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AND-34/BCAR3 Regulates Adhesion-Dependent p130Cas Serine Phosphorylation and Breast Cancer Cell Growth Pattern

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Abstract

NSP protein family members associate with p130Cas, a focal adhesion adapter protein best known as a Src substrate that integrates adhesion-related signaling. Over-expression of AND-34/BCAR3/NSP2 (BCAR3), but not NSP1 or NSP3, induces anti-estrogen resistance in human breast cancer cell lines. BCAR3 over-expression in epithelial MCF-7 cells augments levels of a phosphorylated p130Cas species that migrates more slowly on SDS PAGE while NSP-1 and NSP3 induce modest or no phosphorylation, respectively. Conversely, reduction in BCAR3 expression in mesenchymal MDA-231 cells by inducible shRNA results in loss of such p130Cas phosphorylation. Replacement of NSP3's serine/proline-rich domain with that of AND-34/BCAR3 instills the ability to induce p130Cas phosphorylation. Phospho-amino acid analysis demonstrates that BCAR3 induces p130Cas serine phosphorylation. Mass spectrometry identified phosphorylation at p130Cas serines 139, 437 and 639. p130Cas serine phosphorylation accumulates for several hours after adhesion of MDA-231 cells to fibronectin and is dependent upon BCAR3 expression. BCAR3 knockdown alters p130Cas localization and converts MDA-231 growth to an epithelioid pattern characterized by striking cohesiveness and lack of cellular projections at colony borders. These studies demonstrate that BCAR3 regulates p130Cas serine phosphorylation that is adhesion-dependent, temporally distinct from previously well-characterized rapid Fak and Src kinase-mediated p130Cas tyrosine phosphorylation and that correlates with invasive phenotype.

Keywords

BCAR3; p130Cas; breast cancer; adhesion; phosphorylation; anti-estrogen resistance

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1. Introduction

Phosphorylation of the focal adhesion protein adapter p130Cas regulates epithelial and mesenchymal cell adhesion, motility and response to growth factors. p130Cas was originally identified as a tyrosine-phosphorylated protein in v-Crk and v-Src transformed cell lines [1, 2]. p130Cas binds by its SH3 domain to the focal adhesion kinase (FAK) or the FAK-related kinase PYK2/RAFTK, which in turn binds by a Tyr-397 autophosphorylation site to the SH2 domain of Src family kinases [3-5]. As a result of such FAK/PYK2-mediated recruitment to p130Cas, Src family kinases phosphorylate multiple tyrosines within an interior p130Cas "substrate domain," a modification that can be detected by a reduction in p130Cas mobility in SDS-PAGE gels [6,7]. Phosphorylation of the p130Cas substrate domain results in intracellular redistribution of p130Cas as well as recruitment of the SH2 domain-containing adapter proteins Crk and Crk-L. [8]. Binding of Crk to p130Cas augments cell motility through Crk SH3 domain-mediated recruitment of the Rac GDP exchange factor DOCK180 [9].

AND-34 is a murine SH2 domain-containing protein that binds by a carboxy-terminal GEF-like domain to the carboxy terminus of p130Cas [10,11]. Over-expression of either the human homolog of AND-34, BCAR3, or the human homolog of p130Cas, BCAR1, induces resistance to the growth inhibitory effects of anti-estrogens such as tamoxifen or faslodex in normally estradiol-dependent human breast cancer cell lines [12,13]. Despite AND-34's modest homology to Ras subfamily GDP exchange factors, the GTPases most reproducibly and robustly activated by AND-34 are Rac and Cdc42 [11,14,15]. Such activation correlates with cell shape changes such as membrane ruffles, as well as activation of downstream effector proteins such as the Rac-activated kinase PAK1 [11]. BCAR3 has also been shown to regulate cell motility [16,17].

BCAR3 is a member of a family of three human genes; NSP1, NSP2/BCAR3/AND-34 (hereafter referred to as BCAR3) and NSP3, and two murine genes; AND-34 and SHEP1/CHAT/NSP3 [18-20]. p130Cas is also a member of a family of three genes; p130Cas, HEF1 and Sin. NSP family members bind to the carboxy-termini of all three p130Cas family members [14,20,21]. While initial studies suggested that BCAR3-induced cyclin D1 promoter activation and anti-estrogen resistance were a result of Rac activation, more recent studies have demonstrated that this is not the case [11]. Over-expression of each of the NSP family members in ER α -positive human breast cancer cell lines induces activation of Rac and Cdc42, while only BCAR3 over-expression activates cyclin D1 promoter reporter constructs and induces anti-estrogen resistance [22]. To identify further signaling characteristics of BCAR3 that might underlie its ability to induce anti-estrogen resistance, we examined the role of BCAR3 expression in regulating p130Cas phosphorylation.

2. Materials and methods

2.1. Cell culture

ER α -positive MCF-7, T-47D and ZR-75 and ER α -negative BT-549, MDA-MB-231 and MDA-MB-435S human breast cancer cell lines were obtained from ATCC. The HA-BCAR3 stably-transfected MCF-7 cell line II-6 has been previously described [11]. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Mediatech, Inc.) supplemented with 10% heat-inactivated fetal calf serum (Biomed), 2.2 mM L-glutamine, 100 U/mL penicillin and 100 μ g/mL streptomycin.

2.2 Antibodies and immunoblotting

The following antibodies were used in this study: anti-p130Cas (BD Biosciences), anti-FAK and anti-phosphotyrosine pY100 (Cell Signaling Technology), monoclonal anti-hemagglutinin

(Covance), HRP-conjugated goat anti-mouse IgG and anti-c-Src (Santa Cruz Biotechnology). Peptide affinity-purified polyclonal rabbit anti-BCAR3 antisera have been previously described [22]. Adherent cells were treated with lysis buffer (1% Triton X-100, 20 mM Hepes pH 7.4, 10% glycerol, 150 mM NaCl, 50 mM glycerophosphate, 2 mM EDTA, 2 mM EGTA, 1 mM sodium vanadate, 1 mM benzamidine, 1 mM dithiothreitol, and 1 mM PMSF). Cell lysates were clarified by centrifugation and either used for immunoblot analysis or immunoprecipitated with protein G Sepharose and anti-p130Cas antibody. Heat-denatured proteins were electrophoretically separated in SDS low-bis-polyacrylamide gels (30% T, 0.67% C). After immunoblotting, signals were detected with SuperSignal West Pico Chemiluminescent Substrate kit (Pierce) and HRP activities on membranes were digitized using a model 4000 MM Kodak Image Station.

2.3. Protein phosphatase reaction assays

Precipitated immune complexes were washed with protein phosphatase reaction buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 2 mM MnCl₂, 2 mM DTT, 0.01 % Brij 35, 0.1 mM EGTA) or reaction buffer containing 50 mM EDTA. Reaction buffer volumes of washed immune complexes were adjusted to 35 μ l and 5 μ l of λ protein phosphatase (40 U/ml: New England Biolabs) was added. Samples were incubated at 30°C for 20 min. Reactions were terminated with 4xSDS-PAGE sample buffer.

2.4. Light and immunofluorescent microscopy

MDA-231 cell lines stably transfected with BCAR3 or GFP shRNA lentiviral constructs were cultured with or without 6 μ g/ml doxycycline hyclate (Sigma Aldrich) for 4 days prior to replating on coverslips coated with 15 mg/ml fibronectin (BD Biosciences). After fixation with 3.7% paraformaldehyde at 4 °C, cells were immunostained with p130Cas antibody followed by Alexa Fluor 594 goat anti-mouse IgG (Invitrogen) and monoclonal vinculin-FITC antibody (Sigma). Alternatively, cells were immunostained with Alexa Fluor 594 phalloidin (Invitrogen) and paxillin-FITC antibody (BD Biosciences). Cells were counterstained with 4',6-diamidino-2-phenylindole (Invitrogen). Imaging was performed on a Nikon TE2000-E scope equipped with a Photometrics CoolSnap HQ² camera.

2.5. Fak, NSP family member, p130Cas and Src expression constructs

Wildtype and dominant negative FAK (Y397F) were a gift from Dr. Jean van Seventer (Boston University School of Public Health) [23]. Plasmids pcDNA-HA-NSP1, pcDNA-HA-BCAR3, and pcDNA-HA-NSP3 have been previously described (Near *et al.*, 2007). The HA-tagged constructs, AND-34, SH2, SH2/Pro, Δ SH2, and Δ GEF have been previously described except that they were all subcloned into pcDNA3 [24]. To generate chimeric NSP3/AND-34 constructs, we defined the AND-34 SH2 region as amino acids 142–249 and the proline-serine rich domain (PS) as amino acids 259–517. In NSP3, the corresponding amino acids are 57–164 (SH2) and 174–403 (PS). Using chimeric oligonucleotides that contained overlapping NSP3 and AND-34 sequences as primers, we used a PCR-based strategy to insert the AND-34 domain sequences SH2/PS, SH2-only and PS-only to replace the corresponding sequences of pcDNA-HA-NSP3. HA-tagged rat p130Cas as well as the p130Cas deletion mutants Δ SH3 and Δ SD and the p130Cas SBD point mutants RPLP and Y762F were generously provided by Dr. Tetsuya Nakamoto and Dr. Hisamaru Hirai (University of Tokyo) and have been described previously [25]. Serine residues 436, 437, 439 and 638 of the rat HA-p130Cas construct were mutated to alanine using a QuikChange Site-Directed Mutagenesis Kit (Stratagene). Wildtype and dominant negative Src (K296R/Y528F) were from Upstate Biotechnology.

2.6. Inducible shRNA cell lines

The generation of MDA-231 doxycycline-inducible BCAR3 shRNA cell lines was based on a published protocol [26]. A tetracycline repressor (TetR) starter cell line was made by infecting wild-type MDA-231 cells (ATCC) with pLenti6/TR lentivirus (V480–20, Invitrogen), followed by selection with blasticidin at 10 µg/ml. An shRNA sequence previously demonstrated to down-regulate BCAR3 expression when used in transient transfection experiments

(5'GATCCCGGAAGAACCTCGCTCAGCATTCAAGAGATGCTGAGCGAGGTTCTTCCTTTTTC 3') was introduced into the pLenti4/Block-It-DEST vector (K4925–00, Invitrogen) according to the vendor's protocol. Lentivirus stocks were prepared with helper virus generously provided by Dr. William Hahn's laboratory (Dana Farber Cancer Institute) and used to infect the TetR MDA-231 cell line [27]. Single clones of BCAR shRNA MDA-231 cells were selected and maintained in DMEM medium supplemented with Zeocin at 800 µg/ml. MDA-231 cells infected with a lentivirus targeting GFP were a gift from Dr. Sam Thiagalingam (Boston University School of Medicine).

2.7. Metabolic labelling and immunoprecipitation of p130Cas from cells

Cell monolayers in 100 mm tissue culture plates were incubated for 4 h in phosphate-free media DMEM (Thermo Scientific HyClone) containing 10% phosphate-free dialyzed FBS (Hyclone), followed by ³²P-labelling for 4 h in fresh phosphate-free media supplemented with 1 mCi/ml of ³²P_i (PerkinElmer). Radio-labelling media was removed and cells harvested in 500 µl ice-cold lysis buffer. Cell lysates were cleared by centrifugation. Immunoprecipitated p130Cas was separated by SDS-PAGE, electroblotted to PVDF membranes and exposed to X-OMAT Kodak X-ray films to visualize locations of ³²P-labelled proteins. The location of p130Cas was determined by blotting with anti-p130Cas and ECL.

2.8. Acid hydrolysis and two-dimensional thin layer chromatography electrophoresis (TLCE)

Membrane slices containing immobilized p130Cas proteins were treated with methanol, washed with water and hydrolyzed for 60 min at 110°C in 5.7 M HCl [28,29]. After hydrolysis, ³²P-labeled products were recovered from the membrane and dried by Speedivac. Phospho-amino acid analysis was performed using a Hunter Thin Layer Peptide Mapping Electrophoresis system (C.B.S. Scientific Company, model # HTLE-7002) [28,29]. The dried ³²P-labeled products were dissolved in 10 µl of first dimension TLCE buffer pH 1.9 (2.2% formic acid, 7.8 % acetic acid) containing 1 µg each of cold phosphoserine, phosphothreonine and phosphotyrosine (Sigma-Aldrich) standard and spotted onto cellulose TLC plates (EMD Chemicals). A first dimension separation was performed at 1.5 kV for 20 min in TLCE pH 1.9 buffer and a second dimension separation at 1.3 kV for 16 min in TLCE pH 3.5 buffer (5 % acetic acid, 0.5 % pyridine, 0.5 mM EDTA). TLC plates were dried and sprayed with ninhydrin solution (Sigma-Aldrich) to stain the amino acids. Exposed films were overlain onto corresponding stained chromatograms to map locations of ³²P-amino acids.

2.9. Immuno-affinity purification of p130Cas protein

A p130Cas immuno-affinity column was prepared using 150 mg of monoclonal p130Cas antibody that was diafiltered against PBS and concentrated using an ICON concentrator (Pierce) before covalent coupling to a matrix support. The immuno-affinity matrix was prepared with a Seize Primary Immunoprecipitation kit (Pierce). P130Cas was affinity purified from Triton-soluble protein lysates (3 mg/ml) using procedures outlined in the manufacturer's instructions (Pierce). Neutralized eluants from the p130Cas affinity column were concentrated with an ICON concentrator for SDS-PAGE analysis. Concentrated eluants were electrophoretically separated through 8% SDS-PAGE gels (Invitrogen). After visualization

with SilverSNAP stain reagents (Pierce), proteins separating within the relative molecular weight range of 120 to 150 kD were submitted for mass spectrometric analysis.

2.10. In-solution and in-gel reduction, alkylation and digestion of SDS-PAGE-separated proteins, phosphopeptide enrichment, purification and mass spectrometric analysis of their tryptic peptides

In-solution reduction, alkylation and digestion of proteins was carried out by suspending the samples in 50 mM Tris buffer, pH 8.0 with 5 mM DTT for 1 h at 55 °C, followed by 20 mM (final concentration) iodoacetamide. After the reaction was quenched with 50 mM DTT, the proteins were digested with Trypsin Gold (Promega, Madison, WI, USA) at an enzyme/substrate weight ratio of 1:50 overnight at 37 °C. The digestion was quenched by addition of 10 µl TFA (20%) and the samples were dried in a SpeediVac. In other experiments, the proteins in excised, destained and washed gel pieces were reduced in 20 mM dithiothreitol, 100 mM NH_4HCO_3 at 55 °C for 1 h, alkylated with iodoacetamide (100 mM in 100 mM NH_4HCO_3), and digested with Trypsin Gold at 1:10 trypsin/protein ratio, overnight at 37 °C, with multiple washings after each step. The entire process was carried out in duplicate. Phosphopeptide enrichment was carried out using two protocols: A) a combined procedure employing calcium chloride precipitation followed by passage over an IMAC mini-spin column containing a SwellGel Gallium-Chelated Disc (Pierce, Rockford, IL) and B) passage over titanium dioxide beads [30,31]. Capillary HPLC with electrospray ionization (ESI) tandem mass spectrometry (MS/MS) was performed using a nanoAcquity system (Waters Corp.) coupled to an LTQ-Orbitrap (Thermo Fisher Scientific, San Jose, CA) equipped with a TriVersa NanoMate (Advion, Ithaca, NY). Sample concentration and desalting were performed online using a trapping column (0.18 × 20 mm, packed with 5 mm, 100 Å Symmetry C18 (Waters Corp., Milford, MA). Separation was on a 100 µm × 100 mm capillary column (C₁₈, 1.7 µm, 130 Å) nanoAcquity UPLC Column (Waters Corp.). Mass spectra were acquired in the positive-ion mode in the Orbitrap over the range m/z 300–2000 at a resolution of 60,000. Mass accuracy was within 4 ppm. MS/MS spectra were acquired with the Linear Ion Trap for the five most abundant peaks in the MS spectrum if these had signal intensities of >8000 NL, using Multistage Activation. MS/MS spectra were acquired twice at a m/z range dependent on the precursor ion. Xcalibur software (Thermo Fisher Scientific) was used for data analysis; peptide and protein assignments were conducted by the Mascot search engine against the Swiss-Prot database (51.6 release May 2007) employing a 6 ppm error window on the precursor ions and a 0.6 Da window on the fragment ions. All the potential phosphopeptides assigned by Mascot were verified manually.

3.0 Results

3.1. BCAR3 regulates basal p130Cas phosphorylation in human breast cancer cell lines

In a panel of three estrogen-receptor alpha (ER α)-positive human epithelial breast cancer cell lines examined for BCAR3 and p130Cas expression, we noted that the line with the most abundant level of BCAR3, T-47D cells, contained predominantly a form of p130Cas that migrated more slowly on SDS-PAGE analysis, suggestive of higher basal phosphorylation (Fig. 1A, left three lanes). The line with the lowest expression of BCAR3, MCF-7, had the smallest fraction of slowly migrating p130Cas protein. Similarly, among three ER α -negative mesenchymal breast cancer cell lines, the line with the most abundant BCAR3 expression, MDA-MB-231 cells, also contained the most of the slowly migrating form of p130Cas (Fig. 1A, right three lanes), whereas the line with the least BCAR3, MDA-MB-435S, expressed the smallest fraction of this form of p130Cas. In contrast, BCAR3 did not correlate with slowly migrating p130Cas when epithelial and mesenchymal breast cancer cell lines were compared. Immunoprecipitation of p130Cas, followed by treatment with lambda protein phosphatase, a

phosphatase that dephosphorylates serine, threonine and tyrosine residues, demonstrated that dephosphorylation eliminates the more slowly migrating p130Cas in these cell lines (Fig. 1B).

In MCF-7 cells, BCAR3 levels are low and endogenous p130Cas runs as a doublet with the majority of the protein in the faster migrating lower band (Fig. 1A). Over-expression of hemagglutinin (HA) epitope-tagged BCAR3 markedly shifted the migration of endogenous p130Cas to the slowly migrating upper band (Fig. 1C, upper panel). Comparable results were obtained when HA-tagged p130Cas was examined in the presence or absence of co-transfected HA-BCAR3 (Fig. 1C, lower panel). Transfection of the BCAR3 homologue NSP1 increased the fraction of both endogenous and co-transfected HA-tagged p130Cas running as the slowly migrating band to a more modest extent. Transfection with NSP3 did not alter p130Cas migration (Fig. 1C). The reduction in PAGE migration of HA-p130Cas in BCAR3-transfected MCF-7 cells was due to phosphorylation as phosphatase treatment of the immunoprecipitates resulted in a single lower band (data not shown).

To assess the role of BCAR3 in basal p130Cas phosphorylation in mesenchymal breast cancer cell lines, we isolated nine MDA-MB-231 cell line clones stably transduced with two lentiviruses, one constitutively expressing tetracycline repressor and the other, in a tetracycline-repressor-regulated manner, an shRNA sequence we had previously determined could down-regulate BCAR3 expression. Following addition of doxycycline, expression of BCAR3 was markedly diminished (Fig. 1D, lower panel). When the same MDA-MB-231 cell lysates were assessed for p130Cas PAGE migration, it was apparent that reduction in BCAR3 levels resulted in a loss of the slowly migrating phosphorylated p130Cas species and enrichment of the rapidly migrating p130Cas species (Fig. 1D, upper panel). Treatment of MDA-231 cells infected with a lentivirus targeting GFP with doxycycline had no effect on p130Cas PAGE migration (Fig. 1D). In aggregate, these results demonstrate that BCAR3 expression regulates a form of basal phosphorylation of p130Cas in breast cancer cell-lines that results in reduced PAGE migration.

BCAR3 over-expression results in EGFR-independent p130 Cas tyrosine phosphorylation

p130Cas is best known as an adapter protein that coordinates adhesion and growth factor receptor signaling pathways through Src family kinase-mediated tyrosine phosphorylation of the p130Cas substrate domain. In order to determine whether NSP family members induce p130Cas tyrosine phosphorylation, MCF-7 cells were transfected with vector alone or HA-NSP1, BCAR3 or NSP3, followed by immunoprecipitation of endogenous p130Cas and Western blotting with the anti-phosphotyrosine antibody PY100. BCAR3 over-expression induced tyrosine phosphorylation of both components of the p130Cas doublet to a greater degree than that observed following over-expression of NSP1 (Fig. 2A). p130Cas tyrosine phosphorylation was also observed following over-expression of NSP3, although to a lower level than that observed with either other NSP family member. Treatment of the p130Cas immunoprecipitates with lambda phosphatase eliminated detectable tyrosine phosphorylation (Fig. 2A).

EGF receptor (EGFR) signaling has been reported to induce tyrosine phosphorylation of p130Cas [32]. To determine whether BCAR3-mediated p130Cas tyrosine phosphorylation occurred by an EGF receptor-dependent signaling pathway, we examined immunoprecipitated p130Cas in wildtype MCF-7 cells, MCF-7 cells transiently transfected with BCAR3 and MCF-7 cells stably transfected with HA-BCAR3 (II-6 cells) in the presence or absence of the EGFR tyrosine kinase inhibitor AG1478. Tyrosine-phosphorylated p130Cas was detected in MCF-7 cells either transiently or stably transfected with HA-BCAR3, but not in wildtype MCF-7 cells (Fig. 2B). Treatment of the transiently or stably BCAR3-transfected MCF-7 cells with AG1478 failed to alter BCAR3-mediated p130Cas tyrosine phosphorylation. To establish that the MCF-7 cells were responsive to EGF and that AG1478 blocked EGFR signaling, we treated the same three cell types with EGF (10 min, 10 pM) in the presence or absence of

AG1478. Although EGF-induced p130Cas tyrosine phosphorylation was not detected in untransfected MCF-7 cells under these experimental conditions, EGF treatment resulted in robust ERK phosphorylation in all three sets of cells that was completely abrogated by AG1478 pre-treatment (Fig. 2B). We conclude from these studies that among NSP family members, BCAR3 over-expression preferentially induces p130Cas tyrosine phosphorylation in an EGFR-independent fashion.

3.2 Inhibition of Src kinase reduces BCAR3 over-expression-induced p130Cas tyrosine phosphorylation but not altered p130Cas PAGE migration

The experiments described above suggested that Src family kinase-mediated p130Cas tyrosine phosphorylation might account for the accumulation of a slowly migrating form of p130Cas in BCAR3-expressing cells. To address this hypothesis, we transfected MCF7 cells with HA-BCAR3 and/or HA-p130Cas in the presence or absence of the Src family kinase inhibitor PP2 (10 μ M for 20 h), followed by immunoprecipitation with an anti-HA antibody. While transfected HA-p130Cas had no detectable tyrosine phosphorylation, BCAR3 over-expression induced tyrosine phosphorylation of the upper, more slowly-migrating p130Cas band as well as a yet more slowly migrating band that could represent another tyrosine phosphorylated protein that associates with either HA-p130Cas or HA-BCAR3 (Fig. 2C, third panel, lane 7). Pre-treatment of the transfected MCF-7 cells with PP2 eliminated the BCAR3-mediated induction of either tyrosine phosphorylated band. In contrast, PP2 treatment had no effect whatsoever on the BCAR3-mediated reduction in migration of HA-p130Cas (Fig. 2C, fourth panel, lanes 7 and 8). MCF-7 cells were also transfected with BCAR3 alone and the tyrosine phosphorylation and PAGE migration of endogenous p130Cas in anti-HA BCAR3 immunoprecipitates was examined in the presence and absence of PP2 (first four lanes of Fig. 2C). PP2 treatment did not significantly alter tyrosine phosphorylation of the upper, more slowly migrating endogenous p130Cas band in BCAR3-transfected cells (Fig. 2C, third panel, lanes 3 and 4). Once again, PP2 had no effect on the fraction of endogenous p130Cas that migrated as the upper band (Fig. 2C, second panel, lanes 3 and 4). These experiments suggest that, while BCAR3 over-expression induces modest Src family kinase-mediated tyrosine phosphorylation of p130Cas, such tyrosine phosphorylation does not account for the BCAR3-mediated and phosphorylation-associated reduction in p130Cas migration.

Src family kinases have been reported to induce p130Cas phosphorylation in a FAK-dependent manner, with p130Cas binding by its SH3 domain to FAK, which in turn binds by a Tyr-397 autophosphorylation site to the SH2 domain of Src family kinases [3-5]. Transfection of MCF-7 cells with wildtype Src led to striking tyrosine phosphorylation of p130Cas, far greater than that observed following over-expression of HA-BCAR3 (Fig. 3A, lanes 2 and 5). While transfection of MCF-7 cells with FAK alone led to very modest p130Cas tyrosine phosphorylation, co-transfection with FAK and Src augmented such tyrosine phosphorylation and also accentuated the shift of p130Cas to a more slowly migrating species that co-migrated with that observed in BCAR3-transfected cells (Fig. 3A, lanes 4 and 5). As previously reported by others, FAK/Src transfection also induced the appearance of a range of yet more slowly migrating p130Cas species that also appear to be tyrosine phosphorylated (Fig. 3A, third panel, lane 4), suggesting that progressive substrate domain phosphorylation further alters p130Cas conformation [7]. Such p130Cas species were not observed in MCF-7 cells transfected with BCAR3.

Given the importance of Src signaling to p130Cas biology, we sought a non-pharmacologic experimental approach to confirm that Src family kinases were not responsible for the BCAR3-mediated reduction in p130Cas PAGE migration. MCF-7 cells were co-transfected with p130Cas and BCAR3 alone or in combination with dominant negative forms of Src or FAK, followed by assessment of p130Cas PAGE migration. Transfection with dominant negative

mouse Src (K296R Y528F) at two molar ratios reduced expression of co-transfected HA-p130Cas but did not reduce the ability of HA-BCAR3 to alter p130Cas migration (Fig. 3B). Entirely similar results were obtained with dominant negative FAK (Y397F) (Fig. 3C) [23]. These studies support the conclusion that Src-mediated signaling is not required for the BCAR3-regulated phosphorylation of p130Cas that results in altered p130Cas PAGE migration.

3.3. Mapping of BCAR3 and p130Cas domains required for BCAR3-mediated p130Cas phosphorylation

In order to identify the p130Cas structural determinants required for AND-34/BCAR3-mediated p130Cas phosphorylation, we transfected MCF-7 cells with HA-BCAR3 in combination with either wildtype p130Cas or a series of p130Cas mutants in which structural domains had been deleted [6] [25]. Deletion of the p130Cas SH3 domain (Δ SH3) reduced but did not eliminate the ability of BCAR3 to retard p130Cas migration (Fig. 4A). Elimination of the p130Cas substrate domain (Δ SD) appeared to have relatively little effect on the BCAR3-mediated alteration in p130Cas migration; the interpretation of the data provided by this variant was complicated by the fact that under basal conditions, this protein runs as a doublet [25]. Mutation of the carboxy-terminal p130Cas Src SH3 domain-binding motif from RPLP to RLGS (RPLP) had no effect on BCAR3-mediated reduction in p130Cas migration (Fig. 4A) [25]. Similarly, replacement of the p130Cas tyrosine 762 shown to bind to the Src SH2 domain with phenylalanine (Y762F) also had no effect on BCAR3-mediated retardation of p130Cas migration (Fig. 4A). These studies suggest that deletion of components of either of two domains previously implicated in Src binding to p130Cas or the principal region phosphorylated by Src do not abrogate the form of BCAR3-mediated p130Cas phosphorylation that is associated with a shift in p130Cas PAGE migration.

Transfection of MCF-7 cells with AND-34, the murine homolog of BCAR3, induces a shift in p130Cas migration identical to that observed following BCAR3-induced p130Cas phosphorylation (Fig. 5A). In order to identify the domains in this family of proteins required for p130Cas phosphorylation, we took advantage of a series of mutant expression constructs we had previously created in which AND-34 structural domains had been deleted. MCF-7 cells were transfected with full-length or mutant AND-34 constructs, followed by analysis of both whole cell lysates and p130Cas immunoprecipitates. Transfection of Δ SH2, a construct in which the AND-34 amino-terminus including the SH2 domain (amino acid residues 1–256) is deleted, eliminated AND-34-induced p130Cas phosphorylation (Fig. 5A, lanes 3 vs. 5). When the amino-terminus of AND-34 was expressed containing the SH2 domain but not the subsequent proline/serine-rich domain, no p130Cas phosphorylation was observed (Fig. 5B, lanes 2 vs. 8)). In contrast, transfection of SH2-PS, a construct containing both the SH2 domain and the proline/serine-rich domain but lacking the carboxy-terminal 400 amino acids of AND-34 robustly induced p130Cas phosphorylation (Fig. 5A, lanes 3 vs. 7). As expected from our prior studies demonstrating that AND-34 binds to p130Cas through AND-34's carboxy-terminal GEF-like domain, immunoprecipitation of p130Cas demonstrated association of p130Cas with full-length AND-34 and Δ SH2, whereas no association of p130Cas with SH2-PS was observed (Fig. 5A, lanes 7 vs. 8) [24]. Thus, at least as judged by these over-expression studies, stable association with p130Cas is not required for AND-34-induced p130Cas phosphorylation.

To further refine the region of AND-34 responsible for p130Cas phosphorylation, we generated a construct driving expression of human NSP3 in which NSP3's SH2 domain and serine/proline-rich region were replaced with the corresponding AND-34 sequence (SH2PS/N3). Unlike NSP3 itself that induces no shift in p130Cas migration, this chimeric construct induced phosphorylation of p130Cas indistinguishable from that obtained with full-length AND-34

(Fig. 5B, lane 2 vs. 5). In order to determine whether it was AND-34's SH2 domain or its serine/proline-rich domain that conferred the ability to induce p130Cas phosphorylation, we generated two further NSP3/AND-34 chimeras in which NSP3's SH2 domain or serine/proline-rich domain were replaced with the corresponding region of AND-34 (SH2/N3 and PS/N3). Transfection of these constructs into MCF-7 cells demonstrated that in the presence of the AND-34 serine/proline-rich domain, the NSP3 SH2 domain could substitute for AND-34's SH2 domain to induce p130Cas phosphorylation (PS/N3, Fig. 5B, lane 5). In contrast, the serine/proline-rich domain of NSP3 could not substitute for the corresponding AND-34 domain for induction of p130Cas phosphorylation (SH2/N3, Fig. 5B, lane 4). Our studies suggest a model in which both the SH2 domain and serine/proline-rich domain of AND-34 are required to induce p130Cas phosphorylation but in which the specificity of the AND-34 effect resides in the serine/proline-rich domain.

3.4. BCAR3 over-expression leads to p130Cas serine phosphorylation

In order to determine the type of p130Cas phosphorylation induced by BCAR3 over-expression, we carried out 2-dimensional phospho-amino acid analysis. MCF-7 cells or their BCAR3-overexpressing counterparts, clone II-6 cells, were *in vivo* labeled with ^{32}P for four hours, followed by p130Cas immunoprecipitation. An autoradiogram of the PAGE-separated and transferred immunoprecipitate demonstrated increased phosphate incorporation into p130Cas derived from II-6 cells relative to MCF-7 cells (Fig. 6A). The two p130Cas bands were cut out of the membrane, hydrolyzed and analyzed by 2 dimensional thin-layer chromatography in combination with unlabelled serine, threonine and tyrosine standards. Relative to MCF-7 cells, a marked increase in serine phosphorylation was observed from the upper band of p130Cas derived from II-6 cells, with more modest serine phosphorylation noted in the lower band (Fig. 6A: $n = 2$). Basal serine phosphorylation was noted in both forms of p130Cas derived from MCF-7 cells, but at markedly lower levels than that observed in II-6 cells. BCAR3 over-expression in II-6 cells also induced detectable tyrosine phosphorylation of both the upper and lower p130Cas bands, while basal phosphotyrosine was undetectable in MCF-7 cells. However, the level of phosphotyrosine observed in II-6 cells for either the upper or lower p130Cas band were far lower than phosphoserine. No clear evidence of phosphothreonine was observed in either cell line.

In order to identify specific sites of BCAR3-mediated p130Cas phosphorylation, we carried out tandem mass spectrometric analyses of tryptic digests of immunoaffinity-purified endogenous p130Cas isolated from wildtype MCF-7 cells and from stably BCAR3-transfected II-6 cells [33,34]. Given that the purification was carried out with a commercial "high bis" (29:1) PAGE gel system, each preparation contained both slow and fast-migrating p130Cas species. Tryptic fragments corresponding to 88% of the II-6-derived p130Cas and 75% of MCF-7-derived p130Cas sequence were identified in this analysis (Fig. 6B). In both p130Cas preparations, three tryptic peptides that had undergone serine phosphorylation were identified. In the first case, serine 139 was phosphorylated in a tryptic peptide encompassing residues 123–143. In the second case, the serine phosphorylation could be localized to one of three serines that are close to the amino terminus, residue 437, 438 or 440, in the p130Cas tryptic peptide SSQSASSLEVAGPGR (residues 437–451; Fig. 6B). In the third case, the phosphorylated, p130Cas residue, serine 639, is the sole serine in the observed tryptic peptide (residues 629–642) and lies within a proline motif, RPLPSPP, previously identified as the p130Cas binding site for the Src kinase SH3 domain [25]. No tyrosine or threonine phosphorylated peptides were definitively identified in either p130Cas preparation.

To assess whether phosphorylation at serines 139, 639 or 437/438/440 might account for the altered migration of human p130Cas in cells expressing high levels of BCAR3, we carried out site-directed mutagenesis of the corresponding serines in the HA-tagged rat p130Cas

expression construct. Mutation of rat p130Cas serine 138 (S138A corresponding to human residue 139) or 638 (S638A corresponding to human serine 639) to alanine did not alter the BCAR3-mediated shift in p130Cas. Among the three candidate human p130Cas serines 437/438/440, mutation of the rat p130Cas serine 436 (corresponding to human residue 437) to alanine (S436A) reduced the BCAR3-induced shift in p130Cas migration while mutation of the rat p130Cas residues 437 and 439 (S437A and S439A corresponding to human serines 438 and 440) had no reproducible effect (Fig. 6C). Combined mutation of rat serines 138, 436 and 638 led to further reduction in the p130Cas PAGE shift relative to that observed with S436A alone (Fig. 6C). Our results demonstrate phosphorylation of p130Cas serine residues 139, 437 and 639 and suggest that phosphorylation of Ser 437 contributes to reduced p130Cas PAGE migration in BCAR3-overexpressing cell lines.

3.5. BCAR3 regulates adhesion-associated and actin filament-dependent p130Cas serine phosphorylation

As p130Cas is known to play a role in regulating cell signaling pathways related to adhesion to extracellular matrix proteins, we examined BCAR3 regulation of p130Cas phosphorylation in breast cancer cell lines allowed to grow in a normal adherent state or maintained in suspension on HEMA-coated plates. In the mesenchymal MDA-231 cell line, culture on HEMA-coated plates for 24 h led to a pronounced reduction in the phosphorylated p130Cas species (Fig. 7A). In the epithelial MCF-7 cell line that normally has only a modest fraction of such phosphorylated p130Cas, maintenance on HEMA essentially eliminated this species (Fig. 7A). When MDA-231 cells previously maintained on HEMA were then transferred to a new set of either HEMA or fibronectin-coated plates, augmented autophosphorylation of FAK at tyrosine 397, a signaling event known to correlate with integrin-mediated adhesion, occurred immediately following plating of MDA-231 cells on fibronectin (Fig. 7B). In contrast, the previously observed high basal ratio of slow to fast migrating p130Cas species in this cell line was reestablished only after four to six h of further culture on fibronectin but not HEMA (Fig. 7B). Such late-phase adherence-dependent p130Cas phosphorylation proved to require BCAR3 as the accumulation of the slowly migrating form of p130Cas was markedly attenuated in an inducible BCAR3 shRNA MDA-231 clone in the presence of doxycycline (Fig. 7C). As MDA-231 cells became adherent to the fibronectin-coated plates well before the four to six hours required for accumulation of the slowly migrating p130Cas, we conclude that adhesion is required but not sufficient BCAR3-regulated p130Cas serine phosphorylation.

Given that following adhesion of MDA-231 cells to fibronectin, there is a time-dependent accumulation of stress fibers and focal adhesions (data not shown), we next tested whether inhibition of actin polymerization with cytochalasin D would reduce BCAR3-dependent late-phase p130Cas phosphorylation. MDA-231 cells were cultured for 24 h on HEMA followed by re-plating on fibronectin-coated plates and treatment with vehicle or cytochalasin D (2 μ M) for varying periods of time. The previously observed time-dependent p130Cas phosphorylation was greatly diminished by cytochalasin D treatment despite the fact that the cells adhered to the plate (Figure 7D).

3.6. BCAR3 expression regulates p130Cas location and breast cancer cell line growth pattern

Doxycycline-induced reduction in BCAR3 expression had a striking effect on the growth pattern of the shRNA MDA-231 clones. All nine of the untreated BCAR3 shRNA-transduced inducible clones grew similarly to wildtype mesenchymal MDA-231 cells as angular cells that frequently lacked cell-cell cohesion (Fig. 8A). Following treatment with doxycycline, the MDA-231 clones developed a more epithelial-like morphology, growing as tightly coherent “islands” of cells with a smooth external border that lacked any cellular projections. In contrast to the elongated or angular morphology of the untreated MDA-231 cells, the doxycycline-treated cells grew in a “cobblestone” pattern (Fig. 8A). Comparable morphologic changes were

not observed in the doxycycline-treated MDA-231 cells stably transduced with a lentiviral shRNA construct targeting GFP (Fig. 8A). The morphologic changes observed upon reduction of BCAR3 expression in MDA-231 cells were complementary to those we previously observed following over-expression of BCAR3 in MCF-7 cells [22]. Stable over-expression of BCAR3 in this epithelial breast cancer cell line results in a marked reduction in homotypic adhesion and the acquisition of an invasive mesenchymal-like growth pattern (Fig. 8A).

To assess whether the alterations in cell growth pattern observed following knockdown of BCAR3 expression were associated with changes in focal adhesions and the localization of p130Cas, we carried out immunofluorescence studies on the MDA-231 shRNA clones in the presence and absence of doxycycline. In untreated MDA 231 cells, mature focal adhesions were easily identified with anti-paxillin antibodies as peripherally-located structures oriented perpendicular to the cell membrane along actin stress fibers (Fig. 8B). Following down-regulation of BCAR3 with doxycycline, stress fibers were lost, actin filaments were restricted to a cortical location and paxillin staining was now identified in plasma membrane-associated structures that were parallel to the axis of the cell membrane (Fig. 8B). Analysis with fluorochrome-labeled antibodies against p130Cas and vinculin, two other focal adhesion-associated proteins, confirmed and extended these findings. In untreated MDA 231 clones, both p130Cas and vinculin were once again associated at the edges of cells with radially-distributed focal adhesions. Upon doxycycline treatment, p130Cas and vinculin were now found in complexes parallel to and associated with the plasma membrane (Fig. 8B). Doxycycline treatment of the control GFP shRNA MDA 231 cells had no effect on actin filament, paxillin, vinculin or p130Cas distribution (Fig. 8B and data not shown). These studies demonstrate that, in mesenchymal breast cancer cell lines, BCAR3 is required for both basal p130Cas serine phosphorylation and localization of p130Cas to stress fiber-associated focal adhesion complexes.

4.0 Discussion

The focal adhesion protein p130Cas regulates adhesion-related signaling as a result of Src family kinase-mediated p130Cas substrate domain phosphorylation. Here we demonstrate that increased expression of BCAR3, either naturally in the case of the mesenchymal breast cancer cell line MDA-231, or artificially as in the case of stable MCF-7 BCAR3 transfectants, results in augmented basal serine phosphorylation of p130Cas that normally occurs during adhesion of breast cancer cells to fibronectin. While our studies do identify some modest BCAR3-associated tyrosine phosphorylation of p130Cas, studies with a Src family kinase inhibitor, dominant negative Fak and Src expression constructs and a substrate domain-deleted form of p130Cas suggest that Src family kinase-mediated tyrosine phosphorylation is unlikely to be responsible for the observed BCAR3-regulated shift in p130Cas migration detected by PAGE. Consistent with these experiments, phospho-amino acid analysis demonstrates substantial p130Cas serine phosphorylation following stable transfection of MCF-7 cells with BCAR3. In contrast, levels of BCAR3-associated p130Cas tyrosine phosphorylation, while detectable, are far more modest. Our mass spectrometry studies of tryptic digests of phosphorylated p130Cas derived from MCF-7 cells expressing substantial levels of BCAR3 identified three serine-phosphorylated peptides, but no detectable tyrosinephosphorylated peptides were observed. The majority of the p130Cas substrate domain previously shown to be the principal site of Src family tyrosine phosphorylation fell within non-phosphorylated peptides identified in the mass spectrometric analysis of the BCAR3-shifted form of p130Cas, including nine of fifteen substrate domain “YXXP” motifs. It thus appears that the BCAR3-associated p130Cas tyrosine phosphorylation is either markedly sub-stoichiometric and therefore not easily detected by mass spectrometry or occurs on tryptic fragments that were not identified in this analysis [7]. An alternative explanation for the discrepancy between our mass spectrometry

data and our Western blot analyses that cannot be ruled out is that PY100, the antibody used to detect tyrosine-phosphorylated proteins, is not in fact entirely specific for phosphotyrosine.

Although the kinase responsible for BCAR3-associated p130Cas serine phosphorylation remains unidentified, our studies do offer some clues as to role that BCAR3 might play in regulating p130Cas phosphorylation. ShRNA-induced reduction of BCAR3 in MDA-231 cells results in altered intracellular localization of p130Cas, suggesting that BCAR3 regulates the recruitment of p130Cas to specific intracellular sites where its serine phosphorylation may be enhanced. While the simplest model would be that BCAR3's SH2 domain serves to direct BCAR3-associated p130Cas to a tyrosine-phosphorylated ligand, our domain mapping studies with the murine BCAR3 homolog AND-34 surprisingly do not entirely support such a hypothesis. AND-34's SH2 domain was indeed found to be critical for AND-34's ability to induce p130Cas phosphorylation in MCF-7 cells. In contrast, association with p130Cas through AND-34's carboxy-terminal GEF-like domain was not necessary for p130Cas phosphorylation. Further domain-swapping studies with NSP3, an NSP family member whose over-expression does not induce p130Cas phosphorylation in MCF-7 cells, demonstrated that while both AND-34's SH2 domain and a subsequent serine/proline-rich domain were required for the ability to induce p130Cas phosphorylation, the basis for the specificity of the AND-34 effect lay in the serine/proline-rich domain (residues 259–425). While the precise role that this domain plays in regulating p130Cas phosphorylation remains undetermined, the amino acid sequence of this region is notable for several proline-rich motifs that could serve as binding sites for SH3 or WW-domain-containing proteins [35]. Thus, one model for BCAR3-mediated regulation of p130Cas serine phosphorylation is that BCAR3 binds to a tyrosine phosphorylated substrate through its SH2 domain and recruits a proline-motif-binding molecule that subsequently activates p130Cas phosphorylation.

p130Cas has previously been reported to undergo serine phosphorylation and tyrosine dephosphorylation during mitosis when adherent cells detach from extracellular matrix. Such p130Cas serine phosphorylation occurs during the coordinated dissolution of focal adhesion complexes during mitosis and is associated with concurrent serine phosphorylation of Fak and paxillin [36] [37]. The specific p130Cas serine residues phosphorylated during mitosis have not been reported. As doxycycline-treated MDA-231 shRNA clones in which BCAR3 levels have been substantially reduced do continue to grow, it would appear that BCAR3-associated p130Cas serine phosphorylation is not required for progression through the cell cycle and that BCAR3-associated and mitosis-associated p130Cas serine phosphorylation may differ in the specific serines targeted and the signaling consequences of such phosphorylation. However, only detailed mapping of the p130Cas serine residues phosphorylated in these two signaling environments can substantiate this conclusion.

p130Cas serine phosphorylation has previously been identified in fibroblasts freshly adherent to fibronectin [38]. Consistent with this, p130Cas associates with the phosphoserine-binding 14–3–3 adapter proteins in an integrin-dependent manner, predominantly but not entirely through phosphorylation of p130Cas's serine-rich domain (SR) [39] [40]. Mapping studies have suggested that rat p130Cas serine 592 lies within a consensus 14–3–3 binding site and mutation of this serine abolished binding of 14–3–3 to this region of p130Cas [41]. A tryptic peptide that includes serine 494, the residue corresponding to rat serine 592, was identified in the BCAR3-shifted form of human p130Cas assessed in the current mass spectrometry assay and was not found to be phosphorylated. Plating on fibronectin augments 14–3–3 ζ association with p130Cas in Rat-1 fibroblasts, while detachment from extracellular matrix reduces it [39]. In preliminary studies, we did not find any BCAR3-induced augmentation of 14–3–3 ζ association with phosphorylated p130Cas (data not shown). We did, however, find that detachment of MCF-7 or MDA-231 cells from extracellular matrix proteins by seeding cells on HEMA-coated plates reduces the fraction of serine-phosphorylated p130Cas. However, in

contrast to the rapid serine phosphorylation previously reported in fibroblasts following adhesion to fibronectin, MDA-231 cells required at least 4 h of adhesion to fibronectin-coated plates in order to fully reestablish the basal high ratio of slow to fast migrating p130Cas normally observed in this cell line. Such fibronectin-induced p130Cas phosphorylation was markedly compromised in MDA-231 cells in which doxycycline-regulated shRNA expression reduced BCAR3 expression. Inhibition of actin filament formation with cytochalasin also severely reduced BCAR3-mediated p130Cas phosphorylation. These time-course experiments support the hypothesis that BCAR3 regulates a form of p130Cas phosphorylation that is adhesion and actin filament-dependent but distinct from the previously well-characterized rapid Fak and Src kinase-mediated p130Cas substrate domain tyrosine phosphorylation.

Only stable transfection of BCAR3, as opposed to other NSP family members, results in MCF-7 cell resistance to the ER α antagonist ICI 182,780 as well as a shift in MCF-7 cell growth pattern from epithelial to mesenchymal [22]. Such a growth pattern transition is associated with loss of cadherin homotypic adhesion as well as an increase in fibronectin production by the BCAR3 over-expressing clones. In the current study, only over-expression of BCAR3 induces the most substantial levels of p130Cas phosphorylation. Conversely, shRNA-mediated reduction in BCAR3 expression in the ER α -negative breast cancer cell line MDA-231 results in loss of p130Cas phosphorylation and a shift from a mesenchymal to an epithelioid phenotype. A partial epithelial to mesenchymal transition has previously been documented in MCF-7 cells that have acquired tamoxifen resistance *in vitro* after prolonged exposure to the anti-estrogen [42,43]. In such cells, EGFR and Her2/neu transcript and proteins are up-regulated, a phenomenon that has been noted in ICI 182,780-resistant MCF-7 cells as well [44]. The mesenchymal transition in Tam-R MCF-7 cells was characterized by EGFR-dependent dissolution of cadherin/beta catenin-containing adherens junctions and concurrent up-regulation of beta-catenin/TCF/LEF-mediated transcription [43]. While it is striking that BCAR3-mediated anti-estrogen resistance is also associated with a partial epithelial to mesenchymal shift, our AG1478 inhibitor studies demonstrate that BCAR3-mediated p130Cas phosphorylation is likely independent of EGFR signaling. Whether BCAR3-mediated EMT is associated with activation of the beta-catenin/TCF/LEF pathway will require further study.

Mutation to alanine of the three sites of p130Cas serine phosphorylation identified by mass spectrometry reduces the fraction of p130Cas that migrates slowly following BCAR3 over-expression. The fact that there is only a partial reduction in the altered p130Cas migration in the S138A/S436A/S638A triple mutant suggests that phosphorylation at these sites are not the only molecular events that account for this phenomenon. It is therefore likely that other sites of serine phosphorylation exist on the 12% of the p130Cas sequence that has not yet been covered by peptide assignments in the mass spectrometric analysis.

In MDA-231 cells, knockdown of BCAR3 reduces both basal and adhesion-dependent p130Cas serine phosphorylation and results in a redistribution of p130Cas from radially distributed actin stress fibers to a cortical location. These immunofluorescence results differ somewhat from a report by Schrecongost et al in which the authors report that transient knockdown of BCAR3 in BT549 cells induced a loss of membrane-associated p130Cas [17]. In our studies employing stable inducible lentiviral knockdown of BCAR3, MDA-231 cells are transformed from a typical invasive and motile phenotype to islands of epithelioid cells with striking cohesiveness and lack of cell membrane projections at their borders. In contrast to the transient BCAR3 knockdown studies cited above, stable knockdown of BCAR3 in MDA-231 cells results in a redistribution of p130Cas from stress fiber-associated focal adhesions to an array of vinculin-containing structures that are parallel to and associated with the free edge of such epithelioid cell colonies. The contrast between the results from these two studies regarding the effects of BCAR3 expression on p130Cas localization may arise from transient vs. stable knockdown models used or the differing levels of cell confluence examined.

In a recent study, we found that elimination of AND-34/BCAR3 expression in mice by homologous recombination resulted in postnatal posterior lens rupture with migration of visible fragments of cortical lens into the ocular anterior chamber [45]. Western blot analysis and in situ hybridization demonstrated the presence of AND-34 RNA and protein in lens epithelial cells, particularly at the lens equator. Consistent with the findings presented in the current report, while substantial levels of phosphorylated p130Cas as judged by PAGE migration were observed in wildtype lens epithelial cells, p130Cas in AND-34^{-/-} lens epithelial cells demonstrated little or no such basal phosphorylation [45]. Our future studies will focus on establishing the role of BCAR3-mediated p130Cas serine phosphorylation in focal adhesion biology and breast cancer anti-estrogen resistance.

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Fig. 1. BCAR3 regulates p130Cas phosphorylation in human breast cancer cell lines

Panel A: Three epithelial ER α -positive and three ER α -negative mesenchymal breast cancer cell lines were assessed for BCAR3 and p130Cas expression by Western analysis. **Panel B:** p130Cas was immunoprecipitated from the indicated breast cancer cell lines, treated with lambda phosphatase in the presence or absence of EDTA, and examined by Western analysis. **Panel C:** Upper panel: MCF-7 cells were transfected with vector alone or the same vector driving expression of NSP1, NSP2 (BCAR3) or NSP3, followed by Western blotting with an anti-p130Cas antibody. Lower panel: MCF-7 cells were transfected with a construct driving expression of hemagglutinin epitope-tagged p130Cas (HA-Cas) in combination with vectors driving expression of HA-tagged NSP1, NSP2 or NSP3, as indicated, followed by Western

blotting with an anti-HA antibody. The vector panel is shown at shorter exposure and the NSP1 panel at longer exposure than the NSP2 and NSP3 panel due to varying levels of HA-Cas expression in their respective whole cell lysates. Panel D: Three MDA-231 cell lines were isolated in which the Tet-repressor inhibits expression of an shRNA targeting BCAR3 expression in the absence of doxycycline. The three lines, as well as a control MDA-231 line containing a lentiviral shRNA construct targeting GFP, were treated for four days in the presence or absence of doxycycline, followed by Western analysis for expression of BCAR3 and p130Cas.

Fig. 2. Over-expression of BCAR3 induces p130Cas tyrosine phosphorylation but inhibition of such phosphorylation does not alter the BCAR3-mediated reduction in p130Cas PAGE migration

Panel A: MCF-7 cells were transiently transfected with a construct driving constitutive expression of HA-Cas in combination with vectors driving expression of NSP1, BCAR3 or NSP3, as indicated, followed by immunoprecipitation with anti-p130Cas. Immunoprecipitates were treated with lambda phosphatase in the presence or absence of EDTA, as indicated, followed by Western blotting with PY100, an anti-phosphotyrosine antibody. **Panel B:** MCF-7 cells transiently transfected with vector alone (left four lanes) or HA-BCAR3 (middle four lanes) or stably transfected with HA-BCAR3 (II-6 cells: right four lanes). Each cell type was treated with vehicle (-), EGF (10 nM for 10 min), the EGF tyrosine kinase inhibitor AG1478

(10 μ M, 30 min) or both drugs. p130Cas immunoprecipitates were assessed by Western analysis for tyrosine phosphorylation (Y-PO₄), p130Cas and HA-BCAR3 (HA). In addition, whole cell lysates were assessed for phospho-ERK and total ERK. Panel C: Top panel: MCF-7 cells were transiently transfected with expression constructs driving expression of HA-tagged BCAR3, HA-tagged p130Cas or both in the presence or absence of the Src inhibitor PP2 (20 μ M), followed by Western analysis with an anti-HA antibody. Upper middle panel: A subset of the same whole cell lysates were Western blotted with an anti-p130Cas antibody. Lower middle panel: Lysates were immunoprecipitated with an anti-HA antibody and Western blotted with an anti-phosphotyrosine antibody. Bottom: Lysates were immunoprecipitated with an anti-HA antibody and Western blotted with an anti-hemagglutinin antibody.

Fig. 3. Dominant-negative forms of Src and FAK do not block BCAR3-mediated p130Cas phosphorylation

Panel A: Co-transfection with Src and FAK results in accumulation of more slowly migrating p130Cas species, although the level of p130Cas tyrosine phosphorylation observed is far greater following transfection with Src than with BCAR3. MCF-7 cells were transiently transfected with HA-tagged p130Cas (HA-Cas) in combination with wildtype Fak, Src or HA-BCAR3, followed by Western blotting for anti-phosphotyrosine (anti-P-Y), HA, Src or FAK, as indicated. **Panel B:** Co-expression of dominant negative Src (DN-Src) does not alter the BCAR3-mediated reduction in p130Cas migration. MCF-7 cells were transiently transfected with HA-Cas, HA-BCAR3, and dominant-negative Src (DN-Src). Either one-half (0.5) or one-

quarter (0.25) as much DN-Src plasmid was transfected as HA-Cas and HA-BCAR3. Panel C: Co-expression of dominant negative FAK (DN-FAK) does not alter the BCAR3-mediated reduction in p130Cas migration. MCF-7 cells were transfected as in Panel B but with DN-FAK rather than DN-Src.

Fig. 4. p130Cas Src-binding domain mutants are robustly phosphorylated following over-expression of BCAR3

Panel A: Either wildtype HA-p130Cas or mutants lacking the SH3 domain (Δ SH3), substrate domain (Δ SD) or components of the Src-binding domain (RPLP, Y762F) were transfected into MCF-7 cells in the presence or absence of co-transfected BCAR3, followed by Western analysis for phosphorylation-associated shifts in p130Cas migration. **Panel B:** Cartoon depicting the p130Cas mutants utilized in Panel A.

Fig. 5. AND-34/BCAR3's SH2 and proline/serine-rich domains are required for induction of phosphorylation of p130Cas while its p130Cas-binding GEF-like domain is not

Panel A: Wildtype HA-AND-34, mutants lacking the SH2 domain (ΔSH2) or the carboxy-terminal 400 amino acids of AND-34 (SH2-PS), or a chimeric construct in which the SH2 domain and the proline/serine-rich domains of NSP3 were replaced by the corresponding domains of AND-34 (SH2PS/N3), were transfected into MCF-7 cells with HA-p130Cas. Either whole cell lysates or anti-p130Cas immunoprecipitates were then assessed by Western analysis with anti-HA to determine the ability of these AND-34 expression constructs or chimera to associate with p130Cas and to induce HA-p130Cas phosphorylation as judged by a reduction in PAGE mobility. **Panel B:** AND-34 mutants or chimeric expression constructs in which the

SH2 domain, the proline/serine-rich domain or both domains of NSP3 were replaced with those of AND-34, were transfected along with an HA-p130Cas construct into MCF-7 cells. The ability of the AND-34 expression constructs to induce phosphorylation of p130Cas was assessed by Western analysis of whole cell lysates with anti-HA. Panel C: Cartoon depicting the HA-AND-34 mutants and the AND-34/NSP3 chimeras utilized in Panels A and B.

Fig. 6. BCAR3 over-expression leads to augmented serine phosphorylation of p130Cas
Panel A: At the top of the panel is an autoradiogram of immunoprecipitated p130Cas from *in-vivo* ^{32}P -labeled MCF-7 cells and BCAR3-over-expressing II-6 cells. Beneath this is shown a 2-dimensional phospho-amino acid analysis of the upper and lower p130Cas bands from MCF-7 cells and II-6 cells. The location of serine (S), threonine (T) and tyrosine (Y) is shown as determined by concomitant addition of the unlabelled amino-acids and development of the chromatography plates with ninhydrin. **Panel B:** Tryptic peptides identified by mass spectrometry from purified endogenous p130Cas in MCF-7 cells are shown with a dotted underline. Tryptic peptides identified from purified endogenous p130Cas from a stably BCAR3-transfected MCF-7 cell line, II-6 cells, are shown with a single underline. An asterisk has been placed above the three identified phosphorylation sites: serines 139, 437 and 639. **Panel C:** Western analysis of lysates of MCF-7 cells transfected with either wildtype rat HA-p130Cas or rat HA-p130Cas in which serines 143, 441 and 643 were mutated to alanine, either alone or in combination. The same cells were also transfected with HA-BCAR3 or vector. Rat p130Cas is four residues longer than human p130Cas and thus rat serine 143 corresponds to human serine 139 etc.

Fig. 7. Among NSP family members, over-expression of AND-34/BCAR3/NSP2 (BCAR3) preferentially induces an adherence and actin microfilament-dependent phosphorylation-associated reduction in PAGE migration of p130Cas

Panel A: Epithelial MCF-7 cells and mesenchymal MDA-231 cells were grown as adherent cells under routine tissue culture conditions (A) or were detached from their culture plates with a chelator, followed by culture for 20 h on HEMA-coated tissue culture vessels (NA). Whole cell lysates were assessed for p130Cas expression by Western analysis. **Panel B:** MDA-231 cells previously cultured for 24 h on HEMA-coated plates (0) were transferred to a new set of fibronectin-coated or HEMA-coated plates for the indicated number of hours. Whole cell lysates were analyzed for p130Cas expression and for FAK autophosphorylation (pY397). **Panel C:** An MDA-231 cell line stably expressing a doxycycline-regulated shRNA against BCAR3 was cultured for 24 h on HEMA (suspended control) or standard tissue culture plates (adherent control) in the presence or absence of doxycycline. Additionally, the same cell line initially cultured on HEMA for 24 h was then cultured a further 4 or 6 h, as indicated, on fibronectin-coated plates in the presence or absence of doxycycline. Whole cell lysates were analyzed for p130Cas expression by Western analysis. **Panel D:** MDA-231 cells were cultured as in Panel E except that cytochalasin D (2 μ M) was added to the indicated cell cultures for varying periods of time.

Fig. 8. AND-34/BCAR3 regulates breast cancer cell line growth pattern as well as p130Cas localization

Panel A: Mesenchymal MDA 231 cells in which a lentiviral vector reduces expression of endogenous BCAR3 through Tet repressor-regulated shRNA transcription were grown in the presence or absence of doxycycline for the indicated number of days, followed by light microscopy (10X objective). As a control, an MDA-231 cell line stably transduced with a shRNA lentiviral vector targeting GFP was also grown in the presence or absence of doxycycline. A converse shift in growth pattern was observed following comparison of wildtype epithelial MCF-7 cells or II-6, an MCF-7 cell line stably over-expressing BCAR3. **Panel B:** MDA 231 cells stably transduced with shRNA lentivirus targeting either BCAR3

(Line #1) or GFP (control) were treated with doxycycline or vehicle alone for six days, followed by immunofluorescent microscopy with Alexa Fluor 594 phalloidin to identify F actin filaments and FITC-paxillin to identify adhesion complexes. Panel C: MDA 231 cells stably transduced with shRNA lentivirus targeting BCAR3 (Line #1) were treated with doxycycline or vehicle alone for six days, followed by immunofluorescent microscopy with anti-vinculin (FITC) and anti-p130Cas (Alexa Fluor 594) to identify adhesion complexes as well as the location of p130Cas. Merged immunofluorescent images are shown to demonstrate co-localization of F-actin and paxillin as well as vinculin and p130Cas in Panels B and C, respectively. All images in panels B and C were obtained at 60X magnification.