376-83.
[54] S. Lehoux, J. Abe, J.A. Florian, B.C. Berk, 14-3-3 Binding to Na^+/H^+ exchanger isoform-1 is associated with serum-dependent activation of Na^+/H^+ exchange, J Biol Chem 276(19) (2001) 15794-15800.

[55] F. Mraiche, T. Oka, X.T. Gan, M. Karmazyn, L. Fliegel, Activated NHE1 is required to induce early cardiac hypertrophy in mice, Basic Res Cardiol 106 (2011) 603-616.
[56] E. Takahashi, J. Abe, B. Gallis, R. Aebersold, D.J. Spring, E.G. Krebs, B.C. Berk, p90(RSK) is a serum-stimulated Na^+/H^+ exchanger isoform-1 kinase. Regulatory phosphorylation of serine 703 of Na^+/H^+ exchanger isoform-1., J. Biol. Chem. 274(29) (1999) 20206-20214.
[57] A.K. Snabaitis, F. Cuello, M. Avkiran, Protein kinase B/Akt phosphorylates and inhibits the cardiac Na^+/H^+ exchanger NHE1, Circ Res 103(8) (2008) 881-90.
[58] A.M. Yeves, M.C. Villa-Abrille, N.G. Perez, A.J. Medina, E.M. Escudero, I.L. Ennis, Physiological cardiac hypertrophy: critical role of AKT in the prevention of NHE-1 hyperactivity, J Mol Cell Cardiol 76 (2014) 186-95.
[59] M.E. Meima, B.A. Webb, H.E. Witkowska, D.L. Barber, The sodium-hydrogen exchanger NHE1 is an Akt substrate necessary for actin filament reorganization by growth factors, J Biol Chem 284(39) (2009) 26666-75.
[60] S.M. Yoo, S.J. Cho, Y.Y. Cho, Molecular Targeting of ERKs/RSK2 Signaling Axis in Cancer Prevention, Journal of cancer prevention 20(3) (2015) 165-71.
[61] N. Tejeda-Munoz, M. Robles-Flores, Glycogen synthase kinase 3 in Wnt signaling pathway and cancer, IUBMB life 67(12) (2015) 914-22.
[62] P. De, J.H. Carlson, H. Wu, A. Marcus, B. Leyland-Jones, N. Dey, Wnt-beta-catenin pathway signals metastasis-associated tumor cell phenotypes in triple negative breast cancers, Oncotarget (2016).
[63] S.X. Yang, E. Polley, S. Lipkowitz, New insights on PI3K/AKT pathway alterations and clinical outcomes in breast cancer, Cancer Treat Rev 45 (2016) 87-96.
[64] A.M. Petrilli, C. Fernandez-Valle, Role of Merlin/NF2 inactivation in tumor biology, Oncogene 35(5) (2016) 537-48.
[65] A. Patel, H. Sabbineni, A. Clarke, P.R. Somanath, Novel roles of Src in cancer cell epithelial-to-mesenchymal transition, vascular permeability, microinvasion and metastasis, Life Sci (2016).
[66] J. Liu, J.X. Shen, X.F. Wen, Y.X. Guo, G.J. Zhang, Targeting Notch degradation system provides promise for breast cancer therapeutics, Crit Rev Oncol Hematol (2016).

[67] X. Yuan, M. Zhang, H. Wu, H. Xu, N. Han, Q. Chu, S. Yu, Y. Chen, K. Wu, Expression of Notch1 Correlates with Breast Cancer Progression and Prognosis, PloS One 10(6) (2015) e0131689.
[68] J.Y. Yang, M.C. Hung, Deciphering the role of forkhead transcription factors in cancer therapy, Curr Drug Targets 12(9) (2011) 1284-90.
[69] E. Furuta, H. Okuda, A. Kobayashi, K. Watabe, Metabolic genes in cancer: their roles in tumor progression and clinical implications, Biochim Biophys Acta 1805(2) (2010) 141-52.
[70] M.D. Larrea, J. Liang, T. Da Silva, F. Hong, S.H. Shao, K. Han, D. Dumont, J.M. Slingerland, Phosphorylation of p27Kip1 regulates assembly and activation of cyclin D1-Cdk4, Mol Cell Biol 28(20) (2008) 6462-72.
[71] Y. Umezawa, T. Kurosu, H. Akiyama, N. Wu, A. Nogami, T. Nagao, O. Miura, Down regulation of Chk1 by p53 plays a role in synergistic induction of apoptosis by chemotherapeutics and inhibitors for Jak2 or BCR/ABL in hematopoietic cells, Oncotarget (2016).
[72] L. Kassem, M. Deygas, L. Fattet, J. Lopez, T. Goulvent, E. Lavergne, S. Chabaud, N. Carrabin, N. Chopin, T. Bachelot, G. Gillet, I. Treilleux, R. Rimokh, TIF1gamma interferes with TGFbeta1/SMAD4 signaling to promote poor outcome in operable breast cancer patients, BMC Cancer 15 (2015) 453.
[73] J. Yuan, A. Kramer, Y. Matthess, R. Yan, B. Spankuch, R. Gatje, R. Knecht, M. Kaufmann, K. Strebhardt, Stable gene silencing of cyclin B1 in tumor cells increases susceptibility to taxol and leads to growth arrest in vivo, Oncogene 25(12) (2006) 1753-62.

Figure Legends:

Figure 1: Validation of MS-determined protein interaction partners of NHE1 by co-immunoprecipitation. Potential protein interaction partners of NHE1 as determined by affinity chromatography, were evaluated by co-immunoprecipitation with HA-tagged NHE1 in stimulated (+, 0.2% serum) and unstimulated (−, 10% serum) culture conditions as described in the "Materials and Methods". Validated interaction partners of NHE1 were: 14-3-3, AKT (protein kinase B, PKB), CHP1 (calcineurin B homologous protein 1), α-Enolase, and heat shock proteins HSP70 and HSP90 [N=3].

Figure 2: The Cancer Genome Atlas (TCGA) analysis of *NHE1* mRNA expression in primary patient breast cancer tumours *versus* normal breast tissue. *a,* *NHE1* mRNA expression is higher in breast cancer tumours *versus* associated tissue controls. Comparison of the mean expression values (RSEM normalized values) of breast cancer tumours (N=1062) and associated normal tissue (N=113). Bars represent means +/− SEM. Significance was determined by Student's t-test [*P<0.001]. ***b,*** *NHE1* mRNA expression values in breast cancer tumours by subtype. Comparison of the mean expression values (RSEM normalized values) of Normal (N=23), Luminal A (N=421), Luminal B (N=192), HER2 enriched (N=67), and basal (N=141) breast cancer subtypes. Significance was determined by a one-way ANOVA followed by Tukey's HSD [*P<0.001].

Figure 3. *NHE1* expression correlates with phospho-p90^{RSK} levels in basal, but not luminal, primary breast cancer samples. Phospho-p90^{RSK} (T359/S363) values (RPPA values) were analyzed for correlation with *NHE1* expression values (RSEM normalized counts) in (***a***) luminal

breast cancer samples (n=141) and (***b***) basal breast cancer samples (n=79). The grey line is the regression line and grey shading depicts the 95% confidence interval of the fit. Spearman correlation coefficients (r) with calculated P values are indicated.

Tables:

Table 1: List of NHE1-interacting peptides unique to NHE1-GST versus GST-control analysis.

Accession #	Protein *[GENE]*	PSM	Function:
P19634	Sodium/hydrogen exchanger 1 *[SLC9A1]*	57	Homodimer
P10809	60 kDa heat shock protein, mitochondrial *[HSPD1]*	42	Biosynthesis
P29401	Transketolase *[TKT]*	35	Biosynthesis
O43707	Alpha-actinin-4 *[ACTN4]*	31	Cytoskeletal activity
P07900	Heat shock protein HSP 90-alpha *[HS90AA1]*	32	Signal transduction
P08107	Heat shock 70 kDa protein 1A/1B *[HSPA1]*	29	Signal transduction
P00558	Phosphoglycerate kinase 1 *[PGK1]*	24	Metabolism
P08865	40S ribosomal protein SA *[RPSA]*	19	Biosynthesis
P19338	Nucleolin *[NCL]*	20	Biosynthesis
P09651	Heterogeneous nuclear ribonucleoprotein A1 *[HNRNPA1]*	18	Biosynthesis
P00367	Glutamate dehydrogenase 1, mitochondrial *[GLUD1]*	19	Biosynthesis
P09382	Galectin-1 *[LGALS1]*	14	Signal transduction
P04075	Fructose-bisphosphate aldolase A *[ALDOA]*	17	Metabolism
P0CG48	Polyubiquitin-C *[UBC]*	15	Biosynthesis
P07339	Cathepsin D *[CTSD]*	14	Signal transduction
P26038	Moesin *[MSN]*	13	Cytoskeletal activity
P14625	Endoplasmin *[HSP90B1]*	12	Biosynthesis
P32119	Peroxiredoxin-2 *[PRDX2]*	12	Metabolism
P07237	Protein disulfide-isomerase *[P4HB]*	10	Biosynthesis
P08758	Annexin A5 *[ANXA5]*	10	Signal transduction
P62258	14-3-3 protein epsilon *[YWHAE]*	9	Signal transduction
O00299	Chloride intracellular channel protein 1 *[CLIC1]*	8	Metabolism
P21796	Voltage-dependent anion-selective channel protein 1 *[VDAC1]*	8	Biosynthesis
P27824	Calnexin *[CANX]*	7	Biosynthesis
O00303	Eukaryotic translation initiation factor 3 subunit F *[EIF3F]*	7	Biosynthesis
P27348	14-3-3 protein theta *[YWHAQ]*	6	Signal transduction
P15311	Ezrin *[EZR]*	6	Cytoskeletal activity
P27797	Calreticulin *[CALR]*	6	Biosynthesis
P31947	14-3-3 protein sigma *[SFN]*	4	Signal transduction
P52907	F-actin-capping protein subunit alpha-1 *[CAPZA1]*	4	Cytoskeletal activity
P52565	Rho GDP-dissociation inhibitor 1 *[ARHGDIA]*	4	Biosynthesis
Q9Y490	Talin-1 *[TLN1]*	3	Cytoskeletal activity
A8MWD9	Putative small nuclear ribonucleoprotein G-like protein 15 *[SNRPGP15]*	2	Biosynthesis
P10599	Thioredoxin *[TXN]*	2	Metabolism
P45880	Voltage-dependent anion-selective channel protein 2 *[VDAC2]*	2	Biosynthesis

Table 2: List of NHE1-interacting peptides showing a two-fold increase or greater expression in NHE1-GST analysis versus GST-control.

Accession #	**Protein *[GENE]***	**log2[$PSM_{NHE1\text{-}GST}/PSM_{GST}$]**	**Function:**
P06733	Alpha-enolase *[ENO1]*	4.7	Metabolism
P63104	14-3-3 protein zeta/delta *[YWHAZ]*	3.5	Signal transduction
P11021	78 kDa glucose-regulated protein *[HSPA5]*	3.1	Biosynthesis
P07737	Profilin-1 *[PFN1]*	3.0	Cytoskeletal activity
P08238	Heat shock protein HSP 90-beta *[HS90AB1]*	2.5	Signal transduction
P12814	Alpha-actinin-1 *[ACTN1]*	2.4	Cytoskeletal activity
P32969	60S ribosomal protein L9 *[RPL9]*	2.1	Biosynthesis
P04406	Glyceraldehyde-3-phosphate dehydrogenase *[GAPDH]*	2.0	Metabolism

Table 3: List of NHE1 reverse phase protein array (RPPA) correlations in primary tumour samples from patients with the basal clinical subtype of breast cancer (N=79). Significant Spearman correlation coefficient (*r*, positive and negative) values are presented with corresponding P values > 0.05.

Antibody	Function	*r*	P Value
$p90^{RSK}$ (pT359/pS363)	Serine/threonine signal kinases activated by ERK1/2 [60].	0.31	0.005
pGSK3 α(pS21), β(pS9)	Serine/threonine kinase that phosphorylates and inactivates glycogen synthase kinase (GSK); dysregulation of GSK3 associated with cancer [61].	0.31	0.006
β-Catenin	Key component of Wnt signalling pathway, and regulator of cell-cell adhesion and epithelial-mesenchymal transition [62].	0.27	0.017
AKT (pS473)	Serine/threonine kinase involved in regulation of metabolism, cell survival, and growth; pAKT detected at high frequency in breast cancer [63].	0.26	0.020
NF2	Encodes tumour suppressor protein Merlin (Moesin-ezrin-radixin-like-protein); NF2 mutations may be linked with breast cancer [64].	0.24	0.031
Src (pY416)	Tyrosine kinase involved in regulation of cell growth and differentiation, and cancer cell epithelial-mesenchymal transition. Phosphorylation at Y416 is indicative of the active enzyme [65].	0.23	0.038
Notch1	Receptor for Jagged1/2 and delta-like proteins 1/3/4 [66]. Higher Notch1 activity in basal breast cancer; overexpression of Notch1 correlates with poor patient survival [67].	-0.22	0.051
FOXO3a	Transcription factor involved in cell death, cell cycle arrest and DNA damage repair. Inactivation of FOXO proteins is linked to breast cancer tumourigenesis [68].	-0.23	0.044
ACC1	Acetyl-CoA carboxylase; involved in lipogenic pathways and up-regulated in breast cancer [69].	-0.23	0.044
p27 (pT157)	Ubiquitously expressed kinase inhibitor protein involved in cell cycle regulation [70].	-0.24	0.032
Chk1	Serine/threonine kinase involved in G2/M cell cycle arrest and DNA damage repair [71].	-0.25	0.028
Smad4	Involved in TGF β signalling which plays a role in breast cancer carcinogenesis [72].	-0.25	0.025
Cyclin B1	Regulatory subunit of cyclin-dependent kinase 1 (CDK1); important for entry into mitosis [73].	-0.26	0.020

FIGURE 1

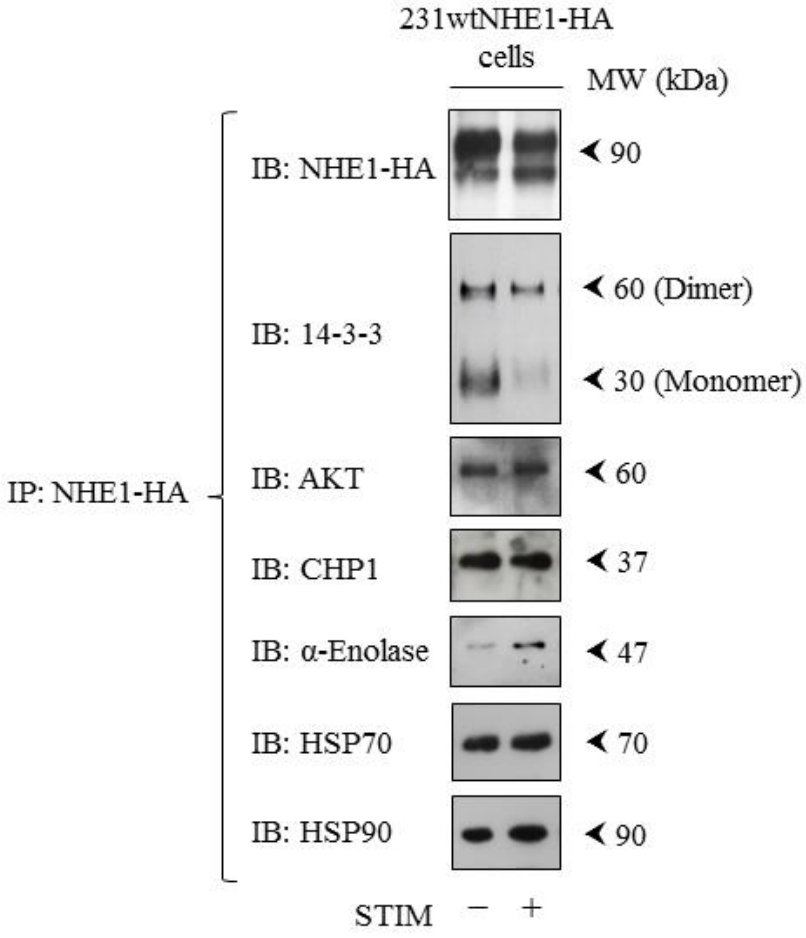

231wtNHE1-HA
cells
MW (kDa)
IP: NHE1-HA
IB: NHE1-HA
90
IB: 14-3-3
60 (Dimer)
30 (Monomer)
IB: AKT
60
IB: CHP1
37
IB: α-Enolase
47
IB: HSP70
70
IB: HSP90
90
STIM
−
+

FIGURE 2

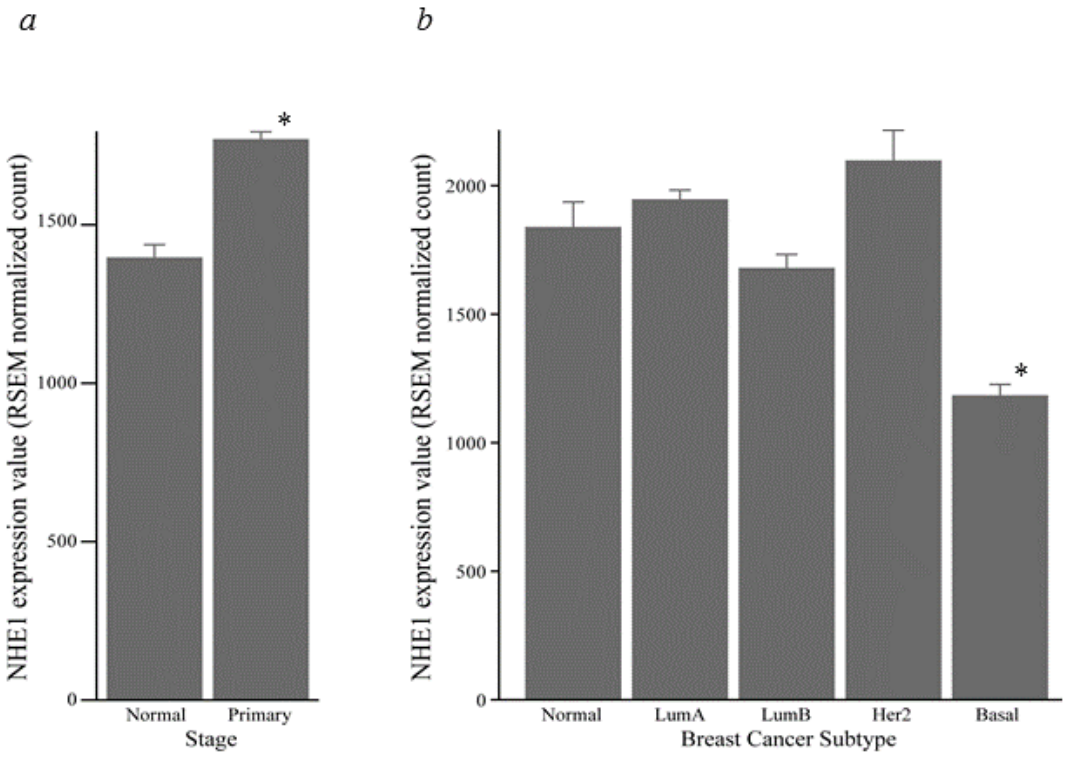
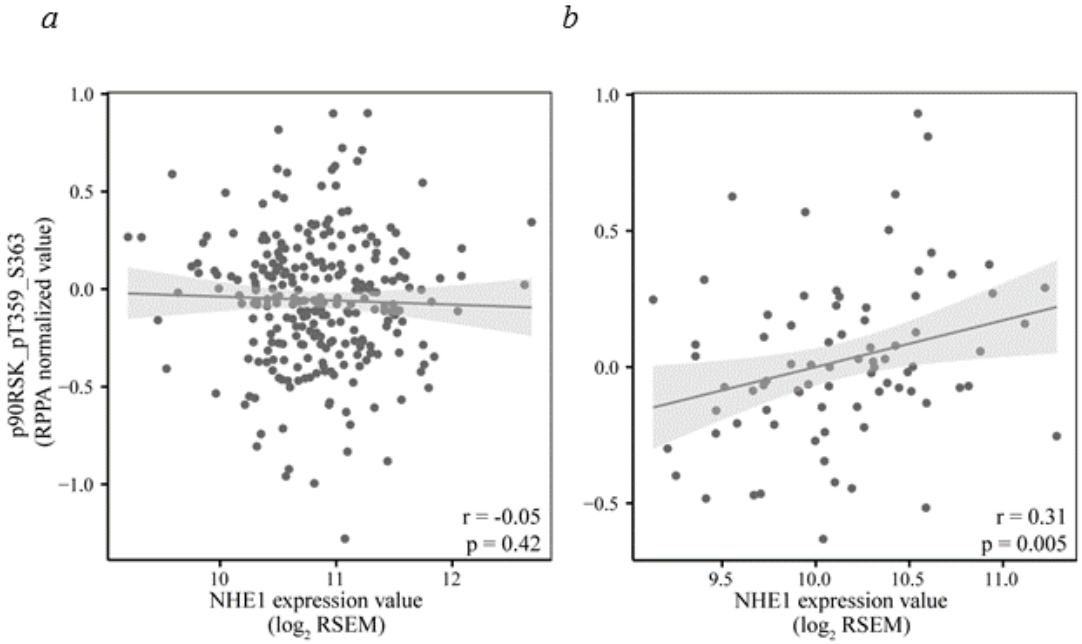

a
b
NHE1 expression value (RSEM normalized count)
0
500
1000
1500
2000
*
*
Normal
Primary
Stage
Normal
LumA
LumB
Her2
Basal
Breast Cancer Subtype

FIGURE 3


a
b
p90RSK_pT359_S363
(RPPA normalized value)
1.0
0.5
0.0
−0.5
−1.0
10
11
12
NHE1 expression value
(log2 RSEM)
r = -0.05
p = 0.42
1.0
0.5
0.0
−0.5
9.5
10.0
10.5
11.0
NHE1 expression value
(log2 RSEM)
r = 0.31
p = 0.005