The Adherens Junction Protein Afadin Is an AKT Substrate that Regulates Breast Cancer Cell Migration

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Abstract

The phosphoinositide 3-kinase (PI3K) and Akt signaling pathway regulates all phenotypes that contribute to progression of human cancers, including breast cancer. Akt mediates signal relay by phosphorylating numerous substrates, which are causally implicated in biologic responses such as cell growth, survival, metabolic reprogramming, migration, and invasion. Here we report the identification of the adherens junction protein Afadin, which is phosphorylated by Akt at Ser1718. Importantly, under conditions of physiologic IGF-1 signaling and oncogenic PI3K and Akt, Afadin is phosphorylated by all Akt isoforms, and this phosphorylation elicits a relocation of Afadin from adherens junctions to the nucleus. Also, phosphorylation of Afadin increased breast cancer cell migration that was dependent on Ser1718 phosphorylation. Finally, nuclear localization of Afadin was observed in clinical breast cancer specimens, indicating that regulation of Afadin by the PI3K–Akt pathway has pathophysiologic significance.

Implications: Phosphorylation of the adhesion protein Afadin by Akt downstream of the PI3K pathway, leads to redistribution of Afadin and controls cancer cell migration. Mol Cancer Res; 1–13. ©2013 AACR.

Introduction

The phosphoinositide 3-kinase (PI3K) and Akt signaling pathway orchestrates virtually all aspects of epithelial and tumor cell behavior, from initial transformation to dysplasia and ultimately the dissemination of cancer cells to distant metastatic sites (1). In addition, mutations in genes that encode proteins that are rate-limiting for transducing the PI3K and Akt signaling are frequent mutated in human cancers. This is most evident in breast cancer, whereby according to molecular subtype, the most frequent genetic lesions are oncogenic mutations in the PIK3CA catalytic subunit, PIK3CA, inactivation or loss of heterozygosity of the tumor suppressor PTEN, and INPP4B, and amplification or somatic-activating mutations in one of the three Akt genes AKT1, AKT2, and AKT3 (2, 3). All of these lesions ultimately result in hyperactivation of Akt and phosphorylation of downstream substrates that transduce the signal to secondary effector pathways and in turn the modulation of phenotypes associated with malignancy, including cell growth, proliferation, survival, metabolic reprogramming, and cell migration and invasion (4). Moreover, because most of the proteins that function to transduce PI3K and Akt signaling are enzymes with catalytic pockets, this pathway is highly druggable and numerous phase I and II clinical trials are underway with small-molecule inhibitors targeting PI3K or Akt isoforms for single agent or combination therapy, including in breast cancer (5).

Increased Akt activity is detected in aggressive human breast cancers and is associated with poor prognosis and higher probability of relapse accompanied by distant metastases in patients (6-8). The ability of cancer cells to migrate requires signals that lead to the rearrangement of the actin cytoskeleton as well as proteolysis of the extracellular matrix (9, 10). Importantly, molecular genetics as well as in vivo studies have demonstrated that Akt isoforms play unique roles in modulating breast cancer cell invasion leading to metastatic dissemination, such that Akt2 is a metastasis enhancer, whereas Akt1 either does not promote metastasis or can actually block this process and thus function as a suppressor (11, 12). Yet, in other cell types and tissues, Akt isoforms either have no specificity in modulating cell migration, or even have opposing roles to those identified in epithelial cells, such as that reported for fibroblast migration (9). Regardless, the signaling specificity attributed to Akt isoforms highlights the importance of a complete understanding of the mechanism that govern cancer cell phenotypes such as invasive migration and metastasis, if specific drugs are to be developed for effective cancer therapy. In terms of mechanisms that explain the function of Akt in the control of migration, invasion, and metastasis, a number of specific substrates have been identified recently. These include the actin-bundling protein palladin, a unique Akt1 substrate that functions to mediate the inhibitor activity of...
this Akt isofrom in cell migration (13). Other substrates include girdin that following phosphoryluation occupies in the leading edges of migrating cells and is essential for the integrity of the actin cytoskeleton and cell migration (9). Also included in this list are ACAP1, whose phosphorylation controls the recycling of integrin-β1 and cell migration, and the G-protein–coupled receptor EDG-1 that is required for endothelial cell chemotaxis (14, 15). Recent global phosphoproteomic studies from cancer cell lines and tissues have identified thousands of novel phosphoproteins with phosphorylation sites that conform to the optimal Akt consensus motif, RxRxxS/T, greatly accelerating the discovery of Akt targets that transduce the signal (16).

Afadin, a tumor suppressor-like protein encoded by the MLLT4 gene, is a multi-domain F-actin–binding protein that is expressed in epithelial cells, neurons, fibroblasts, and endothelial cells (17, 18). There exist two splice variants: l-Afadin and s-Afadin (18). The longer splice variant, l-Afadin (herein referred to as Afadin unless otherwise specified) has two Ras associating-domains, a Forkhead-associating domain, a Dilute domain, a PDZ domain, three proline-rich domains, and the F-actin binding domain at the carboxyl terminus (see Fig. 1A). s-Afadin, the shorter splice variant, lacks the F-actin–binding domain and the third proline-rich domain and its expression is restricted to neuronal tissues (19). Human s-Afadin is identical to the gene product of AF6, an ALL-1 fusion partner involved in acute myeloid leukemia (20, 21).

Afadin is localized at epithelial adherens junctions (18), consisting of two adhesion complexes, the Nectin-Afadin and the E-cadherin-Catenin complexes (20). The role of Afadin in the adherens junction complex is not completely understood. Afadin interacts with cell adhesion molecules, cytoskeletal components, signaling molecules, and is generally considered to function as an adaptor protein. Knockout of Afadin in mice results in embryonic lethality due to disorganization of the ectoderm, impaired migration of the mesoderm, and impaired gastrulation. Moreover, loss of cell polarity due to improperly assembled adherens junction and tight junctions is observed (17, 19, 20, 22). Afadin has also been shown to regulate integrin-mediated cell adhesion and cell migration, although it appears that the function of Afadin in positively or negatively regulating cell motility is context-dependent (23–27).

Here, we show that Afadin is a substrate of Akt whose phosphorylation leads to its relocalization form the plasma membrane to the nucleus, coconmitant with an enhancement of breast epithelial and cancer cell migration.

Materials and Methods
Cell culture
HEK293T, HeLa, MCF7, MDA-MB-231, MDA-MB-453, MDA-MB-468, SKBR3, BT-549, ZR-75-1, MCF10A, T47D, and ZR-75-30 were obtained from the American Type Culture Collection and authenticated using short tandem repeat (STR) profiling. Cells were maintained in culture not more than 6 months. Cells were routinely screened for mycoplasma contamination. Cell lines were maintained as follows: HEK293T, HeLa, MCF7, MDA-MB-231, MDA-MB-453 and MDA-MB-468, Dulbecco’s Modified Eagle Medium (DMEM; Cellgro) supplemented with 10% FBS (HyClone); SKBR3, McCoy’s 5A medium (Cambrex) supplemented with 10% FBS; BT-549 and ZR-75-1, RPMI-1640 (Cellgro) supplemented with 10% FBS; MCF10A DMEM/Ham’s F12 supplemented with 5% equine serum (GIBCO), 10 μg/mL insulin (Sigma-Aldrich), 500 ng/mL hydrocortisone (Sigma-Aldrich), 20 ng/mL EGF (R&D Systems), and 100 ng/mL cholaer toxin (List Biological Labs); T47D and ZR-75-30 in RPMI-1640 supplemented with 10% FBS and 10 μg/mL insulin.

Growth factors and inhibitors
Cells were stimulated with recombinant human IGF-1 (R&D Systems) at a final concentration of 100 ng/mL for 20 minutes unless otherwise specified. CGK733 (Sigma-Aldrich) was added to cells for 4 hours in a final concentration of 10 μmol/L before IGF-1 stimulation. All other inhibitors were added to cells for 20 minutes before stimulation at the following final concentrations: 100 nmol/L wortmannin (Sigma-Aldrich), BEZ235 (Cayman Chemical company), and 1 μmol/L MK2206 (Active Biochem), 0.7 μmol/L A66 (Symansis), 100 nmol/L rapamycin (Sigma-Aldrich), and 10 μmol/L PF4708671 (Sigma-Aldrich), 5 μmol/L GSK650394 (R&D Systems), and cycloheximide (Sigma-Aldrich) was used for 3 to 6 hours at 20 μg/mL.

Antibodies
Anti-phospho-Afadin Ser1718 antibody, anti-pan-Akt antibody, anti-Akt1, anti-Akt2, anti-Akt3, anti-phospho-Akt Ser473, anti-GSK3β, anti-phospho-GSK3β Ser9, anti-p110α, anti-S6K, anti-phospho-S6K Thr389, anti-E-cadherin, anti-NDRG1, anti-phospho-NDRG1 Thr346, anti-CENP-A, anti-NuP98, anti-Fibrillarin, and anti-Histone H3 were from Cell Signaling Technology. Anti-Myc antibody, anti-Tubulin, and anti-lamin A/C antibodies were from Santa Cruz Biotechnology. Anti-Afadin antibodies used for immunoblotting were from Bethyl laboratories and used for immunofluorescence from BD Biosciences. Horseradish peroxidase-conjugated anti-mouse and anti-rabbit immunoglobulin G (IgG) antibodies were from Chemicon. Cy3-conjugated anti-mouse IgG antibody and Alexa-Flour 488 anti-rabbit antibody were from Jackson ImmunoResearch Laboratories. Anti-β-actin monoclonal antibody was purchased from Sigma-Aldrich. Anti-HA monoclonal antibody was purified from the 12CA5 hybridoma. The anti-p85 antibody has been described (28).

Plasmids
The Afadin University of Leeds, UK cDNA constructs pEGFP-N2-AF6i3-Myc and pEGFP-N2-AF6i1-Myc were a gift from Mihaela Latorre University of Leeds, UK and have been described (24). shRNA rescue mutants were generated by introduction of 6 silent mutations using the following primer: 5'- GGA AC CAG CCA GCG TCT TTT TTC ACA AGG ACA GGA CGT CTC TAA AAG GT-3'.

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The shRNA-resistant mutants with phosphorylation site mutations were generated by site-directed mutagenesis using the following primers: S1718A: 5′-GAA CGC CAG CGT CTT TTT GCA CAA GGA CAG GAC G 3′; S1718D: 5′-ACA TTC AAG GAA CGC CAG CGT CTT TTT GAT CAA GGA CAG GAC GTC 3′; S1718E: 5′-ACA TTC AAG GAA CGC CAG CGT CTT TTT GAG CAA GGA CAG GAC GTC 3′. All sequences were verified by sequencing. pcDNA3-Myr-HA-Akt1, pcDNA3-Myr-HA-Akt2, pcDNA3-Myr-HA-Akt3, from William Sellers (Addgene plasmids 9008, 9016, 9017; ref. 29). HA-GSK3β has been described (30). JP1520-GFP, JP1520-PK3CA-WT-HA, JP1520-PK3CA-H1047R-HA, and JP1520-PK3CA-E545K-HA were from Joan Brugge (Addgene plasmids 14570, 14571, 15572; ref. 31).

RNA interference
For shRNA silencing, a set of single-stranded oligonucleotides encoding the Akt1 or Akt2 target shRNA and its complement were previously described (11). Akt3 sense, 5′-GAA TTC TCG 3′.

GAA AGC TTC TCG 3′.
CCG GCT GCC TTG GAC TAT CTA CAT TCT CGA GAA TGT AGA TAG TCC AAC GCA TTT TTT G 3'; Akt3 antisense, 5' AAT TCA AAA ACT GCC TTG GAC TAT CTA CAT TCT CGA GAA TGT AGA TAG TCC AAG GCA G 3' (Sigma-Alrich). For silencing of Afadin, specific sequences for l-Afadin were used: shAfadin #2, sense, 5' CCG GAA GGT CAA GAT GTA TCC AAT ACT CGA GTA TGG GAT ACA TGG GTC TCA CTT TTT TTT G 3'; shAfadin #2, antisense, 5' AAT TCA AAA AAA GGT CAA GAT GTA TCC AAT ACT CGA GTA TGG GAT ACA TCT TGA CCA GTT TTT TTT G 3'; shAfadin #3, sense: 5' CCG GAA ACT TGA CAT TCA AGG AAC GCT CGA GCG TTC CTT GGA TGT CAA GTT TTT TTT G 3'; shAfadin #3, antisense: 5' AAT TCA AAA AAA ACT TGA CAT TCA AGG AAC GCT CGA GCG TTC CTT GGA TGT CAA GTT TTT TTT G 3'. The oligonucleotide pair for each target was annealed and inserted into pLKO. To produce lentiviral supernatants, 293T cells were cotransfected with control or shRNA-containing pLKO vectors, VSVG, and psPAX2 for 48 hours.

In vitro kinase assays

HeLa cells were transfected with Myc-Afadin-wild-type or Myc-Afadin-S1718A. Twenty-four hours after transfection, cells were serum starved for 16 hours. Afadin was immunoprecipitated from cell extracts and incubated with 500 ng recombinant Akt1 or Akt2 (Cell Signaling Technology) in the presence of 250 μmol/L cold ATP in a kinase buffer for 1 hour at 30°C. The kinase reaction was terminated by addition of SDS-PAGE sample buffer.

Transwell migration assay

Cells (1 × 10⁵) in serum-free medium containing 0.1% bovine serum albumin (BSA) were added to top chambers of Transwell filters (8 μm pore size; BD Biosciences) in triplicate. NIH 3T3 cell-conditioned medium or growth medium from MCF10A was added to bottom chambers. After 2- to 16-hour incubation at 37°C, nonmigrated cells were removed and cells that had migrated to the bottom of the filters were fixed and stained using the Hema-3 Stain Set (Fisher Scientific).

Immunofluorescence

Cells plated on coverslips were fixed with 2% paraformaldehyde for 10 minutes, permeabilized with 0.5% Triton X-100, and blocked with 1% BSA in 20 mmol/L Tris-HCl (pH 7.5) for 20 minutes, then incubated with antibodies for 1 hour (anti-Afadin, anti-pAfadin, anti-pAkt S473, anti-E-cadherin, anti-CENP-A, anti-NuP98, anti-Fibrillarin, anti-Histone H3; 1:200). After washing twice with PBS, cells were incubated with Cy3-conjugated anti-mouse IgG antibody or Alexa-flour 488 anti-Rabbit IgG for 1 hour. F-actin was visualized with Alexa Fluor 647-conjugated phallolid (Life Technologies). Cells were then rinsed twice with PBS and mounted with Prolong Gold antifade reagent 4',6-diamidino-2-phenylindole (DAPI; Life Technologies). Images of cells were acquired using a fluorescence microscope (Nikon Eclipse Ti) and digital image analysis software (NIS-Elements, Nikon). Magnification plan Apo VC 60×/1.40 oil. Experiments to determine staining with phospho-Afadin S1718 antibody always included costaining with total Afadin antibody to ensure specificity of staining.

Immunoblotting and immunoprecipitation

Cells were lysed in radioimmunoprecipitation assay as previously described (13). Lysates were resolved on 6%–10% acrylamide gels by SDS-PAGE and transferred to polyvinylidene difluoride membrane (EMD Millipore). The blots were blocked in TBST buffer (10 mmol/L Tris-HCl (pH 8), 150 mmol/L NaCl, 0.2% Tween 20) containing 5% (w/v) non-fat dry milk for 30 minutes and then incubated with the specific primary antibody diluted in blocking buffer at 4°C for 16 hours. Membranes were washed three times in TBST and incubated with horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature. Membranes were washed three times and developed using enhanced chemiluminescence substrate (EMD Millipore). For immunoprecipitation, lysates were incubated with 1–2 μg antibody for 2 to 4 hours at 4°C followed by incubation with 15 μL protein A/G Sepharose beads (Amersham Biosciences). Immune complexes were washed with NETN buffer [0.5% NP-40, 1 mmol/L EDTA, 20 mmol/L Tris-HCl (pH 8), 100 mmol/L NaCl]. Precipitates were resolved by SDS-PAGE.

Subcellular fractionation

Cells were fractionated using the Subcellular Protein Fractionation Kit for cultured cells (Thermo Fisher Scientific) according the manufacturer’s instructions.

Tissue microarrays

Tissue microarrays (TMA) containing normal breast tissue (2 cores per case) and invasive breast cancer (2 cores per case) were constructed from archival formalin-fixed, paraffin-embedded breast tissue specimens obtained from Beth Israel Deaconess Medical Center (Boston, MA) under an institutionally approved IRB protocol for discarded deidentified tissues. Double immunofluorescence for Afadin and E-cadherin was performed. Antigen retrieval was performed by boiling the slides for 10 minutes in 10 mmol/L sodium citrate pH 6 with a pressure cooker. The sections were then incubated with 1 mg/mL sodium borohydride (MP Biomedicals) for 5 minutes at room temperature. Sections were incubated with 5% normal donkey serum (Jackson Immunoresearch) for 1 hour at room temperature. Slides were then incubated with mouse anti-Afadin (1:100, BD Biosciences) and Rabbit anti-E-Cadherin (1:100, Cell Signaling Technology) overnight at 4°C. The slides were washed and incubated with Alexa 488 conjugated Donkey anti-rabbit or anti-mouse secondary antibodies (Jackson Immunoresearch Lab, 1:200). Samples were then washed and mounted with Prolong Gold anti-fade mounting media containing DAPI (Invitrogen). We digitally acquired 98 microscopic images of normal breast tissue and 98 microscopic images of invasive breast cancer at × 63 magnification. Features were extracted from the digital images in ImageJ and statistical
analyses were performed using Jython and R. To determine the statistical significance of the difference in Afadin nuclear localization score in normal breast as compared with invasive breast cancer, we performed a two-sided Student \( t \) test.

**Results**

**Akt phosphorylates Afadin at Ser1718**

Global phosphoproteomic analyses have revealed that the adherens junction protein Afadin is phosphorylated at serine 1718 (Ser1718; ref. 16), in a sequence within the actin-binding domain that conforms to the optimal Akt consensus motif RXRXXS/T (32). The motif surrounding Ser1718 is evolutionarily conserved from *Drosophila* to mammals (Fig. 1A). Because the PI3K/Akt pathway modulates all cellular processes associated with breast cancer and does so by phosphorylating substrate proteins to transduce the signal, we evaluated Afadin protein expression in breast cancer cell lines. Afadin is highly expressed in various breast cancer cell lines, including basal and luminal cellular subtypes as well as the non-tumorigenic line MCF10A (Fig. 1B).

To determine whether Afadin is a substrate of Akt, MCF10A (Fig. 1C) and HeLa cells (Supplementary Fig. S1A) were serum-starved and stimulated with IGFl-1. Stimulation leads to phosphorylation of Afadin at Ser1718 as detected by a phoso-specific anti-pSer1718 antibody. Ser1718 phosphorylation induced by IGFl-1 is significantly inhibited by wortmannin (a pan-PI3K inhibitor), BEZ-235 (a dual PI3K and TORC1 inhibitor), A66 (a p110\( \alpha \)-specific inhibitor), and MK2206 (an allosteric pan-Akt inhibitor; refs. 33–36; Fig. 1C). In contrast, Ser1718 phosphorylation is not blocked by rapamycin (an mTOR inhibitor), GSK650394 [an SGK (serum and glucocorticoid-induced kinase) inhibitor], or PF4708671 [an S6K1 (p70 S6-kinase-1) inhibitor], or CGK733 [an ATM/ATR (Ataxia Telangiectasia Mutated/ATM-related; ATR) inhibitor]; refs. 37–41. Akt phosphorylates Afadin specifically at Ser1718 because a Ser1718Ala mutant is not phosphorylated in response to IGF-1, and no additional Akt consensus motifs are found in the Afadin amino acid sequence. Moreover, the short isoform of Afadin (s-Afadin) is not phosphorylated in response to IGF-1 (Fig. 1D), consistent with the fact that it lacks the Ser1718 motif (Fig. 1A).

To determine whether one or more Akt isoform can phosphorylate Afadin in cells, Akt1, Akt2, and Akt3 were silenced using specific shRNAs introduced into MCF10A cells (Fig. 2A) and HeLa cells (Supplementary Fig. S1B). Silencing individual Akt isoforms partially attenuates Ser1718 phosphorylation, whereas combination silencing of Akt1, Akt2, and Akt3 leads to a complete abrogation of the pSer1718 signal (16% in pLKO vs. 100% in pLKO cell stimulated with IGFl-1; Akt1 silencing (104%), silencing Akt2 (45%), silencing Akt3 (25%), and combined Akt1/ Akt2/Akt3 silencing (34%), normalized relative to total Afadin). Moreover, coexpression of constitutively active, myristoylated Akt1, Akt2, and Akt3 alleles leads to enhanced Afadin Ser1718 phosphorylation (Fig. 2B), and purified recombinant Akt1 or Akt2 can directly phosphorylate Afadin at Ser1718 in an in vitro protein kinase assay (Fig. 2C). Similarly, coexpression of the oncogenic *PIK3CA* alleles H1047R and E545K that stimulate hyperactivation of Akt also induces Afadin Ser1718 phosphorylation in MCF10A cells (Fig. 2D) and HeLa cells (Supplementary Fig. S1C). In aggregate, these data demonstrate that Afadin is phosphorylated by all Akt isoforms downstream of PI3K, but is not a substrate for other AGC kinases, including SGKs and S6K1.

**Phosphorylation of Afadin at Ser1718 promotes nuclear localization**

Because Afadin is an adherence junction protein, we next evaluated the consequence of Ser1718 phosphorylation by Akt on cellular localization. Using immunofluorescence of IGFl-1–stimulated MCF10A cells, we detect phosphorylated, activated Akt (pS473) within 20 minutes of stimulation (Supplementary Fig. S2). Under these conditions, total Afadin shows a predominantly membrane-restricted localization. However, within 1 hour of stimulation, Afadin membrane localization is significantly diminished, concomitant with the appearance of nuclear localization as evidenced by co-staining with DAPI (Fig. 3A and Supplementary Fig. S2). Nuclear localization of Afadin is most evident by 6 hours post-stimulation, with a punctate nuclear staining pattern (Fig. 3A; IGFl-1, 6 hours). Nuclear translocation of total Afadin is dependent on PI3K and Akt activity, as wortmannin, MK2206, A66, and BEZ235 block nuclear localization in favor of membrane localization (Fig. 3A and Supplementary Fig. S3A). Quantification of the nuclear translocation in response to IGFl-1 and Akt inhibitor is depicted in the bar graph (Fig. 3A). The pSer1718 antibody also reveals Afadin nuclear localization in response to IGFl-1 stimulation (Fig. 3B, quantification shown in the corresponding bar graph).

To explore the contribution of Ser1718 in plasma membrane to nucleus translocation, an shRNA silencing and rescue experiment was performed. MCF10A cells were transduced with Afadin shRNA and non-silenceable wild-type, Ser1718Ala (S1718A) and Ser1718Glu (S1718E) mutants transiently expressed. As predicted, in full serum conditions, wild-type Afadin is localized to the plasma membrane and nucleus, Ser1718Ala Afadin is localized predominantly to the plasma membrane, whereas the phosphomimetic Ser1718Glu mutant shows an exclusively nuclear localization (Fig. 4A, quantification shown in the corresponding bar graph). Moreover, coexpression of constitutively activated, myristoylated Akt1, Akt2, or Akt3 alleles also promotes Afadin nuclear localization compared with control cells (Fig. 4B and corresponding bar graph, and Supplementary Fig. S3B). Therefore, Akt signaling promotes the relocation of Afadin from the plasma membrane to the nucleus in a manner that depends on Ser1718 phosphorylation.

To further explore the mechanism of Afadin nuclear translocation, cell fractionation was performed. In agreement with the immunofluorescence data, IGFl-1 stimulation of MCF10A cells results in a time-dependent decrease of total Afadin from the cytoplasm and membrane compartments.
concomitant with an increase of Afadin in the nuclear compartment, most dramatically evident 6 hours post-stimulation (Fig. 5A, left). Treatment with the Akt inhibitor MK2206 attenuates this translocation (Fig. 5A, right). We also evaluated the consequence of Afadin phosphorylation on protein stability. Serum-starved cells were stimulated over time with IGF-1 in the presence or absence of the protein synthesis inhibitor cycloheximide as well as Akt inhibitor. As observed in Fig. 5B, prolonged treatment of cells with MK2006 results in a reduction of total Afadin expression, which is further enhanced in the presence of cycloheximide. Similar results are observed when the same cells are examined by immunofluorescence (Fig. 5C and corresponding bar graph). These data indicate that Akt signaling, in addition to promoting nuclear relocalization, also promotes stabilization of Afadin.

Phosphorylation of Afadin at Ser1718 enhances migration and perturbs cell to cell adhesion

We next reasoned that because Akt promotes relocalization of Afadin from adherens junctions to the nucleus, this would likely have a profound impact on cell adhesion and cell migration, phenotypes that are dependent on intact adherens junctions. In this context, previous studies have shown that Afadin shRNA enhances migration of MCF7, SK-BR3, and MDA-MB-231 cells (27). Yet in other studies, Afadin silencing reportedly enhances cell adhesion in T cells (42). The contribution of Afadin to cell migration is therefore likely to be context dependent. In BT549 and MDA-MB-468 breast cancer cells that express high levels of Afadin and exhibit PI3K pathway activation due to PTEN inactivation and consequently predominantly nuclear localized Afadin (Supplementary Fig. S5A and

Figure 2. PI3K and Akt signaling promotes Afadin phosphorylation in breast cancer cells. A, Akt1, Akt2, and Akt3 shRNA transduced alone or in combination in MCF10A cells. Cells were stimulated with IGF-1 and whole-cell lysates immunoblotted with the indicated antibodies. B, MCF10A cells transfected with control vector or myristoylated Akt alleles (MyrAkt1,2,3), cells were serum starved for 16 hours and whole-cell lysates immunoblotted with the indicated antibodies. C, in vitro kinase assay using wild-type Afadin, Afadin Ser1718Ala (S1718A), or control immunoprecipitated with Myc antibody. Akt substrate GSK-3β expressed in HeLa cells and immunoprecipitated with HA antibody and served as a positive control. Precipitates were incubated with purified recombinant Akt1 or Akt2. Whole-cell lysates were immunoblotted with the indicated antibodies. D, MCF10A cells infected with vector control, PIK3CA wild-type, PIK3CA H1047R, PIK3CA E545K, cells serum starved, and whole-cell lysates immunoblotted with the indicated antibodies. In all cases, p85 served as loading control. Results are representative of at least three independent experiments.
Silencing Afadin with specific shRNA leads to a profound inhibition of cell migration (Supplementary Fig. S5B). Conversely, expression of wild-type or phospho-mimetic Ser1718Asp (S1718D) or Ser1718Glu (S1718E) Afadin in T47D cells, which do not express Afadin, leads to enhanced cell migration in a manner that is not phenocopied by the Ser1718Ala mutant (Fig. 6A). The cellular localization of these mutants expressed in T47D cells is in agreement with the localization observed in MCF10A cells (Supplementary Fig. S6 compared with Fig. 4A). Consistent with the finding that Afadin promotes cell migration of breast cancer cells, silencing Afadin in MCF10A cells profoundly blocks migration in a manner that is partially rescued by reexpression of wild-type and Ser1718Asp (S1718D), but not Ser1718Ala (S1718A) mutants (Fig. 6B). Taken together, these results demonstrate that Afadin promotes breast cancer cell migration in a manner that depends, at least in part, on Ser1718 phosphorylation mediated by Akt.

Finally, because Afadin is localized to adherens junctions (20), we evaluated the consequence of Afadin relocalization on cell to cell adhesion using E-cadherin staining measured by immunofluorescence. In control serum-starved MCF10A cells, both Afadin and E-cadherin show restricted membrane localization with defined cell to cell adhesion (Fig. 6C). In contrast, in cells transduced with Afadin shRNA, E-cadherin staining is significantly disrupted. A similar phenotype is observed in cells in which Afadin is silenced and the nuclear localized Ser1718Asp (S1718D) mutant is re-expressed (Fig. 6C, controls of wild-type Afadin, S1718A and S1718E Afadin are shown in Supplementary Fig. S7). We conclude that membrane-localized Afadin is required for maintaining intact adherens junctions and productive cell to cell adhesion, such that loss of membrane localization and nuclear relocalization disrupts adhesion, concomitant with an increase in cell migration.

To address the specific nuclear compartment that Afadin localizes to, we performed colocalization experiments using a

Figure 3. IGF-1 stimulation promotes Afadin nuclear localization. A, MCF10A cells were serum starved and stimulated with IGF-1 for 1 or 6 hours, either alone or following treatment with MK2206 (1 μmol/L). Immunofluorescence was performed with the indicated antibodies. Quantification of the nuclear staining is presented in the bar graph. (Student t test; *, P < 0.05; **, P < 0.01). B, MCF10A cells serum starved or stimulated for 6 hours with IGF-1 and immunofluorescence performed with the indicated antibodies. In all cases, nuclei were stained with DAPI. Images are representative of multiple fields and of at least three independent experiments. Quantification of the nuclear staining is presented in the bar graph. (Student t test; **, P < 0.01). Magnified single channel staining for Afadin is presented in Supplementary Fig. S4.

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number of established nuclear markers: CENP-A, a centromere marker; NuP98, a nuclear envelope marker; Fibrillarin, a nucleolar marker; and Histone H3, a nucleosome or chromatin marker (Fig. 6D). The punctate nuclear pattern of Afadin does not colocalize with any of these markers. Future studies will address the specific nuclear compartment that Afadin localizes to and the importance of this localization for the nuclear function of Afadin.

Afadin localization in breast cancer

We next assessed Afadin localization in human breast cancer. Tissue microarrays containing normal breast epithelium and invasive breast cancer tissue obtained from archival pathology specimens from 49 patients were used for localization evaluated by immunofluorescence using Afadin and E-cadherin staining. The quantification protocol and analysis is summarized in Supplementary Fig. S8. We identified significantly increased nuclear localization of Afadin in invasive breast cancer as compared with normal breast, with 53% higher nuclear localization in invasive breast cancer (mean Afadin nuclear localization score in normal = 0.019 vs. mean Afadin nuclear localization score in cancer = 0.029; P, 0.02). Figure 7 shows representative images of normal breast tissue and invasive breast cancer specimens. From these data, we conclude that the nuclear localization of Afadin, regulated by phosphorylation at Ser1718 by the Akt pathway, is clinically relevant for breast cancer progression.

Discussion

We have identified and characterized a new substrate of Akt, the adherens junction protein Afadin. This finding
adds to the list of the more than 200 currently identified substrates of Akt kinases that transduce the PI3K/Akt signal to a plethora of biologic and pathophysiologic responses, particularly in the context of cancer (43). We have shown that Akt phosphorylates Afadin at Ser1718 in a motif that is evolutionarily conserved, indicating that this phosphorylation has evolved to modulate a key biologic event. We have shown that physiologic signaling in nontumorigenic MCF10A cells stimulated with IGF-1 leads to Afadin phosphorylation, and that in breast cancer cell lines harboring pathway mutations such as oncogenic PIK3CA and PTEN inactivation, Afadin is phosphorylated in an Akt-dependent manner. Moreover, although several other AGC kinases such as a S6K and SGKs have an overlapping optimal consensus phosphorylation motif to Akt, only Akt is capable of phosphorylating Afadin in cells. Although a number of Akt isoform-specific substrates have recently been identified, Afadin does not appear to be an isoform-specific substrate, at least in the breast cancer cell lines tested here.

Because mutations in genes that encode proteins in the PI3K and Akt signaling pathway are among the most common and frequent in human cancers, especially breast cancer, there are currently numerous clinical trials targeting both PI3K and Akt for therapeutic benefit. Hyperactivation of Akt due to oncogenic PIK3CA mutations as well as amplification and somatic mutations in Akt genes are common events in breast cancer etiology, and have been shown to result in cell transformation and cancer progression using mouse models. While the mechanisms by which PI3K and Akt promote cell transformation are well understood, the mechanisms by which this pathway promotes cancer progression at the level of tumor dissemination, invasion, and metastasis are not as well characterized. In this context, it is now well established that Akt isoforms play differential roles in promoting breast cancer cell invasion and metastasis,
whereby Akt1 does not enhance metastasis or can actually function as an invasion and metastasis suppressor, yet Akt2 promotes invasive migration leading to metastatic dissemination (12, 44). A role for Akt3 in breast cancer progression has yet to be defined. The mechanistic basis for the differential role of Akt isoforms in mediating breast cancer progression is likely complex, and involves both differential intracellular localization as well as accessibility to specific substrates that control cell migration and invasion. A number of such substrates have been identified, including the actin-bundling protein palladin, Girdin, and ACAP1 (9, 13, 14). In this context, it is interesting to note that Ser1718 lies within a previously identified actin-binding domain (18), and as such, it is possible that Ser1718 phosphorylation may modulate actin binding to promote cell migration, though this remains to be determined. Regardless, identifying the specific mechanisms by which the Akt pathway controls the phosphorylation of substrates that mediate cell migration is critical for a complete understanding of the contribution of this pathway in cancer progression, and in turn, the development of drugs to target Akt kinases therapeutically.

We have shown that phosphorylation of Afadin promotes relocalization from adherens junctions to the nucleus. This is most evident when evaluating Afadin localization by immunofluorescence, whereby IGF-1 stimulation leads to a relocalization of total and pSer1718 Afadin from the plasma membrane to the nucleus (Fig. 3). Similarly, a Ser1718Ala non-phosphorylatable mutant is membrane restricted and moreover a phosphomimetic Ser1718Asp.
mutant is constitutively localized to punctate nuclear structures in breast epithelial cells (Fig. 4). Most importantly, this localization phenocopies cell migration, whereby the Ser1718Ala cannot rescue the deficit in Transwell migration induced by Afadin shRNA, whereas the Ser1718Asp mutant can (Fig. 6). We conclude that Afadin phosphorylation at Ser1718 by Akt promotes cell migration, concomitant with a relocalization from the membrane to the nucleus. Although localization of Afadin to the nucleus is dependent on productive Akt signaling and was observed in all experiments and in many cells visualized by immunofluorescence, it was not a quantitative event observed in 100% of the cell population. This is not surprising, however, as previous studies have reported cell-to-cell variability with respect to the activation status of Akt in a population of cells as result of PIK3CA heterogeneity (45). The proposed model is a bimodal distribution of Akt activation that is an invariable characteristic of exponentially growing cells. Limiting Akt activity to only 20% to 30% of cells in a population serves, according to this study, two related purposes: it prevents senescence and maintains suboncogenic levels of PI3K activity in large populations.

What is more surprising is the relocalization of an adherens junction protein from the membrane to the nucleus in response to a single posttranslational modification, most obviously identified by the localization pattern of the Ser1718Asp and Ser1718Glu mutant Afadin. However there is some precedent to Afadin nuclear localization, as the short form of Afadin, s-Afadin, has been shown to be a dual residency protein that localizes to the nucleus and to the plasma membrane in a manner dependent on growth factor signaling (46). In this study, l-Afadin was not detected in the nucleus, although this is likely due to distinct experimental conditions and antibodies used to detect Afadin localization. What specifically mediates the translocation of Afadin from adherens junctions to the nucleus remains to be defined and likely involves a multi-step process of nuclear import and retention.

However, nuclear translocation is associated with an increase in breast epithelial and cancer cell migration. There are likely to be several mechanisms by which changes in Afadin localization mediate cell migration, because knocking out Afadin using specific shRNA decreases cell migration. At the same time, expression of wild-type or Ser1718Asp mutant Afadin that is nuclear-restricted robustly enhances cell migration (Fig. 6B), indicating the simple removal of Afadin from adherens junction is not the only mechanism that affords cell migration. How a nuclear localized Afadin promotes cell migration remains to be determined, but could involve the induction of a transcriptional program. Although there is no evidence that Afadin can function as a transcriptional coactivator, this is reminiscent of β-catenin, also a component of adherens junctions that upon Wnt signaling translocates to the nucleus and functions as a transcriptional coactivator for the TCF/LEF transcription factor complex, and that in turn initiates a range responses, including the epithelial to mesenchymal transition (EMT; ref. 47). Whether Afadin functions in a similar manner remains to be determined. However, it is intriguing to note that either knocking out Afadin with shRNA or expression of a nuclear restricted Afadin mutant (Ser1718Asp) results in significant disruption of E-cadherin staining at the membrane (Fig. 6C).

Interestingly, loss of Afadin has been suggested to be a marker of poor prognosis in breast cancer, such that loss of Afadin actually promotes cell migration of MCF7, MDA-MB-231, and SKBR3 cells as measured in nondirectional wound-healing assays (27). In our studies performed in Transwell assays, in MCF10A, BT549 and MDA-MB-468–specific Afadin shRNA suppresses cell migration towards chemoattractants, and this can be effectively rescued by introduction of wild-type or phosphomimetic Afadin.
alleles (Fig. 6B). Therefore, the specific contribution of Akt signaling to Afadin and in turn cell migration is likely to be highly context-dependent, including the level of Afadin expression as well as the genetic background, in particular PI3K/Akt pathway mutations.

In summary, our study identifies Afadin as a new substrate of Akt that mediates cell migration in a manner that is dependent on cellular localization. Moreover, we show that nuclear-localized Afadin is a feature of human tumors as evident from localization studies from tissue microarrays. We propose that the phosphorylation of Afadin, an adherens junction protein that is traditionally thought to reside exclusively at cell to cell adhesions and whose phosphorylation modulates cell migration, is a previously uncharacterized mechanism by which the Akt pathway promotes cancer progression. In this context, although Afadin was originally defined as a "tumor-suppressor-like" protein, it may also serve to function as a "tumor-promoting" protein at least in situations of pathophysiologic PI3K and Akt signaling.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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