

AWARD NUMBER: W81XWH-15-1-0306

TITLE: Predicting Sensitivity of Breast Tumors to Src-Targeted
Therapies through Assessment of Cas/Src/BCAR3 Activity

PRINCIPAL INVESTIGATOR: Amy H. Bouton, PhD

CONTRACTING ORGANIZATION: Rector and Visitors of the University of Virginia
Charlottesville, VA 22903

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PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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14. ABSTRACT Purpose: The purpose of this research is to assess the role of a signaling pathway comprised of the protein tyrosine kinase c-Src (Src) and two adaptor molecules, Cas and BCAR3, in promoting breast tumor growth, metastasis and therapeutic resistance toward Src-targeted small molecule inhibitors. Scope: The proposed research employs 2- and 3-dimensional tissue culture models, transplantable mouse models of breast cancer, and analysis of human breast tumor samples. Major Findings: Key results from the first year of support include (1) BCAR3 and Cas are co-expressed in multiple subtypes of breast cancer but not in normal mammary epithelial cells; (2) the Cas/Src/BCAR3 signaling complex regulates breast tumor cell adhesion dynamics and invasion; (3) BCAR3 is an essential regulator of tumor initiation in a transplantable murine model of breast cancer; and (4) BCAR3 is essential for the ability of mammary epithelial cells to develop ex vivo into budding breast organoids. These data provide evidence for the crucial activity of the Cas/Src/BCAR3 signaling node in breast cancer progression.					
15. SUBJECT TERMS Breast tumor invasion, adhesion signaling, breast organoids, breast tumor initiation,					
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1. INTRODUCTION

The goal of this project is to (1) understand the cooperating pathways through which signaling by the Cas/Src/BCAR3 signaling node drives tumor growth, metastasis and therapeutic resistance; and (2) be poised to begin clinical trials that will test BCAR3 expression as a predictor of response to the Src inhibitor dasatinib in combination with estrogen receptor (ER) and/or EGFR/HER2-targeted therapies. During the first year of this grant, we have achieved several key milestones. We have shown that Cas-BCAR3 interactions are prevalent in breast tumor cells and that they control adhesion dynamics and invasion. We have gained support for the co-expression of BCAR3-Cas in multiple subtypes of human breast tumors, and have shown that BCAR3 is required for tumor initiation in an orthotopic transplantable murine model of breast cancer. We have developed a breast organoid system to specifically test the role for BCAR3 and the Cas/Src/BCAR3 signaling node in breast morphogenesis and tumor initiation. Finally, we have acquired a large collection of human breast tumor samples and have begun to analyze them for BCAR3 expression. These accomplishments, together with the robust cellular reagents and technical approaches that we have developed, place us in an excellent position to continue along the strong trajectory of progress that has been achieved during this first year of support.

2. KEY WORDS

Breast tumor invasions, adhesions signaling, breast organoids, breast tumor initiation.

3. ACCOMPLISHMENTS

Specific Aim 1; Task 1. *Evaluate a cohort of tumors obtained from patients on a Phase II clinical trial (BMS CA180261).* See Section 5 below.

Specific Aim 1; Task 2. *Expand the pilot study to evaluate BCAR3 expression in a larger cohort of clinical breast tumor samples.* We have now obtained a collection of 190 breast tumor and 21 normal mammary gland clinical samples (Table 1). All of these samples have been stained for H&E, BCAR3, and in some cases Cas and Src. They have all been scanned in their entirety and are currently undergoing analysis by two independent investigators (one pathologist, one researcher) to determine the extent, intensity, and localization of BCAR3 expression. These data will then be analyzed by our biostatistician to determine whether there is a correlation between BCAR3 expression and a particular subtype and/or grade of ductal carcinoma in situ (DCIS) or invasive ductal carcinoma.

Specific Aim 2; Task 3. *Determine whether dasatinib sensitivity can be modulated by BCAR3 expression and Cas/Src/BCAR3 signaling in tissue culture models.* We have generated cell lines depleted for BCAR3 in three claudin-low, triple-negative cell backgrounds that contain moderate levels of BCAR3: MDA-MB-231, Hs578T, and MDA-MB-436 (Table 2). This is an expanded set of cell lines from those proposed in the original grant application. Downregulation of BCAR3 expression in Hs578T (Figure 1) and MDA-MB-436 (data not shown) cells results in a modest increase in the IC₅₀ of dasatinib.

Table 1. Clinical Breast Tissue Samples	
Tumor Type	Number of Samples
DCIS	54
E+H+ IDC	13
ER+ IDC	51
H+ IDC	22
TN IDC	49
ER+PR- IDC	1
TOTAL	190
Normal	21
DCIS = Ductal carcinoma in situ; IDC = Invasive ductal carcinoma; ER = Estrogen receptor; PR = Progesterone receptor; H = HER2; TN = Triple-negative	

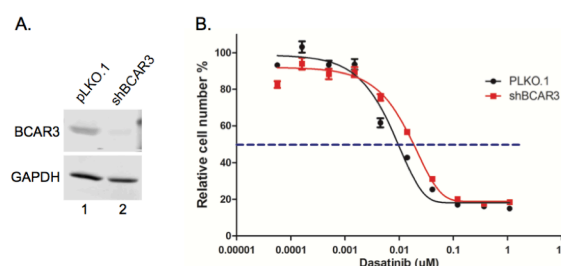


Figure 1. Depletion of BCAR3 reduces sensitivity of Hs578T breast cancer cells to dasatinib. A) Representative immunoblot showing depletion of BCAR3 in shBCAR3 but not vector-controlled (pLKO.1) cells. B) Cells were plated in triplicate, treated for 72 hours with the indicated concentrations of dasatinib, and analyzed by AlamarBlue (ThermoFisher Scientific) to determine cell viability.

Table 2. Engineered Cell Lines						
Parental Cell Line	Molecular Subtype	ER/PR/HER2 (BCAR3)	Knockdown	Rescue		Over-expression
MDA-MB-231	Claudin-low	Neg/Neg/Neg (Moderate)	shBCAR3	WT BCAR3	L744E/R748E BCAR3	
Hs578T	Claudin-low	Neg/Neg/Neg (Moderate)	shBCAR3	WT BCAR3	L744E/R748E BCAR3	
MDA-MB-436	Claudin-low	Neg/Neg/Neg (Moderate)	shBCAR3			WT Venus-BCAR3
T47D	Luminal A	Pos/Pos/Neg (Moderate)				WT Venus-BCAR3
BT474 (low	Luminal B; HER2+	Pos/Pos/Pos (Low)				WT Venus-BCAR3
SKBR3	HER2+	Neg/Neg/Pos (Low)				WT Venus-BCAR3

We have overexpressed wildtype Venus-BCAR3 in 4 cell lines that express moderate to low levels of BCAR3: MDA-MB-436, T47D, BT474, and SKBR3 cells (**Table 2**). Preliminary data indicate that overexpression of BCAR3 in MDA-MB-436 cells results in a modest decrease in the IC50 (data not shown). We were somewhat surprised at the modest changes in IC50 as a function of BCAR3 knockdown or over-expression. To address this issue, we are now adapting the drug sensitivity studies to a 3D matrigel tissue culture model based on reports indicating that drug sensitivities can be substantially altered when the cells are grown under more physiological conditions.

Specific Aim 2; Task 4. Determine whether dasatinib sensitivity can be modulated by BCAR3 expression and Cas/Src/BCAR3 signaling in mouse models. As a first step in this aim, we have performed two experiments examining the role of BCAR3 in tumor initiation and progression. Using an orthotopic transplantable model, we found that MDA-MB-231 breast cancer cells depleted for BCAR3 are significantly impaired in tumor initiation (**Figure 2**). In fact, the few tumors that ultimately grew out in these mice, albeit with much delayed kinetics, were found to re-express BCAR3 (data not shown). These data strongly implicate BCAR3 as a regulator of tumor initiation. These data also indicate that subsequent experiments designed to test dasatinib sensitivity in the mouse as a function of BCAR3 will require conditional

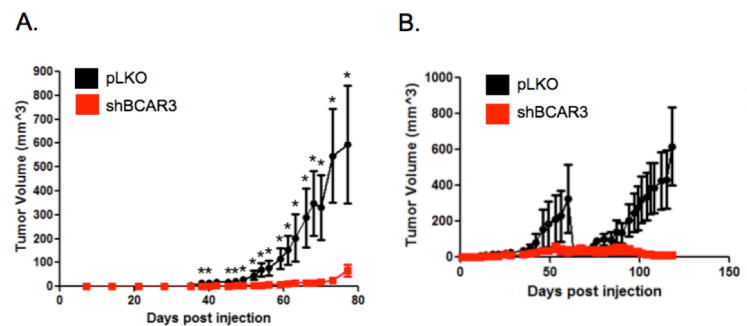


Figure 2. BCAR3 regulated tumor initiation/progression in MDA-MB-231 cells. 1×10^6 vector-controlled (pLKO) or BCAR3-depleted (shBCAR3) MDA-MB-231 cells were implanted into the 5th (A) or 4th (B) mammary fat pad of nude mice. Tumors were measured by caliper 2-3 times per week for the indicated times. (A) Data presented are the mean \pm SEM for 7 pLKO and 10 shBCAR3 tumors. (B) Data shown are for individual tumors (n= 4 for pLKO and 6 for shBCAR3 injected mammary glands). The two curves for the pLKO cells represent mice that were euthanized early or later due to reaching humane endpoints.



Figure 3. BCAR3 expression is down-regulated in MDA-MB-231 cells expressing a tetracycline-inducible shBCAR3 construct. Representative immunoblots containing lysates from tetracycline-inducible vector controlled (TET-pLKO) or BCAR3-depleted (TET-shBCAR3) MDA-MB-231 cells treated for the indicated times with 1 μ g/ml doxycycline.

knockdown of BCAR3 such that the tumors are allowed to form in the presence of BCAR3. BCAR3 will then be depleted and tumor growth determined in the presence or absence of dasatinib. This has required regeneration of our cell lines to include inducible shRNA constructs; luciferase expressing MDA-MB-231 cells expressing doxycycline-inducible shRNA constructs have been generated and are now ready for implantation in mice (**Figure 3**).

Specific Aim 3; Tasks 5 and 6. No progress to date.

Research Team Meeting. The most recent research team meeting was held on September 15, 2016. At this meeting, strategies for analysis of the clinical samples were discussed.

4. IMPACT

While Src inhibitors have shown some activity in breast cancer patients, their lack of consistent efficacy has been somewhat surprising in light of the fact that elevated Src activity is a feature of many breast cancers. Nonetheless, Src inhibitors continue to be considered as potentially viable options for the treatment of breast cancer, largely because Src activity has been implicated in tumor and tumor cell behaviors associated with proliferation, survival, invasion, metastasis, and therapeutic resistance. Thus, there is a desperate need for new approaches that can guide their application to patients who will most likely benefit from this class of drugs. With the extensive collection of clinical breast tumor samples that we have acquired (**Table 1**), the robust phenotypes that we have identified demonstrating a role for BCAR3 in tumor initiation/growth (**Figure 2**) and organoid proliferation and structure (see below, **Figure 4**), the genetic and cellular tools that we have developed (**Table 2**), and the combined expertise of our team, we will be well positioned to rapidly translate this work into the clinic and thus provide benefit to these patients.

5. CHANGES/PROBLEMS

Specific Aim 1; Task 1. Evaluate a cohort of tumors obtained from patients on a Phase II clinical trial (BMS CA180261). Despite providing a letter indicating their willingness to share samples from this clinical trial and frequent back and forth between UVA and Bristol-Myers Squibb (BMS) during this past year, we were informed on September 7, 2016 that BMS would not provide us with the clinical samples that they had promised because of issues related to inadequate consent (**Appendix 1 and 2**). While this was very disappointing, the large cohort of clinical samples that we have amassed from the UVA archives (see **Table 1**) and compelling data from the mouse xenograft studies (see **Figure 2**)

provide strong support for the potential impact of this project. In addition, we have acquired BCAR3 knockout (KO) mice from Dr. Adam Lerner (Boston University School of Medicine), which have provided an additional tool with which to study the role of the Cas/Src/BCAR3 pathway in the mammary epithelium. These mice will allow us to expand the scope of our work with the use of wildtype (WT) and BCAR3 KO mammary organoids. Interestingly, organoids isolated from BCAR3 KO mice fail to bud in response to Fibroblast Growth Factor (FGF) (**Figure 4**). These data provide a

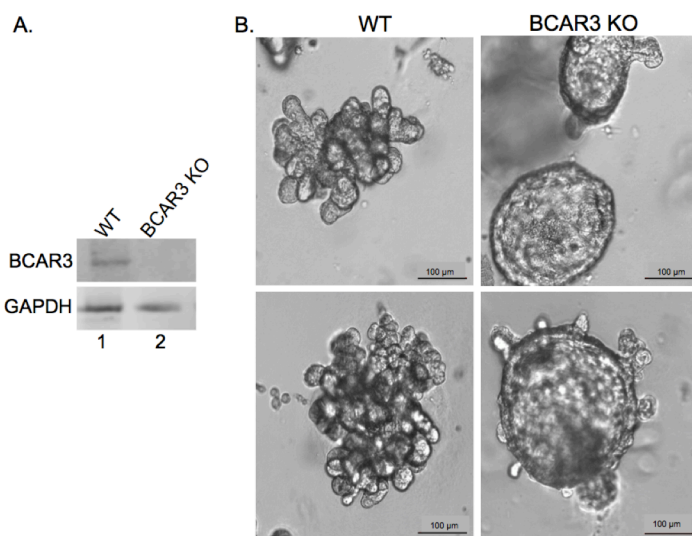


Figure 4. BCAR3 regulates FGF-dependent budding in breast organoids. (A) Representative immunoblot of cell lysates isolated from organoid cultures generated from WT and BCAR3 knockout (BCAR3 KO) mice. (B) Two representative images of organoids grown from 8-week old WT (left panels) or BCAR3 KO (right panels) animals treated with 2.5 nM FGF for 7 days.

platform for identifying signaling pathways impacted by Cas/Src/BCAR3 that may contribute to tumor initiation and progression.

Specific Aim 2; Task 4. Determine whether dasatinib sensitivity can be modulated by BCAR3 expression and Cas/Src/BCAR3 signaling in mouse models. Based on our data showing that knockdown of BCAR3 in breast tumor cells causes significant defects in tumor initiation, we have generated inducible constructs for BCAR3 knockdown that will allow us to directly test the mechanisms through which this pathway controls tumor growth, progression, and response to Src-targeted therapies (see **Figure 3**).

6. PRODUCTS

Cross, AM, Wilson, AL, Guerrero, MS, Thomas, KS, Bachir, AI, Kubow, KE, Horwitz, AR, and Bouton, AH. 2016. Breast cancer antiestrogen resistance 3-p130^{Cas} interactions promote adhesion disassembly and invasion in breast cancer cells. *Oncogene* Apr 25. Doi:10.1038/onc.2016.123. Epub ahead of print. (See Appendix 4)

7. PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

Participant	Role	Salary Support*
Amy H. Bouton, Ph.D.	Principal Investigator	10%
Kristen Atkins, M.D.	Co-Investigator	5%
Mark Conaway, Ph.D.	Co-Investigator	4%
Patrick Dillon, M.D.	Co-Investigator	2%
Carol Gold, J.D., LLM	Consumer Advocate	
Barbara Dziegielewska	Research Scientist	47%
Allison Cross	Graduate Research Assistant	52%
Ryan Llewellyn	Graduate Research Assistant	14%
Keena Thomas, M.S.	Laboratory Specialist	87%

***NOTE:** % effort is greater than the salary support in some instances due to cost-sharing and/or salary caps.

Other support: There has been no change in other support for the PI or Dr. Atkins. Please see the revised “Other Support” documents for Drs. Conaway and Dillon (**Appendix 3**).

8. SPECIAL REPORTING REQUIREMENTS – None to report.

9. APPENDICES

- **Appendix 1.** Letter of Support from Bristol-Myers Squibb (BMS).
- **Appendix 2.** Email from BMS indicating that clinical samples are not available due to consent issues.
- **Appendix 3.** Revised “Other Support” for Drs. Conaway and Dillon.
- **Appendix 4.** Cross *et al.*, 2016. Breast cancer antiestrogen resistance 3-p130^{Cas} interactions promote adhesion disassembly and invasion in breast cancer cells. *Oncogene* Apr 25. Doi:10.1038/onc.2016.123. Epub ahead of print.



Bristol-Myers Squibb Company

P.O. Box 5100 Wallingford, CT 06492-7660

Amy H. Bouton, Ph.D.
Associate Dean of Graduate and Medical Scientist Programs
Professor of Microbiology, Immunology, and Cancer Biology
Box 800734
University of Virginia School of Medicine
Charlottesville, VA 22908

12 June 2014

Dear Amy,

This letter is to affirm our strong enthusiasm for collaborating with you on your grant entitled, "Cas/Src/BCAR3 Signaling Drives Breast Tumor Progression and Therapeutic Responses." Your efforts to understand whether Cas/Src/BCAR3 pathway activity contributes to the aggressiveness of breast cancers, and might predict sensitivity to dasatinib, fit well with our interest in understanding responses to dasatinib in the clinic.

Bristol-Myers Squibb has implemented three randomized Phase II trials using dasatinib to treat patients with advanced breast cancer; archived tissue collection was included in these trials in order to support exploratory biomarker studies. We have performed immunohistochemical analysis for a number of candidate biomarkers chosen to reflect ER/src interaction, including a second member of your signaling complex, Cas (as well as H&E, ER & PR, Ki67, and others). Thus, we are excited about your interest in measuring BCAR3 levels in tumor samples obtained from patients on our study, with the goal of linking them to outcomes data (clinical benefit rate and progression-free survival distribution).

To this end, we will provide paraffin-embedded, formalin-fixed tumor tissue from patients on BMS study CA180261 (exemestane +dasatinib vs +placebo in patients who had progressed on or after a non-steroidal aromatase inhibitor). At this time, we expect to provide you with pre-treatment samples from 84 patients (of 155 treated patients), essentially all of which were ER+ (~75% also PR+). These samples will be provided either as unstained slides (n=2 per subject) or as blocks which are to be returned to BMS upon slide preparation. As we discussed, you will stain one slide for BCAR3 and the other for H&E.

We will then jointly link our existing outcome data, by patient, with BCAR3 expression (H-score) in order to determine whether there is a correlation between this biomarker and outcome, testing the prognostic impact of this biomarker. As approximately half of the patients received dasatinib and half a placebo, we will also assess any correlation between BCAR3 expression and clinical benefit from dasatinib, i.e. to detect a predictive effect. Contingent on findings in this first set of samples, additional tumor samples may become available from the other two studies.

Finally, pending completion of a material transfer agreement, we will be happy to provide dasatinib for laboratory use, furthering understanding of this important signalling pathway.

We look forward to a productive collaboration and wish you luck on your grant application to NIH.

A handwritten signature in black ink, appearing to read "Lewis C. Strauss".

Lewis C. Strauss, M.D.
Global Clinical Research
Bristol-Myers Squibb
Wallingford CT 06492

Subject: RE: CA180261 breast cancer samples

Date: Thursday, September 8, 2016 at 2:06:56 PM Eastern Daylight Time

From: Geese, William

To: Bouton, Amy Hollander (ahb8y)

CC: Strauss, Lewis

Amy,

Unfortunately, it's not a matter of where the samples are tested. It's a matter of whether they permitted to be tested or not. And in this case, the only samples that we would be able to analyze would be from the same three US sites, regardless of staining at BMS or UVa.

Sorry to be the bearer of bad news. I am also sorry that this has taken over a year to finally reach resolution. I was very hopeful that we could collaborate on this, especially since we had generated so much additional (negative) exploratory biomarker results that might have lived to see the light of day if these extended studies were positive. Many thanks again for your patience on this.

Best,

Bill

From: Bouton, Amy Hollander (ahb8y) [mailto:ahb8y@eservices.virginia.edu]

Sent: Wednesday, September 07, 2016 2:57 PM

To: Geese, William <william.geese@bms.com>

Cc: Strauss, Lewis <lewis.strauss@bms.com>

Subject: Re: CA180261 breast cancer samples

Bill,

Would you be able to stain the samples if I send antibody?

Amy

--

Amy H. Bouton, Ph.D.

Associate Dean of Graduate and Medical Scientist Programs

Professor of Microbiology, Immunology, and Cancer Biology

Box 800734

University of Virginia School of Medicine

Charlottesville, VA 22908

Tel: (434) 924-2513

Fax: (434) 982-1071

From: "Geese, William" <william.geese@bms.com>

Date: Wednesday, September 7, 2016 at 12:16 PM
To: Amy Bouton <ahb8y@eservices.virginia.edu>
Cc: "Strauss, Lewis" <lewis.strauss@bms.com>
Subject: CA180261 breast cancer samples

Hi Amy,

I have finally received the information on the samples that you were interested in for the -261 study. Unfortunately, due to myriad reasons (consent, time elapsed after study closure, country-specific requirements etc etc), it came down to our ability to send samples from just 3 sites in the US. Unfortunately, those were among the lowest enrolling sites, yielding only 3 pts. I think we are now at a close with regard to the feasibility of getting samples sent for IHC.

I am extremely disappointed at this outcome, and greatly apologize for the length of time involved to get to the bottom of this. Unfortunately, all of the operational review of ICFs etc were completely out of my hands, so I was at the mercy of our sample ops group. I hope you understand the reasoning behind this unfortunate outcome, but welcome any additional questions, should you have any.

Best,
Bill

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OTHER SUPPORT**CONAWAY, M.**ACTIVE

R01CA142859-04A1 (Conaway)	05/01/2014-04/30/2018	2.7 calendar
NIH/NCI	\$213,739	

Designs for phase I trials in heterogeneous groups

The goal of this project is to develop and test statistical designs for dose-finding trials conducted in heterogeneous groups of patients.

P30CA044579-23 (Loughran)	03/14/2014-01/31/2017	0.6 calendar
NIH/NCI	\$1,561,689	

Cancer Center Support Grant (Biostatistics Core)

Role: Co-investigator

This is to support investigators in the UVA Cancer Center in the design and analysis of studies related to cancer.

R01HD068345-02 (Ramey/Stevenson/Case-Smith)	09/27/2012-07/30/2017	0.6 calendar
NIH/NICHHD	\$583,094	

Multi-Site RCT of Pediatric Constraint-Induced Movement therapy (CIMT)

Role: Co-investigator

The proposed study is a multi-site, randomized controlled trial (RCT) of 144 children (3 - 6 yrs old) with hemiparetic CP

K24AI102972-02 (Houpt)	07/01/2013-06/30/2018	0.78 calendar
NIH/NIAID	\$152,048	

Molecular Diagnostic Tools for Patient-Oriented Field Studies in ID

Role: Co-investigator

The goal of this project is to develop patient-oriented studies in order to inform clinically important areas such as the cause of diarrhea in developing countries and proper treatment for MDR Tb.

R01CA178338-01A1 (Gioeli)	05/01/2014-04/30/2019	0.6 calendar
NIH/NCI	\$207,500	

Checkpoint Signaling and Androgen Receptor Function in Prostate Cancer

Role: Co-investigator

This project seeks to determine the mechanism of CHK2 regulation of AR activity and CRPC cell growth.

P01HL120840-01A1 (Ravichandran)	08/01/2014-05/31/2019	0.6 calendar
NIH/NHLBI	\$1,492,275	

Role of pannexins in vascular physiology and inflammation

Role: Co-investigator

This project comprises several research projects surrounding the role of pannexin in vascular physiology. My specific role will be to assist with biostatistical issues in the design and analysis of experiments.

BC141713 (Bouton)	9/15/15-9/14/18	0.36 calendar
DOD Breast Cancer Research Program	\$225,000	3 cal. mos.

Predicting sensitivity of breast tumors to Src-targeted therapies through assessment of Cas/Src/BCAR3 activity. CURRENT PROPOSAL

Doris Duke Foundation (PI: R Scharf) **08/01/2014-07/31/2018** **0.6 calendar**
 Early Predictors and Biomarkers of Cognition and Growth in Impoverished Children
 Role: Co-investigator \$150,000
 Goal: This study explores the relationship of nutrition and illness to cognitive development in children.

P01HL055798-16 (Hedrick) **07/01/2012-06/30/2017** **0.12 calendar**
 NIH/NHLBI \$2,006,008
 Immune Cells in Atherosclerosis and Vascular Disease
 Role: Co-investigator
 This program project grant centers around the immunobiology of atherosclerosis, with the goal of understanding how macrophages and lymphocytes communicate in the aortic wall to modulate atherogenesis.

1U01NS088034-01 (Kapur) **09/30/2014-02/29/2020** **0.12 calendar**
 NIH \$6,170,975
 Established Status Epilepticus Treatment Trial (ESETT)
 Role: Co-investigator
 This is a multi-site randomized clinical trial of 3 current treatments in epilepsy: fosphenytoin, levetiracetam and valproic acid.

1R01HL128492-01A1 (Lau) **04/12/2016-03/31/2021** **1.2 calendar**
 NIH \$687,033
 Prevention of lung transplant injury with adenosine 2a receptor agonist
 Role: Co-investigator
 This is a phase I, dose-finding clinical trial of an adenosine 2a receptor agonist.

Overlap

There is no scientific or budgetary overlap with any of these projects with the exception of the current grant (BC141713).

PENDING

P01. Non-coding RNAs in gliomas (Abounader) **09/01/2016-08/31/2021** **1.2 calendar**
 NIH/NCI
 Role: Co-investigator
 This is a program project application in non-coding RNAs for brain tumors

R01 (Cox) **07/01/2016-06/30/2020** **1.2 calendar**
 Treating type 2 diabetes by reducing postprandial glucose elevations: A paradigm shift in lifestyle modification
 NIH
 Role: Co-investigator
 This is a randomized trial of 4 behavioral interventions for the treatment of type II diabetes.

R01 (Salerno) **09/01/2016-08/31/2020** **0.6 calendar**
 High-Resolution Whole Heart Quantitative CMR Perfusion Imaging in Ischemic Heart Disease
 Role: Co-investigator
 This project investigates the use of high resolution imaging in diagnosing ischemic heart disease.

OTHER SUPPORT

DILLON, PATRICK M.

ACTIVE

U.S. Dept of Defense - Army Medical Command Bouton (PI), Dillon (Sub-Investigator) 9/15/15-9/14/18
GG11879/149747 \$ 1,089,482.68

"Predicting Sensitivity of Breast Tumors to Src-targeted Therapies Through Assessment of Cas/Src/BCAR3 Activity"

This project will seek to develop methods to determine which tumors will respond to a given drug and which combinations of drugs will produce the most benefit for the patient. This requires detailed knowledge of the intracellular signaling pathways that drive tumor growth and metastasis in specific tumor subtypes. In this proposal, we focus on a signaling network comprised of three proteins, the adapter molecules p130^{Cas} (Cas) and Breast Cancer Antiestrogen Resistance-3 (BCAR3), and the tyrosine kinase c-Src (Src). The rationale for concentrating on this signaling node stems from extensive data in support of these molecules controlling tumor cell proliferation, survival, invasion, tumor growth, metastasis, and therapeutic response. The project thus addresses two overarching challenges: 1) Identify what drives breast cancer growth: determine how to stop it and 2) Eliminate the mortality associated with metastatic breast cancer. (CURRENT GRANT)

U.S. Dept of Defense - Army Medical Command Dzielgowski (PI), Dillon (Sub-I) 9/15/15-9/14/18
GG11882/149829 \$1,098,569 0.36 cal mos.

"Low-Voltage Activated Calcium Channels - Their Role in HER2-Driven Breast Cancer and Potential as a New Therapeutic Target"

This project focuses on the role of low voltage activated calcium channels (LVA, also called T-type channels) in breast cancer development, progression and resistance to therapy. Preliminary results suggest that overexpression/aberrant activation of LVA channels is an important pro-survival and anti-therapy mechanism for breast cancer, especially for the subtype overexpressing human epidermal growth factor receptor 2 (HER2+). Therefore, the study of LVA channels may identify what drives breast cancer growth and determine how to stop it. Importantly, an efficient, safe, and clinically available inhibitor of LVA channels, mibefradil, is currently being developed as anti-cancer agent and will be studied in mouse models in this project with plans to translate results to a future clinical trial at the University of Virginia. (NO OVERLAP)

PENDING

None

OVERLAP

There is no scientific or budgetary overlap with any of these projects.

COMPLETED WITHIN THE LAST 5 YEARS

CMS Innovation Challenge Award Read (PI), Dillon (Sub-Investigator) 8/1/2012-8/1/2015
1C1CMS331031 GC12175/140949 \$ 2,571,322 0.6 cal mos.

"Rapid Response supportive and Palliative Care Program for Patients with Advanced Cancer"

This program will integrate data from multiple sources to help providers proactively identify opportunities for evidence-based care interventions that have been shown to improve quality of care, increase survival, and reduce costs. In addition to various aspects of care, the program includes a specific redesign of radiation therapy to provide highly effective single-day treatment for cancer that has spread to the bone. By limiting unnecessary travel for frequent radiation treatments and delivering more rapid radiation therapy to reduce tumor size this initiative is expected to decrease complications from metastatic disease. Specific Aims include, 1) To improve access to palliative care services and to improved multidisciplinary care through a Supportive Care Tumor Board; 2) To develop the MyCourse Symptom Assessment tool and assess whether it can help identify those who need palliative and supportive care services; 3) To assess whether Stat-Rad rapidly deployed radiation therapy can be administered and effective in managing symptomatic bone metastases in advanced cancer patients.

Charlottesville Women's 4-Miler Fund

Dillon (PI)

6/1/2013-5/31/2015

\$29,000

"A phase II trial of oral calcitriol with docetaxel or paclitaxel in patients with advanced breast cancer"

This IRB approved protocol will assess the efficacy of high dose calcitriol with taxane chemotherapy as a route to prevent the development of chemotherapy resistance and enhance chemoresponse. This biomarker driven study will assess whether 2 biomarkers can predict response and inform future vitamin D therapeutic trials in the metastatic breast cancer setting. The specific aims are, 1) To determine objective response to calcitriol and taxane therapy in patients with metastatic breast cancer, using PERCIST (Positron Emission Tomography Response Criteria in Solid Tumors); 2) To estimate the time to progression in response to calcitriol in combination with either docetaxel or paclitaxel; 3) To evaluate the safety of calcitriol in combination with docetaxel or paclitaxel; 4) To examine the correlation of biomarkers of calcitriol activity pre-therapy in tumor tissue by immunohistochemistry for VDR and CYP24 with response to therapy; 5) To evaluate change in VDR and CYP24 expression between pre-therapy and 4-week tissue samples, and to examine associations with response; 6) To evaluate response by RECIST 1.1 in patients with measurable disease, and compare response classification to that for PERCIST 1.0.

Burroughs Wellcome Foundation

Sharpless (PI)

7/1/08-6/30/2012

Clinical Scientist Award in Translational Research:

"p16INK4a Tumor Suppressor in Stem Cell Aging"

This award funds research into aging as mediated by the tumor suppressor senescence pathway.

Role: sub-investigator from 7/1/2008-6/30/2010.

Charlottesville Women's 4-Miler Fund

Dillon (PI)

6/1/2011-6/1/2013

\$16,000

The Effect of the Nia Technique on quality of life for breast cancer survivors

This philanthropic grant allows the measurement of effects of exercise interventions on quality of life, depression, and well-being for survivors of breast cancer. The clinical trial measures quality of life scores for breast cancer survivors who participate in proscribed Nia exercise and mindfulness classes.

University of Virginia Cancer Center

Dillon (PI)

8/1/2010-8/1/2013

Start-up award

\$125,000 annual

Primary Research Project title is "A pilot study of the addition of poly-ICLC to a 9-peptide breast cancer vaccine for adjuvant breast cancer."

ORIGINAL ARTICLE

Breast cancer antiestrogen resistance 3–p130^{Cas} interactions promote adhesion disassembly and invasion in breast cancer cellsAM Cross^{1,5}, AL Wilson^{1,5}, MS Guerrero², KS Thomas¹, AI Bachir³, KE Kubow⁴, AR Horwitz³ and AH Bouton¹

Adhesion turnover is critical for cell motility and invasion. We previously demonstrated that the adaptor molecule breast cancer antiestrogen resistance 3 (BCAR3) promotes adhesion disassembly and breast tumor cell invasion. One of two established binding partners of BCAR3 is the adaptor molecule, p130^{Cas}. In this study, we sought to determine whether signaling through the BCAR3–Cas complex was responsible for the cellular functions of BCAR3. We show that the entire pool of BCAR3 is in complex with Cas in invasive breast tumor cells and that these proteins colocalize in dynamic cellular adhesions. Although accumulation of BCAR3 in adhesions did not require Cas binding, a direct interaction between BCAR3 and Cas was necessary for efficient dissociation of BCAR3 from adhesions. The dissociation rates of Cas and two other adhesion molecules, α -actinin and talin, were also significantly slower in the presence of a Cas-binding mutant of BCAR3, suggesting that turnover of the entire adhesion complex was delayed under these conditions. As was the case for adhesion turnover, BCAR3–Cas interactions were found to be important for BCAR3-mediated breast tumor cell chemotaxis toward serum and invasion in Matrigel. Previous work demonstrated that BCAR3 is a potent activator of Rac1, which in turn is an important regulator of adhesion dynamics and invasion. However, in contrast to wild-type BCAR3, ectopic expression of the Cas-binding mutant of BCAR3 failed to induce Rac1 activity in breast cancer cells. Together, these data show that the ability of BCAR3 to promote adhesion disassembly, tumor cell migration and invasion, and Rac1 activity is dependent on its ability to bind to Cas. The activity of BCAR3–Cas complexes as a functional unit in breast cancer is further supported by the co-expression of these molecules in multiple subtypes of human breast tumors.

Oncogene advance online publication, 25 April 2016; doi:10.1038/onc.2016.123

INTRODUCTION

Cell motility is an essential feature of processes involved in development and tissue repair, as well as in pathological states such as inflammation and cancer. A signaling node comprised of the adaptor molecules breast cancer antiestrogen resistance 3 (BCAR3) and p130^{Cas} (Cas; also known as BCAR1) has been established as a regulator of several aspects of motility, including cell protrusion, adhesion, migration and invasion.^{1–5} BCAR3 is a member of the novel SH2 domain-containing protein (NSP) family of adaptor molecules, and contains an N-terminal SH2 domain and a C-terminal guanine nucleotide exchange factor (GEF)-like domain with sequence homology to the Cdc25 family of Ras GEFs.^{6–8} These domains promote the interaction between BCAR3 and its two established binding partners, protein tyrosine phosphatase α and Cas, respectively.^{3,9} Cas contains multiple protein interaction domains that contribute to its localization to focal adhesions and its activity as a regulator of cell motility.¹⁰

BCAR3 and Cas bind directly to one another at their C-termini. The C-terminal domain of BCAR3 adopts a ‘closed’ conformation, which is not only necessary for its binding to Cas but also prevents the C-terminus of BCAR3 from functioning as a GEF.⁹ BCAR3 association with Cas has been shown to stabilize each protein and

enhance Cas–c-Src (Src) interactions, Src kinase activity and Src-mediated Cas tyrosine phosphorylation.^{3,5,11}

Previous work from our group and others showed that BCAR3 promotes migration and invasion in breast cancer cell lines.^{2,4} One of the first steps in cell migration is the formation of nascent adhesions at the leading edge of a migrating cell.¹² These nascent adhesions can either undergo disassembly (turnover) or they mature into focal complexes and focal adhesions. Adhesion turnover is initiated when there is a lack of tension to reinforce the adhesion. This is mediated through adaptor molecules and kinases that function in adhesions to locally activate Rac1 and inhibit RhoA GTPase signaling, thereby reducing tension and promoting adhesion disassembly.^{13,14} In order for the cell to move forward, adhesions in the rear of the cell must also undergo disassembly. We have previously demonstrated that the adaptor molecule BCAR3 promotes Rac1 activity and adhesion disassembly in invasive breast cancer cells.⁴ However, the mechanism(s) through which BCAR3 contributes to these activities remained to be elucidated.

In this study, we sought to determine the role of the BCAR3–Cas complex in BCAR3-mediated adhesion dynamics, migration and invasion of breast cancer cells. We found that all of the BCAR3 in invasive breast cancer cells is present in a complex with Cas and

¹Department of Microbiology, Immunology and Cancer Biology, University of Virginia School of Medicine, Charlottesville, VA, USA; ²Fujifilm Diosynth Biotechnologies, USA, Inc., Cary, NC, USA; ³Department of Cell Biology, University of Virginia School of Medicine, Charlottesville, VA, USA and ⁴Department of Biology, James Madison University, Harrisonburg, VA, USA. Correspondence: Dr AH Bouton, Department of Microbiology, Immunology and Cancer Biology, University of Virginia School of Medicine, Box 800734, Charlottesville, VA 22908, USA.

E-mail: ahhb8y@virginia.edu

⁵These authors contributed equally to this work.

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that both proteins colocalize in focal adhesions. BCAR3 entry into adhesions did not require a direct interaction with Cas or an intact SH2 domain. However, the kinetics of BCAR3 dissociation from adhesions was impaired in the absence of Cas binding. This paralleled a similar delay in the dissociation of other adhesion proteins, indicating that BCAR3–Cas interactions have an important role in adhesion complex disassembly. The BCAR3–Cas complex was also found to be important for BCAR3-dependent Rac1 activation, migration and invasion in three-dimensional (3D) matrices. Finally, BCAR3 and Cas were found to be co-expressed in multiple subtypes of human breast tumors. Collectively, these data highlight the importance of a functional BCAR3–Cas complex in invasive breast cancer cells.

RESULTS

The entire cellular pool of BCAR3 is in complex with Cas in invasive breast cancer cells

Given the evidence of a strong functional relationship between BCAR3 and Cas, we measured the steady-state levels of BCAR3–Cas complexes in invasive breast cancer cells. Lysates from BT549 and MDA-MB-231 cells were subjected to serial immunoprecipitations with either Cas or BCAR3 antibodies (Figure 1). BCAR3 was present in Cas immune complexes (Figure 1a, lanes 5–6 and 12–13) and coincidentally lost from the lysates following immune depletion of Cas (lanes 2–4 and 9–11), indicating that the majority of BCAR3 present in BT549 and MDA-MB-231 cells is in complex with Cas. In contrast, although Cas was also present in BCAR3 immune complexes (Figure 1b, lanes 5 and 12), significant amounts of Cas remained in the lysates following immune depletion of BCAR3 (lanes 2–4 and 9–11). Together, these data show that, although a substantial pool of Cas is free of BCAR3, the majority of BCAR3 in invasive breast cancer cells is in complex with Cas. Based on these dynamics, it is likely that the interaction between these molecules is critical for the biological functions of BCAR3.

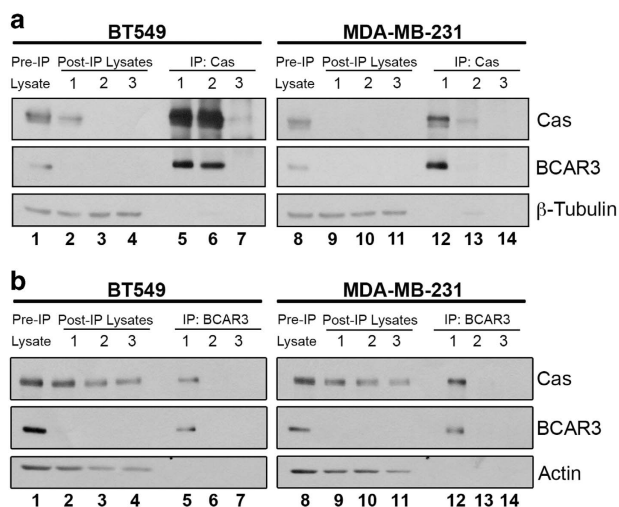


Figure 1. The entire cellular pool of BCAR3 is in complex with Cas. BT549 and MDA-MB-231 cell lysates were subjected to three serial Cas (a) or BCAR3 (b) immunoprecipitations (IP). Pre-IP lysates were separated by 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (lanes 1 and 8) together with the proteins present in the IPs (lanes 5–7 and 12–14) and post-IP lysates (2–4 and 9–11). Proteins were immunoblotted with antibodies recognizing the designated proteins. The pre-IP lysate is 10% of the amount of protein used for the initial IP.

Localization of BCAR3 to adhesions does not require a functional SH2 domain or direct interaction with Cas

As discussed above, BCAR3 and Cas have substantial roles in motility and invasion. BCAR3 has been reported to localize to vinculin-containing adhesions in mouse embryo fibroblasts.³ To determine whether BCAR3 also localizes to adhesions in human breast cancer cells, GFP-BCAR3 was expressed in BT549 invasive breast cancer cells and adhesions were visualized by total internal reflection fluorescence (TIRF) microscopy. Similar to mouse embryo fibroblasts, GFP-BCAR3 was present in adhesions in BT549 cells (Figure 2A, panel a). In addition, GFP-BCAR3 colocalized with endogenous Cas in these adhesions (panels a–c).

To determine which domains of BCAR3 are required for localization to adhesions, we generated functional domain mutants and expressed them in BT549 cells (Figure 2B). As the SH2 domain was previously demonstrated to be critical for BCAR3 localization to adhesions in mouse embryo fibroblasts,³ we first investigated whether a mutant of this domain (R171V GFP-BCAR3) could localize to adhesions in breast cancer cells. This molecule was found to be present in adhesions and, like WT BCAR3, it colocalized with endogenous Cas (Figure 2A; panels d–f). This shows that the SH2 domain of BCAR3 is not the sole determinant of adhesion targeting in breast cancer cells. As a direct interaction between BCAR3 and Cas was reported to be important for their reciprocal stability,⁵ and all of the BCAR3 in these cells is bound to Cas (Figure 1), we next asked whether localization of BCAR3 to adhesions requires association with Cas. This was addressed using a BCAR3 molecule containing two point mutations, L744E and R748E, which were recently shown to prevent the interaction between BCAR3 and Cas.⁵ To verify that these point mutations abrogated Cas binding, Cas immune complexes were isolated from BT549 cells expressing WT GFP-BCAR3 or L744E/R748E GFP-BCAR3 (Figure 2B). As expected, endogenous BCAR3 (lower bands in lower panel, lanes 6–10) and WT GFP-BCAR3 (upper band, lane 7) were present in Cas immune complexes. However, L744E/R748E GFP-BCAR3 (L/R) failed to interact with Cas (lane 9). Despite the fact that this mutant was unable to bind to Cas, it was present in adhesions and colocalized with endogenous Cas (Figure 2A, panels g–i). This demonstrates that BCAR3 localization to adhesions does not require direct association with Cas.

Although neither the SH2 domain nor the Cas-binding domain were found to be solely responsible for BCAR3 localization to adhesions, these data do not discount the possibility that both domains could contain adhesion-targeting activity. To test this, a triple BCAR3 mutant (R171V/L744E/R748E GFP-BCAR3) that lacks both a functional SH2 domain and the Cas-binding site was expressed in BT549 cells. This molecule failed to associate with Cas (Figure 2B, lane 10); however, as was the case for the individual mutants, the triple mutant was present in adhesions and colocalized with Cas (Figure 2A, panels j–l). Together, these data show that, although protein tyrosine phosphatase α (through the SH2 domain) and/or Cas (through the C-terminus) may facilitate BCAR3 localization to adhesions, other mechanisms must be available in the absence of these interactions to recruit BCAR3 to adhesion sites in breast cancer cells.

Direct interaction between BCAR3 and Cas is required for efficient adhesion disassembly in BT549 breast cancer cells

Although BCAR3 localization to adhesions does not require a direct association with Cas, BCAR3 function may be dependent on this interaction. In a previous study, we demonstrated that BCAR3 promotes adhesion disassembly in invasive breast cancer cells.⁴ To test whether this function is dependent on BCAR3–Cas interactions, live TIRF imaging was performed on BT549 cells that were co-transfected with plasmids encoding mCherry-tagged Cas and either WT or L744E/R748E GFP-BCAR3. Under these conditions, both WT and L744E/R748E GFP-BCAR3 colocalized with Cas in

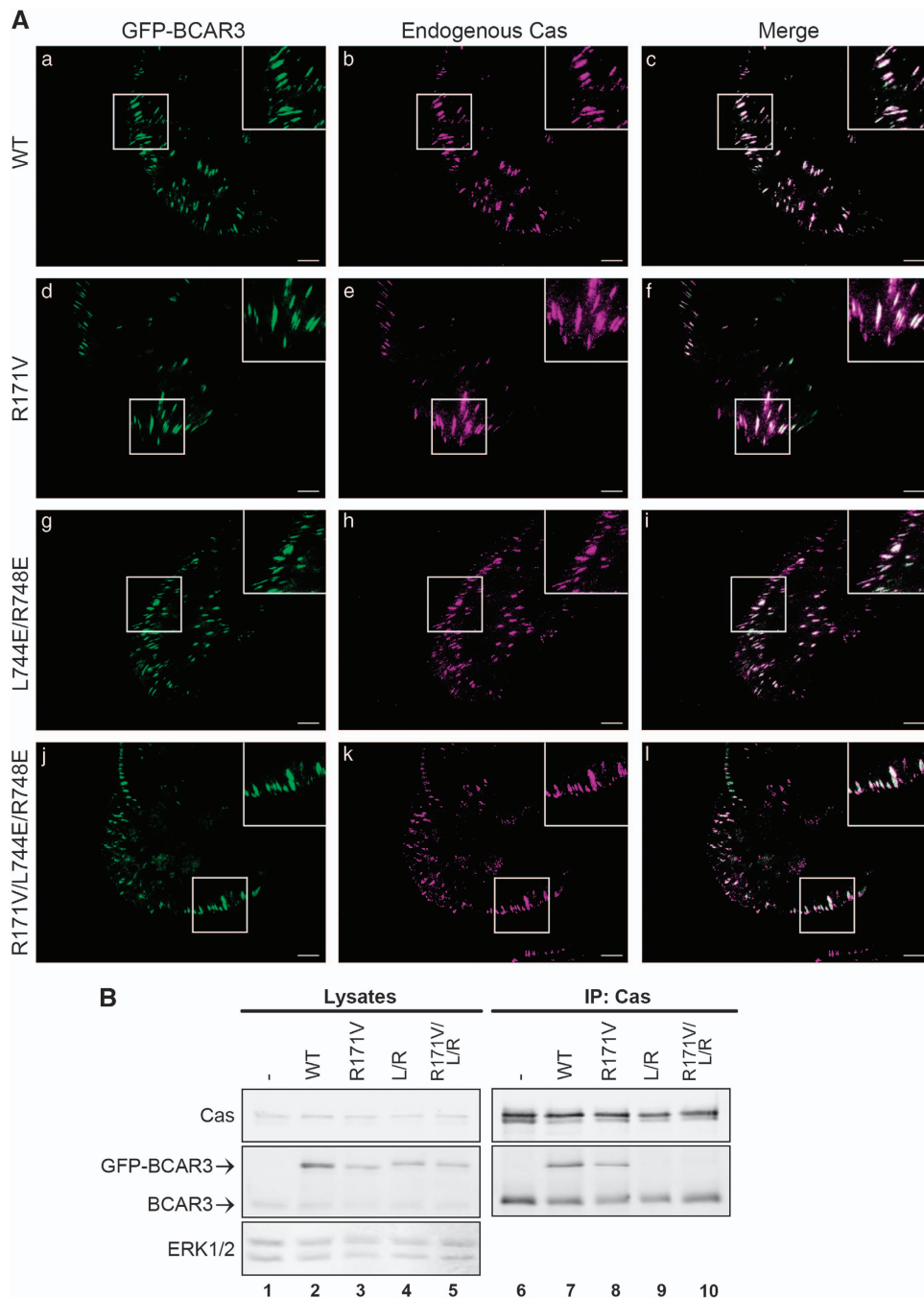


Figure 2. BCAR3 localization in adhesions does not require a functional SH2 domain or interaction with Cas. **(A)** BT549 cells were transfected with plasmids encoding WT GFP-BCAR3, R171V GFP-BCAR3, L744E/R748E GFP-BCAR3 or R171V/L744E/R748E GFP-BCAR3. Cells were incubated for 24 h before plating on 10 μ g/ml fibronectin-coated coverslips for 4 h. Cells were fixed, stained with polyclonal Cas antibodies (panels b, e, h and k), and subjected to TIRF microscopy to visualize adhesions. Merged images are shown in the right panels and insets show higher magnifications of the designated areas. **(B)** BT549 cells were transfected with plasmids encoding GFP, WT GFP-BCAR3, R171V GFP-BCAR3, L744E/R748E GFP-BCAR3 or R171V/L744E/R748E GFP-BCAR3 and lysed in a non-denaturing buffer 24 h post transfection. Total cell protein and Cas immune complexes (generated from 50X more protein than the lysates) were immunoblotted with antibodies to detect the indicated proteins. Left and right panels are identical exposures from the same film.

dynamic adhesions (Figure 3). To quantify adhesion turnover, adhesions at peripheral, protruding edges of a cell were selected for analysis. Time-lapse images show incorporation (arrowheads) and dissociation (arrows) of BCAR3 and Cas into and from representative adhesions co-expressing Cas and either WT (Figure 3a) or L744E/R748E GFP-BCAR3 (Figure 3b). By measuring fluorescence intensity over time (Figures 3c and d), BCAR3

and Cas were found to incorporate into adhesions at similar rates (Figure 3e, compare bars 1 and 3). This was independent of the ability of BCAR3 to bind to Cas, as L744E/R748E GFP-BCAR3 entered adhesions at a rate similar to that of WT BCAR3 (compare bars 1 and 2). Moreover, when Cas was co-expressed with mutant BCAR3, it entered adhesions at a similar rate to when it was co-expressed with WT GFP-BCAR3 (compare bars

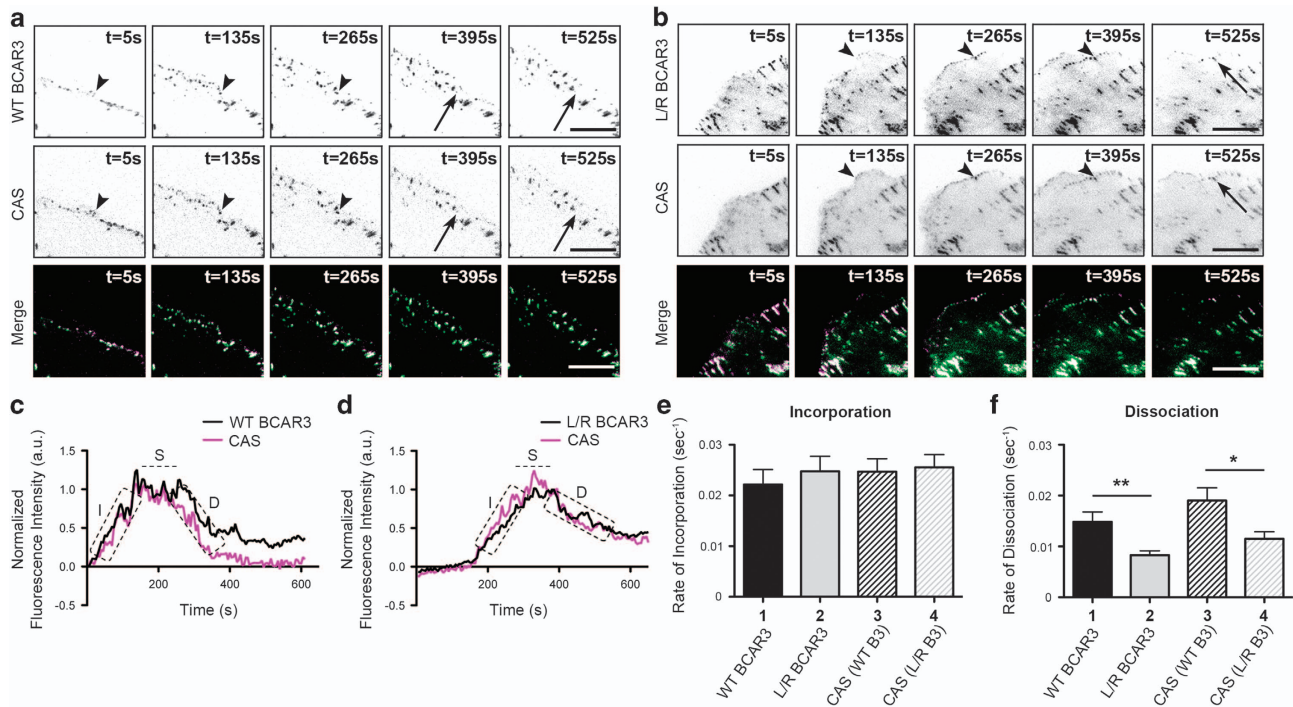


Figure 3. Direct interaction between BCAR3 and Cas is required for efficient dissociation of BCAR3 from adhesions. BT549 breast cancer cells were co-transfected with plasmids encoding WT or L744E/R748E (L/R) GFP-BCAR3 and mCherry-Cas, incubated for 24 h, and then plated on 2 μ g/ml fibronectin-coated glass-bottomed TIRF dishes for 30–40 min before visualizing adhesion dynamics via live-imaging TIRF microscopy. (**a, b**) Representative time-lapse images show incorporation into adhesions (arrowheads) and dissociation (arrows) of the indicated proteins over the specified time course. Scale bars = 100 μ m. (**c, d**) Representative fluorescence intensity time tracings of BCAR3 (black) and Cas (magenta) present in adhesions from cells expressing WT (**c**) or L744E/R748E (**d**) GFP-BCAR3. Dashed boxes/line indicate the incorporation (I), stability (S) and dissociation (D) phases of adhesion dynamics. (**e, f**) Quantitative analysis of the incorporation (**e**) and dissociation (**f**) rates of WT GFP-BCAR3 (bar 1), L744E/R748E (L/R) GFP-BCAR3 (bar 2), Cas co-expressed with WT GFP-BCAR3 (bar 3), and Cas co-expressed with L744E/R748E (L/R) GFP-BCAR3 (bar 4). Data presented are the mean \pm s.e.m. of ≥ 35 adhesions from three WT and L744E/R748E GFP-BCAR3 expressing cells from three independent experiments. * $P < 0.05$, ** $P < 0.01$.

3 and 4). Together, these data demonstrate that BCAR3 can efficiently incorporate into adhesions without being directly bound to Cas.

Using a similar approach to measure adhesion disassembly, we found that BCAR3 and Cas dissociate from adhesions at similar rates (Figure 3f, compare bars 1 and 3). However, the rate of L744E/R748E GFP-BCAR3 dissociation was significantly reduced compared with WT GFP-BCAR3 (compare bars 1 and 2), and dissociation of Cas from these adhesions was similarly impaired (compare bars 3 and 4). This suggests that direct binding between BCAR3 and Cas is required for efficient dissociation of BCAR3 and Cas from adhesions.

The reduced dissociation rate of Cas and L744E/R748E GFP-BCAR3 from adhesions could be the result of a specific delay in the dissociation of Cas and mutant BCAR3 from the adhesions, a more generalized stabilization of adhesion proteins in the adhesion complexes, or a reduction in the turnover rate of mutant BCAR3 and Cas. The latter possibility seems unlikely, as ectopic WT and L744E/R748E BCAR3 were found to have similar half-lives (Supplementary Figure S1). Moreover, these half-lives, as well as the half-life of Cas (data not shown), were found to be over 20 h, which is far greater than the 10- to 12-min timespan of the videos used to quantify adhesion disassembly.

To distinguish between the first two possibilities, we examined the adhesion dynamics of another well-established adhesion protein, talin, in the presence of WT or L744E/R748E GFP-BCAR3. Unlike Cas, talin does not associate with WT BCAR3 (Supplementary Figure S2). Live TIRF imaging was performed to visualize adhesion dynamics in cells expressing mCherry-talin and either WT or L744E/R748E GFP-BCAR3, and adhesion

turnover was quantified as described above (Figure 4). As before, the incorporation of BCAR3 into adhesions was not dependent on its ability to bind to Cas (Figure 4e, compare bars 1 and 2), but its rate of dissociation from adhesions was significantly reduced in the absence of Cas binding (Figure 4f, compare bars 1 and 2). The rates at which BCAR3 and talin entered and left adhesions were not significantly different (Figures 4e and f, bars 1 and 3). However, as was the case for Cas, the rate at which talin dissociated from adhesions was significantly reduced in the presence of L744E/R748E GFP-BCAR3 (Figure 4f, compare bars 3 and 4). This was also the case for a third adhesion protein, α -actinin (Supplementary Figure S3), which similarly does not interact with BCAR3 (Supplementary Figure S2).

It is important to note that, for all of the adhesion proteins analyzed, the reduced rate at which they dissociated from adhesions in the presence of L744E/R748E BCAR3 was similar to the rate at which the mutant BCAR3 molecule left adhesions (Figures 3f and 4f, and S3f, compare bars 2 and 4). This is consistent with a stabilization of the entire adhesion complex under these conditions, suggesting that a direct interaction between BCAR3 and Cas is required for efficient adhesion complex disassembly and turnover.

Our previous work showed that loss of BCAR3 in breast cancer cells resulted in a reduction of Rac1 activity coincident with an increase in RhoA activity, stress fiber stabilization and slower adhesion turnover.⁴ As proper control of adhesion dynamics by BCAR3 required an intact Cas-binding site, we hypothesized that the ability of BCAR3 to promote Rac1 activity may be dependent on its association with Cas. To test this hypothesis, active GTP-bound Rac1 was measured in extracts from BT549 cells expressing WT or L744E/R748E GFP-BCAR3

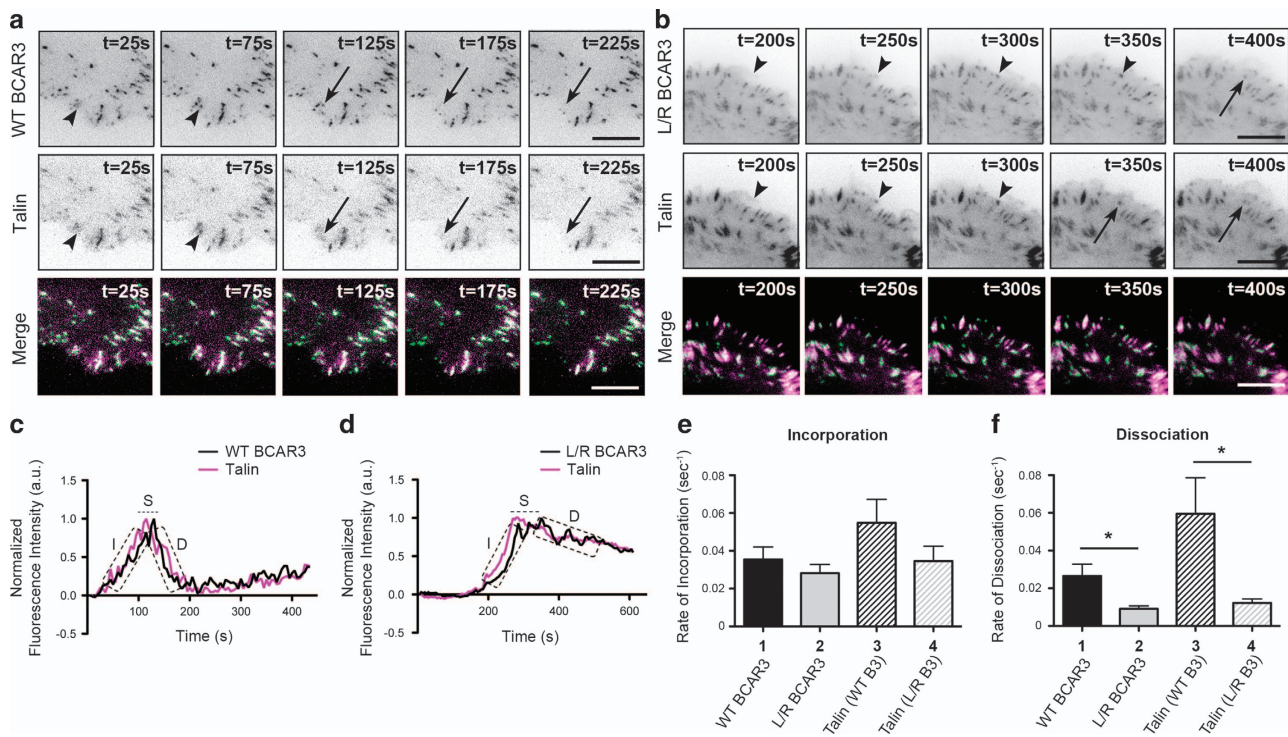


Figure 4. Direct interaction between BCAR3 and Cas is required for efficient dissociation of talin from adhesions. BT549 invasive breast cancer cells were co-transfected with plasmids encoding WT or L744E/R748E (L/R) GFP-BCAR3 and mCherry-talin, incubated for 24 h, and then plated on 2 μ g/ml fibronectin-coated glass-bottomed TIRF dishes for 30–40 min before visualizing adhesion dynamics via live-imaging TIRF. **(a, b)** Representative time-lapse images show incorporation into adhesions (arrowheads) and dissociation (arrows) of the indicated proteins over the specified time course. Scale bars = 100 μ m. **(c, d)** Representative fluorescence intensity time tracings of BCAR3 (black) and talin (magenta) present in adhesions from cells expressing WT **(c)** or L744E/R748E (L/R) GFP-BCAR3 **(d)**. Dashed boxes/line indicate the incorporation (I), stability (S) and dissociation (D) phases of adhesion dynamics. **(e, f)** Quantitative analysis of the incorporation **(e)** and dissociation **(f)** rates of WT GFP-BCAR3 (bar 1), L744E/R748E (L/R) GFP-BCAR3 (bar 2), talin co-expressed with WT GFP-BCAR3 (bar 3) and talin co-expressed with L744E/R748E (L/R) GFP-BCAR3 (bar 4). Data presented are the mean \pm s.e.m. of ≥ 14 adhesions from five separate WT BCAR3/talin or three separate L744E/R748E BCAR3/talin movies generated from three independent experiments. * $P < 0.05$.

(Figure 5). Overexpression of WT BCAR3, but not the Cas-binding mutant, was found to increase Rac1 activity in the cell. Together, these data show that the BCAR3–Cas complex promotes increased Rac1 activation and adhesion disassembly/turnover in breast cancer cell lines.

Direct interaction between BCAR3 and Cas promotes breast tumor cell invasion in 3D and chemotaxis toward serum

Previous studies have shown that BCAR3 promotes breast tumor cell motility and invasion.^{2,4} Considering that adhesion turnover is a critical facet of motility/invasion, and that BCAR3 promotes adhesion disassembly through its interaction with Cas (see above), we hypothesized that BCAR3-mediated breast tumor cell invasion and migration would similarly be dependent upon the ability of BCAR3 to bind to Cas. To test this hypothesis, stable MDA-MB-231 cells were generated that express either empty vector (pLKO) or a BCAR3-targeted short hairpin RNA (shBCAR3) (Figure 6A, lanes 1 and 2). The stable shBCAR3 cell lines were then infected with the lentiviral vector pLV-Venus (Figure 6A, lane 3) or shRNA-resistant wobble versions of pLV-Venus WT BCAR3 (lane 4) or the Cas-binding mutant of BCAR3 (L744E/R748E, L/R) (lane 5). The expected Cas-binding capabilities of these molecules were confirmed through analysis of Cas immune complexes (lanes 7–10). Each cell line was seeded in 3D Matrigel cultures to assess whether BCAR3–Cas interactions were necessary for BCAR3-mediated invasion. As has been reported previously for parental MDA-MB-231 cells,¹⁵ control cells formed highly invasive structures when grown in 3D Matrigel culture (Figure 6B, panel a). In contrast, knockdown of BCAR3 resulted in a significant

reduction in the percentage of invasive structures observed at day 8 in culture (Figure 6B, panel b, Figure 6C, compare bars 1 and 2). Cells infected with a second shRNA construct that resulted in a less robust knockdown of BCAR3 (Supplementary Figure S4A) exhibited an intermediate invasive phenotype (Supplementary Figures S4B and C). The reduced invasive phenotype exhibited by cells expressing shBCAR3 was rescued by expression of WT BCAR3 protein but not the empty vector or Cas-binding mutant (Figure 6B, panels c–e, Figure 6C, bars 3–5). This requirement for direct BCAR3–Cas binding in BCAR3-mediated invasion was confirmed with a second cell line, HS-578 T (Supplementary Figure S5). To determine the importance of direct binding between BCAR3 and Cas in promoting BCAR3-mediated migration, the MDA-MB-231 cells described above were plated in a modified Boyden chamber and allowed to migrate toward serum for 6 h. Knockdown of BCAR3 resulted in a loss of migration as previously described² (Figure 6D, bars 1 and 2). The reduced migration observed in cells expressing shBCAR3 was similarly observed in cells re-expressing the empty vector and the Cas-binding mutant of BCAR3 (Figure 6D, bars 3 and 5) but not in cells re-expressing WT BCAR3 (bar 4). Collectively, these data show that BCAR3 promotes both chemotaxis toward serum and invasion through its interactions with Cas.

BCAR3 is co-expressed with Cas in multiple subtypes of human breast tumors

Considering the strong functional relationship between BCAR3 and Cas in breast cancer cell lines *in vitro*, we next sought to determine whether there was evidence for a similar functional

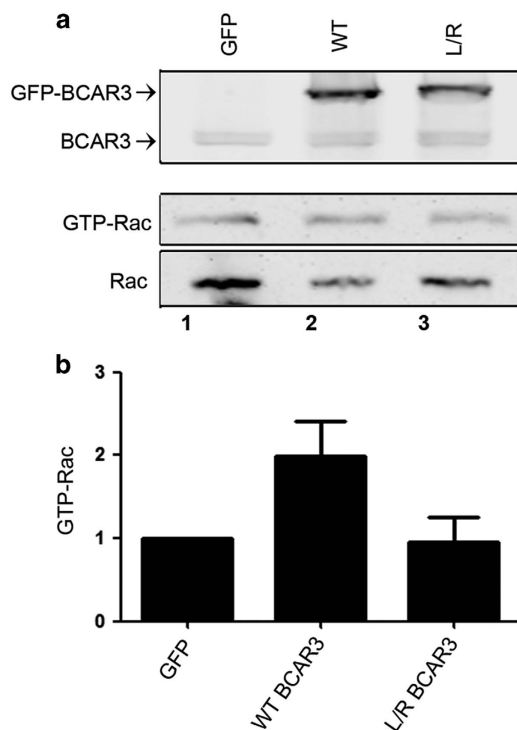


Figure 5. BCAR3–Cas interactions are required for BCAR3-dependent Rac activity. BT549 cells were transfected with plasmids encoding GFP, WT GFP-BCAR3 or L744E/R748E GFP-BCAR3 and incubated for 24 h. Cells were held in suspension for 90 min, then plated on 10 μ g/ml fibronectin for 1 h. (a) GTP-bound Rac1 was isolated from whole-cell lysates by incubation with PAK-1-binding domain agarose. Bound proteins (middle panel) and total Rac1 (bottom panel) were detected by immunoblotting with a Rac1 antibody, and BCAR3 expression was confirmed with a BCAR3-specific antibody (top panel). (b) Quantification of the relative GTP-Rac1 level is shown. Data presented are the mean \pm s.e.m. of three independent experiments.

association in human breast tumors. Sequential sections of tumor tissue were stained with hematoxylin and eosin or antibodies recognizing BCAR3 or Cas. BCAR3 expression was found to be low to non-detectable in normal breast tissue (Figure 7, top panels) but upregulated in multiple breast tumor subtypes (bottom 3 panels). Moreover, BCAR3 was found to be co-expressed with Cas in localized regions of tumor tissue (see insets), suggesting that these two molecules may indeed function as a unit in breast cancers.

DISCUSSION

BCAR3 expression is upregulated in invasive breast cancer cell lines and has been shown to promote migration and invasion in these cells.^{2,4,16} Work from the Pasquale group demonstrated that direct binding between BCAR3 and Cas is required for enhanced Src activity and Cas phosphorylation.⁵ In this study, we sought to further elucidate the importance of BCAR3–Cas complexes in BCAR3-dependent functions, particularly those associated with cell motility and invasion. The functional nature of this protein complex is underscored by our finding that all of the BCAR3 is in complex with Cas in invasive breast cancer cells.

BCAR3 targeting to adhesions is multifactorial

As all of the BCAR3 in BT549 and MDA-MB-231 breast cancer cells is present in BCAR3–Cas complexes, it is formally possible that, in the absence of any perturbation, endogenous BCAR3 enters adhesions together with Cas. However, there must also be Cas-independent mechanisms for adhesion targeting of BCAR3 as ectopically

expressed L744E/R748E GFP-BCAR3 readily localized to adhesions despite its inability to associate with Cas (Figure 8a). The SH2 domain has been reported to mediate BCAR3 targeting in mouse embryo fibroblasts through its interaction with protein tyrosine phosphatase α ,³ however, the SH2 domain was dispensable for adhesion targeting in our system. Moreover, the dual SH2/Cas-binding mutant (R171V/L744E/R748E GFP-BCAR3) also localized to adhesions, indicating that there are other focal adhesion-targeting mechanisms that contribute to BCAR3 localization to these sites, at least in the absence of Cas and protein tyrosine phosphatase α interactions. It is unlikely that this targeting activity is a direct consequence of talin and α -actinin, as neither protein was present in WT or L744E/R748E GFP-BCAR3 immune complexes (Supplementary Figure S2). Whether other adhesion proteins are responsible for adhesion targeting of ectopic BCAR3 molecules in these circumstances remains to be determined.

BCAR3–Cas interactions are required for efficient BCAR3-mediated adhesion disassembly, migration, invasion and Rac1 activity

The data presented in the current report provide the first mechanistic insight into how BCAR3 promotes adhesion disassembly and invasion of breast cancer cells. Under conditions in which BCAR3–Cas complexes were able to form (that is, WT BCAR3), we observed rapid dissociation of multiple proteins from adhesions. However, when BCAR3–Cas interactions were blocked (that is, L744E/R748E BCAR3), the rate of adhesion disassembly was significantly reduced. This suggests that the BCAR3–Cas complex contributes to adhesion disassembly. Recent studies have shown that the ability of BCAR3 to induce membrane ruffling/lamellipodia in 2D also requires Cas binding.⁵ Data presented in this report expand on these findings by showing that interactions between BCAR3 and Cas are required for the invasive phenotype of breast cancer cells in 3D as well as chemotaxis toward serum. Finally, BCAR3 expression in cells grown on plastic promotes Src-mediated Cas phosphorylation in breast cancer cells, leading to Cas/Crk coupling and Rac1 activation.^{2–4,10,11,17,18} We show here that BCAR3-dependent Rac1 activation also requires interaction with Cas. On 3D matrices, Rac1 activity promotes a mesenchymal phenotype, whereas elevated RhoA signaling promotes more rounded cell morphology.¹⁹ It is therefore interesting to speculate that BCAR3–Cas-dependent Rac1 activity may be critical for its ability to promote an invasive phenotype in 3D culture. Whether the adhesion turnover functions of BCAR3–Cas observed in 2D contribute to the BCAR3-dependent invasive phenotype in 3D remains to be determined, particularly as adhesions that form in 2D and 3D may differ significantly in protein composition, dynamics and regulation.^{20,21}

The colocalization of BCAR3 and Cas in adhesions suggests that BCAR3–Cas-mediated Rac1 activation is likely to occur at these sites. This activity, coincident with the possible suppression of RhoA in adhesions, could account for the faster rate of adhesion disassembly and turnover observed when WT GFP-BCAR3 and Cas are expressed in the cells (Figure 8b). Although the Cas-binding mutant of BCAR3 was also seen to efficiently localize to adhesions, it failed to promote Rac1 activity. In the absence of BCAR3–Cas interactions (or upon depletion of BCAR3 as was the case in our previous study), we speculate that the inability to locally activate Rac1, together with a possible rise in RhoA-mediated tension, provides the reinforcement necessary to stabilize adhesions and reduce the rate of disassembly (Figure 8c). This model is supported by work from the Lerner group, who showed that deletion of the C-terminus of BCAR3 abrogated both Cas binding and Rac1 activation.²² They also showed that a mutant of BCAR3 containing a single point mutation in the Cas-binding domain was still able to promote Rac1 activity despite its apparent inability to bind to Cas. It has since been shown, however, that this point mutation may not completely abrogate Cas binding in the cell.⁵

In conclusion, we favor a model wherein BCAR3 promotes Rac1 activation, adhesion disassembly and an invasive phenotype through its binding to Cas, and that interfering with the

interaction between these proteins short circuits signaling network(s) responsible for these activities (Figure 8). An alternative explanation for the data presented in this study is that L744E/748E

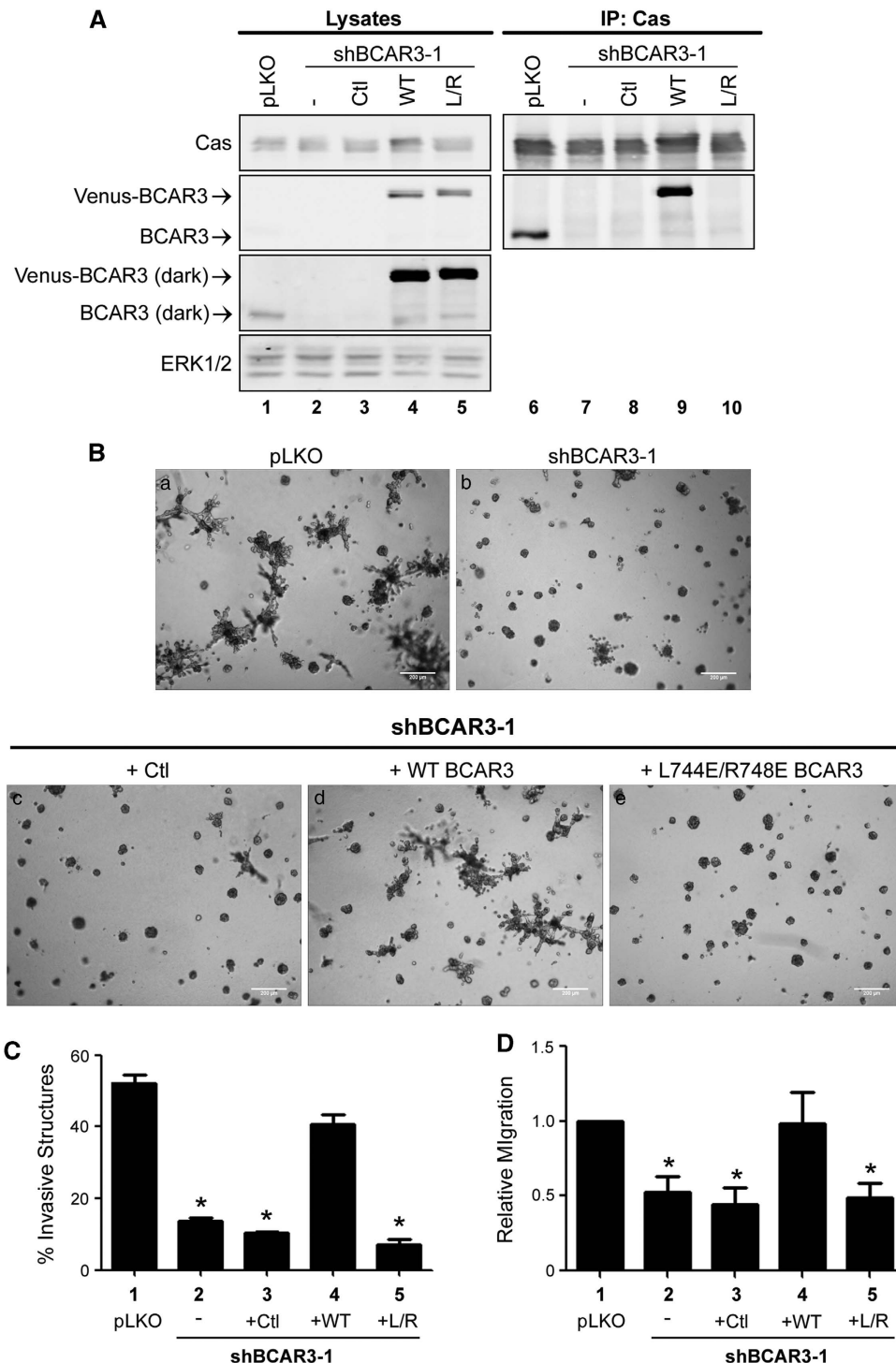


Figure 6. Direct interaction between BCAR3 and Cas is required for invasion of MDA-MB-231 cells in 3D Matrigel culture and chemotaxis toward serum. **(A)** MDA-MB-231 cells stably expressing empty vector (pLKO) or shBCAR3-1 lentiviral constructs were infected with lentiviruses encoding third-base wobble variants of WT Venus-BCAR3 or L744E/R748E (L/R) Venus-BCAR3 or empty vector (pLV-Venus; Ctl). Total cell protein and Cas immune complexes were immunoblotted with antibodies to detect the indicated proteins. Left and right panels are identical exposures from the same film. **(B, C)** The cells described in panel **a** were grown in 3D Matrigel culture for 8 days. Representative phase images **(B)** and quantification of invasive structures **(C)** are shown. Data presented are the mean \pm s.e.m. of three independent experiments, performed in quadruplicate. Scale bars = 200 μ m. **(D)** The cells described in panel **a** were serum-starved overnight and plated (2.5×10^4) in the top of a Boyden chamber (6.5 mm, 8.0- μ m Transwell Costar membrane; Corning Inc.). Cells were allowed to migrate toward 10% serum for 6 h and the cells that migrated to the lower chamber were counted. Data presented are the mean \pm s.e.m. of seven independent experiments. * $P < 0.05$ relative to pLKO.

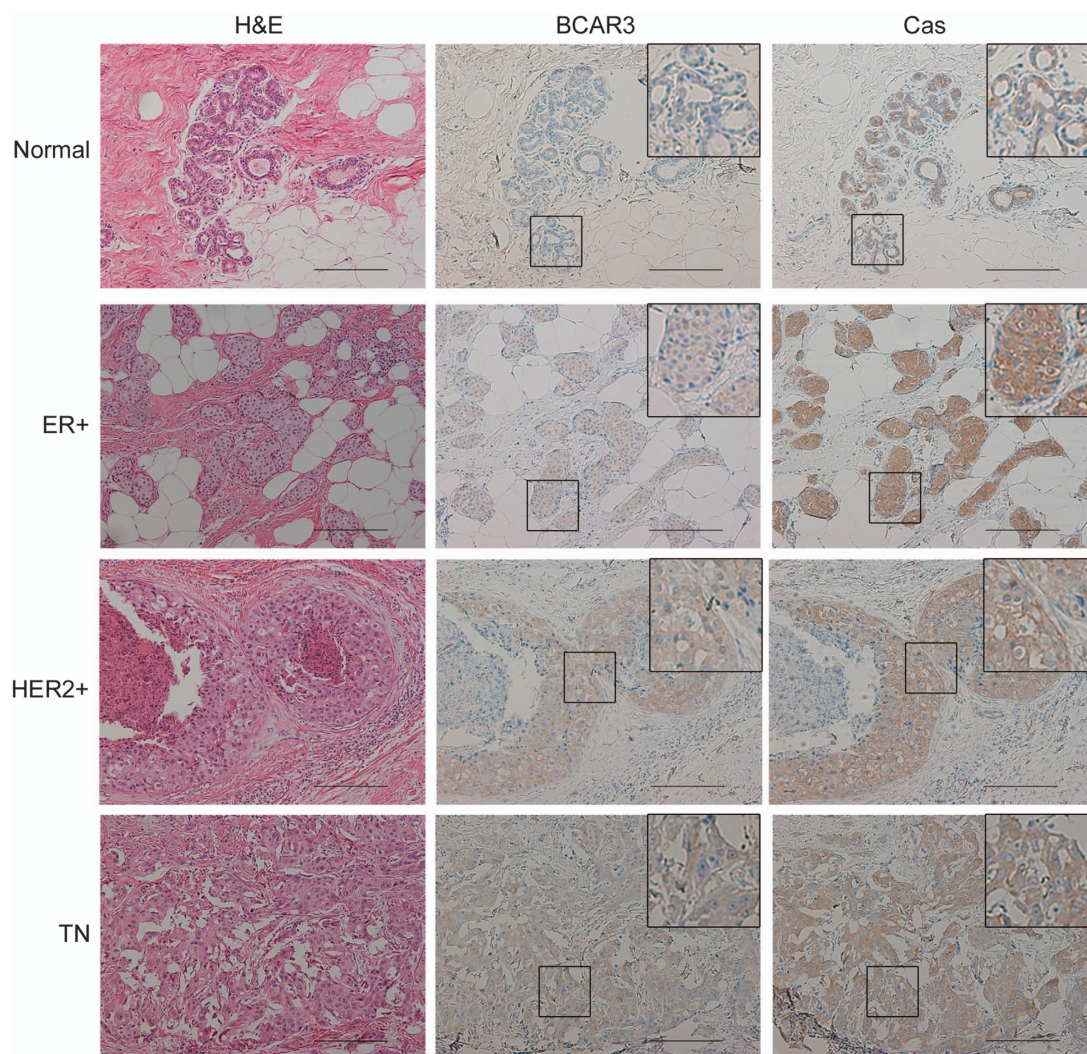


Figure 7. BCAR3 is co-expressed with Cas in multiple subtypes of human breast tumors. Sequential sections of human tissue were stained with hematoxylin and eosin (H&E) (left panels) or immunostained with BCAR3 (middle panels) or Cas (right panels) antibodies. Insets show higher magnifications of the designated areas. Scale bars = 50 μ M. ER, Estrogen Receptor; HER2, Human Epidermal Growth Factor Receptor 2; TN, Triple-Negative

BCAR3 may function independently of Cas to inactivate other molecules/pathways whose functions are critical for these outcomes. We consider this to be unlikely, however, largely because expression of the Cas-binding mutant of BCAR3 phenocopies the effects of BCAR3 knockdown that were reported in our previous study⁴ with respect to the adhesion turnover defect and diminished Rac1 activation.

BCAR3-Cas functions as an oncogenic protein complex in invasive breast tumor cells

Despite strong evidence for BCAR3 as a potent regulator of cell adhesion and invasion in breast cancer cells, the fact that a global knockout of BCAR3 fails to cause any major developmental or phenotypic abnormalities at birth²³ indicates that its expression is largely dispensable for morphogenesis. BCAR3 expression is upregulated in invasive breast cancer cell lines,^{2,16} and it is in this context that BCAR3 appears to have a critical role in adhesion turnover and invasion. Our finding that the majority of BCAR3 in BT549 and MDA-MB-231 cells is associated with Cas suggests that the function of BCAR3 in these cells is dependent on the BCAR3-Cas complex. This is further supported by the data

presented above showing that BCAR3 is co-expressed with Cas in multiple breast tumor subtypes. Together, these data suggest that BCAR3-Cas and/or its downstream effectors may prove to be effective therapeutic targets for tumors that co-express these molecules, particularly because BCAR3 is non-essential for development.

MATERIALS AND METHODS

Antibodies and reagents

Monoclonal antibodies recognizing β -actin (A3854), β -tubulin (T4026), α -actinin (A5044) and talin (T3287) were purchased from Sigma-Aldrich (St Louis, MO, USA). Polyclonal antibodies were obtained from the following sources: BCAR3 (Bethyl Laboratories, Inc., Montgomery, TX, USA; A301-671A); BCAR3 (for immunohistochemistry) (Sigma-Aldrich, HPA014858); GFP (Abcam, Cambridge, UK; AB6673), ERK (Cell Signaling Technology, Inc., Danvers, MA, USA; 9102); Texas red-conjugated goat anti-rabbit (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA USA; 111-075-144); CasB.²⁴ Additional reagents included fibronectin (Sigma-Aldrich, F1141), EGF (Peprotech, Rocky Hill, NJ, USA; AF-100-15) and Matrigel (Corning Inc., Corning, NY, USA; 354230).

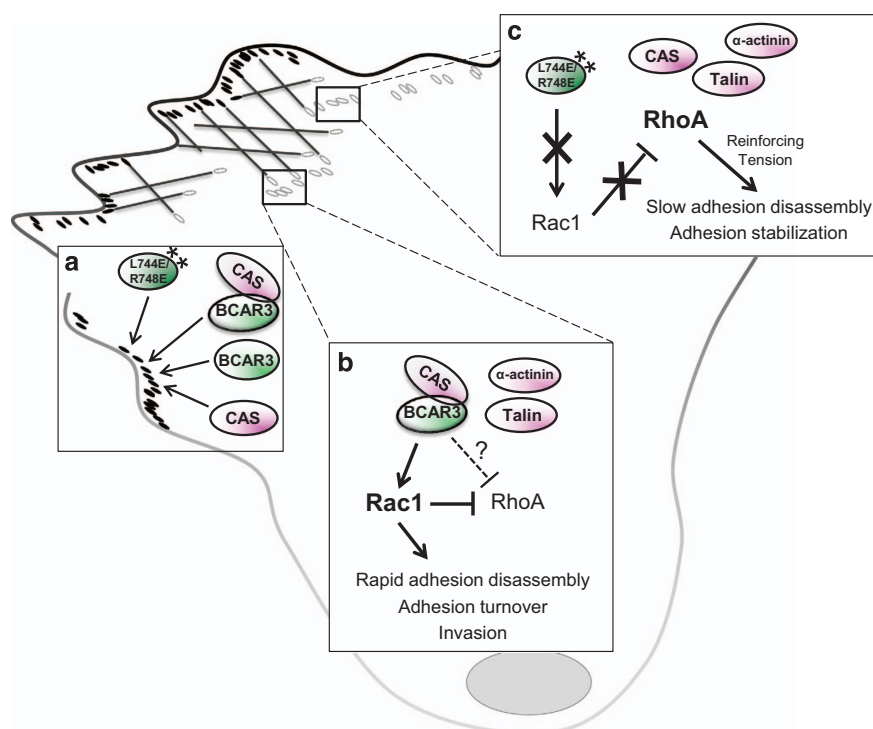


Figure 8. BCAR3-Cas interactions promote efficient adhesion complex disassembly and invasion. **(a)** BCAR3 can efficiently incorporate into adhesions in the absence of a functional Cas binding and/or SH2 domain. **(b)** Under conditions where BCAR3-Cas interactions are enabled (that is, WT BCAR3), rapid disassembly of multiple adhesion proteins is observed. We propose BCAR3-Cas complexes promote localized activation of Rac1 and/or suppression of RhoA under these conditions, therefore initiating rapid adhesion turnover and invasion. **(c)** When BCAR3-Cas interactions are prevented (that is, L744E/R748E BCAR3), local Rac1 activation is diminished, leading to a possible rise in localized RhoA-mediated tension, which provides the reinforcement necessary to stabilize adhesions and slow the rate of disassembly.

Expression vectors

BCAR3 complementary DNA was cloned into the *EcoRI* and *XbaI* sites of pEGFP-C1 (Clontech Laboratories, Inc., Mountain View, CA, USA) to generate pEGFP-BCAR3 (WT GFP-BCAR3). Cas complementary DNA was cloned into the *XbaI* and *BamHI* sites of pm-Cherry-C1 to generate pm-Cherry-Cas.

Mutant R171V, L744E/R748E and R171V/L744E/R748E GFP-BCAR3 proteins were created using the QuickChange II Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA). The following primers were used (changed nucleotides are underlined, and all constructs were confirmed by sequencing): R171V forward: 5'-CGAGATGGTGACTTCCTAGTTGTCGACTCTCTGTCCAGCCCTGGG-3'; R171V reverse: 5'-CCCAGGGCTGGACAGAGAGTCGACA ACTAGGAAGTCAACATCTCG-3'; L744E/R748E forward: 5'-CATGCTGAACCAT GAGCAACAGCGGAATTCATGGCCGAGGCTGC-3'; L744E/R748E reverse: 5'-GC AGCCTCGCCATGAATTCGCTGTTGCCTCATGGTTCAGCATG-3'.

Wobble mutants of BCAR3 were generated in the pLV-Venus vector. WT and L744E/R748E BCAR3 complementary DNA were cloned into the *NotI* and *SpeI* sites of the pLV-Venus vector. Site directed mutagenesis was performed using the QuickChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) to eliminate targeting by shBCAR3-1 without altering the amino-acid sequence of the resultant BCAR3 protein. The following primers were used: (changed nucleotides are underlined, and all constructs were confirmed by sequencing) shB3wobble1 forward: 5'-CCAGATTTAACTGCGCT GTCCCGAAAATTGGAACCTCCTCCTG-3', shB3wobble1 reverse: 5'-CAGGAGGA GTTCCCAATTTTCGGGACAGCGCAGTTAAATCTGG-3'.

shRNA oligonucleotides targeting BCAR3 and cloned into the TRC2-pLKO-puro vector were purchased from Sigma-Aldrich. Hairpin sequences were as follows: shBCAR3-1 shRNA ID: TRCN0000364816, sequence: 5'-CCG GTAACCTGCCCTCTCGCGTAAATCTCGAGATTACGCGAGAGGGCAGTTATTTTG-3', shBCAR3-2 shRNA ID: TRC0000376503, sequence: 5'-CCGTCGGCATTGCAGTGG ACATTCCTCGAGGAATGTCCACTGCAATGCCGATTTTG-3'.

Cell culture, invasion, migration and Rac assays

BT549 and MDA-MB-231 cells (American Type Tissue Culture, Manassas, VA, USA) were cultured as previously described.^{2,11} HS-578 T cells, generously provided by Dr Kevin Janes (UVA), were cultured in

Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 0.01 mg/ml bovine insulin and 1% penicillin/streptomycin. Cells lines were confirmed to be free of mycoplasma. For 3D culture of MDA-MB-231 and HS-578 T cells, Matrigel (50 μ l) was spread evenly on the bottom of eight-well chamber slides. Cells grown in 2D monolayer culture were trypsinized and plated in the chamber slides with Dulbecco's modified Eagle's medium containing 2% (MDA-MB-231) or 10% (HS-578 T) serum, 2% Matrigel, 5 ng/ml EGF, and 0.5 μ g/ml (MDA-MB-231) or 1 μ g/ml (HS-578 T) puromycin. Cells were grown for 6–8 days with media changes every 4 days. Phase images of representative fields were captured using an Olympus CKX41 (Center Valley, PA, USA) or Zeiss Axiovert 40 CFL inverted scope (Thornwood, NY, USA). Transwell migration assays were performed as previously described,² and the cells were stained using Protocol HEMA 3 stain set (Fisher Scientific, Waltham, MA, USA; 122-911). Rac1 assays were performed on BT549 cells transfected with plasmids encoding GFP, WT GFP-BCAR3 or L744E/R748E GFP-BCAR3 as previously described.⁴

Plasmid transfection, lentivirus production and infection

Transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA; 11668019) following the manufacturer's specifications.

Lentiviral particles were produced by calcium phosphate transfection of HEK293T cells with a mixture of the transfer vector (pLKO-shBCAR3 or pLV-Venus-BCAR3), packaging vector (psPAX2) and envelope vector (pMD2.G). Medium containing lentivirus was collected 48 h post transfection, filtered through 0.45 μ m filter and used immediately or frozen at -80°C . Cells were infected with lentivirus in the presence of 8 μ g/ml polybrene.

Immunoprecipitation, immunoblotting and immunofluorescence

Cells grown in 2D were lysed in ice-cold radioimmune precipitation assay (RIPA) buffer supplemented with protease inhibitors and protein concentrations determined as previously described² or in a non-denaturing lysis buffer as described by Wallez *et al.*⁵ Immunoprecipitations, immunoblotting and immunofluorescence were performed as previously described.²

Live-cell imaging and adhesion turnover analysis

BT549 cells were plated on acid-washed 2 µg/ml fibronectin-coated glass bottom TIRF dishes (MatTek Corporation, Ashland, MA, USA) and incubated for 30–40 min at 37 °C, pH 7.4 in CCM1 media (Hyclone, Logan, UT, USA). Images were captured using an inverted TIRF microscope (1X70; Olympus Life Science, Center Valley, PA, USA) with a 60X objective (±1.5X magnification), a cool charged-couple device camera (Retiga EXi; Qimaging, Surrey, BC, Canada), and heated objective/stage (Biopetechs, Butler, PA, USA). Images were captured every 5 s for 10–12 min using MetaMorph software (Nashville, TN, USA). To quantify adhesion turnover, adhesions at peripheral, protruding edges were manually selected for analysis. Complete fluorescence intensity time tracings for individual adhesions were (1) normalized, (2) corrected for background intensity by subtracting an average intensity value corresponding to a background region away from the cell and (3) plotted. Both the increase (incorporation/assembly) and decrease (dissociation/disassembly) in fluorescence intensity were linear as a function of time on semilogarithmic plots, and rate constants were determined from the slopes of these graphs. Rate constant measurements were obtained for a minimum of 13 individual adhesions on 2–5 cells.

Human breast tumor staining

Sequential sections of breast tissue were received from the University of Virginia Biorepository and Tissue Research Facility (BTRF; IRB#HSR17196). Sections were stained with hematoxylin and eosin or immunostained with BCAR3 or Cas antibodies by the BTRF.

Statistical analysis

Statistical analyses were conducted using GraphPad Prism (San Diego, CA, USA) and the sample size was shown to have adequate power. For the adhesion turnover and invasion analysis, a Kruskal–Wallis one-way analysis of variance and a Dunn's multiple comparison *post-test* were used to compare multiple experimental groups. For migration studies, a one-way analysis of variance and the Dunn's *post-test* were used to compare groups, as the data followed Gaussian distribution and passed a Bartlett's test for equal variance.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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Supplementary Information accompanies this paper on the Oncogene website (<http://www.nature.com/onc>)

SUPPLEMENTAL MATERIALS

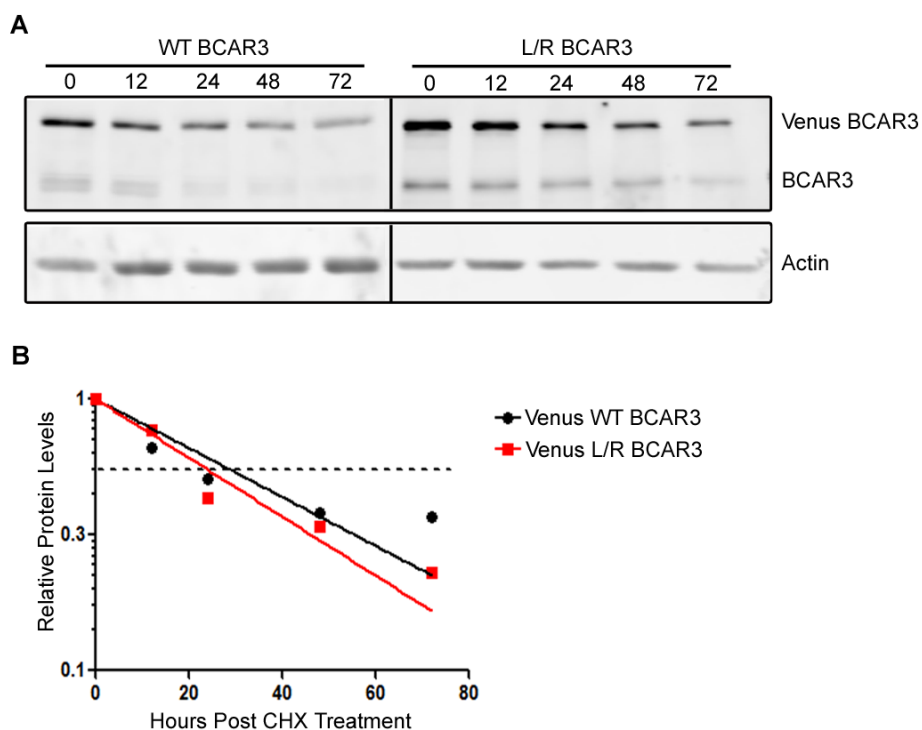


Figure S1. WT and L/R BCAR3 have similar half-lives

(A) BT549 cells were infected with lentiviruses encoding WT Venus-BCAR3 or L744E/R748E (L/R) Venus-BCAR3 and plated in 6-well dishes at 100,000 cells per well. One day after plating, cells were treated with 25 μ g/ml cyclohexamide (CHX) and lysed at the indicated times. Proteins were immunoblotted with antibodies recognizing the designated proteins. Representative blots are shown. (B) Protein levels from the representative blots were normalized to the 0 hour time point and plotted as an exponential decay nonlinear regression.

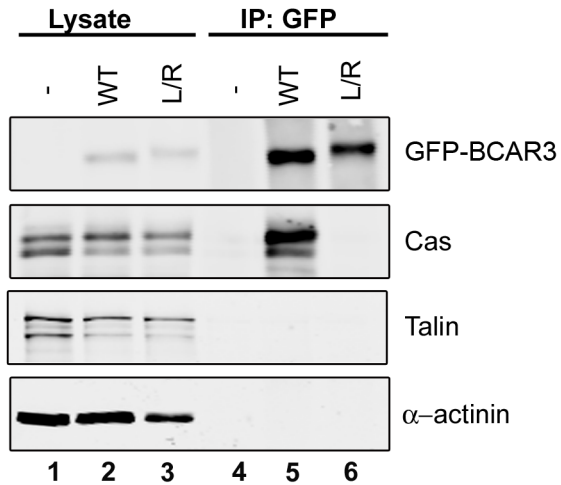


Figure S2. BCAR3 is not in complex with talin or α -actinin

BT549 cells were transfected with plasmids encoding GFP (-), WT GFP-BCAR3, or L744E/R748E GFP-BCAR3 and lysed 24 hours post-transfection. Total cell protein and GFP immune complexes (generated from 50X more protein than the lysates) were immunoblotted with antibodies to detect the indicated proteins.

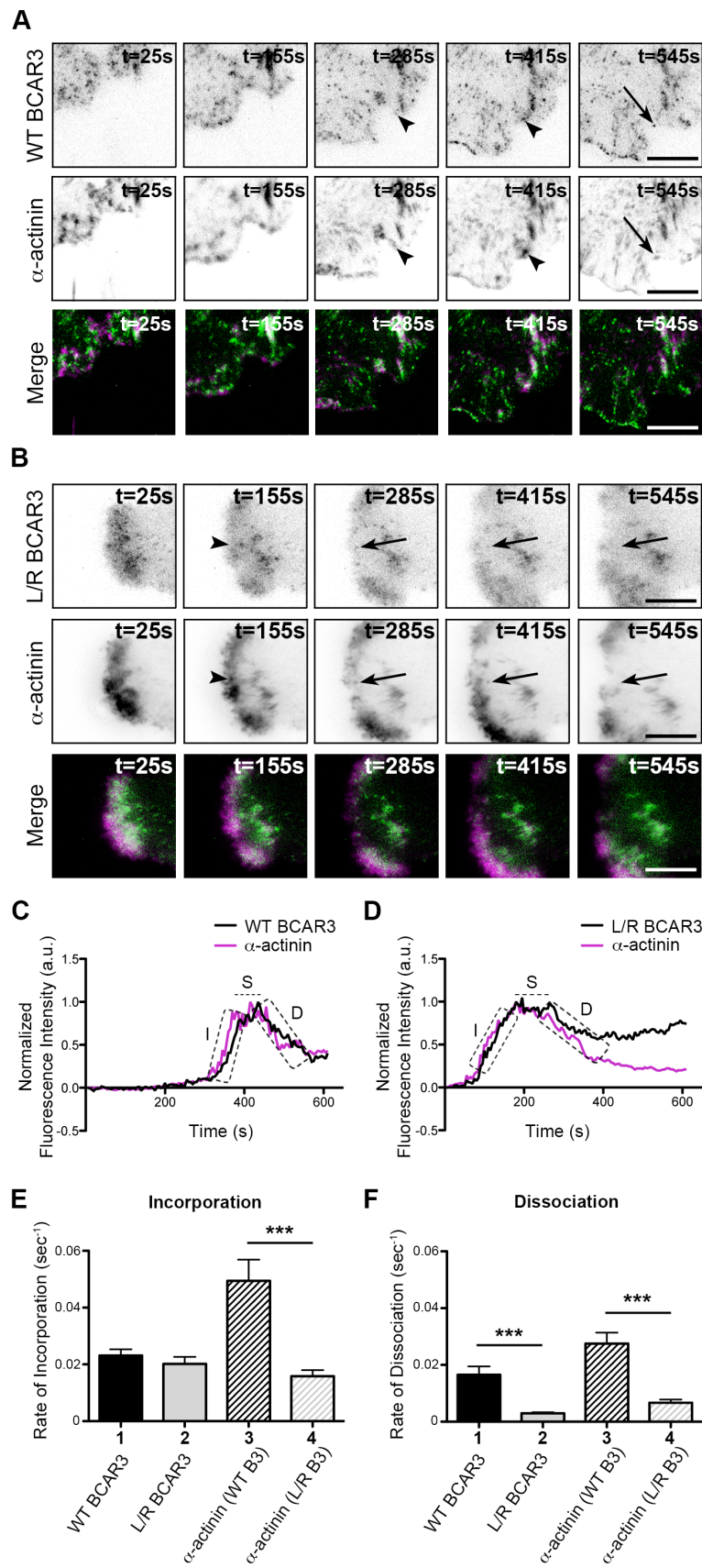


Figure S3

Figure S3. Direct interactions between BCAR3 and Cas are required for efficient incorporation and turnover of α -actinin in adhesions

BT549 breast cancer cells were co-transfected with plasmids encoding WT or L744E/R748E (L/R) GFP-BCAR3 and mCherry- α -actinin, incubated for 24 hours, and then plated on 2 μ g/ml fibronectin-coated glass-bottomed TIRF dishes for 30-40 minutes prior to visualizing adhesion dynamics via live-imaging TIRF. (A, B) Representative time-lapse images show incorporation into adhesions (arrowheads) and dissociation (arrows) of the indicated proteins over the specified time course. Scale bars = 100 μ m. (C, D) Representative fluorescence intensity time tracings of BCAR3 (black) and α -actinin (magenta) present in adhesions from cells expressing WT (C) or L744E/R748E (L/R) GFP-BCAR3 (D). Dashed boxes/line indicate the incorporation (I), stability (S), and dissociation (D) phases of adhesion dynamics. (E, F) Quantitative analysis of the incorporation (E) and dissociation (F) rates of WT GFP-BCAR3 (bar 1), L744E/R748E (L/R) GFP-BCAR3 (bar 2), α -actinin co-expressed with WT GFP-BCAR3 (bar 3), and - -actinin co-expressed with L744E/R748E (L/R) GFP-BCAR3 (bar 4). Data presented are the mean \pm SEM of ≥ 13 adhesions from 4 separate WT BCAR3/ α -actinin or 2 separate L744E/R748E BCAR3/ α -actinin movies generated from 3 independent experiments. ***, $p < 0.001$.

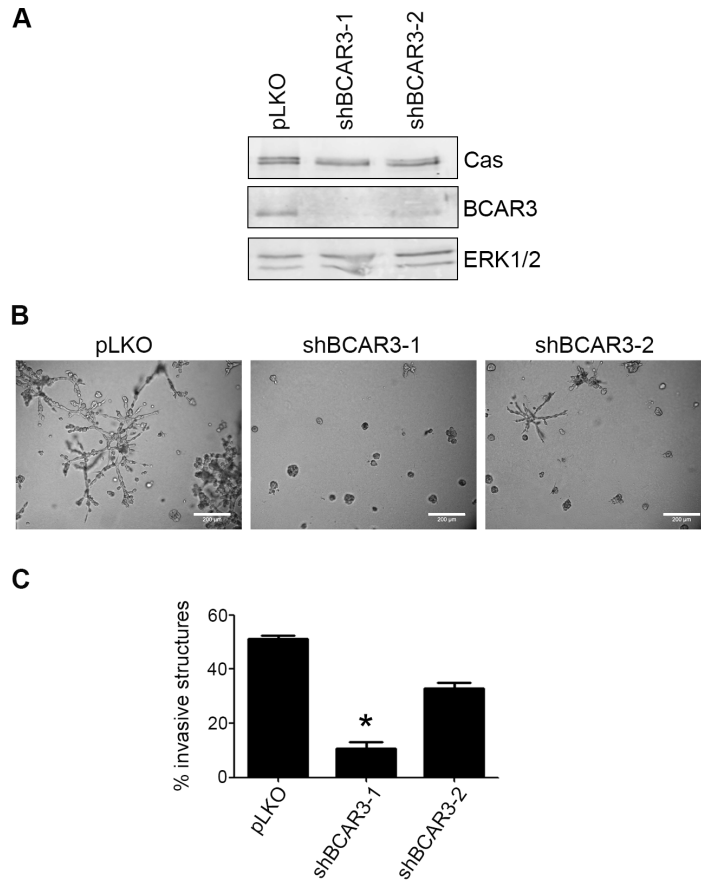


Figure S4. BCAR3 promotes invasion of MDA-MB-231 cells in 3D Matrigel culture

MDA-MB-231 cells stably expressing empty vector (pLKO), shBCAR3-1, or shBCAR3-2 lentiviral constructs were grown in 3D Matrigel culture for 8 days. (A) The cell/Matrigel mixture was collected in 0.25% trypsin, pipetted several times, and incubated for 30 minutes at 37°C. Cells were centrifuged at 150 x g for 3 minutes, washed 1X in DMEM, resuspended in phosphate buffered saline (PBS), and then pelleted by spinning for 3 minutes at 2000 rpm in a micro-centrifuge. The pellet was resuspended in ice-cold RIPA buffer supplemented with protease inhibitors and protein concentrations determined as previously described². A representative immunoblot is shown confirming knockdown of BCAR3 with both shRNA constructs. (B, C) Representative phase images (B) and quantification of invasive structures (C) are shown. Data presented are the mean \pm SEM of 3 independent experiments, performed in quadruplicate... Scale bars = 200 μ m. *, $p < 0.05$ relative to pLKO.

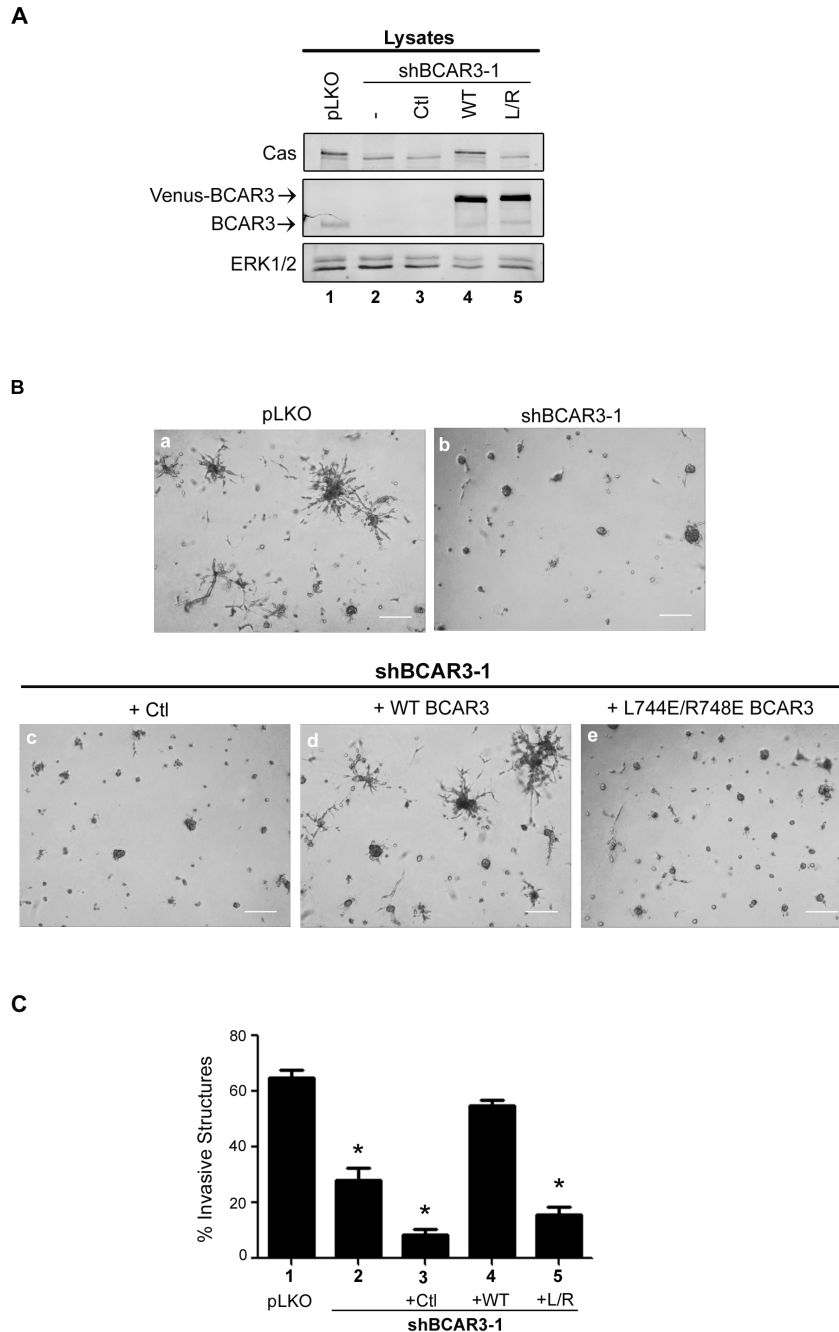


Figure S5: Direct interaction between BCAR3 and Cas is required for invasion of HS-578T cells in 3D Matrigel culture

(A) HS-578T cells stably expressing empty vector (pLKO) or shBCAR3-1 lentiviral constructs were infected with lentiviruses encoding 3rd-base wobble variants of WT Venus-BCAR3, L744E/R748E (L/R) Venus-BCAR3 or empty vector (pLV-Venus; Ctl). Total cell protein was immunoblotted with antibodies to detect the indicated proteins. (B, C) The cells described in

panel A were grown in 3D Matrigel culture for 6 days. Representative phase images (B) and quantification of invasive structures (C) are shown. Data presented are the mean \pm SEM of 6-7 replicates per condition from one experiment. Scale bars = 200 μ m. *, $p < 0.05$ relative to pLKO.