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AND-34/BCAR3 differs from other NSP homologs in induction of anti-estrogen resistance, cyclin D1 promoter activation and altered breast cancer cell morphology

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Abstract

Over-expression of AND-34/BCAR3/NSP2 (BCAR3) or its binding-partner p130Cas/BCAR1 generates anti-estrogen resistance in human breast cancer lines. Here, we have compared BCAR3 to two related homologs, NSP1 and NSP3/CHAT/SHEP, with regards to expression, anti-estrogen resistance, and signaling. BCAR3 is expressed at higher levels in ER α -negative, mesenchymal, than in ER α -positive, epithelial, breast cancer cell lines. Characterization of “intermediate” epithelial-like cell lines with variable ER- α expression reveals that BCAR3 expression correlates with both mesenchymal and ER α -negative phenotypes. Levels of the BCAR3/p130Cas complex correlate more strongly with the ER α -negative, mesenchymal phenotype than levels of either protein alone. NSP1 and NSP3 are expressed at lower levels than BCAR3 and without correlation to ER α /mesenchymal status. Among NSP-transfectants, only BCAR3 transfectants induce anti-estrogen resistance and augment transcription of cyclin D1 promoter constructs. Over-expression of all homologs results in activation of Rac, Cdc42 and Akt, suggesting that these signals are insufficient to induce anti-estrogen resistance. BCAR3 but not NSP1 nor NSP3 transfectants show altered morphology, transitioning from polygonal cell groups to rounded, single cells with numerous blebs. Whereas stable over-expression of BCAR3 in MCF-7 cells does not lead to classic epithelial-to-mesenchymal transition, it does result in down-regulation of cadherin-mediated adhesion and augmentation of fibronectin expression. These studies suggest that BCAR3's ability to induce anti-estrogen resistance is greater than that of other NSP homologs and may result from altered interaction of breast cancer cells with each other and the extracellular matrix.

Keywords

AND-34; BCAR3; anti-estrogen resistance; breast cancer

Introduction

Anti-estrogens such as tamoxifen and fulvestrant can be effective treatment for both localized and metastatic estrogen-receptor alpha (ER α)-positive breast cancer. However in the setting of metastatic disease, resistance to anti-estrogen therapies invariably occurs (Clarke et al., 2003). While the molecular mechanisms by which such clinical resistance develops remain a

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subject of debate, both animal and cell-line studies have repeatedly documented novel growth-promoting signaling pathways in breast cancer cells that acquire anti-estrogen resistance (Lu et al., 2003; McClelland et al., 2001; Meijer et al., 2006).

In an effort to identify changes in gene expression in an unbiased manner that would allow the initially estrogen-dependent breast cancer cell line ZR-75-1 to grow in the presence of the anti-estrogen tamoxifen, Dorssers and colleagues performed random retroviral integration into ZR-75-1 cells, followed by selection in tamoxifen (Dorssers et al., 1993). Among 80 anti-estrogen-resistant ZR-75-1 clones isolated, six were a consequence of independent integration of retrovirus into the promoter for the newly defined BCAR3 gene and its subsequent over-expression (van Aghoven et al., 1998). Subsequent screening of other breast cancer cell lines confirmed higher levels of BCAR3 transcript in ER α -negative than ER α -positive breast cancer cell lines.

As a result of studies from several laboratories, it is now clear that BCAR3 is a member of a family of three related human proteins, NSP1, NSP2/BCAR3 and NSP3, termed the NSP (novel SH2-containing protein) family by Lu et al (Lu et al., 1999b). In mice, two members of this gene family are expressed: AND-34, an NSP2 homolog and CHAT/SHEP, an NSP3 homolog (Cai et al., 1999; Dodelet et al., 1999; Sakakibara and Hattori, 2000). Each of these proteins constitutively associates with the focal adhesion adapter proteins p130Cas and HEF1 and contains an amino-terminal SH2 domain and a carboxy-terminal region with modest homology to the Cdc25 Ras subfamily GDP exchange factor domain. The overall shared identity among the three human NSP family members is between 25 and 39% (Lu et al., 1999b).

In a later study by Dorssers and colleagues, four additional anti-estrogen resistant ZR-75-1 clones were found to be the result of retroviral integration into the BCAR1 gene, the human homologue of p130Cas, with subsequent over-expression of this protein (Brinkman et al., 2000). A high level of expression of BCAR1 in tumor specimens proved to correlate with reduced disease-free and overall survival in breast cancer patients, as well as reduced sensitivity to tamoxifen therapy in patients with relapsed disease (van der Flier et al., 2000). In conjunction with the demonstration that AND-34/BCAR3 physically associates with p130Cas/BCAR1, these studies established that BCAR3 and p130Cas form a signaling complex in which over-expression of either member induces estrogen independence in human breast cancer cell lines (Cai et al., 1999).

Mapping of the domains responsible for association of AND-34/BCAR3 (BCAR3) and NSP3 proteins with p130Cas or HEF1 demonstrated that the carboxy-terminal GEF-like domain of the NSP proteins associates with the carboxy-terminus of p130Cas and HEF1 (Gotoh et al., 2000). The GEF activity of the NSP family of proteins towards small GTPases remains controversial. Dodelet et al reported that SHEP/NSP3 bound Rap1 and R-Ras but not Ral, but GEF activity towards these GTPases was not identified in in vitro assays (Dodelet et al., 1999). While Gotoh et al initially reported that over-expression of BCAR3 led to activation of Ral, Rap1 and R-Ras, subsequent studies using pulldown assays rather than chimeric GST-GTPase assays have not confirmed robust BCAR3-mediated activation of these GTPases (Cai et al., 2003a; Felekis et al., 2005; Gotoh et al., 2000; Yu and Feig, 2002). Sakakibara et al reported that over-expression of CHAT/NSP3 led to activation of Rap1, but this group concluded that such activation was likely to be indirect, as CHAT-mediated Rap1 activation was dependent on p130Cas, Crk and C3G, a known Rap1 GEF (Sakakibara et al., 2002). Our laboratory has found that the most reproducible effect of BCAR3 over-expression is activation of the Rho subfamily GTPases Rac and Cdc42, with resultant alterations in cell morphology and activation of the Rac and Cdc42 effector protein Pak1 (Cai et al., 2003a; Cai et al., 2003b). BCAR3-mediated Rac activation is dependent upon both an intact SH2 domain and a

carboxy-terminal GEF-like domain and can be inhibited by either wortmannin or a dominant negative form of p85, suggesting that such Rac activation occurs in a phosphatidylinositol-3-kinase-dependent manner (Felekkis et al., 2005).

Since BCAR3 associates with p130Cas, an adapter protein known to modulate adhesion-related signaling, and since BCAR3 undergoes tyrosine phosphorylation following adhesion of cells to fibronectin, it is plausible to hypothesize that BCAR3-mediated anti-estrogen resistance is the result of altered adhesion-related signaling (Cai et al., 1999). Breast cancer cells do not grow alone, but within an extracellular matrix (ECM). Signaling by breast cancer cell line integrin receptors that bind to the ECM protein fibronectin has been shown to modulate the subsequent response to growth factors such as EGF (Bill et al., 2004; Matsuo et al., 2006; Moro et al., 1998). Estrogen dependence in breast cancer cell lines correlates with patterns of adhesion signaling, as most ER α -positive and anti-estrogen-sensitive breast cancer cell lines grow as tightly aggregated epithelial sheets with strong homotypic E-cadherin-based adhesion, while most ER α -negative, anti-estrogen insensitive breast cancer cell lines have a mesenchymal pattern of cell growth, with loss of E-cadherin expression, disaggregation of cells and augmented integrin-mediated adhesion (Lacroix and Leclercq, 2004). Signal pathways that regulate a comparable “epithelial to mesenchymal transition” (EMT) in epithelial cell lines have been a focus of research, in part because such a process has been hypothesized to take place when epithelial malignancies hematogenously metastasize to distant sites (Kang and Massague, 2004).

While we and others have confirmed the observation of van Agthoven et al that over-expression of BCAR3 induces resistance to tamoxifen and the pure ER antagonist ICI 182,780 in normally anti-estrogen-sensitive human breast cancer cell lines, the expression of other members of the NSP family has not been systematically analyzed in human breast cancer cell lines, nor has the ability of other NSP proteins to induce anti-estrogen resistance been assessed (Cai et al., 2003b; Yu and Feig, 2002). In this report, we compare the expression and function of NSP1, BCAR3 and NSP3 in breast cancer cells. We find that among NSP family members, BCAR3 is unique in its ability to induce anti-estrogen resistance and activate the cyclin D1 promoter in MCF-7 cells. Surprisingly, the ability of BCAR3 to activate Rac, Cdc42 or Akt is not specific to this family member. Instead, BCAR3-mediated anti-estrogen resistance and cyclin D1 promoter activation correlate with disaggregation of BCAR3 stable transfectants, a reduction in cadherin-based cell-cell adherence and augmented levels of fibronectin production.

Materials and Methods

Cell Culture

Breast cancer cell lines were cultured in DMEM with 10% FCS supplemented with l-glutamine and penicillin/streptomycin. T-47D, MDA-MB-231, MDA-MB-435S, BT-549, MDA-MB-361, MDA-MB-134, and MDA-MB-453 breast cancer cell lines were obtained from ATCC (Manassas, VA). MCF-7 and ZR-75 were obtained from Dr. David Seldin (Dept of Medicine, Boston University School of Medicine) and Hs578T was obtained from Dr. Gail Sonenshein (Dept of Biochemistry, Boston University School of Medicine).

Antisera

Polyclonal rabbit antisera to NSP proteins were generated as follows: Pairs of rabbits were immunized with one of the following peptides: Ac-RSFSEDTLMDGPARG-amide corresponding to amino acid residues 121 to 136 of human NSP1, Ac-RSPLAEHRPDAYQDVSIC-amide corresponding to amino acid residues 31-47 of human NSP2 (AND-34/BCAR3) or Ac-CTALSHKLEPAVRSEL-OH corresponding to amino acid residues 688-703 of human NSP3 (CHAT/SHEP) (QCB, Hopkinton, MA). Whole cell lysates

were made from HEK-293 cells transiently transfected with HA-tagged expression constructs for human NSP1, NSP-2 or NSP3 (see below). The specificity of the resulting peptide immunopurified antisera were then verified by immunoblotting such whole cell lysates with anti-HA and one of the antisera.

The following antibodies were also used in this study: mouse anti-Rac (610651) and anti-Cdc42 (61092) (BD PharMingen, San Diego, CA); rabbit anti-Akt, (9272) antiphospho-Ser43 Akt, anti-phospho Thr202/Tyr204 Erk, anti-phospho Thr183/Tyr185 JNK and anti-phospho Thr180/Tyr182 p38 (Cell Signaling, Beverly, MA); mouse anti-HA (MMS-101P) (Covance, Princeton, NJ); rabbit anti-ER α (Santa Cruz Biotechnology, Santa Cruz, CA); mouse anti-vimentin (Lab Vision, Fremont, CA); mouse anti-E-Cadherin (BD Biosciences, San Diego, CA); and mouse anti-tubulin (Sigma, St. Louis, MO). Rabbit anti-HEF1 (also called R1 (Law et al., 1998)) was generously donated by Jean Maguire van Seventer (Boston University, Boston, MA). Anti-p130Cas (sc-20029) and anti-fibronectin (sc-18825) monoclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

Transient and stable transfections

Plasmids were transfected into MCF-7 cells using FuGene 6 reagent (Roche Diagnostics, Indianapolis, IN). Briefly, MCF-7 cells were grown to 50-70% confluence in six-well cell culture plates. A total of 100 μ L fetal bovine serum-free DMEM medium was mixed with 4 μ L FuGene 6 and left at room temperature for 5 minutes. Then, 1 μ g DNA was added into the FuGene 6 solution and maintained at room temperature for an additional 30-45 minutes before adding into cell cultures. Whole cell lysates were prepared 48 hours after transfection. For stable transfection, MCF-7 cells were transfected as for transient transfection. After 48 hours, cells were trypsinized and plated into 10 cm plates at various dilutions at approximately 1/10 of the original cells. Selection was added 24 hrs later at 0.5mg/ml G418 (Invitrogen) and 2 days later increased to 1mg/ml. Cells were fed every 3 days. Colonies formed in 2-3 weeks and were picked into 24-well plates until 80% confluency. Cells were then split into 2 wells (24-well plate), one well frozen one well lysed in 0.5ml lysis buffer as described below. Lysates were assayed for expression levels and correct size by Western analysis using anti-HA antibody.

Immunoprecipitation and Western blot analysis

Western blot analysis was performed as previously described (Cai et al., 2003a). Briefly, cells were washed in PBS and lysed in Nonidet P-40 (NP40) buffer (1% NP40, 150 mM NaCl, 50 mM TrisCl (pH 7.4), 1 mM NaVO₄, 1 mM EDTA, 1 mM EGTA, 5% glycerol, 25 mM glycerophosphate and protease inhibitors). After centrifugation at 14,000 rpm (relative centrifugal force 16,000) for 5 min, the supernatants were collected. The AND-34 or HEF1/p130Cas protein from 250 to 500 μ g of whole-cell lysate was immunoprecipitated with 2 μ g antibody for 2 h. Twenty μ L of protein A/G agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) were added and incubated for an additional 2 h. The beads were washed 4 times with lysis buffer. Proteins were released from the beads by boiling in protein sample buffer for 5 min and separated on a SDS-PAGE gel. After transfer to a PVDF membrane, Western blots were developed by ECL following the vendor's protocol (Pierce, Rockford, IL).

Plasmid Constructs

NH₂-terminal HA-tagged NSP1, BCAR3 and NSP3 (CHAT/SHEP) expression vectors were cloned and constructed as follows: A) NSP1: Expression (by PCR) of NSP1 was assayed in several cell lines and found in human prostate cancer cell line PC3. cDNA was synthesized from total PC3 RNA using Superscript II (Invitrogen, Carlsbad, CA) according to the manufacturer. RT-PCR was performed with UltraPfu reverse transcriptase (Stratagene, La Jolla, CA) with primers designed to isolate the NSP1 open reading frame in 2 overlapping

fragments. The 5' NSP1 fragment was amplified with CTGGCACCTTCTGTTCCCAAGAG and CCAGATGAGACTCCCATGTTGC and the 3' fragment with CCTCTGATGCCAGATCTGCAG and CAAGACCACAGGCTGAGATCTC oligonucleotides. The resultant fragments were joined together with PCR by taking advantage of the overlap of the fragments by using only the 5' and 3'-most oligonucleotides to join the 5' and 3' cDNA fragments with UltraPfu PCR. The subsequent products were A-tailed (Qiagen) and subcloned into pCR2.1 (Invitrogen, Carlsbad, CA). A hemagglutinin tag (HA) was added to NSP1 by a similar joining-PCR strategy using a chimeric oligonucleotide (TACGCCTCCCTCGGATCCGAGGTGCCAC - containing the 3' area of HA and the 5' area of NSP1), the NSP1 full-length cDNA, and a fragment containing the full HA-tag. The entire HA-NSP1 piece was subcloned into pCDNA3. The insert was sequenced and two mutations were removed (QuikChange Mutagenesis Kit, Stratagene). Functionality was demonstrated by transfection and Western analysis with subsequent detection of a 70kd band with either anti-HA or anti-NSP1 antibodies. **B) BCAR3:** The HA-tagged BCAR3 expression construct has been previously described (Cai et al., 1999) (Cai et al., 2003b), but was subcloned into the expression vector pCDNA3. **C) NSP3:** A strategy similar to that described above for NSP1 was used for the NSP3 construct. As the 702 amino-acid form of NSP3 is expressed in a wide range of tissues (Sakakibara and Hattori, 2000), first-strand cDNA was synthesized from total mouse spleen RNA (Dodelet et al., 1999). RT-PCR was performed to generate the NSP3 open reading frame in 2 overlapping fragments. The 5' NSP3 fragment was amplified with CTTTCAGTTGTGATCAGGGGATG and GGAGGCTGGAGCTGACAGTAGTG, and the 3' fragment with CTATGTCCTGGAAATACTCCAAAG and TGTGGTGCTCTGGAGGGAATG oligonucleotides. The resultant fragments were joined and a hemagglutinin tag (HA) added to by a joining-PCR strategy similar to that described above. The correct sized fragments were isolated, A-tailed and subcloned into pGEM-Teasy (Promega) and later into pCDNA3. The final HA-NSP3 clone was sequenced and had only silent mutations. Functionality was demonstrated by transfection into 293T or MCF-7 cells and Western analysis with subsequent detection of a 70kd band with either anti-HA or anti-NSP3 antibodies. HA-HEF1 and HA-p130CAS constructs have been previously described (Cai et al., 2003a).

RT-PCR

A 464 bp portion of the human NSP1 transcript was amplified with the following two primers: 5' oligonucleotide: 5'- tctgtcccaagagctccat -3', 3' oligonucleotide: 5'- aggtgactgtgtctccact -3' for 35 cycles with the following conditions: 95°C 30", 60°C 45", 72°C 45". A 892 bp portion of the human NSP2 (BCAR3) transcript was amplified with the following two primers: 5' oligonucleotide: 5'- ctgtcagtgaagaacctcgc -3', 3' oligonucleotide: 5'- ggtcttccctgtcatcatc -3' for 32 cycles with the following conditions: 94°C 40", 60°C 50", 72°C 50". A 420 bp portion of the human NSP3 transcript was amplified with the following two primers: 5' oligonucleotide: 5'-gggaagcatgactgctgtggg-3', 3' oligonucleotide: 5'-ttgaagtgcaggcctggtt-3' for 34 cycles with the following conditions: 95°C 30", 60°C 45", 72°C 45". A 366 bp portion of the human NSP3 transcript equivalent to murine CHAT-H was amplified with the following two primers: 5' oligonucleotide: 5'- cttcagcagtggaggtagaacc -3', 3' oligonucleotide: 5'- gaagtgcaggcctggttgcg -3' for 35 cycles with the following conditions: 95°C 30", 60°C 45", 72°C 45". A 550 bp portion of the human β -actin transcript was amplified with the following two primers: 5' oligonucleotide: 5'- ggcacgtgatgactcc -3', 3' oligonucleotide: 5'- gctggaaggtggacagcga -3' for 20 cycles with the following conditions: 95°C 30", 56.5°C 45", 72°C 45".

ICI-resistance assay

MCF-7 cells or their stable NSP family member-transfected clones were plated into 6-well plates at 50,000 cells/well in triplicate. Cells were allowed to adhere for 1 day. Fresh media was added (2ml/well) and cells were untreated or treated in triplicate with ICI 182,780 (Tocris,

Ellisville, MO) at 100 nM. Media and ICI 182,780 were changed every 2 days. After 6 days of treatment, all cells were trypsinized and 1/10 of the cells were transferred to fresh 6-well plates with fresh media with or without ICI 182,780. Media and ICI 182,780 were changed every 2 days. After 6-7 days (at 80-90% confluency) cells were trypsinized and cells counted with a standard hemocytometer also in triplicate.

Rac and Cdc42 GTPase pulldown assays

Levels of activated Rac and Cdc42 were determined by pull-down analysis as described previously (11). Forty-eight hours after transfection, MCF-7 cells were harvested in lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 200 mmol/L NaCl, 5 mmol/L MgCl₂, 1% NP40, 15% glycerol, and protease inhibitors]. Whole cell lysates were incubated for 2 hours with 10 μ L glutathione-Sepharose 4B beads preincubated with 5 μ g GST-PAK. The GST-PAK-RBD construct was a kind gift of Dr. Zhijun Luo and has been described previously (Section of Endocrinology, Boston Medical Center, Boston, MA) (Cai et al., 2003b). The beads were washed 3X with cell lysis buffer and GTP-bound Rac or Cdc42 was released by boiling for 5 minutes in 2X SDS sample buffer. Rac or Cdc42 was then detected by Western blot analysis.

Akt phosphorylation assay

Confluent 10 cm² plates of MCF-7 cells stably transfected with vector only or NSP family members were trypsinized and 1/15, 2/15 or 4/15 of the resulting cell suspension used to seed new 10 cm² plates. After culture for three days, the cells were harvested and counted prior to lysis. The final cell densities were 2.3 $\pm$ 0.2, 5.2 $\pm$ 0.8 and 10 $\pm$ 2.0 million cells/ 10 cm² dish, respectively. Lysates were Western blotted for Akt phosphorylated at serine 473, total Akt, HA-tagged NSP family member or α -tubulin.

Cyclin D1 luciferase promoter assay

The cyclin D1 reporter construct was obtained from Dr's Chris Albanese and Richard Pestell (Albanese et al., 1995). MCF7 cells were seeded into 60-mm dishes at 50–60% confluence in triplicate overnight. Cells were transfected with HA-NSP1, HA-BCAR3, HA-NSP3 or vector (pcDNA1) and the cyclin D1 full-length (1745 CD1luc) construct. Total transfected DNA was kept constant. After 48 h, whole cell lysates were harvested, and luciferase activity was determined with a Promega luciferase assay kit (Madison, WI). Relative luciferase units were calculated by normalizing for total protein.

Disaggregation assay

Vector only or NSP family member stable transfectants of MCF-7 cells were grown to confluence, then split 1:10 and grown for 48 hours. Both total and solitary cells (cells sharing less than 20% of their cell border with another cell) were enumerated in quadruplicate in two transfectants of each type by light microscopy.

Immunohistochemistry

MCF-7 cells stably transfected with a BCAR3 expression construct (clone II-6) or vector alone were grown to 80% confluence on glass coverslips and fixed in 100% methanol overnight at -20° C. After fixation, cells were rinsed with TBS and permeabilized for 10 minutes with 0.5% Triton X100 in TBS. The cells were then rinsed with TBS and blocked in TBS with 2% bovine serum albumin (BSA) for 70 minutes. Anti-E-Cadherin (BD Biosciences, San Diego, CA) was diluted 1:500 in TBS with 2% BSA and the cells were incubated with this primary antibody for 1.5 hours at room temperature. The cells were then rinsed 4 times with TBS and probed with goat anti-mouse IgG-FITC (Santa Cruz Biotechnology, Santa Cruz, CA), at a 1:1000 dilution in TBS with 2% BSA for 1 hour at room temperature. Subsequently, cells were rinsed 3 times with TBS and the coverslips mounted on glass slides using Prolong Gold antifade

reagent with DAPI (Invitrogen, Carlsbad, CA). The slides were allowed to cure in the dark for at least 24 hours before viewing using a Nikon microscope equipped with a mercury lamp and FITC filter.

Statistical analysis

All data were expressed as the mean $\pm$ the standard error of the mean (SEM) of at least three different experiments. Statistical analysis was carried out using GraphPad Prism software, Version 4.0.

Results

Expression of NSP homologs in human breast cancer cell lines

Although AND-34/BCAR3/NSP2 (BCAR3) is known to be expressed in breast cancer cell lines and contribute to anti-estrogen resistance, the two related homologs, NSP1 and NSP3 (CHAT/SHEP) have not undergone a similar systematic analysis. Proteins from multiple breast cancer cell lines with varying ER α expression and representative of epithelial or mesenchymal phenotypes were assayed by Western analysis with antibodies against the three NSP family members (Figures 1 and 2). BCAR3 was detectable in whole-cell lysates of all the cell lines; however, the ER α -positive lines (MCF-7, T-47D, ZR-75-1) showed lower BCAR3 levels than the ER α -negative cell lines (BT-549, MDA-MB-231 and MD-MB-435S).

When NSP1 and NSP3 were assayed, neither was detectable in whole-cell lysates (10 μ g) of the breast cancer cell lines (Figure 2). In all cases, the antisera readily detect the control NSP1 and NSP3-transfected proteins in MCF-7 cells. Since the NSP proteins are constitutively associated with the focal adhesion protein p130Cas, anti-p130Cas antibody was used for immunoprecipitation of 500 μ g of protein from each line in order to concentrate NSP1 or NSP3 protein (Lu et al., 1999a) (Sakakibara and Hattori, 2000) (Dail et al., 2004). In contrast with whole-cell lysates, NSP1 was detected as a 65 kDa and NSP3 was detected as a 78 kDa protein in p130Cas immunoprecipitates (Figure 2A and B). The results show variation from line to line but no correlation with ER α -status. CHAT-H, a 115 kDa splicing isoform of NSP3 previously identified in hematopoietic cells, was not detected in whole-cell lysates or immunoprecipitates of the cell lines (Sakakibara et al., 2003).

Since NSP1 and NSP3 proteins were only detectable in immunoprecipitates, we performed semi-quantitative RT-PCR with three ER α -positive (MCF-7, T47D, ZR-75-1) and three ER α -negative (MDA-231, BT549, Hs-578T) cell lines. The resultant PCR supports the presence of NSP1 as found in immunoprecipitates since transcript was detected in all six lines, although BT549 and Hs578T cells expressed lower levels (Figure 2C). Although NSP3 transcripts were detectable in all lines, analogous to the immunoprecipitate results, disparate results indicated that higher NSP3 transcript levels were present in ER α -positive cell lines. Therefore, post-transcriptional regulation may modify NSP3 expression. RT-PCR for CHAT-H demonstrated no transcript in breast cancer cell lines, although this NSP3 splice variant was easily detected in the human T cell leukemia line Jurkat. As a control, PCR showed that BCAR3 transcript levels were higher in ER α -negative than the ER α -positive cell lines (Figure 2C), confirming results previously reported by van Agthoven et al (van Agthoven et al., 1998) and consistent with the Western analysis results discussed above (Figure 1).

Levels of p130Cas-BCAR3 complex strongly correlate with ER α -status

Since the focal adhesion adapter protein p130Cas forms a complex with NSP proteins and since over-expression of p130Cas, also termed BCAR1, has been reported to induce anti-estrogen resistance, the association of BCAR3 and p130Cas was examined in the breast cancer cell lines (Brinkman et al., 2000). Immunoprecipitates, performed with either anti-p130Cas or anti-

BCAR3 antibodies, were examined for both BCAR3 and p130Cas expression. Immunoprecipitation with anti-p130Cas yielded very little BCAR3 in ER α -positive cell lines (although significant levels were seen in the T-47D line), but high levels in ER α -negative cell lines (Figure 1B). The reciprocal experiment confirmed this result. Immunoprecipitation with anti-BCAR3 yielded small amounts of p130Cas in ER α -positive cell lines and high levels in ER α -negative cell lines (Figure 1C). Given that expression of p130Cas is only marginally higher in ER α -negative than ER α -positive breast cancer cell lines (Figure 1A), as well as the roughly comparable levels of BCAR3 immunoprecipitated from these six cell lines (Figure 1C), the markedly reduced levels of BCAR3-associated p130Cas in ER α -positive cell lines (Figure 1C) would suggest that complex formation by these two proteins in cells may not simply be a function of their abundance. Of note, NSP1 and NSP3 also form p130Cas complexes, but do not show the distinctive differential expression levels of the complex between ER α -positive and negative cell lines (Figure 2).

AND-34/BCAR3 is able to bind to HEF-1, an adapter protein highly related to p130Cas/BCAR1 (Cai et al., 2003a), in B lymphocytes. As human breast cancer cells have been reported to express HEF-1, we examined HEF-1 expression levels relative to that of p130Cas among breast cancer cell lines using a Western assay (O'Neill and Golemis, 2001). HA-p130Cas or HA-HEF1 was transfected into MCF7 cells and lysates were titrated such that equal amounts of HA-p130Cas or HA-HEF1 protein could be loaded onto Western blots as assayed by anti-HA antibody. Multiple Western blot wells containing equal amounts of HA-p130Cas or HA-HEF1 protein were titrated against varying anti-p130Cas or anti-HEF1 antibody such that the amount of these antibodies was determined that gives equal signal intensity against equal amounts of protein. Lysates from breast cancer lines were examined by Western analysis with these predetermined anti-p130Cas or anti-HEF1 antibody concentrations to determine relative HEF-1 versus p130Cas expression levels. The results indicate that endogenous p130Cas is expressed at significantly higher levels (greater than 20-fold) than HEF-1 in both ER α -positive and negative breast cancer cell lines (data not shown). Thus, p130Cas appears to be the predominant member of this family of proteins available for interaction with NSP family members in human breast cancer cell lines.

Breast cancer progression and metastasis has been modeled by the epithelial-to-mesenchymal transition (EMT) hypothesis (Kang and Massague, 2004). During EMT, epithelial cells lose E-cadherin-based cell-cell adhesion and develop a mesenchymal phenotype with increased integrin-mediated motility and invasiveness. EMT is characterized by down-regulation of epithelial markers such as E-cadherin and increased expression of mesenchymal markers such as vimentin and fibronectin. ER α -positive breast cancer lines generally have an epithelial phenotype, whereas ER α -negative lines are frequently mesenchymal in phenotype (Lacroix and Leclercq, 2004). Consistent with this model, the three ER α -positive epithelial lines examined above express E-cadherin while the three ER α -negative mesenchymal lines express vimentin (Figure 1). Since the ER α phenotype does not strictly correlate with the EMT status of breast cancer lines, we next addressed whether the higher levels of BCAR3 /p130Cas complex observed in the ER α -negative lines would correlate with the mesenchymal phenotype and/or with the lack of ER α expression. We obtained 4 “epithelial-like” breast cancer cell lines (MDA-134, MDA-361, MDA-453, and SK-BR3) that possess epithelial markers at reduced levels, have variable ER α phenotype, and lack the mesenchymal marker vimentin. The ER α -positive lines MDA-134 and MDA-361, indeed, have lower levels of the BCAR3/p130Cas complex as compared with the ER α -negative lines MDA-453 and SK-BR3 (Figure 3). However, the level of BCAR3 detected in the BCAR3 /p130Cas complex from these ER α -negative cell lines was significantly less than that observed in the vimentin-positive mesenchymal breast cancer cell lines such as BT549 (Figure 1). Thus, both ER status and mesenchymal phenotype are predictive of levels of p130Cas-associated BCAR3 in breast cancer cell lines.

BCAR3 differs from NSP1 and NSP3 in its ability to induce anti-estrogen resistance and cyclin D1 promoter activation

To determine the relative ability of NSP family members to induce anti-estrogen resistance in normally estrogen-dependent breast cancer cell lines, we stably transfected MCF-7 cells with pcDNA3 expression constructs for NH2-terminal hemagglutinin (HA) epitope-tagged forms of NSP1, BCAR3 or NSP3 or with vector alone. Western analysis with an anti-HA antibody was performed and transfectants with comparable levels of each of the three epitope-tagged NSP proteins were chosen for further analysis (Figure 7 and data not shown). Transfectants were grown in media with or without the addition of the pure ER α antagonist ICI 182,780 (changed every 2 days) at 100 nM in 6-well plates in triplicate. After 1 week of culture, at which time cells had reached 80-90% confluence, cells were passed 1:10 to new wells. Cell growth was determined at the end of two weeks by trypsinizing and manually counting with a hemocytometer. Figure 4A shows the relative cell growth of ICI 182,780-treated cells compared with untreated cells (ICI/Normal). The general trends are easily seen. BCAR3 stable clones demonstrated anti-estrogen resistance, while the homolog clones showed little difference in growth when compared with that observed in vector-only transfected clones. There was wide variability in the degree of anti-estrogen resistance observed among the BCAR3 transfectants, with 3-10 fold greater cell growth than the control transfectants. Among the four BCAR3 stable transfectants examined, Western analysis did not reveal a correlation between the level of BCAR3 expression and the degree of anti-estrogen resistance observed (data not shown). There was a modest correlation between the degree of ICI 182,780 resistance observed and morphologic changes described below (Figure 6), with BCAR3 transfectants that had multiple surface blebs and markedly reduced cell-cell association tending to show the greatest degree of anti-estrogen resistance.

Addition of 17 β -estradiol to human G1 arrested human breast cancer cells leads to cyclin D1 gene transcription and anti-estrogens block such cyclin D1 expression (Altucci et al., 1996). In prior studies, we have determined that over-expression of BCAR3 leads to activation of 1745 and 163 but not 66 bp fragments of the cyclin D1 promoter (Cai et al., 2003b). To examine the relative ability of different NSP family members to induce cyclin D1 promoter activation in human breast cancer cells, we transiently transfected MCF-7 cells with a luciferase construct containing 1745 bp of the cyclin D1 promoter in combination with NSP1, BCAR3, NSP3 or vector only plasmid constructs. As shown in Figure 4B, in pooled data from five such transfection experiments, when compared with MCF-7 cells transfected with vector alone, over-expression of BCAR3 led to cyclin D1 promoter activation (4.1 ± 0.3 -fold increase in mean luminescence relative to vector alone), but neither NSP1 (0.8 ± 0.1) nor NSP3 (0.8 ± 0.1) had such activity (two-tailed t test, $p < .01$). Thus, BCAR3 differs from NSP1 and NSP3 in its ability to activate the cyclin D1 promoter in MCF-7 cells.

Neither Rac, Cdc42, nor Akt activation distinguish NSP homologs

In prior studies, we have implicated AND-34/BCAR3-mediated Rac activation in BCAR3's ability to induce anti-estrogen resistance (Cai et al., 2003b). To examine whether among NSP family members the ability to activate Rac and Cdc42 was unique to BCAR3, we transfected MCF-7 cells with NSP1, BCAR3 or NSP3 and performed pulldown assays to isolate the activated forms of the two GTPases. Surprisingly, these studies demonstrated that over-expression of any of the three NSP family members led to comparable strong Rac and Cdc42 activation (Figure 5A). Given our prior results demonstrating that over-expression of AND-34/BCAR3 uniquely led to anti-estrogen resistance, the implication of the pulldown assays was that while AND-34/BCAR3-mediated Rac activation may be required for anti-estrogen resistance, such activation must not be sufficient, as NSP1 and NSP3-induced Rac and Cdc42 activation does not result in significant anti-estrogen resistance.

Transient transfection with AND-34/BCAR3 activates PI3K and induces AKT serine 473 phosphorylation (Felekakis et al., 2005). AND-34/BCAR3-induced Rac and Cdc42 activation appear to be dependent upon such PI3K activation as co-treatment with PI3K inhibitors or co-transfection with dominant-negative p85 blocks AND-34-induced Rac and Cdc42 activation (Felekakis et al., 2005). To examine whether NSP1 and NSP3 also activate this signaling pathway, we compared Akt serine 473 phosphorylation in vector only, NSP1, BCAR3 and NSP3 stable MCF-7 transfectants. As initial observations suggested that BCAR3-induced Akt phosphorylation was density-dependent, we plated all cell lines at low, intermediate and high cell densities, followed by cell lysis, normalization of protein concentrations and Western analysis. As shown in Figure 5B, relative to a vector-only transfectant, stable transfection of all three members of the NSP family induces Akt serine 473 phosphorylation when cells are examined at low density.

Morphologic alterations, reduced cadherin-mediated cell:cell association, and augmented fibronectin expression distinguish BCAR3 transfectants from other NSP family members

While isolating stable NSP transfectants in MCF-7 cells, we noted that whereas stable clones were readily obtained for NSP1 and NSP3 constructs (about 30-40% of G418-resistant colonies), BCAR3-expressing colonies were relatively rare, with only about 1-5% of drug-resistant colonies expressing BCAR3. We previously observed that BCAR3 expression altered cell morphology in HEK-293 cells (Cai et al., 2003b). Consistent with this, we noted that MCF-7 colonies expressing BCAR3 frequently showed irregular borders and cells within colonies were less tightly organized. Once we recognized this phenotype, we were able to distinguish BCAR3 colonies at about 20% frequency (data not shown). Light microscopic examination of the subsequently cloned BCAR3 stable transfectants revealed that at low cell density, such cells were dispersed, in contrast to the more coherent growth pattern of wild-type MCF-7 cells or vector-only stable transfectants (Figure 6A). BCAR3 transfectants were also frequently noted to have surface blebs. These morphologic alterations were also seen occasionally in NSP1 and NSP3 homolog transfectants, but to a much lesser degree (data not shown). The cell:cell junctions are also much more disjointed in BCAR3 transfectants compared with other NSP transfectants, often displaying blebs between cells even when at higher confluence (Figure 6A, arrows). Indeed, direct counting experiments show that, at low cell density, "solitary cells" (sharing less than 20% of their cell circumference with another cell) made up 56 and 61% of total cells in two BCAR3 transfectants, whereas they made up 1-9% of cells in vector only, NSP1 and NSP3 transfectants ($p < 0.05$).

As the loss of cell-cell adherence observed in AND-34/BCAR3 transfectants is reminiscent of an epithelial to mesenchymal transition (EMT) reported for breast cancer cells in which specific signaling pathways have been activated, we assessed stable transfectants of the three NSP family members for evidence of such a mesenchymal phenotype by Western analysis for markers of EMT status. All transfectants expressed their respective homolog proteins at high levels (Figure 7A). In general, MCF-7 cells transfected with NSP family members maintained an epithelial phenotype, with stable expression of E-cadherin and lack of expression of vimentin (Figure 7A). Of note, expression of p130Cas was augmented in NSP1, AND-34/BCAR3 and NSP3 transfectants. This result suggests that association of p130Cas with transfected NSP family members may stabilize p130Cas and induce higher steady-state levels of the p130Cas/NSP complex (Figure 7A). Stable over-expression of NSP1, BCAR3 or NSP3 did not lead to a pattern of loss of ER α expression, although moderately lower ER α levels were observed in a number of the stable transfectants (Figure 7A).

While overall, these studies did not support the hypothesis that over-expression of NSP family members in MCF-7 cells leads to classic EMT, levels of fibronectin, an extracellular matrix protein typically produced at higher levels by mesenchymal than epithelial breast cancer cell

lines (Figure 7B), were strikingly augmented in two BCAR3 transfectants, but not in NSP1, NSP3 or vector-only transfectants (Figure 7A). When a larger panel of AND-34/BCAR3 stable transfectants was examined to determine the reproducibility of this phenomenon, six out of seven such transfectants over-expressed fibronectin relative to levels present in MCF-7 cells (data not shown). Parallel immunohistochemistry studies demonstrated that in comparison to MCF-7 cells, BCAR3 transfectants demonstrated a marked diminution in cell-cell junctions characterized by concentrated E-cadherin immunofluorescence (Figure 6B). While E-cadherin expression in BCAR3 transfectants was not lost entirely, even at high cell confluence, cell:cell junctions frequently expressed low levels of immunofluorescence. This reduction in E-cadherin immunofluorescence at cell-cell junctions was not observed in stable NSP1 and NSP3 transfectants (data not shown). Thus, in this comparison of three NSP family members, over-expressed BCAR3's consistent ability to induce anti-estrogen resistance and activate the cyclin D1 promoter does not correlate with either Rac, Cdc42 or Akt activation. BCAR3-mediated anti-estrogen resistance does, however, correlate with a reduction in cadherin-based cell:cell association and augmented levels of fibronectin.

Discussion

Dorssers and colleagues used a non-biased retroviral insertional mutagenesis technique to discover that over-expression of either BCAR3 or p130Cas/BCAR1, two proteins that associate with one another through their carboxy termini, induces resistance of estrogen-dependent breast cancer cell lines to anti-estrogens such as tamoxifen and ICI 182,780 (van Agthoven et al., 1998) (Brinkman et al., 2000) (Cai et al., 1999). The signaling pathway by which this protein complex confers estrogen-independent cell growth remains unknown. The current study has taken advantage of the fact that BCAR3 is a member of a family of three human gene products, initially designated NSP1, NSP2 and NSP3, to elucidate the signaling events induced by over-expression of these family members that might be relevant to anti-estrogen resistance. Consistent with the fact that, among anti-estrogen resistant clones, Dorssers and colleagues did not identify either NSP1 or NSP3 as gene products whose over-expression led to anti-estrogen resistance, we find that the effects of BCAR3 over-expression differ markedly from that of the two other NSP homologs.

Our prior studies of AND-34/BCAR3-mediated anti-estrogen resistance have focused on our observation that over-expression of BCAR3 activates the small GTPases Rac and Cdc42. AND-34/BCAR3-induced Cdc42 and Rac activation correlate with both morphologic changes in cells and with activation of a kinase, PAK1, known to be activated by the GTP-bound form of these GTPases (Cai et al., 2003b). We implicated such Rac activation in BCAR3-mediated anti-estrogen resistance by demonstrating that RacN17, a dominant negative form of Rac and NSC23766, a small molecule Rac inhibitor, block BCAR3-mediated cyclin D1 promoter activation and anti-estrogen resistance, respectively (Cai et al., 2003b; Felekis et al., 2005). Further, we found that stable transfection of MCF-7 cells with RacV12, a constitutively active form of Rac1, confers anti-estrogen resistance (Cai et al., 2003b). In the current study, we find that high-level BCAR3 expression consistently induces anti-estrogen resistance in MCF-7 cells, while over-expression of NSP1 or NSP3 do not. Consistent with this observation, among the three NSP family members, only BCAR3 over-expression activates cyclin D1 promoter luciferase reporter constructs.

Given this background, our observation that over-expression of each of the three NSP family members led to activation of Cdc42 and Rac was unexpected. Our prior studies had suggested that BCAR3-mediated Rac activation is the result of PI3K activation, most likely because the PIP3 produced by PI3K results in activation of a pleckstrin homology domain-containing Rac/Cdc42 GEF (Felekis et al., 2005). When stable MCF-7 transfectants of each NSP family member were cultured at low cell densities, we observed comparable activation of Akt, as

judged by Ser 473 phosphorylation, following over-expression of each of the three NSP family members. In combination with our prior studies, the current results would suggest that NSP family member-mediated Rac and/or Cdc42 activation might be necessary but not sufficient for cyclin D1 promoter and anti-estrogen resistance. Further, these results suggest that despite the ability of transiently transfected NSP1 and NSP3 to activate Rac in pulldown assays, the signaling induced by constitutive transfection with RacV12, which consistently induces anti-estrogen resistance, must be of a different nature than the signaling conferred by stable transfection with NSP1 or NSP3, which does not.

If neither Rac/Cdc42 activation nor Akt activation alone account for BCAR3's ability to activate the cyclin D1 promoter and induce anti-estrogen resistance, another property of BCAR3 must account for these phenomena. The results of the current study suggest that high level BCAR3 expression may alter interaction of breast cancer cells with each other and with extracellular matrix proteins such as fibronectin. Our analysis of NSP family member expression revealed that while some level of the BCAR3/p130Cas complex was detected in all human breast cancer cell lines examined, levels were consistently higher in ER α -negative breast cancer cells. Further, when ER α -negative but "epithelial-like" breast cancer cell lines such as MDA-453 and SKBR3 were compared with the mesenchymal ER α -negative cell line BT549, levels of the BCAR3/p130Cas complex were significantly higher in BT549 cells. This suggests that a mesenchymal phenotype (vimentin-positive, E cadherin-negative) is an independent predictor of BCAR3/p130Cas complex expression aside from ER α -status. Conversely, over-expression of BCAR3 in MCF-7 cells induces two mesenchymal attributes in this epithelial cell-line: augmented levels of extracellular fibronectin and an increased tendency of the BCAR3 transfectants to grow as individual cells rather than as clusters of tightly-associated epithelial cells.

The altered characteristics of BCAR3 transfectants described above are not part of a classic "epithelial to mesenchymal transition" as the transfectants do not gain expression of vimentin nor lose expression of E-cadherin. The morphologic and biochemical changes we observe in BCAR3 transfectants are instead reminiscent of reports in which epithelial cells undergo a switch from primarily cadherin-based intercellular adhesion to primarily integrin-based adhesion to the extracellular matrix (Avizienyte and Frame, 2005). Colon cancer cell lines with augmented levels of Src activity, for instance, have a redistribution of components of adherens junctions such as vinculin to integrin-based adhesion complexes (Avizienyte et al., 2002). In this study, Src-induced integrin signaling was shown to induce de-regulation of cadherin localization. Over-expression of the transmembrane tyrosine kinase EphA2 in MCF-10A mammary epithelial cells results in similar inhibition of cadherin-based cell-cell adhesion as well as up-regulation of extracellular matrix fibronectin (Hu et al., 2004). Of note, SHEP1/NSP3 was originally cloned by one group as a result of its ability to bind to activated ephrin receptors (Dodelet et al., 1999).

If up-regulated adhesion to extracellular matrix does play a role in BCAR3-mediated anti-estrogen resistance, how might it do so? $\alpha 5/\beta 1$ integrin-mediated adhesion to fibronectin has been reported to transactivate the EGF receptor, activate MEK/ERK and PI3K/Akt pathways and induce epithelial cell proliferation (Kuwada and Li, 2000) (Matsuo et al., 2006). EGF receptor signaling is well established as a pathway capable of inducing anti-estrogen resistance in human breast cancer cell lines (Van Agthoven et al., 1992). Thus, one plausible hypothesis is that BCAR3 over-expression induces "inside-out" integrin-mediated signaling resulting in activation of an extracellular growth factor receptor that allows cell proliferation in the absence of estrogen receptor signaling. However, gene chip analyses of BCAR3 and BCAR1 (p130Cas) transfected breast cancer cell lines have suggested that the transcriptional outcome of over-expression of these proteins does not overlap with that of the same breast cancer cell line over-expressing the EGFR (Dorssers et al., 2005). Our future studies of BCAR3 will focus on how

altered interaction with the extracellular matrix proteins such as fibronectin influences BCAR3's ability to activate the cyclin D1 promoter and promote growth in the presence of anti-estrogens.

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Figure 1. Expression of BCAR3 and p130Cas in six breast cancer cell lines with varying ER α expression and epithelial/ mesenchymal phenotype
 Three ER α -positive, epithelial breast cancer cell lines (MCF-7, T47D and ZR-75-1) and three ER α -negative, mesenchymal breast cancer cell lines (BT549, MDA-231, and MDA-435S) were assayed by Western analysis and immunoprecipitation (IP) for BCAR3 and p130Cas expression. **Panel A:** Levels of BCAR3 and p130Cas protein present in whole cell lysates (WCL). α tubulin is shown as a control for total protein levels. **Panels B and C:** IP of equal amounts of protein with anti-p130Cas or anti-BCAR3, respectively, and Western analysis of IP products with anti-BCAR3 and anti-p130Cas. CT- control IP with non-immune rabbit antisera. **Panel D:** Western analysis of ER α , E-cadherin and vimentin in WCL.

Figure 2. Expression of NSP1 and NSP3 in six breast cancer cell lines

Panels A and B: Expression levels of NSP1 (A) and NSP3 (B) were assayed in whole cell lysates (WC: 10 µg) or anti-p130Cas immunoprecipitates (IP Cas: 500 µg) of ERα-positive epithelial breast cancer cell lines (MCF-7, T47D and ZR-75-1) and ERα-negative, mesenchymal breast cancer cell lines (BT549, MDA-231, MDA-435S or hs578-T). On the far right of each panel (upper row), whole cell lysates from NSP1 or NSP3-transfected cells were examined as a positive control. On the far right of each panel (lower row), a control immunoprecipitate with non-immune rabbit antisera was examined (CT). **Panel C:** RT-PCR analysis of NSP1, NSP2 (BCAR3) and NSP3 transcript levels in human breast cancer cell lines. Three ERα-positive, epithelial breast cancer cell lines (MCF-7, T47D and ZR-75-1) and three ERα-negative, mesenchymal breast cancer cell lines (MDA-231, BT549 and hs578-T) were assessed by RT-PCR for NSP1, NSP2 and NSP3 expression. For NSP3, an alternatively spliced exon 1 transcript, previously reported in hematopoietic cells, CHAT-H, was also

amplified using the human Jurkat T cell leukemia cell line as a positive control. The relative levels of total cDNA were estimated by RT-PCR with actin-specific oligonucleotides as shown.

Figure 3. The effect of epithelial/mesenchymal phenotype vs. ER α expression on levels of BCAR3/p130Cas complex

Levels of BCAR3/p130Cas complex were assessed in four “epithelial-like” breast cancer cell lines (MDA-134, MDA-361, MDA-453, and SK-BR3) that possess epithelial markers at reduced levels, have variable ER α phenotype, and lack the mesenchymal marker vimentin. Samples were from WCL as probed with anti-BCAR3 or anti-p130Cas (top panel), anti-p130Cas IP probed with anti-BCAR3 (second panel), and WCL probed with anti-vimentin or anti-ER α as an assay for phenotype (third panel). Control for protein level was in WCL with anti-tubulin (last panel).

Figure 4.

Panel A. Analysis of anti-estrogen resistance among stable NSP family transfectants. Stable transfectant clones containing NSP homologs or vector-only (pcDNA3) were plated in triplicate and grown in media with or without ER α antagonist ICI 182,780 at 100 nM. Cell growth was determined at the end of two weeks by trypsinizing and manually counting with a hemocytometer. Relative cell growth (number of cells treated with ICI/number of cells untreated) and standard error are shown. Stable transfection with BCAR3 but not NSP1 or NSP3 led to statistically significant augmentation in anti-estrogen resistance (ANOVA $p = 0.0162$; Tukey's multiple comparison post-test for BCAR3 vs. cDNA clones; $p < 0.05$). The data shown are from the largest independent experiment performed that includes all the above cell lines, and are consistent with at least two prior experiments. **Panel B.** Effects of NSP family member over-expression on activity of a cyclin D1 promoter reporter construct. MCF-7 cells were transiently transfected with expression constructs driving the expression of NSP1, NSP2, NSP3 or vector alone in combination with a luciferase reporter construct containing a 1745 bp fragment of the cyclin D1 promoter. The mean relative luciferase activity observed (RLU), normalized to the control vector samples, along with the SEM, are shown from a total of five

experiments. Transfection with BCAR3 but not NSP1 or NSP3 led to a statistically significant 4.1-fold augmentation in luciferase activity (one-way ANOVA $< .0001$).

Figure 5. Analysis of the ability of over-expressed NSP family members to activate either the small GTPases Rac and Cdc42 or Akt serine 473 phosphorylation

Panel A. Lysates from MCF-7 cells transiently transfected with NSP1, NSP2, NSP3 or vector alone (CT) were incubated with chimeric GST-PAK-RBD protein, followed by “pull-down” analysis of the relative levels of activated GTP-bound forms of Rac or Cdc42 by Western analysis as shown. The total levels of Rac and Cdc42 in the initial cell lysates are shown, as is the expression of the transfected HA-epitope-tagged NSP family members. **Panel B.** Vector only, NSP1, BCAR3 and NSP3 stable MCF-7 transfectants were plated at low, intermediate and high cell densities, followed by cell lysis, normalization of protein concentrations and Western analysis for total Akt (Akt), Ser 473 phosphorylated Akt (P-AKT), hemagglutinin (HA) or α -tubulin as indicated.

Figure 6. Morphological alterations and E-cadherin expression distinguish BCAR3 transfectants from other NSP family members transfectants

Panel A. Stable MCF-7 transfectants containing vector only or BCAR3 were grown in 6-well plates and examined by light microscopy. Photographs were taken at two magnifications. Arrows indicate cell-cell junctions. Panel B. Vector only or BCAR3 transfectants were assessed for E-cadherin expression by immunohistochemical analysis. Photographs were taken at two magnifications. Arrows indicate E-cadherin expression at cell-cell junctions.

Figure 7. Epithelial-mesenchymal phenotypic markers among NSP transfectants: BCAR3 but not NSP1 or NSP3 over-expression results in augmented fibronectin production

Panel A: WCL containing equal amounts of protein from NSP transfectants were analyzed by Western blotting for expression of hemagglutinin (HA), fibronectin (FN), vimentin (Vim), p130Cas (Cas), estrogen receptor alpha (ER), E-cadherin (ECad), and α -tubulin (Tubl). Of note, as indicated by the amount of tubulin detected, substantially less lysate was loaded for BT-549 cells at the far left of the panel than for the other cell samples. **Panel B:** Fibronectin expression was assessed by Western analysis in three epithelial and three mesenchymal breast cancer cell lines, as indicated.