Cancer Research

The Adaptor Protein AMOT Promotes the Proliferation of Mammary Epithelial Cells via the Prolonged Activation of the Extracellular Signal-Regulated Kinases

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Abstract

The asymmetric organization of epithelial cells is a basic counter to cellular proliferation. However, the mechanisms whereby pro-growth pathways are modulated by intracellular factors that control cell shape are not well understood. This study demonstrates that the adaptor protein Amot, in addition to its established role in regulating cellular asymmetry, also promotes extracellular signal-regulated kinase 1 and 2 (ERK1/2)–dependent proliferation of mammary cells. Specifically, expression of Amot80, but not a mutant lacking its polarity protein interaction domain, enhances ERK1/2-dependent proliferation of MCF7 cells. Further, expression of Amot80 induces nontransformed MCF10A cells to overgrow as disorganized cellular aggregates in Matrigel. Conversely, Amot expression is required for proliferation of breast cancer cells in specific microenvironmental contexts that require ERK1/2 signaling. Thus, Amot is proposed to coordinate the dysregulation of cell polarity with the induction of neoplastic growth in mammary cells. *Cancer Res; 71(6); 2203–11. ©2011 AACR.*

Introduction

Growth arrest is a primary feature of mammary cells with an intact apical domain (1). Ductal cells in the mammary gland asymmetrically organize into an apical pole oriented toward the tissue exterior and a basal pole that interfaces with the stroma and vasculature (2). The establishment and maintenance of the apical cortex is controlled by the Par (partition defective) and Crb (crumb) polarity protein complexes (3). These complexes mediate cell polarity by orienting vectorial processes such as protein trafficking to cellular landmarks including intercellular junctions (4). Recent studies indicate that dysregulation of the core proteins that promote apical asymmetry (aka polarity) also disrupts normal cellular growth control mechanisms (5, 6). For instance, amplification of apical polarity proteins Crb, Par-6, and αPKC (α-protein kinase C) promotes mammary cell proliferation (7–9). Conversely, loss of Par-3 and Crb3 is correlated with tumor development (7). This dual role of polarity proteins in oncogenic and tumor suppressive functions likely results in part from a requirement for their intracellular localization to be precisely regulated.

The adaptor protein Amot (aka Angiomotin) controls the spatial distribution of apical polarity proteins to regulate apical asymmetry. Amot encodes 2 well-characterized functional moieties. Through a C-terminal PDZ-binding motif, Amot binds the multi-PDZ domain containing proteins Patj and Mupp1 that in turn scaffold other Par and Crb proteins (10). Amot also directly binds membranes enriched in recycling endosomes and the apical plasma membrane via an Amot coiled–coil homology (ACCH) domain (11). Together, these domains allow the 80 kDa isoform of Amot (Amot80) to bind and redistribute components of the Par and Crb protein complexes from apical intercellular junctions to endosomes (10). Consequently, Amot80 expression induces the disruption of the integrity of apical intercellular junctions; an early event in the loss of cellular differentiation (2). The increased expression of Amot in epithelial cells in the elongating trophoblast (12), in the anterior visceral endoderm (AVE; ref 10) and in endothelial cells undergoing angiogenesis (13) suggest that Amot coordinates polarity proteins to mediate cell migration. Consistently, genetic inactivation of Amot results in embryonic lethality from either cells in the AVE failing to extend to cover the embryo (14) or from later defects in neovascularization (13). While Amot transcript levels correlate with invasive and metastatic breast cancers (15), no studies have investigated a cell autonomous role in the development of carcinomas.

This study describes a novel role for Amot in promoting the prolonged activation of the extracellular signal-regulated kinase isoforms 1 and 2 (ERK1/2). The importance of such signaling in cell proliferation is demonstrated in several contexts. For instance, expression of Amot80 promotes the rate of

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growth of luminal type MCF7 cells by inducing ERK1/2-dependent signaling. Expression of Amot80 also circumvents differentiation-induced growth control of MCF10A cells in Matrigel. Conversely, reduced expression of Amot results in decreased ERK1/2-associated growth of MDA-MB-468 and SKBR3 cells.

Materials and Methods

Cell lines

MDA-MDA-MB-436 and MDA-MDA-MB-231cells (H. Nakshatri, IUSOM) were cultured in L15 with 10% FBS. HCC1937 (B.H.), MCF7 (L. Quilliam, IUSOM), MDA-MDA-MB-468, HS578T, T47D, SKBR3 (H. Nakshatri), and 293 and 293T [American Type Culture Collection (ATCC)] cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS. MCF10A cells (B. Herbert) were cultured in 1:1 Ham's:DMEM with 5% horse serum, 0.5 μg/mL hydrocortisone (Sigma),10 μg/mL human insulin (Sigma), 20 ng/mL epidermal growth factor (EGF; Sigma), and 100 ng/mL cholera toxin (Sigma). BT474 and ZR75 cells (B. Herbert) were cultured in RPMI with 10% FBS. All cell lines were verified by DNA profiling by the original sources. For all experiments, only early passages of these cells (up to 20) were used.

Plasmids

DsRed-Amot80, CFP-Amot80, YFP-Amot 80, 3XFlag-Amot80 (10), CFP-Amot80ΔC, and YFP-Amot80ΔC (16) were previously described. CA-Ras (G12V) mutants and ELK-1 reporter (L. Quilliam), SRE-luciferase and TK-*Renilla* (Clontech), and psRSV-Rev, pMDLg-RRE, and pCMV-VSVG (Addgene) were also used.

RNA interference and short hairpin RNA

Transient knockdown of Amot utilized siRNA (AAGAAAAGCGAGACGACAAUU, Dharmacon Inc.) and control ON-TARGET*plus* (#1 D-001810-01-20, Dharmacon). For stable knockdown, shRNA sequence GACAGAAATCCAGCGCGTCTCG in pLKO.1 (Addgene) and Scramble shRNA control pLKO.1 (Plasmid #1864, Addgene) were transfected into 293T cells with packaging vectors by polyethylenimine (PEI) method. After 48 hours, virus was titered and used for infection. Assays on bulk-infected populations were performed after 1 to 4 passages.

Stable heterologous expression

Retroviral vectors were transfected with pCMV-VSVG into 293 GP cells. Lentivirus vectors were transfected with packaging vectors into 293T cells. After 48 hours, virus was titered and subsequently used for infections. Assays on bulk-infected cell populations were performed after 1 to 4 passages.

Immunoblot analysis

Cells in 1% TX-100, 50 mmol/L HEPES pH 7.5, protease inhibitor cocktail (Sigma), 1 mmol/L $NaVO_4$, and 10 mmol/L NaF were incubated on dry ice for 5 minutes. Defrosted and clarified lysates were boiled in sample buffer resolved by SDS-PAGE and immunoblotted with antibodies at dilutions: MEK1/2, MEK1/2 (S217/S221), ERK1/2, ERK1/2 (Y202/Y204; Cell Signaling), Raf1, Raf1 (Y340/Y341; Santa Cruz), GFP (Invitrogen), and HA (12CA5) at 1:1,000. Glyceraldehyde 3 phosphate dehydrogenase (GAPDH; Millipore), and Flag/M2 (Sigma) at 1:5,000.

Imaging

Confocal images were acquired on Zeiss Axio ObserverZ1 as structured light via an Apotome and processed with Axiovision 4.7. Stereo images were acquired using a Nikon SMZ1500 microscope. Immunohistochemistry was performed as described in (11) with the antibody dilutions: α-Par-3 1:800 (Zymed), α-Amot 1:200, α-PO_4-ERK1/2 1:500, and α- ERK1/2 1:500.

Reporter assays

Cells were passaged for 24 hours, transfected with an ELK-1 reporter or with the SRE-luciferase (Clontech) as well as a TK-*Renilla* normalization construct. Eight hours following transfection, the indicated treatments were performed before luciferase activities were measured by the dual luciferase method (Promega).

Accumulation assays

A total of 50,000 cells were plated into 6 cm dishes in triplicate for each time point. At the indicated times, the cells were harvested with trypsin and counted with a hemacytometer.

lrECM assays

MCF10A cells (5,000) were grown on growth factor-reduced Matrigel as described (17) in 8-well culture slides (BD-Biosciences). The MCF10A media containing 2.5% Matrigel and 5 ng/ml EGF was replaced every 4 days. A total of 10,000 SKBR3 cells were seeded onto Matrigel containing DMEM and 10% serum.

Tritiated thymidine incorporation assays

5,000 cells were seeded into a 96-well plate. Within 24 hours, a 6-hour pulse of 1.0 μCi of [^{3}H] thymidine was applied before being automatically harvested (Brandel, Gaithersburg, MD), and the level of [^{3}H] thymidine incorporated into nucleic acids (isolated by filter binding) was determined as counts per minute (CPM).

Results

Amot is required for prolonged activation of ERK1/2 in HEK 293T cells

The effects of Amot on growth factor-induced migration (18) and cell polarity suggest that it may also regulate mitogen-activated protein kinase (MAPK) signaling. Growth factor receptors signal via Ras GTPases through a 3 component MAPK cascade. This consists of the top-level serine/threonine Raf kinases (19) that phosphorylates the MAPK/ERK kinases (MEK; ref 20). MEK then dually phosphorylates the 42 and 44 kDa isoforms of ERK at a threonine and tyrosine residue resulting in a several thousand fold activation of kinase activity (21). The effects of ERK1/2 are in turn largely determined by whether they are activated at the plasma membrane where they mediate transient cytosolic process versus at

endosomes where they undergo prolonged activation linked to pro-growth nuclear signaling (22).

The role of Amot in MAPK signaling was initially examined in 293T cells as they express high levels of Amot. The fraction of phosphorylated Raf1 (340Y/341Y), MEK1 (217S/221S), and ERK1/2 (202T/204Y) were reduced in cells treated with siRNA against Amot versus control siRNA at all time points following activation by EGF or serum (Fig. 1A and Supplementary Fig. S1A). To pinpoint where Amot modulates growth factor signaling, the requirement for Amot for the activation of ERK1/2 by H- and N-Ras was examined. Cells stably silenced for Amot and transfected with a constitutively active N-Ras mutant had an approximate 80% to 90% reduction in ERK1/2 phosphorylation versus control cells. Similarly, reduced levels of Amot inhibited the effects of H-Ras on phosphorylation of ERK1/2 by 30% to 40% (Fig. 1B). Thus, Amot likely acts by promoting the activation of Raf by Ras.

The finding that basal levels of phospho-ERK1/2 were strongly reduced versus control cells (Figure S1B), indicates that Amot is important for sustained ERK activity. To further examine the relative impact of loss of Amot on early versus later times following ERK1/2 activation, relative phospho-ERK1/2 levels were measured by immunoblot of lysates from cells infected with Amot shRNA lentivirus or with control virus (Supplementary Fig. S1C). These cells were then serum starved for 24 hours before incubation with DMEM containing 10% serum for 5 and 20 minutes. Cells with reduced Amot showed a modest reduction of phospho-ERK1/2 levels versus control cells at 5 minutes but an approximate 75% reduction in ERK1/2 levels at 20 minutes (Fig. 1C). This is consistent with Amot preferentially regulating the pool of ERK1/2 that is mainly targeted to the nucleus (23) where it phosphorylates the ternary complex transcription factor ELK-1 (24). This induces the transcriptionally active complex containing ELK-1 and

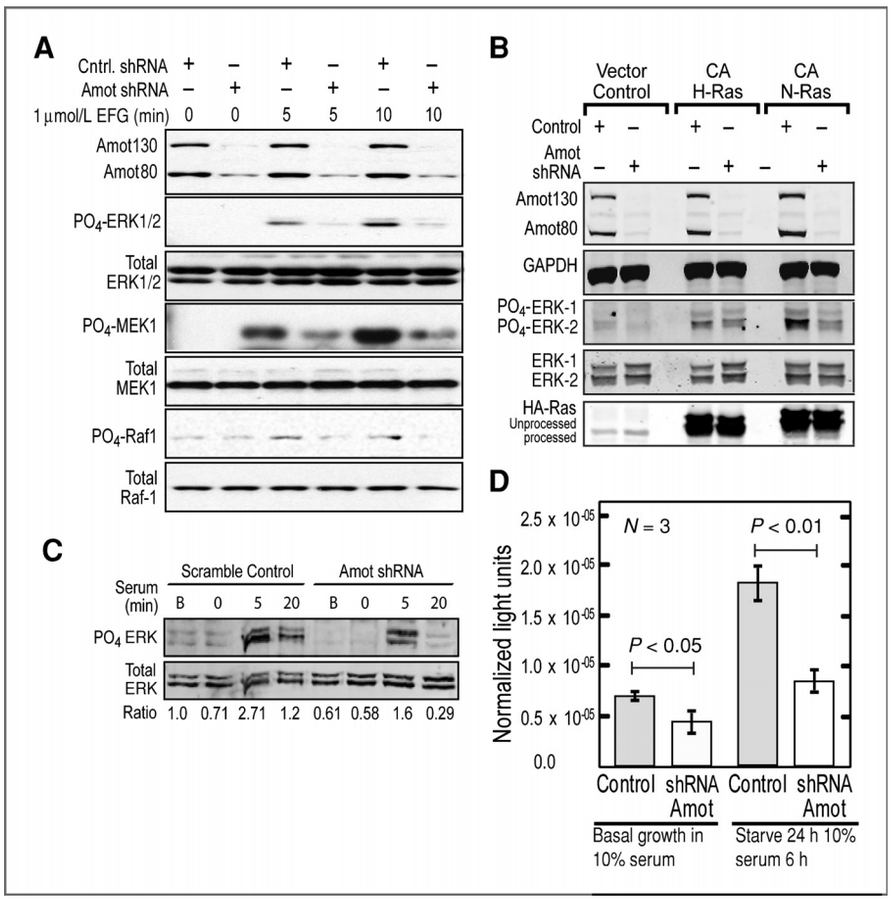

Figure 1. Amot is required for prolonged activation of ERK1/2. A, HEK 293T cells were serum starved for 24 hours and then stimulated for EGF for 5 and 10 minutes. Levels of Phospho- and total Raf-1, MEK1, and ERK1/2 as well as Amot were measured by immunoblot. B, constitutively active (CA) mutants of H- and N-Ras were transiently expressed in HEK293T cells stably infected with lentivirus encoding scramble control or shRNA against Amot. The levels of Amot, GAPDH, HA-Ras, and phospho- and total ERK1/2 were measured by immunoblot. C, a bulk population of HEK293T cells stably silenced for Amot expression by infection with shRNA lentivirus were cultured in 10% serum (basal-B) or in DMEM alone and then treated with 10% serum for the indicated times. The levels of phospho- and total ERK were measured by immunoblot (Amot and GAPDH levels are shown in Supplementary Fig. S1C). The ratio of phospho- over total ERK1/2 (fluorescence intensities measured by LiCOR Odyssey) is listed below the bottom. D, the normalized levels of luciferase expressed from an ELK-1 reporter plasmid in control or Amot silenced cells are plotted as the mean of 3 independent samples. Assays were performed using cells cultured under basal conditions or starved for 24 hours and then in serum for 4 hours. Error was computed as the standard deviation of the mean. *P* values are derived from unpaired/2-tailed student *t* tests.

serum response factor (SRF) to promote the expression of progrowth genes (25–27). Consistent with Amot regulating nuclear signaling, silencing of Amot resulted in an approximate 30% reduction of luciferase expression from an ELK-1 reporter plasmid in cells cultured in 10% serum and over a 50% decrease in luciferase expression in cells grown in 1% serum versus control cells (Fig. 1D).

Comparative expression of Amot in human mammary cell lines

Because the proliferation of ductal mammary cells requires ERK1/2-dependent signaling (28), the relative levels of Amot protein were measured in a panel of human breast cell lines with differing hormone and growth factor receptor status (29). The levels of the 80 and 130 kDa isoforms of Amot (Amot130) were highest in MDA-MB-468, MDA-MB-436, and BT474 cells. MCF7, T47D, Hs578T, SKBR3, and ZR75 cells expressed moderate levels of Amot130 and low levels of Amot80. Amot was barely detectable in HCC1937 and MDA-MB-231 cells. A band that migrates between 80 and 130 kDa isoforms was also variably detected that may either represent a degradation product or a nonspecific band. No Amot was detected in nontransformed MCF10A mammary cells (Fig. 2). The correlation of Amot80 expression with the susceptibility of these cells to be growth inhibited by UO126 is consistent with Amot having a role in ERK1/2-dependent growth (Supplementary Fig. S2B).

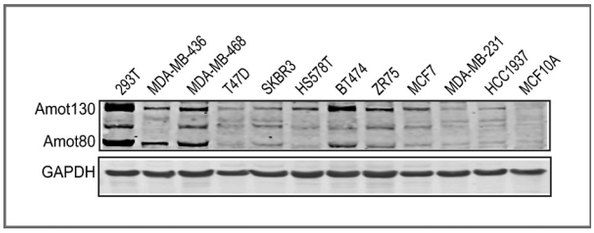

Figure 2. Amot is most highly expressed in breast cancer cell lines that utilize ErbB type receptors for growth. The relative levels of endogenous Amot protein were measured in the lysates derived from the indicated cell lines by immunoblot analysis.

Amot80 expression increases ERK1/2-dependent signaling to enhance the rate of proliferation of MCF7 cells

While MCF7 cells do not typically require ERK1/2 for growth (30), they switch to such signaling following prolonged estrogen deprivation or anti-estrogen treatment (31). We therefore examined the effects of heterologous expression of Amot80 on MAPK signaling and mitotic rate of MCF7 cells. MAPK activation in MCF7 cells infected in duplicate with retroviruses that express Flag-tagged Amot80 showed enhanced basal levels of phospho-Raf1 and -ERK1/2 versus control cells (Fig. 3A). Consistent with these effects being linked to transcription, MCF7 cells expressing CFP-tagged Amot80 expressed luciferase driven by the ELK-1 and SRF

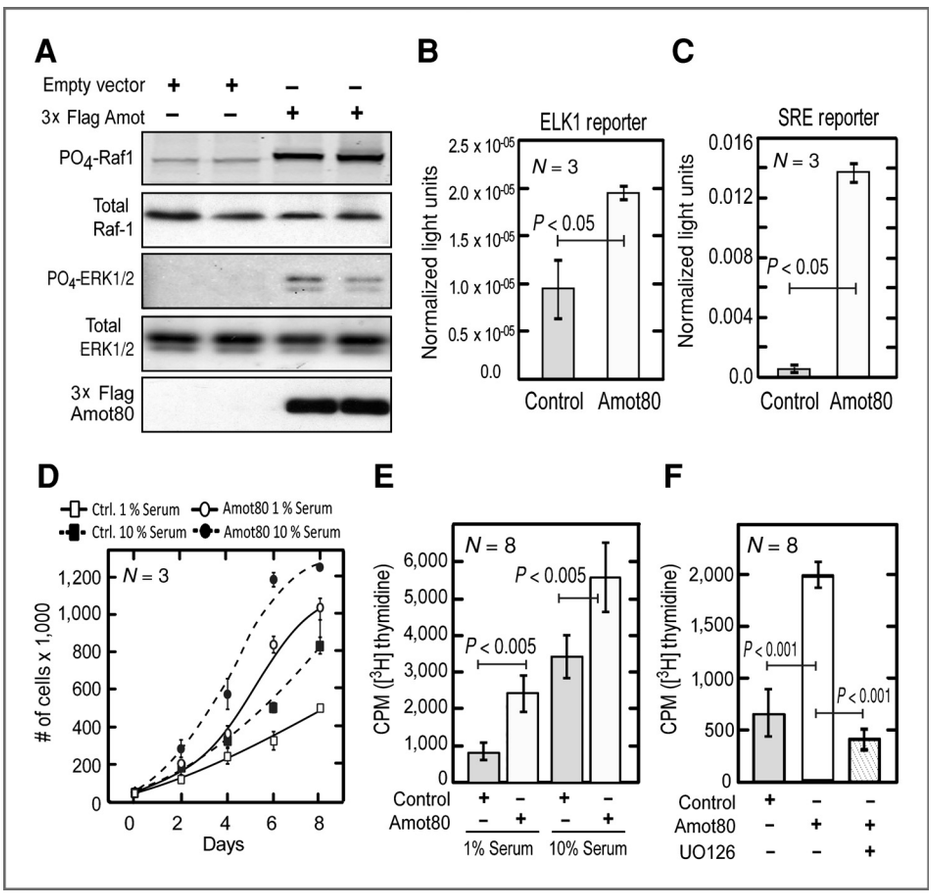

Figure 3. Amot induces ERK1/2-dependent proliferation of MCF7 cells. A, the relative levels of Flag-tagged Amot80, phospho- and total Raf1, and ERK1/2 were measured in lysates from MCF7 cells stably expressing control or Flag-tagged Amot80. B, plot of the normalized luciferase activities of MCF7 cells expressing CFP or CFP-tagged in Amot80 and the ELK-1 reporter construct. C, plot of the normalized luciferase activities of MCF7 cells expressing CFP or CFP-tagged in Amot80 and the SRE reporter construct. D, the mean number of MCF7 cells at the indicated days from 3 independent experiments are plotted where cells grown in 1% serum (solid lines) and expressing CFP (□) or CFP-tagged Amot80 (○) as well as cells grown in 10% serum (dashed lines) and expressing control CFP (■) or CFP-tagged Amot80 (●) are depicted. E, the mean levels of [^{3}H] thymidine incorporated into nucleic acids from 8 replicates of MCF7 cells expressing control (grey bars) or Amot80 (white bars). F, MCF7 cells expressing the indicated constructs were grown in 1% serum and 10 μmol/L UO126 or DMSO were assayed as described in E. Error represents the standard deviation of the mean. *P* values are from unpaired/2-tailed student *t* tests.

reporter elements by over 2-fold and 20-fold, respectively (Fig. 3B and C).

Based on the effects of Amot80 on MAPK signaling, its impact on cell proliferation was measured. MCF7 cells infected in triplicate with either a retrovirus encoding CFP-tagged Amot80 or CFP alone were counted over 8 days. Cells expressing Amot80 accumulated 2- to 3-fold faster in 1% serum and approximately 70% faster in 10% serum versus control cells (Fig. 3D). Further, MCF7 cells expressing CFP-tagged Amot80 incorporated 2- to 3-fold more [^{3}H] thymidine into nucleic acids over 6 hours versus control cells in a manner that was inhibited by the anti-MEK compound UO126 (Fig. 3E and F). Thus, Amot80 expression strongly induces ERK1/2-dependent growth in MCF7 cells.

Amot requires an intact C-terminal PDZ-binding motif to promote chemotactic migration and to disrupt apical junctions (10, 18). Here, the requirement for this motif in promoting ERK1/2-dependent cell growth was investigated. Basal phospho-ERK1/2 levels were measured by immunoblot in MCF7 cells infected with retrovirus expressing CFP, CFP-tagged Amot80 or CFP-tagged Amot80 truncated by 4 C-terminal residues (Amot80ΔC). Cells expressing Amot80 but not cells expressing Amot80ΔC had enhanced basal levels of phospho-ERK1/2 (Fig. 4A). Similarly, Amot80 but not Amot80ΔC expressing cells incorporated [^{3}H] thymidine into DNA at a 2-fold greater degree than control cells (Fig. 4B).

Immunofluorescence-based analysis further indicates that the effects of Amot on ERK1/2 likely occur, at least in part, in a cell autonomous fashion. MCF7 cells transiently expressing YFP-tagged Amot80 or YFP-tagged Amot80ΔC were fixed and immunostained for phospho- and total ERK1/2 (Fig. 4C, D). Cells expressing Amot80 but not Amot80ΔC selectively showed increased staining of phospho-ERK1/2. Higher resolution imaging revealed that phospho-ERK1/2 staining co-distributes at about a 60% frequency with DsRed-tagged Amot80 at intracellular compartments (Supplementary Fig. S3A–C). Further, YFP-tagged Amot80 co-distributes at intracellular puncta with Par-3 (Supplementary Fig. S3E). This is strongly reminiscent of previous reports showing that Amot80 and Par-3 co-distribute at recycling endosomes in MDCK cells (10, 11). Thus, it is predicted that Amot80 promotes the activation of ERK1/2 at recycling endosomes to which it also redistributes apical polarity proteins.

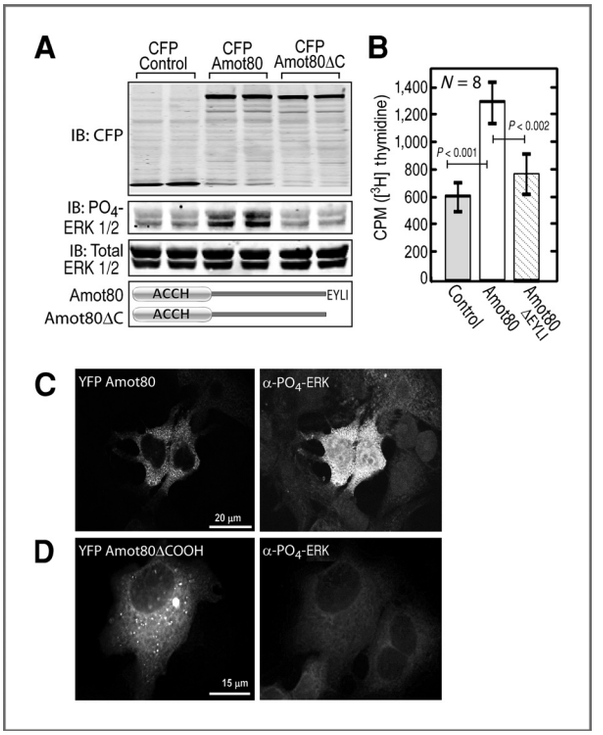

Figure 4. Amot80 requires an intact C-terminus to activate ERK1/2 and cell proliferation. A, lysates from MCF7 cells infected with retrovirus encoding CFP, CFP-tagged Amot80, or CFP-tagged Amot80ΔC were immunoblotted for CFP, phospho-ERK1/2, and total ERK1/2. A cartoon representation of Amot80 and Amot80ΔC proteins is depicted in the bottom. B, the levels of [^{3}H] thymidine incorporated into nucleic acids by MCF7 cells grown in 1% serum and expressing CFP control (grey bars), CFP-tagged Amot80 (white bars), or CFP-Amot80ΔC (hatched bars). C, a confocal image of fluorescence in MCF7 cells expressing YFP-tagged Amot80 (left) and immunostained for phospho-ERK1/2 (right). D, confocal image of YFP-tagged Amot80ΔC (left) and an immunostain of phospho-ERK1/2 (right) in MCF7 cells.

Expression of Amot80 induces non-polarized growth of MCF10A cells in Matrigel

Studies on pro-growth signaling in cells grown on 2-dimensional surfaces poorly capture the inhibitory effects of differentiation on proliferation (32). However, nontransformed mammary cells grown in a laminin-rich matrix of extracellular proteins proliferate and assemble into ordered hollow fluid filled acini that in many ways resemble normal mammary ducts (17). To examine the impact of expression of Amot80 in such an environment, the growth of MCF10A cells stably expressing CFP-tagged Amot80 (Fig. 5A) in Matrigel was monitored. Within 4 days, MCF10A cells expressing Amot80 grew into colonies that were visibly larger than control cells. By 8 days, this difference was over 4-fold greater in size (Fig. 5B and C and Supplementary Fig. S4 A, B). Confocal imaging of 4′, 6 diamidino 2 phenylindole (DAPI)-stained nuclei indicates that the spheroids formed by MCF10A cells expressing Amot80 contained a greater number of cells and lacked a hollow lumen (Fig. 5C) reminiscent of defects produced from an inability to down regulate ERK1/2 signaling (28).

SKBR3 cells require Amot expression for ERK1/2 but not Akt-dependent growth

The differential requirement for ERK and Akt signaling for the growth of SKBR3 cells was exploited to probe whether Amot selectively functions in ERK-dependent growth. Mammary cancers often escape a requirement for ERK1/2 signaling for growth by alternatively activating Akt (33, 34). Increased levels of phosphatidylinositol-(3,4,5)-trisphosphate produced by the phosphatidylinositol-3-kinase (PI3K) triggers the activation of the serine/threonine kinase AKT that in turn promotes cell proliferation, survival and motility (35). SKBR3 cells utilize Akt signaling for growth on a plastic surface (an

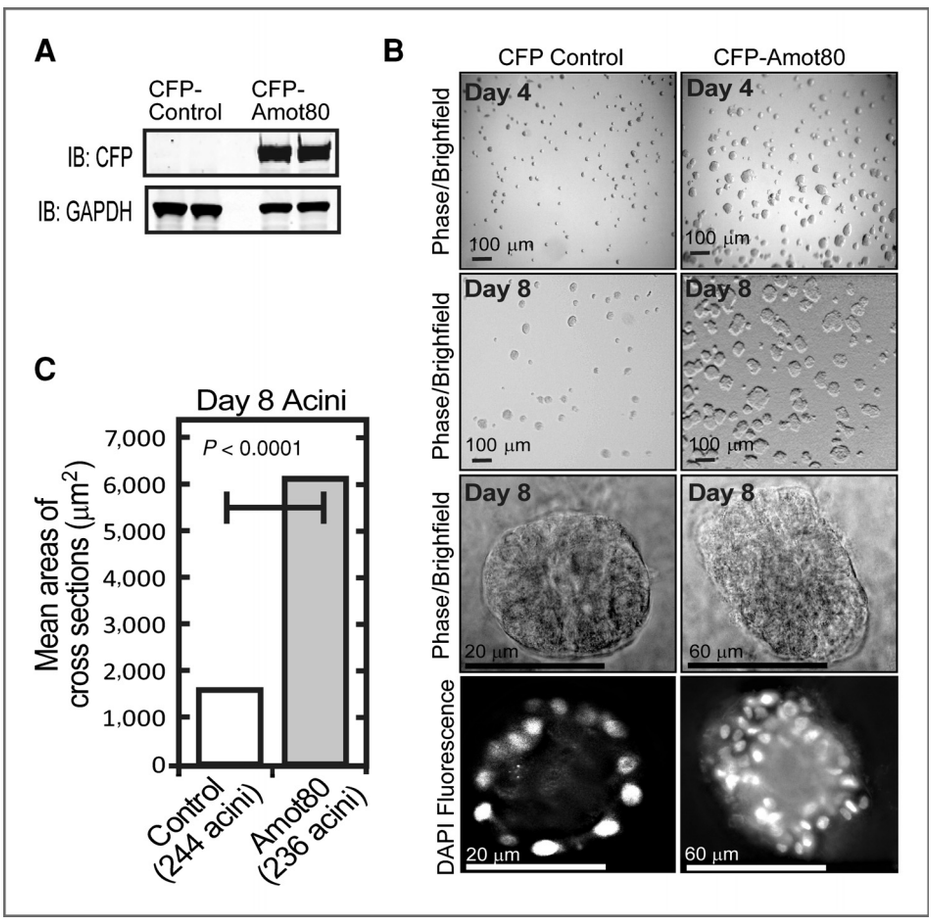

Figure 5. Heterologous expression of Amot80 promotes the poorly differentiated outgrowth of MCF10A cells cultured in Matrigel. A, the protein levels of CFP, CFP-tagged Amot80, and GAPDH in the indicated MCF10A lines was measured by immunoblot. B, brightfield stereo images of the indicated cells after 4 days (top row) and 8 days (second row) of growth in Matrigel. A high-resolution image of cells after 8 days was imaged by brightfield (third row) and a reconstructed z-stack of confocal images of nuclei stained with DAPI from the cells depicted above (fourth row). C, the mean cross-sectional area (μm^2) of acini from 8 filters per condition was plotted. *P* value represents unpaired/2-tailed *t* test assuming unequal variance.

environment conducive to poorly differentiated growth), whereas they switch to a dependence on ERK signaling for growth when cultured in Matrigel (34, 36).

Consequently, the effects of both heterologous expression and silencing of Amot on the growth of SKBR3 cells grown on plastic was investigated. SKBR3 cells expressing CFP-tagged Amot80 or CFP alone were cultured on plastic and stimulated with serum for the indicated times. The levels of GFP, GAPDH, phospho- and total ERK1/2 in lysates was then measured by immunoblot. Unlike MCF7 cells, expression of Amot80 had little effect on the basal levels of phospho-ERK1/2; however, expression of Amot80 promoted higher levels of phospho-ERK1/2 at 30 minutes (Fig. 6A). Conversely, stable silencing of Amot expression by infection with shRNA lentivirus had no significant effect on the constitutive levels of phospho-ERK1/2 (Fig. 6B). However, SKBR3 cells expressing Amot80 showed a modest but significant increase in cellular accumulation (Fig. 6C) as well as increased incorporation of [^{3}H] thymidine into DNA in a UO126 sensitive manner (Fig. 6D). Basal growth rates in this same assay, however, were not reduced by loss of Amot expression or by treatment with UO126. Thus, expression of Amot80 modestly induces ERK1/2-dependent growth of SKBR3 cells on plastic, but loss of Amot expression has no discernible impact on basal rates of growth.

The requirement for Amot expression in SKBR3 cells grown in Matrigel was also defined. Consistent with previous reports that SKBR3 cells utilize ERK1/2 signaling in Matrigel (34, 36), treatment with U0126 but not DMSO strongly inhibited their growth (Supplementary Fig. S5A). Further, SKBR3 cells silenced for Amot expression that grew normally on plastic exhibited a complete loss of growth in Matrigel (Fig. 6E and F). This analysis was further extended in MDA-MB-468 cells that are highly sensitive to growth inhibition by UO126 (30). In these cells, the level of Amot silencing directly correlated with the degree of inhibition of ERK1/2 phosphorylation (Supplementary Fig. S6A and B). While adhesion defects confounded attempts to measure cell growth on plastic, an approximate 50% silencing of Amot resulted in a significant retardation of growth in Matrigel (Supplementary Fig. S6C and D). Taken together, Amot appears to be selectively required for ERK1/2-dependent growth of mammary epithelial cells.

Discussion

This study describes a novel role for the adaptor protein Amot in ERK1/2-dependent proliferation of mammary epithelial cells. This requires the polarity protein interaction motif within Amot and likely results, at least in part, by coupling the

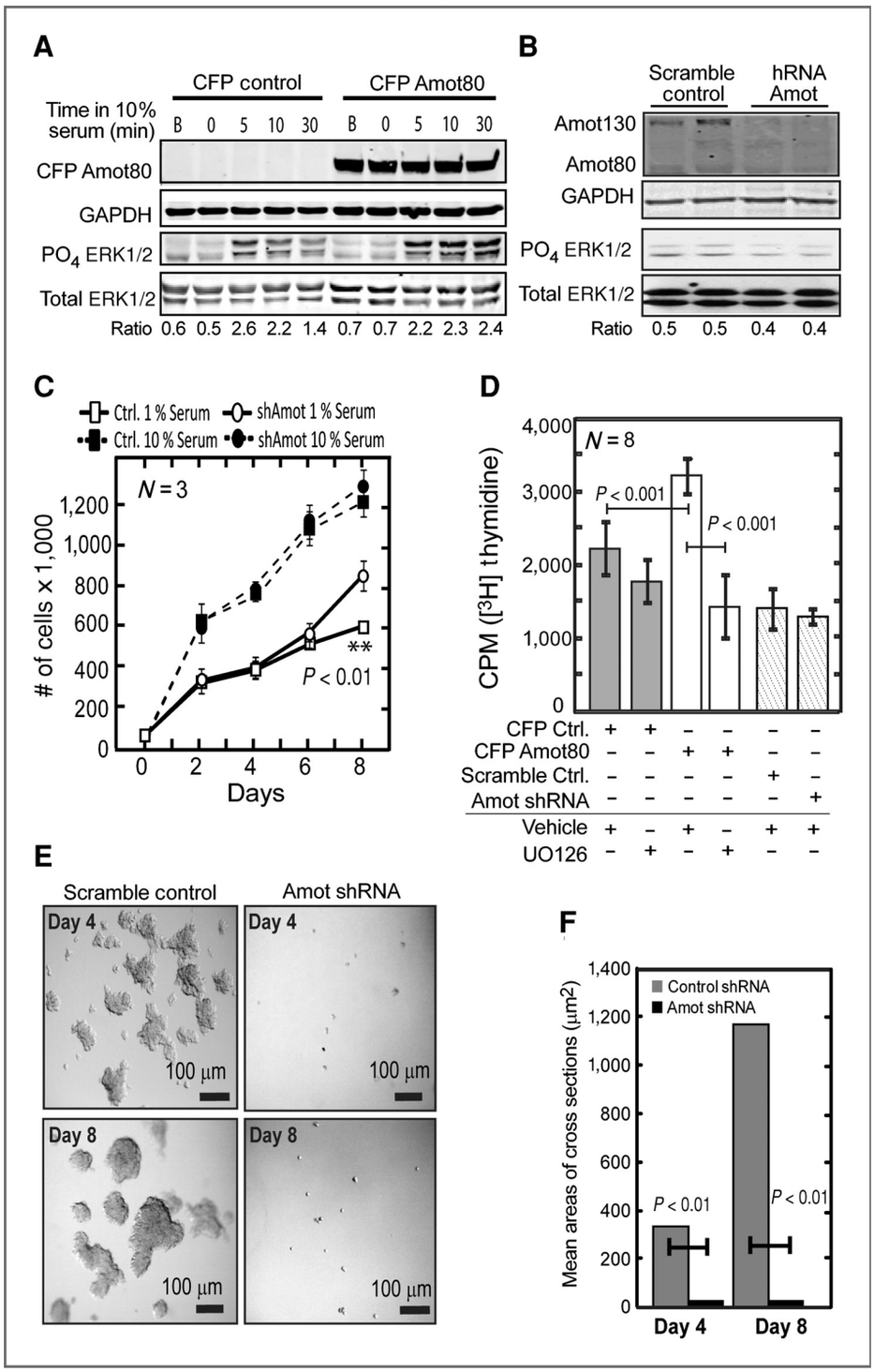

Figure 6. Amot is essential for ERK-dependent proliferation of SKBR3 Cells. A, control and Amot80 expressing SKBR3 cells were stimulated with serum before the levels GFP, GAPDH, and phospho- and total ERK1/2 were measured by immunoblot. The ratio of fluorescence intensities of phospho- and total ERK1/2 are given below. B, bulk populations of SKBR3 cells infected with lentivirus-encoding scramble control or Amot shRNA were immunoblotted for Amot, GAPDH, and phospho- and total ERK1/2. C, after the indicated number of days of growth cells were counted from 3 independent experiments. The mean number cells grown in 1% serum (solid line) control vector (□) or CFP-tagged AMOT80 (○) or 10% serum (dashed line) control vector (■) or CFP-tagged Amot80 (●) are plotted. D, SKBR3 cells from A and B were plated in 10% serum with vehicle or U0126 for 24 hours before [^{3}H] thymidine incorporation was measured. The mean from 8 replicates was plotted. Error in C/D was computed as standard deviation of the mean. *P* values were derived from unpaired/2-tailed student *t* tests assuming equal variance. E, brightfield stereo images of SKBR3 cells from B grown in Matrigel for 4 and 8 days. F, the mean cross-sectional areas (μm^2) of colonies described in E from 8 filters per condition were plotted. *P* values were derived from unpaired/2-tailed *t* tests assuming unequal variance. **, $P < 0.01$.

activation of Raf by Ras. Consistent with Amot coordinating the induction of proliferation with de-differentiation, its expression enhances the rate of proliferation of MCF7 cells and induces MCF10A cells to grow as enlarged disorganized spheroids in Matrigel. Conversely, loss of Amot expression reduces ERK1/2-related growth of MDA-MB-468 and SKBR3 cells.

Amot expression likely promotes cell growth by fostering the prolonged activation of MAPK signaling by Ras. Amot expression enhances the basal levels of phospho-ERK1/2 and

phospho-Raf1; whereas, loss of Amot expression inhibits increased levels of phospho-Raf1, -MEK1 and -ERK1/2 induced by EGF or serum. The preferential effects of Amot on constitutive or prolonged phosphorylation of ERK1/2 likely allows sustained activation of ternary complex transcription factors required to drive the cell to enter S-phase (26, 27). This is strongly supported by the observations that Amot80 expression activates ELK-1 and SRF transcription as well as enhances the rate of cell proliferation of MCF7 cells.

Amot may coordinate the activation of MAPKs with the redistribution of apical polarity proteins to endosomes. The apical polarity proteins Crb3 and Par-6, in addition to their roles in cellular asymmetry, also promote cell growth (8, 37); where Par-6 acts via ERK1/2 signaling (8). One explanation for how these proteins may promote both differentiation and proliferation is that they alternatively promote differentiation on the apical plasma membrane but induce cell growth at intracellular compartments. The common requirement of the C-terminus of Amot for binding and redistributing apical polarity proteins to the endosomal recycling compartment (ERC; refs 10, 11) and for ERK1/2-dependent proliferation suggests that Amot may be responsible for repurposing apical polarity proteins to promote pro-growth signaling at endosomes. This might explain how the "slow" trafficking pathway involving cholesterol-enriched vesicles that move between the plasma membrane and the ERC mediate ERK1/2-dependent cell growth (38). Consistently, Amot80 and phospho-ERK1/2 co-distribute at intracellular compartments. The strong role of Amot downstream of N-Ras also fits with this premise as N-Ras binds cholesterol-enriched vesicular membranes (39). Further, Raf is only activated by Ras with a multi-hour lifespan associated with pro-growth signaling at endosomes (40, 41). Future work relating the mechanisms whereby MAPKs are scaffolded and targeted (22, 42) with the regulation of specific polarity proteins by Amot will likely uncover the biochemical and cellular mechanisms for such signaling. An intriguing possibility is that this is coordinated with Hippo signaling, a key pathway controlling cell growth (43) that is regulated by apical polarity proteins (44) and predicted to be upstream of ERK1/2 signaling (45).

The effects of expression of Amot80 on MCF7 cells recapitulate aspects of anti-estrogen resistance in luminal-type breast carcinomas. MCF7 cells via the estrogen receptor alpha (ERα) grow in response to estrogen and are mainly refractory to anti-MEK drugs (30). However, acquired resistance to the anti-estrogens such as Fulvestrant results in increased ERK1/2-dependent growth (46). Thus increased Amot expression could explain 1 mechanism whereby luminal tumors acquire estrogen independence. This also fits with the observation that the levels of Amot correlate with high tumor grade and metastatic risk (15), features that are common to recurrent estrogen independent tumors that present with increased MAPK signaling (47–49).

High levels of Amot expression may indicate breast cancers that utilize ERK1/2 signaling for growth. ErbB2 positive tumors or basal-like breast tumors that express ErbB1 and/or ErbB3 generally activate either RAF-MEK-ERK1/2 or PI3K-Akt-dependent signaling for growth. A significant problem in treating these cancers is that drugs such as Trastuzumab often lose effectiveness when the tumor cells switch from ERK- to Akt-dependent growth (33). The correlation between Amot expression and sensitivity to UO126 in the basal-like (MDA-MB-468 and MDA-MB-436) and ErbB2-positive (BT474 and SKBR3) breast cancer cell lines (30) indicates that Amot80 expression may be associated with tumors that utilize ERK1/2 signaling for growth. A lack of expression of Amot in non-transformed cells may also, in part, explain how signaling by oncogenes such as ErbB1, ErbB2, and Ras are uncoupled from pro-growth effects by differentiation. Future investigations defining how the expression of different splice forms of Amot are induced in tumor subtypes may therefore lead to a better understanding of how these tumors coordinately couple a loss of differentiation with pro-growth signaling.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Bissell MJ, Rizki A, Mian IS. Tissue architecture: the ultimate regulator of breast epithelial function. Curr Opin Cell Biol 2003;15:753–62.
2. Itoh M, Bissell MJ. The organization of tight junctions in epithelia: implications for mammary gland biology and breast tumorigenesis. J Mammary Gland Biol Neoplasia 2003;8:449–62.
3. Shin K, Fogg VC, Margolis B. Tight junctions and cell polarity. Annu Rev Cell Dev Biol 2006;22:207–35.
4. Nejsum LN, Nelson WJ. Epithelial cell surface polarity: the early steps. Front Biosci 2009;14:1088–98.
5. Rubin H. Cell-cell contact interactions conditionally determine suppression and selection of the neoplastic phenotype. Proc Natl Acad Sci U S A 2008;105:6215–21.
6. Feigin ME, Muthuswamy SK. Polarity proteins regulate mammalian cell-cell junctions and cancer pathogenesis. Curr Opin Cell Biol 2009;21:694–700.
7. Huang L, Muthuswamy SK. Polarity protein alterations in carcinoma: a focus on emerging roles for polarity regulators. Curr Opin Genet Dev 20:41–50.
8. Nolan ME, Aranda V, Lee S, Lakshmi B, Basu S, Allred DC, et al. The polarity protein Par6 induces cell proliferation and is overexpressed in breast cancer. Cancer Res 2008;68:8201–9.
9. Aranda V, Haire T, Nolan ME, Calarco JP, Rosenberg AZ, Fawcett JP, et al. Par6-aPKC uncouples ErbB2 induced disruption of polarized epithelial organization from proliferation control. Nat Cell Biol 2006;8:1235–45.

10. Wells CD, Fawcett JP, Traweger A, Yamanaka Y, Goudreault M, Elder K, et al. A Rich1/Amot complex regulates the Cdc42 GTPase and apical-polarity proteins in epithelial cells. Cell 2006;125:535–48.
11. Heller B, Adu-Gyamfi E, Smith-Kinnaman W, Babbey C, Vora M, Xue Y, et al. Amot recognizes a juxtanuclear endocytic recycling compartment via a novel lipid binding domain. J Biol Chem 285:12308–20.
12. Ross JW, Ashworth MD, Stein DR, Couture OP, Tuggle CK, Geisert RD. Identification of differential gene expression during porcine conceptus rapid trophoblastic elongation and attachment to uterine luminal epithelium. Physiol Genomics 2009;36:140–8.
13. Aase K, Ernkvist M, Ebarasi L, Jakobsson L, Majumdar A, Yi C, et al. Angiomotin regulates endothelial cell migration during embryonic angiogenesis. Genes Dev 2007;21:2055–68.
14. Shimono A, Behringer RR. Angiomotin regulates visceral endoderm movements during mouse embryogenesis. Curr Biol 2003;13:613–7.
15. Jiang WG, Watkins G, Douglas-Jones A, Holmgren L, Mansel RE. Angiomotin and angiomotin like proteins, their expression and correlation with angiogenesis and clinical outcome in human breast cancer. BMC Cancer 2006;6:16.
16. Colwill K, Wells CD, Elder K, Goudreault M, Hersi K, Kulkarni S, et al. Modification of the Creator recombination system for proteomics applications—improved expression by addition of splice sites. BMC Biotechnol 2006;6:13.
17. Debnath J, Muthuswamy SK, Brugge JS. Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. Methods 2003;30:256–68.
18. Levchenko T, Aase K, Troyanovsky B, Bratt A, Holmgren L. Loss of responsiveness to chemotactic factors by deletion of the C-terminal protein interaction site of angiomotin. J Cell Sci 2003;116 (Pt 18):3803–10.
19. Avruch J, Khokhlatchev A, Kyriakis JM, Luo Z, Tzivion G, Vavvas D, et al. Ras activation of the Raf kinase: tyrosine kinase recruitment of the MAP kinase cascade. Recent Prog Horm Res 2001;56:127–55.
20. Ahn NG. The MAP kinase cascade. Discovery of a new signal transduction pathway. Mol Cell Biochem 1993;127–128:201–9.
21. Cobb MH, Goldsmith EJ. How MAP Kinases Are Regulated. J Biol Chem 1995;270:14843–14846.
22. Brown MD, Sacks DB. Protein scaffolds in MAP kinase signalling. Cell Signal 2009;21:462–9.
23. Burack WR, Shaw AS. Live cell imaging of ERK and MEK: simple binding equilibrium explains the regulated nucleocytoplasmic distribution of ERK. J Biol Chem 2005;280:3832–7.
24. Gille H, Kortenjann M, Thomae O, Moomaw C, Slaughter C, Cobb MH, et al. ERK phosphorylation potentiates Elk-1-mediated ternary complex formation and transactivation. EMBO J 1995;14:951–62.
25. Shaw PE, Saxton J. Ternary complex factors: prime nuclear targets for mitogen-activated protein kinases. Int J Biochem Cell Biol 2003;35:1210–26.
26. Murphy LO, Blenis J. MAPK signal specificity: the right place at the right time. Trends Biochem Sci 2006;31:268–75.
27. Brunet A, Roux D, Lenormand P, Dowd S, Keyse S, Pouysségur J. Nuclear translocation of p42/p44 mitogen-activated protein kinase is required for growth factor-induced gene expression and cell cycle entry. EMBO J 1999;18:664–74.
28. Turley EA, Veiseh M, Radisky DC, Bissell MJ. Mechanisms of disease: epithelial-mesenchymal transition—does cellular plasticity fuel neoplastic progression? Nat Clin Pract Oncol 2008;5:280–90.
29. Lacroix M, Leclercq G. Relevance of breast cancer cell lines as models for breast tumours: an update. Breast Cancer Res Treat 2004;83:249–89.
30. Mirzoeva OK, Das D, Heiser LM, Bhattacharya S, Siwak D, Gendelman R, et al. Basal subtype and MAPK/ERK kinase (MEK)-phosphoinositide 3-kinase feedback signaling determine susceptibility of breast cancer cells to MEK inhibition. Cancer Res 2009;69:565–72.
31. Gee JM, Howell A, Gullick WJ, Benz CC, Sutherland RL, Santen RJ, et al. Consensus statement. Workshop on therapeutic resistance in breast cancer: impact of growth factor signalling pathways and implications for future treatment. Endocr Relat Cancer 2005;12 Suppl 1:S1–7.
32. LaBarge MA, Petersen OW, Bissell MJ. Of microenvironments and mammary stem cells. Stem Cell Rev 2007;3:137–46.
33. Sergina NV, Rausch M, Wang D, Blair J, Hann B, Shokat KM, et al. Escape from HER-family tyrosine kinase inhibitor therapy by the kinase-inactive HER3. Nature 2007;445:437–41.
34. Weigelt B, Lo AT, Park CC, Gray JW, Bissell MJ. HER2 signaling pathway activation and response of breast cancer cells to HER2-targeting agents is dependent strongly on the 3D microenvironment. Breast Cancer Res Treat 2010;122:35–43.
35. Citri A, Yarden Y. EGF-ERBB signalling: towards the systems level. Nat Rev Mol Cell Biol 2006;7:505–16.
36. Pickl M, Ries CH. Comparison of 3D and 2D tumor models reveals enhanced HER2 activation in 3D associated with an increased response to trastuzumab. Oncogene 2009;28:461–8.
37. Lu H, Bilder D. Endocytic control of epithelial polarity and proliferation in Drosophila. Nat Cell Biol 2005;7:1232–9.
38. Muralidharan-Chari V, Hoover H, Clancy J, Schweitzer J, Suckow MA, Schroeder V, et al. ADP-ribosylation factor 6 regulates tumorigenic and invasive properties in vivo. Cancer Res 2009;69:2201–9.
39. Matallanas D, Arozarena I, Berciano MT, Aaronson DS, Pellicer A, Lafarga M, et al. Differences on the inhibitory specificities of H-Ras, K-Ras, and N-Ras (N17) dominant negative mutants are related to their membrane microlocalization. J Biol Chem 2003;278:4572–81.
40. Casar B, Arozarena I, Sanz-Moreno V, Pinto A, Agudo-Ibáñez L, Marais R, et al. Ras subcellular localization defines extracellular signal-regulated kinase 1 and 2 substrate specificity through distinct utilization of scaffold proteins. Mol Cell Biol 2009;29:1338–53.
41. Chiu VK, Bivona T, Hach A, Sajous JB, Silletti J, Wiener H, et al. Ras signalling on the endoplasmic reticulum and the Golgi. Nat Cell Biol 2002;4:343–50.
42. Willard MD, Willard FS, Li X, Cappell SD, Snider WD, Siderovski DP. Selective role for RGS12 as a Ras/Raf/MEK scaffold in nerve growth factor-mediated differentiation. EMBO J 2007;26:2029–40.
43. Zhang J, Ji JY, Yu M, Overholtzer M, Smolen GA, Wang R, et al. YAP-dependent induction of amphiregulin identifies a non-cell-autonomous component of the Hippo pathway. Nat Cell Biol 2009;11:1444–50.
44. Grusche FA, Richardson HE, Harvey KF. Upstream regulation of the hippo size control pathway. Curr Biol 2010;20:R574–82.
45. Bakal C, Linding R, Llense F, Heffern E, Martin-Blanco E, Pawson T, et al. Phosphorylation networks regulating JNK activity in diverse genetic backgrounds. Science 2008;322:453–6.
46. Frogne T, Benjaminsen RV, Sonne-Hansen K, Sorensen BS, Nexo E, Laenkholm AV, et al. Activation of ErbB3, EGFR and Erk is essential for growth of human breast cancer cell lines with acquired resistance to fulvestrant. Breast Cancer Res Treat 2009;114:263–75.
47. Harari D, Yarden Y. Molecular mechanisms underlying ErbB2/HER2 action in breast cancer. Oncogene 2000;19:6102–14.
48. Milde-Langosch K, Bamberger AM, Rieck G, Grund D, Hemminger G, Müller V, et al. Expression and prognostic relevance of activated extracellular-regulated kinases (ERK1/2) in breast cancer. Br J Cancer 2005;92:2206–15.
49. Adeyinka A, Nui Y, Cherlet T, Snell L, Watson PH, Murphy LC. Activated mitogen-activated protein kinase expression during human breast tumorigenesis and breast cancer progression. Clin Cancer Res 2002;8:1747–53.