The Adaptor Proteins p66Shc and Grb2 Regulate the Activation of the GTPases ARF1 and ARF6 in Invasive Breast Cancer Cells*

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Background: ADP-ribosylation factors (ARF) 1 and 6 play an important role in breast cancer cell proliferation, migration, and invasion.

Results: In invasive breast cancer cells, EGF-mediated ARF1 and ARF6 activation is regulated by p66Shc and Grb2.

Conclusion: Adaptor proteins are required to promote ARF activation.

Significance: Understanding the signaling mechanisms leading to ARF activation may identify novel therapeutic targets for the treatment of breast cancer.

Signals downstream of growth factor receptors play an important role in mammary carcinogenesis. Recently, we demonstrated that the small GTPases ARF1 and ARF6 were shown to be activated downstream of the epidermal growth factor receptor (EGFR) and act as a key regulator of growth, migration, and invasion of breast cancer cells. However, the mechanism via which the EGFR recruits and activates ARF1 and ARF6 to transmit signals has yet to be fully elucidated. Here, we identify adaptor proteins Grb2 and p66Shc as important regulators mediating ARF activation. We demonstrate that ARF1 can be found in complex with Grb2 and p66Shc upon EGF stimulation of the basal-like breast cancer MDA-MB-231 cell line. However, we report that these two adaptors regulate ARF1 activation differently, with Grb2 promoting ARF1 activation and p66Shc blocking this response. Furthermore, we show that Grb2 is essential for the recruitment of ARF1 to the EGFR, whereas p66Shc hindered ARF1 receptor recruitment. We demonstrate that the negative regulatory role of p66Shc stemmed from its ability to block the recruitment of Grb2/ARF1 to the EGFR. Conversely, p66Shc potentiates ARF6 activation as well as the recruitment of this ARF isoform to the EGFR. Interestingly, we demonstrate that Grb2 is also required for the activation and receptor recruitment of ARF6. Additionally, we show an important role for p66Shc in modulating ARF activation, cell growth, and migration in HER2-positive breast cancer cells. Together, our results highlight a central role for adaptor proteins p66Shc and Grb2 in the regulation of ARF1 and ARF6 activation in invasive breast cancer cells.

The epidermal growth factor receptor (EGFR), one of the best characterized tyrosine kinase receptors, has been shown to be highly expressed in certain breast cancer patients (1). Activation of this receptor by the binding of a variety of ligands, including the epidermal growth factor (EGF), has been implicated in breast cancer cell proliferation, survival, migration, and invasion (2). Upon binding of EGF, the EGFR can homodimerize or heterodimerize with other EGFR family members, ErbB2 and/or ErbB3 (3–5). This leads to the auto-phosphorylation of several tyrosine residues on the intracellular domains of the receptor. These residues serve as docking sites for a variety of adaptor proteins that are essential for the initiation of downstream signaling (6–8), such as the phosphoinositide 3-kinase (PI3K) and mitogen-activating protein kinase (MAPK) pathways (2, 9, 10).

One family of adaptors that are recruited to the EGFR are the Src homology 2 domain-containing proteins (Shc), which consists of four members, ShcA, -B, -C, and -D (11–15). Although ShcB and -C have been shown to be primarily present within the central nervous system and ShcD has only been identified in mice, ShcA is ubiquitously expressed and has been implicated in breast cancer (11–13, 15). ShcA consists of three isoforms: p46Shc, p52Shc, and p66Shc, which result either from alternative translational initiation sites (p46Shc and p52Shc) or mRNA splicing (p66Shc) (16, 17). Although ShcA is generally considered as an adaptor protein mediating EGFR-dependent activation of the MAPK pathway (16), the function of each isoform, especially p66Shc, in different physiological and pathological settings, remains controversial. Like p52Shc, p66Shc has also been reported to be recruited to the EGFR and associate with Grb2 upon stimulation (16). However, unlike the other Shc isoforms, p66Shc blocked the recruitment of Grb2 to the EGFR and insulin-like growth factor receptor. This was shown to lead to an inhibition of the Ras/MAPK pathway (18, 19). Furthermore, the expression of p46Shc and p52Shc was shown to be

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The abbreviations used are: EGF, epidermal growth factor receptor; Shc, Src homology domain 2-containing protein; ARF, ADP-ribosylation factor.
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Elevated in tumors isolated from transgenic breast cancer mouse models, whereas p66Shc levels were undetectable (20, 21). In fact, recent studies have reported that p66Shc expression in breast cancer patients may be predictive of node negativity, reduced disease stage, and decreased incidence of patient relapse (22, 23). However, the same group demonstrated that p66Shc expression was associated with a poor prognosis in colorectal cancer patients (24). Interestingly, p66Shc expression has been demonstrated to be up-regulated by steroid hormones in differentiated hormone-sensitive cancer cells and elevated in the highly invasive breast cancer MDA-MB-231 cell line, as well as in invasive prostate cancer cells (25–27) suggesting a role for this isoform in cancer progression. Moreover, LNCaP cell proliferation and motility was shown to be significantly hindered upon the depletion of p66Shc (27). In summary, the role of p66Shc in cancer remains highly controversial and the exact role for p66Shc in invasive breast cancer has yet to be examined.

Recently, members of the ADP-ribosylation factor (ARF) family of small GTPases have been shown to be activated downstream of the EGFR in highly invasive breast cancer cells such as MDA-MB-231, MDA-MB-435, and SKBr3. Moreover, these ARF proteins play an essential role in the proliferation, migration, and invasion of these cells (28, 29). Briefly, ARFs are members of the Ras superfamily of small monomeric G proteins and consist of six isoforms divided into three distinct classes: Class I, consisting of ARF1–3, which is known to regulate the secretory pathway; Class II, ARF4 and ARF5, in which their role has not yet to be fully elucidated; and Class III, ARF6, known to modulate intracellular trafficking between the plasma membrane and the endosomes and play an essential role in the organization of the actin cytoskeleton (30). In breast cancer, isoforms ARF1 and ARF6 have been the best characterized. Both GTPases play critical roles in the proliferation and migration of invasive breast cancer cells (28, 29). Although ARF6 has been shown to exert its oncogenic properties via the ERK1/2 pathway, we demonstrated that ARF1 signals primarily via the PI 3-kinase/AKT signaling axis (28, 29, 31). Little is known on the molecular mechanism downstream of the EGFR that leads to activation of ARFs. It was suggested that for ARF6, the guanine nucleotide exchange factor GEP100 (BRAG2) directly bound to the EGFR to mediate the activation of this small GTPase (32). However, it is important to define whether classical adaptor proteins contribute to regulate ARF activation.

Here, for the first time, we show that the adaptor proteins p66Shc and Grb2 are key proteins controlling EGFR-dependent ARF1 and ARF6 activation in invasive breast cancer cells. We demonstrate that whereas p66Shc attenuates ARF1 activation, it potentiates ARF6 activation. Furthermore, we demonstrate that another adaptor, Grb2, is essential for the activation of both ARF1 and ARF6. More specifically, we show that p66Shc mediates ARF1 activation by blocking recruitment of the Grb2-ARF1 complex to the EGFR. Conversely, we demonstrate that p66Shc potentiates ARF6 activation by favoring its Grb2-dependent recruitment to the EGFR.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Lipofectamine 2000™ was purchased from Invitrogen. EGF was purchased from Fitzgerald Industries International, Inc. (Concord, MA). Monoclonal antibodies used in the study were ARF1 (Sigma), p66Shc (Invitrogen), ARF6 (Santa Cruz Biotechnology, Dallas, TX), and phosphotyrosine (Millipore, Billerica, MA). Polyclonal antibodies used were EGFR, HER2, Pan-actin, pERK1/2, pAKT, AKT (Cell Signaling, Danvers, MA), ARF1 (Proteintech Group, Chicago, IL), Grb2, HA tag, H-Ras, ERK1/2 (Santa Cruz Biotechnology), and Shc (BD Transduction Laboratories, Mississauga, Ontario, Canada). Other reagents used were goat anti-mouse antibody-horseradish peroxidase and goat anti-rabbit antibody-horseradish peroxidase (R & D Systems, Minneapolis, MN) and protein G-agarose plus beads (Santa Cruz Biotechnology).

DNA Plasmids and siRNAs—HA-p66Shc cloned into a pcDNA3 vector was a gift from Dr. Nagamine (Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland) (33). Double-stranded scrambled with 19-nucleotide duplex RNA and 2-nucleotide 3′-dTdT overhangs were previously described (34). The 19-nucleotide sequence for the human Grb2 siRNA target was 5′-GAA AGG AGC TTG CCA CGG G-3′. The 21-nucleotide sequence for the human p66Shc siRNA target was 5′-GAA UGA GUC UCU GUC AUC GUC-3′ as previously described (33). All siRNA include 2-nucleotide 3′-dTdT overhangs and were purchased from Dharmacon Inc. (Lafayette, CO).

Cell Culture and Transfection—MDA-MB-231, SkBr3, and MCF7 cells were maintained at 37 °C, 5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS). HCC70 cells were maintained at 37 °C, 5% CO2 in Roswell Park Memorial Institute medium (RPMI) supplemented with 10% FBS. Cells were transfected with plasmid cDNA and/or siRNA using Lipofectamine 2000 according to the manufacturer’s instructions. Briefly, for overexpression experiments, cells were mock transfected or transfected with HA-p66Shc cDNA for 6 h prior to being serum starved overnight and then stimulated with EGF for the indicated time points. In siRNA experiments, MDA-MB-231 cells were transfected with 50 nM siRNA for 72 h, serum-starved overnight, and then stimulated with EGF for the indicated time points.

Co-immunoprecipitation and Western Blot Analysis—Serum-starved cells from confluent 10-cm dishes were harvested in 500 μl of lysis buffer (20 mM Tris-HCl, pH 8, 1% Triton X-100, 10% glycerol, 140 mM NaCl, 5 mM EDTA, 1 mM sodium orthovanadate (Na3VO4) complemented with protease inhibitors aprotinin (5 μg/ml), benzamidine (150 μg/ml), leupeptin (5 μg/ml), pepstatin (4 μg/ml), and phenylmethylsulfonyl fluoride (20 mg/ml)). Cell lysates were solubilized at 4 °C for 30 min and total soluble proteins were run on polyacrylamide gels and transferred onto nitrocellulose membranes. The transferred proteins were detected using specific primary antibodies (see each experiment for details). Secondary antibodies were all horseradish peroxidase-conjugated, and chemiluminescence was used to detect protein expression. The quantification of the digital images obtained was performed using ImageQuant TL.
Briefly, cells were lysed in 200 μl of ice-cold lysis buffer E (pH 7.4, 50 mM Tris-HCl, 1% Nonidet P-40, 137 mM NaCl, 10% glycerol, 5 mM MgCl₂, 20 mM NaF, 1 mM NaPP₆, 1 mM Na₃VO₄, and the protease inhibitors: aprotinin (5 μg/ml), benzamidine (150 μg/ml), leupeptin (5 μg/ml), pepstatin (4 μg/ml), and phenylmethylsulfonyl fluoride (20 mg/ml)). Samples were spun for 5 min at 10,000 rpm. GST-Raf-binding domain coupled to glutathione-Sepharose 4B was added to each tube, and the samples were rotated at 4 °C for 45 min. Proteins were eluted in 20 μl of SDS-sample buffer by heating to 65 °C for 15 min. The detection of ARF1-GTP or ARF6-GTP was performed by immunoblot analysis using specific antibodies to ARF1 and ARF6, respectively.

**Ras Activation Assay**—Cells were plated into 10-cm dishes, transfected for the indicated times, and serum-starved overnight. Cells were then stimulated with EGF (10 ng/ml) at 37 °C for the indicated times, and the activation of ARF1 was measured as previously described (34). Briefly, cells were lysed in 400 μl of ice-cold lysis buffer E (pH 7.4, 50 mM Tris-HCl, 1% Nonidet P-40, 137 mM NaCl, 10% glycerol, 5 mM MgCl₂, 20 mM NaF, 1 mM NaPP₆, 1 mM Na₃VO₄, and the protease inhibitors: aprotinin (5 μg/ml), benzamidine (150 μg/ml), leupeptin (5 μg/ml), pepstatin (4 μg/ml), and phenylmethylsulfonyl fluoride (20 mg/ml)). Samples were spun for 5 min at 10,000 rpm. GST-GGA3-(1–316) (35) coupled to glutathione-Sepharose 4B was added to each tube, and the samples were rotated at 4 °C for 45 min. Proteins were eluted in 20 μl of SDS-sample buffer by heating to 65 °C for 15 min. The detection of ARF1-GTP or ARF6-GTP was performed by immunoblot analysis using specific antibodies to ARF1 and ARF6, respectively.

**Ras Activation Assay**—Cells were plated into 6-well plates, transfected for the indicated times, and serum-starved overnight. Cells were then stimulated with EGF (10 ng/ml) at 37 °C for the indicated times, and the activation of Ras was measured. Briefly, cells were lysed in 200 μl of ice-cold lysis buffer E (pH 7.4, 50 mM Tris-HCl, 1% Nonidet P-40, 137 mM NaCl, 10% glycerol, 5 mM MgCl₂, 20 mM NaF, 1 mM NaPP₆, 1 mM Na₃VO₄, and the protease inhibitors: aprotinin (5 μg/ml), benzamidine (150 μg/ml), leupeptin (5 μg/ml), pepstatin (4 μg/ml), and phenylmethylsulfonyl fluoride (20 mg/ml)). Samples were spun for 5 min at 10,000 rpm. GST-Raf-binding domain coupled to glutathione-Sepharose 4B was added to each tube, and the samples were rotated at 4 °C for 45 min. Proteins were eluted in 20 μl of SDS-sample buffer by heating to 65 °C for 15 min. The detection of Ras-GTP was performed by immunoblot analysis using a specific antibody to H-Ras.

**Cell Counting Assay**—Cells were transiently transfected with 50 mM scrambled siRNA or p66Shc siRNA for 48 h for knockdown experiments or an empty vector or HA-p66Shc cDNA for 24 h for overexpression experiments, trypsinized, and an equal cell number (1 × 10⁶ cells) were reseeded in a 6-cm dish for 24, 48, and 72 h. For each indicated time point, cells were trypsinized, stained with trypan blue, and live cells were manually counted.

**Cell Viability Assay (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide)**—Cells were transiently transfected with 50 μM scrambled siRNA or p66Shc siRNA for 48 h for knockdown experiments or an empty vector or HA-p66Shc cDNA for 24 h for overexpression experiments, trypsinized, and equal cell numbers (1000 cells) were reseeded in a 96-well plate for 72 h. Cells were then stained with thiazolyl blue tetrazolium bromide (Sigma) for 2 h before being solubilized in 20% SDS, 50% dimethylformamide solution overnight. Absorbance was measured at 570 nm with a reference wavelength at 450 nm using a plate reader.

**Cell Migration Assay**—Cells were transiently transfected with 50 mM scrambled siRNA or p66Shc siRNA for 72 h or an empty vector or HA-p66Shc cDNA for 24 h. Cells were then seeded onto Boyden Chambers (8 μm pores) (Corning, New York) and incubated with or without EGF (10 ng/ml) for 6 h at 37 °C. Cells were fixed in 4% paraformaldehyde and stained with crystal violet for 16 h. Cells present in the upper chamber were removed with a cotton swab and the migrated cells, present in the lower chamber, were quantified by manual counting. Images were acquired using an epifluorescent inverted microscope (Carl Zeiss Axio Observer A1) with ZEN Pro 2011 software Blue edition.

**Statistical Analysis**—Statistical analysis was performed using either a one-way or two-way analysis of variance followed by a Bonferroni’s multiple comparison test using GraphPad Prism (version 5, San Diego, CA).

**RESULTS**

**p66Shc Modulates ARF1 Activation in Invasive Breast Cancer Cells**—Knowing that ARF1 is activated downstream of the EGFR, we sought to determine whether key adaptor proteins, such as Grb2 and Shc, may play a role in the recruitment of this GTPase to the activated receptor. Therefore, we first evaluated the expression levels of Grb2 and the three isoforms of ShcA (p46Shc, p52Shc, and p66Shc) in the non-invasive MCF7 cells (low EGFR, ARF1/ARF6-expressing) and the invasive MDA-MB-231 cell line (high EGFR, ARF1/ARF6-expressing) (Fig. 1A). Although we observed no significant difference in expression of Grb2, p46Shc, and p52Shc between the two cell types, p66Shc was found to be present only in the MDA-MB-231 cells. This is in accordance with previously published data highlighting an increased expression of p66Shc in this invasive breast cancer cell line (26). We next evaluated whether ARF1 could form a complex with p66Shc. As shown in Fig. 1B, ARF1 co-immunoprecipitated with p66Shc and this association was enhanced upon EGF stimulation. Additionally, we detected an association between ARF1 and Grb2 and p52Shc, but not p46Shc. These associations were also enhanced by EGF treatment. Because the function of p66Shc still remains ill-defined in breast cancer and this isoform is specifically expressed in MDA-MB-231 breast cancer cells, we further examined the role of this Shc isoform in the activation process of ARF proteins. Therefore, we next assessed the importance of p66Shc in the regulation of EGF-induced ARF1 activation. To do this, we measured the levels of GTP-bound ARF1 in EGF-stimulated MDA-MB-231 cells that were either transfected with scrambled or p66Shc siRNA. As shown in Fig. 1C, EGF induced the activation of ARF1 in cells transfected with a control siRNA. However, a significant increase in ARF1 activation was observed in cells where endogenous expression of p66Shc was reduced suggesting that this Shc isoform, in MDA-MB-231 cells, might act to limit ARF1 activation. For all experiments described in this study, we observed an average inhibition of p66Shc expression by 67%, when cells were transfected with a specific siRNA. To further evaluate the role of p66Shc, we next overexpressed an HA-tagged p66Shc in MDA-MB-231 cells. In these conditions, acti-
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**FIGURE 1.** p66Shc negatively regulates ARF1 activation. A, the endogenous expression of Shc isoforms (p46Shc, p52Shc, and p66Shc), Grb2, EGFR, ARF1, ARF6, and actin were measured by Western blot (IB, immunoblot) analysis of lysates obtained from either confluent MCF7 or MDA-MB-231 cells. B, endogenous ARF1 was immunoprecipitated from lysates obtained from serum-starved MDA-MDA-231 cells that were stimulated with EGF (10 ng/ml) for the given time points and associated Shc isoforms and Grb2 were detected by Western blot analysis. The upper arrow indicates the p66Shc isoform, the middle arrow the p52Shc, and the lower arrow the p46Shc. Results presented are representative of three independent experiments and the inputs represent 5% of the total protein present in each sample. C, MDA-MB-231 cells transfected with a scrambled (CTL) or p66Shc siRNA were stimulated with EGF (10 ng/ml) for the indicated times. Cells were lysed, and a GST pulldown assay using GST-GGA3 coupled to glutathione-Sepharose 4B beads was used to capture activated ARF1. Endogenous levels of activated ARF1 and the total protein levels of ARF1 in cell lysates were assessed by Western blot analysis. Additionally, Western blot analysis was used to confirm the depletion of p66Shc. Results presented are representative of three independent experiments and the inputs represent 5% of the total protein present in each sample. The quantifications of each experiment are presented as fold-increase over basal, and are normalized to total protein content and are the mean ± S.E. with (*) p < 0.05 and (**) p < 0.001, compared with the control condition. D, MDA-MB-231 cells transfected with an empty vector (CTL) or HA-p66Shc were stimulated with EGF (10 ng/ml) for the indicated times. Endogenous levels of activated ARF1 and total protein levels of ARF1 were captured and detected as in C. Western blot analysis was used to confirm the overexpression of HA-p66Shc. Results presented are representative of three independent experiments and the inputs represent 5% of the total protein present in each sample. The quantifications of each experiment are presented as fold-increase over basal, and are normalized to total protein content, and are the mean ± S.E. with (***p < 0.001. E, HCC70 cells were transfected and stimulated as in D. Endogenous levels of activated ARF1 and total protein levels of ARF1 were captured and detected as in C. Western blot analysis was used to confirm the overexpression of HA-p66Shc. Results presented are representative of three independent experiments and the inputs represent 5% of the total protein present in each sample. The quantifications of each experiment are presented as fold-increase over basal, and are normalized to total protein content, and are the mean ± S.E. with (***p < 0.001. Indeed, similar to what was observed in MDA-MB-231 cells, ARF1 activation was significantly reduced when p66Shc was overexpressed compared with control cells. In summary, high expression of p66Shc in invasive breast cancer cell models, such as HCC70, MDA-MB-231, and MCF7, results in decreased ARF1 activation. Further, the endogenous expression of p66Shc was significantly decreased (Fig. 1D), further supporting the role of p66Shc in controlling ARF1 activation downstream of the EGFR. Next, we confirmed our finding in another basal-like breast cancer cell model, the HCC70 cell line (Fig. 1E). Indirectly, similar to what was observed in MDA-MB-231 cells, ARF1 activation was significantly reduced when p66Shc was overexpressed compared with control cells. In summary, high expression of p66Shc in invasive breast cancer cell models, such as HCC70, MDA-MB-231, and MCF7, results in decreased ARF1 activation.
cells acts to negatively regulate ARF1 activation, upon EGF stimulation.

_p66Shc Regulates the Activation of the Ras/MAPK and AKT Pathways_—To further characterize the regulation of signaling cascades downstream of the EGFR, we next aimed to define the role of p66Shc on the regulation of ARF-dependent signals such as the PI 3-kinase and MAPK pathways (29). First, we determined whether p66Shc mediated the activation of another small GTPase known to be activated by the EGFR, Ras (36). To do this, we measured the levels of GTP-bound Ras in EGF-stimulated MDA-MB-231 cells that were either transfected with scrambled or p66Shc siRNA (Fig. 2A). Interestingly, knockdown of p66Shc significantly decreased Ras activation compared with control cells. Next, we compared the Ras activation profile to that of ERK1/2 activation. As shown in Fig. 2A, whereas the initial activation of ERK1/2 by EGF stimulation was diminished by depletion of p66Shc, no significant alterations in ERK activation were observed during later time points. These findings differ from previous observations in other cell types in which p66Shc has been shown to negatively regulate the activation of both Ras and ERK1/2 (16, 19, 37, 38). No significant effects on AKT phosphorylation were observed in p66Shc knockdown cells. To further evaluate the role of p66Shc in regulation Ras/MAPK activation in MDA-MB-231 cells, we next overexpressed a HA-tagged p66Shc in these cells. Surprisingly, overexpression of p66Shc resulted in a significant increase in the basal activation of both Ras and ERK1/2 (Fig. 2B). However, a significant decrease in Ras and MAPK activation that is consistent with previous reports was observed at later time points (16, 19, 37, 38). Additionally, we observed a delay in the phosphorylation of AKT in cells overexpressing p66Shc compared with control cells. Together, our results demonstrate that p66Shc mediates important signaling cascades known to be regulated by ARF proteins.

_p66Shc Mediates Breast Cancer Cell Growth and Migration_—Because ARF1 and p66Shc have been previously reported to mediate cell growth and migration (29, 37, 39, 40), we next evaluated the physiological role of p66Shc in MDA-MB-231 cells. First, we evaluated the importance of p66Shc expression in cell proliferation using a cell counting assay. As shown in Fig. 3A, knockdown of p66Shc, by siRNA significantly reduced both the basal and EGF-mediated growth rate of MDA-MB-231 cells at both 48 and 72 h compared with the control scrambled siRNA-transfected cells. Next, we overexpressed p66Shc in MDA-MB-231 cells and evaluated cell growth at 24, 48, and 72 h (Fig. 3B). A significant decrease in basal and EGF-dependent cell growth was observed in cells overexpressing HA-p66Shc for all tested time points. More interestingly, the EGF-independent cell number of HA-p66Shc overexpressing cells was found to be lower than the cell number originally seeded. This would suggest that overexpression of p66Shc may induce MDA-MB-231 cell death. We further confirmed the above regulation of cell growth using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell viability assay. Although no significant difference in basal cell growth was observed in p66Shc siRNA-transfected cells compared with control, EGF-induced cell growth was significantly reduced upon p66Shc knockdown (Fig. 3C). Additionally, we also observed a decrease in both basal and EGF-dependent proliferation in HA-p66Shc overexpressing conditions (Fig. 3D). Together, our results show that whereas p66Shc plays an important role in breast cancer cell growth, elevated expression of p66Shc may promote cell death.

Next, we examined the role of p66Shc in cellular migration using Boyden chambers. As illustrated in Fig. 3E, EGF induced the migration of control MDA-MB-231 cells. However, EGF-mediated migration was significantly reduced upon the depletion of p66Shc. Furthermore, we were able to enhance the basal, but not EGF-dependent, migration of MDA-MB-231 cells by overexpressing p66Shc (Fig. 3E). Together, our results demonstrate that p66Shc is an important mediator of invasive breast cancer cell growth and migration.

_p66Shc Regulates ARF1 Activity, Cell Growth, and Migration of HER2-positive Breast Cancer Cells_—Thus far, we have demonstrated that p66Shc is an important regulator of the growth and migration of triple negative breast cancer cells. Moreover, we show that p66Shc is an important mediator of signaling events downstream of the EGFR such as ARF1 and Ras/MAPK activation and AKT phosphorylation. However, triple negative breast cancer represents only ~15% of all breast cancer cases (41). Therefore, we next evaluated the role of p66Shc in a more prominent cellular model of HER2 positive breast cancer, the SKBR3 cell line. In fact, approximately, 20–40% of breast cancer patients have an amplified HER2 receptor expression (42). First, we assessed the expression of p66Shc in SKBR3 cells (HER2 and EGFR positive) compared with MDA-MB-231 cells (HER2 negative and EGFR positive). As shown in Fig. 4A, MDA-MB-231 cells express high levels of p66Shc compared with SKBR3 cells. These findings are in accordance with previously published observations showing that p66Shc expression is negatively correlated with expression of the HER2 receptor (43). Furthermore, we previously reported that SKBR3 cells were shown to have a higher expression level of ARF1 compared with MDA-MB-231 cells (29). Comparable expression of EGFR, Grb2, and ARF6 were observed for both cell lines. Next, we examined the effect of overexpressing HA-tagged p66Shc on ARF1 activation in this cell type (Fig. 4B). In control cells, EGF stimulation induced the activation of ARF1. Interestingly, overexpression of p66Shc was shown to also reduce the activation of ARF1 further emphasizing the role of p66Shc as a negative regulator of ARF1 activation. Knowing that p66Shc influenced ARF1 activation in these cells and that we previously found that the proliferation and migration of SKBR3 cells was dependent on ARF1 expression (29), we next assessed the physiological role of p66Shc in this HER2 positive background. First, we evaluated its regulation of SKBR3 cell growth. As shown in Fig. 4D, EGF stimulation of control cells significantly promoted proliferation. Whereas, cells overexpressing p66Shc were shown to have an increased growth rate after 24 h. However, a significant decrease in both basal and EGF-dependent cellular growth was observed by 72 h. Second, we assessed the migration of mock-transfected or p66Shc overexpressing SKBR3 cells in the absence and presence of EGF stimulation (Fig. 4E). EGF was shown to enhance the migration of control SKBR3 cells. Interestingly, both EGF-independent and -dependent migration was enhanced in cells overexpressing p66Shc.
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A

MDA-MB-231

| EGF | 0 | 1 | 2 | 5 | 15 | 30 | 60 | 0 | 1 | 2 | 5 | 15 | 30 | 60 |
|-----|---|---|---|---|----|----|----|---|---|---|---|----|----|----|
| IB H-Ras-GTP | | | | | | | | | | | | | | |
| IB pErk1/2 | | | | | | | | | | | | | | |
| IB pAkt | | | | | | | | | | | | | | |
| IB H-Ras (Input) | | | | | | | | | | | | | | |
| IB Erk1/2 (Input) | | | | | | | | | | | | | | |
| IB Akt (Input) | | | | | | | | | | | | | | |
| IB p66Shc (Input) | | | | | | | | | | | | | | |

B

MDA-MB-231

| EGF | 0 | 1 | 2 | 5 | 15 | 30 | 60 | 0 | 1 | 2 | 5 | 15 | 30 | 60 |
|-----|---|---|---|---|----|----|----|---|---|---|---|----|----|----|
| IB H-Ras-GTP | | | | | | | | | | | | | | |
| IB pErk1/2 | | | | | | | | | | | | | | |
| IB pAkt | | | | | | | | | | | | | | |
| IB H-Ras (Input) | | | | | | | | | | | | | | |
| IB Erk1/2 (Input) | | | | | | | | | | | | | | |
| IB Akt (Input) | | | | | | | | | | | | | | |
| IB HA-Tag (Input) | | | | | | | | | | | | | | |
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FIGURE 3. p66Shc mediates MDA-MB-231 cell growth and migration. A, MDA-MB-231 cells transfected with a scrambled (CTL) or p66Shc siRNA were left untreated or stimulated with EGF (10 ng/ml) for the indicated times. Cell numbers were determined by a trypan blue exclusion assay via manual counting. Results presented are representative of three independent experiments. The quantifications of each experiment are presented as fold-increase in absorbance over basal absorbance and are the mean ± S.E. with (*) p < 0.05 and (**) p < 0.001. B, MDA-MB-231 cells transfected with an empty vector (CTL) or HA-p66Shc were left untreated or stimulated with EGF (10 ng/ml) for the indicated times. Cell numbers were determined as in A. The quantifications of each experiment are presented as fold-increase over basal and are the mean ± S.E. with (***) p < 0.001. C, MDA-MB-231 cells were transfected and stimulated as in A. Cell growth at 72 h was determined via a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described under “Experimental Procedures.” The quantifications of each experiment are presented as fold-increase in absorbance over basal absorbance and are the mean ± S.E. with (***) p < 0.001. D, MDA-MB-231 cells were transfected and stimulated as in B. Cell growth was determined as in C. The quantifications of each experiment are presented as fold-increase in absorbance over basal absorbance and are the mean ± S.E. with (****) p < 0.001 and (*****)) p < 0.001. E, MDA-MB-231 cells were transfected as in A. Cells were then seeded onto Boyden chambers and stimulated or not with EGF (10 ng/ml). Migration was assessed after 6 h. Results presented are representative of three independent experiments. The quantifications of each experiment are presented as fold-increase over basal and are the mean ± S.E. with (****) p < 0.001. F, MDA-MB-231 cells were transfected as in B and migration was assessed as described in E. Results presented are representative of three independent experiments. The quantifications of each experiment are presented as fold-increase over basal and are the mean ± S.E. with (****) p < 0.001. The depletion of p66Shc and the overexpression of HA-tagged p66Shc was confirmed by Western blot analysis for all physiological assays. IB, immunoblot.
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FIGURE 4. Signals downstream of the HER2 receptor are mediated by p66Shc. A, the endogenous expression of p66Shc, Grb2, EGFR HER2, ARF1, ARF6, and actin were measured by Western blot (IB, immunoblot) analysis of lysates obtained from either confluent SKBR3 or MDA-MB-231 cells. B, SKBR3 cells transfected with an empty vector (CTL) or HA-p66Shc were stimulated with EGF (10 ng/ml) for the indicated times. Cells were lysed, and a GST pulldown assay using GST-GGA3 coupled to glutathione-Sepharose 4B beads was used to capture activated ARF1. Endogenous levels of activated ARF1 and the total protein levels of ARF1 in cell lysates were assessed by Western blot analysis. Additionally, Western blot analysis was used to confirm the overexpression of HA-p66Shc. Results presented are representative of three independent experiments and the inputs represent 5% of the total protein present in each sample. The quantifications of each experiment are presented as fold-increase over basal, are normalized to total protein content, and are the mean ± S.E. with (**) p < 0.01. C, SKBR3 cells transfected as in A were left untreated or stimulated with EGF (10 ng/ml) for the indicated times. Cell numbers were determined by a trypan blue exclusion assays via manual counting. Results presented are representative of three independent experiments. The quantifications of each experiment are presented as fold-increase over basal and are the mean ± S.E. with (***)) p < 0.001. D, SKBR3 cells were transfected as in A. Cells were then seeded onto Boyden chambers and stimulated or not with EGF (10 ng/ml). Migration was assessed after 6 h. Results presented are representative of three independent experiments. The quantifications of each experiment are presented as fold-increase over basal and are the mean ± S.E. with (*) p < 0.05 and (**) p < 0.01.

Together, our results demonstrated that whereas p66Shc negatively regulated EGF-mediated signaling cascades such as ARF1 activation and cellular growth in HER2 positive breast cancer cells, it significantly enhanced cellular migration. These findings would suggest that p66Shc would be positively acting on other key proteins mediating this important cellular response.

p66Shc Attenuates the Recruitment of ARF1 to the EGFR—Having characterized the role of p66Shc in mediating ARF1 activation, cell growth, and cell migration in both a cellular model of triple negative and HER2 positive breast cancer, we next sought to delineate the mechanism via which p66Shc regulated ARF1 activation. Because p66Shc is endogenously expressed and ARF1 activation has been previously described in MDA-MB-231 cells, we used this cellular model to characterize the mechanism of negative regulation of ARF1 activity by p66Shc. First, we asked whether this ARF isoform was recruited to the EGFR in p66Shc-depleted cells (Fig. 5A). To do this, we immunoprecipitated EGFR from lysates of cells either transfected with control scrambled or p66Shc siRNA and immunodetected associated ARF1 using specific antibodies. EGF induced the recruitment of ARF1 to the EGFR in control siRNA cells. However, knockdown of p66Shc resulted in an increased EGFR recruitment of ARF1 suggesting that p66Shc blocks ARF1 receptor recruitment. To further confirm these results, we either mock transfected MDA-MB-231 cells or overexpressed HA-tagged p66Shc. As shown in Fig. 5B, the overexpression of p66Shc significantly attenuated the EGFR recruitment of ARF1 compared with the mock transfected condition. Together, our findings suggest that p66Shc attenuates ARF1 activation by blocking the recruitment of this GTPase to the EGFR.

Recruitment of the Grb2-ARF1 Complex to the EGFR Is Attenuated by p66Shc—It has been previously demonstrated that p66Shc can block the recruitment of Grb2 to the EGFR and insulin-like growth factor receptor leading to decreased receptor signaling (18, 19). Therefore, we next evaluated whether in our cellular model p66Shc could block the recruitment of Grb2 to the EGFR thereby impacting the recruitment and activation of ARF1. In MDA-MB-231 cells transfected with a control siRNA or p66Shc-specific siRNA, we immunoprecipitated the EGFR and examined Grb2 (Fig. 6A). As expected Grb2 was recruited to the EGFR upon its activation in control cells. However, the recruitment of Grb2 to the EGFR was enhanced upon...
Grb2 Is Essential for the Recruitment of ARF1 to the EGFR and Activation of GTPase—With evidence demonstrating that p66Shc blocks the recruitment of Grb2 to the EGFR, we next examined the influence of p66Shc on association between ARF1 and Grb2. Here, we immunoprecipitated ARF1 from lysates obtained from either scrambled or p66Shc siRNA-transfected cells and measured the level of associated Grb2 (Fig. 7A). Interestingly, knockdown of p66Shc resulted in an increased association between Grb2 and ARF1 compared with control cells. Alternatively, we next determined the influence of Grb2 on interaction between p66Shc and ARF1. To do this, we immunoprecipitated ARF1 from lysates obtained from cells depleted of Grb2. As shown in Fig. 7B, the association between p66Shc and ARF1 was decreased in cells depleted of this adaptor. For this study, the average percent inhibition of Grb2 expression of siRNA was 95%. Together, our results suggest that regulation of ARF1 activation by p66Shc may stem from its negative effect on the Grb2/ARF1 interaction. Our results further suggest that the formation of a complex between p66Shc and ARF1 may occur indirectly, via the adaptor Grb2.

Subsequently, we sought to determine whether Grb2 was essential for ARF1 activation and the recruitment of this GTPase to the EGFR. As shown in Fig. 7C, depletion of Grb2 significantly suppressed EGF-induced ARF1 activation compared with control conditions further suggesting that p66Shc may inhibit ARF1 activation by blocking the actions of Grb2.

Next, we determined whether Grb2 was required for the recruitment of ARF1 to the EGFR. To do this, we immunoprecipitated the EGFR from cells either transfected with scrambled or Grb2 siRNA and immunodetected the presence of ARF1. As shown in Fig. 7D, EGF-induced recruitment of ARF1 to the EGFR in control conditions. However, the recruitment of this ARF isoform to the receptor was significantly blocked in cells depleted of Grb2. Together, these findings highlight the importance of Grb2 in receptor recruitment and activation of ARF1.

ARF6 Activation and Its Recruitment to the EGFR Is Potentiated by p66Shc—Because ARF6 has also been shown to be activated downstream of the EGFR (29, 31), we next examined whether p66Shc could regulate ARF6 activation in highly invasive breast cancer cells. First, EGF stimulation promoted GTP loading on this other ARF isoform in control MDA-MB-231 cells (Fig. 8A). Although, knockdown of p66Shc expression attenuated ARF6 activation. The contribution of p66Shc in ARF6 activation was confirmed by overexpressing HA-tagged p66Shc. As illustrated in Fig. 8B, overexpression of p66Shc increased ARF6 activation. In fact, the potentiated activation of ARF6 was shown to be independent of EGF stimulation as observed by a significant increase in ARF6 activation at the basal level that was not altered upon EGF stimulation. Next, we examined the role of p66Shc in mediating ARF6 activation in the HER2 positive breast cancer SKBR3 cell line (Fig. 8C). Similar to what was observed in MDA-MB-231 cells, a significant increase in basal ARF6 activation was observed in SKBR3 cells overexpressing HA-p66Shc compared with control conditions. Together, our results demonstrate that activation of both ARF1 and ARF6 are regulated by p66Shc. However, whereas p66Shc blocked both the basal and EGF-dependent activation of ARF1, it significantly increased EGF-independent ARF6 activation.
We next examined the role of p66Shc in the recruitment of ARF6 to the EGFR. As seen in Fig. 8D, EGFR stimulation of control cells induced the recruitment of this ARF isoform to the EGFR. However, knockdown of p66Shc expression reduced the ability of this ARF isoform to associate with the receptor. In contrast, overexpression of p66Shc resulted in an increase in ARF6 recruitment to the EGFR.
increased recruitment of ARF6 to the EGFR (Fig. 8E). This recruitment to the EGFR, in cells overexpressing p66Shc, was shown to be independent of EGF stimulation as an increased association with the EGFR was equally observed in untreated and EGF-treated cells. Collectively, our results illustrate that whereas p66Shc attenuates ARF1 activation by blocking its recruitment to the EGFR, ARF6 activation and recruitment to the activated receptor is dependent on p66Shc.

**Grb2 Is Required for Activation of ARF6 and Its Recruitment to the EGFR**—We next examined whether Grb2 was also required for ARF6 activation. Interestingly, similar to what was observed for ARF1, the depletion of Grb2 was associated with a decreased activation of ARF6 (Fig. 9A), suggesting that the activation process of both ARF isoforms requires this adaptor. Last, we evaluated the role of Grb2 in recruitment of ARF6 to the EGFR. Once again, as observed for ARF1, depletion of Grb2 was associated with a diminished association between the receptor and ARF6 (Fig. 9B). These data show that Grb2 is essential for activation and EGFR recruitment of both ARF isoforms. To further define the role of p66Shc in the activation process of ARF6, we examined its ability to regulate the Grb2/ARF6 interaction. As illustrated in Fig. 9C, depletion of p66Shc blocked the ability of ARF6 to associate with Grb2 suggesting that Grb2-dependent recruitment of ARF6 to the EGFR may be acting through p66Shc. Together, our results suggest that Grb2 is required for activation and receptor recruitment of ARF6 and that this small GTP-binding protein is in complex with Grb2 via its association with p66Shc.

 Altogether, these results demonstrate that the adaptor proteins p66Shc and Grb2 regulate both ARF1 and ARF6 activation. We show that ARF1 is recruited to the EGFR and activated through its association with Grb2. Furthermore, we demonstrate that p66Shc decreases the activation of ARF1 by blocking
recruitment of the Grb2-ARF1 complex to the EGFR. Additionally, we demonstrate that ARF6 activation and EGFR recruitment are dependent on both p66Shc and Grb2, where in which ARF6 is recruited to the EGFR via Grb2 by means of its association with p66Shc.

**DISCUSSION**

Because ARF1 and ARF6 are small GTPases activated downstream of the EGFR in invasive breast cancer cells, we aimed at defining the molecular mechanisms by which EGF stimulation leads to their activation. Classically, stimulation of the EGFR by...
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FIGURE 9. Grb2 is also essential for ARF1 activation and its recruitment to the EGFR. A, MDA-MB-231 cells transfected with a scrambled (CTL) or Grb2 siRNA were stimulated with EGF (10 ng/ml) for the indicated times. Cells were lysed, and a GST pulldown assay using GST-GGA3 coupled to glutathione-Sepharose 4B beads was used to capture activated ARF6. Endogenous levels of activated ARF6 and the levels of ARF6 protein in total cell lysates were assessed by Western blot (IB, immunoblot) analysis. Additionally, Western blot analysis was used to confirm the depletion of Grb2. Results presented are representative of three independent experiments and the inputs represent 5% of the total protein present in each sample. The quantifications of each experiment are presented as fold-increase over basal, are normalized to total protein content, and are the mean ± S.E. with (*) \( p < 0.05 \) and (**) \( p < 0.001 \). B, MDA-MB-231 cells were transfected and stimulated as in A. Cells were lysed and endogenous EGFR was immunoprecipitated (IP). Associated ARF6 was detected by Western blot analysis. Western blot analysis was also used to confirm the protein expression of ARF6 and EGFR as well as the depletion of Grb2. Results presented are representative of three independent experiments and the inputs represent 5% of the total protein present in each sample. The quantifications of each experiment are presented as fold-increase over basal, are normalized to total protein content, and are the mean ± S.E. with (*) \( p < 0.05 \) and (**) \( p < 0.001 \). C, MDA-MB-231 cells transfected with a scrambled (CTL) or p66Shc siRNA were stimulated with EGF (10 ng/ml) for the indicated times. Cells were lysed and endogenous ARF6 was immunoprecipitated. Associated p66Shc was detected by Western blot analysis. Western blot analysis was also used to confirm the protein expression of p66Shc and ARF6 as well as the depletion of p66Shc. Results presented are representative of three independent experiments and the inputs represent 5% of the total protein present in each sample. The quantifications of each experiment are presented as fold-increase over basal, are normalized to total protein content, and are the mean ± S.E. with (*** \( p < 0.001 \).
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was a downstream effector of ARF1 signals in MDA-MB-231 cells and the overexpression of a constitutively active form of Rac1 could reverse the inhibitory effect of ARF1 depletion on cell migration (50). Therefore, p66Shc may promote cell migration via the activation of Rac1. Together, our findings demonstrate that p66Shc is a key regulator of breast cancer cell growth and migration and may play an important role in breast cancer progression.

The majority of mechanistic studies have examined the signaling role of p52Shc. Upon EGFR stimulation, this Shc isoform is recruited to the receptor and assembled into a complex with Grb2 (51–53). However, p66Shc has also been reported to be recruited to both EGFR and Grb2 (16). In smooth muscle cells, p66Shc sequesters Grb2 away from the insulin-like growth factor receptor and EGFR blocking the activation of downstream signals (18, 54). Here, we show that p66Shc can also block the recruitment of Grb2 to the EGFR in invasive breast cancer cells. Additionally, unlike p46Shc and p52Shc, which activate ERK1/2 when overexpressed in HeLa cells, the overexpression of p66Shc has been shown to have little effect on ERK1/2 activation (16). Meanwhile, p66Shc was shown to block ERK1/2 activation in a variety of other cell lines such as mouse renal proximal tube cells, mouse splenic T cells, and porcine smooth muscle cells (18, 47, 54). In MDA-MB-231 cells, overexpression of p66Shc potentiated the basal ERK1/2 phosphorylation, a process we and others have shown to be mediated by ARF6 (29, 31). Furthermore, this increase in basal ERK activation correlated with an increase in Ras activation. We therefore propose that this increase in Ras/ERK activation observed in p66Shc overexpressing cells stems from an increased ARF6 activation. Interestingly, overexpression of p66Shc was shown to block the activation of both Ras and ERK1/2 following prolonged stimulation with EGF (15–60 min). Our findings also show that p66Shc can negatively regulate AKT phosphorylation, an effect previously described in a variety of cell types (55, 56). This attenuation of AKT phosphorylation has been associated with the apoptotic properties of p66Shc (56), thus suggesting a possible role for p66Shc in mediating MDA-MB-231 cell survival. Together, our data reveals a mechanism through which p66Shc attenuates ARF1/AKT activation by blocking the recruitment of Grb2 to the EGFR and potentiates basal ARF6/Ras/ERK activation by promoting the receptor recruitment of ARF6.

Similar to p66Shc, the role of the adaptor Grb2 in breast cancer is ill-defined. The expression of Grb2 has been shown to be elevated in primary breast tumors (57) as well as in estrogen receptor positive breast cancer cells (58). In our experiments, we observed a slightly higher expression of Grb2 in MCF7 cells (prototypical estrogen receptor positive cell line) compared with the MDA-MB-231 cells (prototypical triple negative cell line). Although it has previously been reported that overexpression of this adaptor alone is insufficient to transform cells, Grb2 is well known to promote the activation of the Ras/MAPK pathway (59–61). Furthermore, delayed Poly Middle T Antigen-induced mammary tumor formation was observed in Grb2 knock-out mice suggesting a role for this adaptor in mammary tumorigenesis (62). Here, we show that Grb2 is essential for the activation of both ARF1 and ARF6 and thus may contribute in mediating the cellular responses associated with activation of this GTPase: proliferation, migration, and invasion.

It was proposed that GEP100 (BRAG2), an ARF GEF, could directly interact with the phosphorylated Tyr-1068 and Tyr-1086 residues on the EGFR through its PH domain and therefore act as an intermediate mediating ARF activation following EGF stimulation (32). Interestingly, we show that overexpression of p66Shc enhances total phosphorylation of the EGFR, including residues Tyr-1068 and Tyr-1086 (data not shown). This suggests that p66Shc may enhance ARF6 activation by potentiating the tyrosine phosphorylation of the EGFR. The conclusions demonstrated by Morishige and colleagues (32) were obtained from in vitro evidences, where phosphopeptides that mimicked residues Tyr-1068 and Tyr-1086 of EGFR could directly interact with the PH domain of GEP100 (63). In vivo, these two phosphorylated residues have been characterized as Grb2 binding sites (64, 65). Here, we show that classical EGFR adaptors do play an important role in the activation process of ARF1 and ARF6 similar to what has been reported for other GEFs. Namely, Grb2 is well known to interact with the GEF Sos to promote its recruitment to the EGFR (66, 67). Through its PH domain, Sos interacts with the plasma membrane, where it activates the small GTPase Ras (67, 68). Interestingly, we show here that p66Shc also plays a role in mediating Ras activation, possibly by regulating the recruitment of a GEF. Additionally, Grb2 has also been reported to recruit another PH domain containing GEF, Vav2, to HER2 to promote activation of both Ras and Rac1 (69). Together, this suggests that Grb2 may play a conserved role in the recruitment of GEFs to the EGFR. Furthermore, p66Shc may also modulate ARF1 activation selectively by either blocking the recruitment of ARF1/GEFs or promoting the recruitment of ARF6/GEFs. Additionally, p66Shc may function to regulate the association of ARF GTPases with their GEFs. This adaptor may promote ARF6/GEF interactions and dissolve ARF1/GEF interactions. Altogether, the recruitment of GEFs and ARF activation may be mediated by both EGFR tyrosine phosphorylation and adaptor recruitment.

Altogether, we demonstrate a role for adaptor proteins p66Shc and Grb2 in mediating EGF-induced ARF1 and ARF6 activation, as well as their recruitment to the EGFR. More specifically, whereas we demonstrate that p66Shc negatively regulates ARF1 signals, ARF6 activation was potentiated by this adaptor. Furthermore, we show that the adaptor Grb2 plays an essential role in the activation of both ARF1 and ARF6. We propose that p66Shc blocks recruitment of the ARF1-Grb2 complex to the EGFR by either competing with Grb2 for common recruitment sites on the receptor or by sequestering Grb2 away from it. This could allow for an increased recruitment of p66Shc/ARF6 to the EGFR and increased ARF6 activation. Based on our findings, we propose the following model of ARF activation in MDA-MB-231 cells (Fig. 10). (a) When the expression levels of Grb2 and p66Shc are in equilibrium, ligand binding induces the activation of the EGFR leading to its auto-phosphorylation. This first event allows recruitment of the adaptor Grb2. Grb2 acts to recruit ARF1 to the EGFR where it becomes activated by a guanine nucleotide exchange factor leading to activation of the PI3K/AKT pathway. Grb2 also recruits ARF6 to the EGFR, via p66Shc, resulting in its activa-
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FIGURE 10. Model of ARF1 and ARF6 activation downstream of the EGFR in MDA-MB-231 cells. A, in normal conditions, the expression of the adaptors p66Shc and Grb2 is at equilibrium. Upon activation of the EGFR, Grb2 is recruited to the EGFR. This leads to the recruitment of ARF1 and the activation of this GTPase. Furthermore, Grb2 recruits the p66Shc-ARF6 complex to the EGFR leading to the activation of ARF6. B, the depletion of p66Shc is associated with an increased recruitment of Grb2 and ARF1 to the EGFR and an increase in ARF1 activation. Alternatively, a decrease in ARF6 activation stemming from a decreased EGFR recruitment of this GTPase was observed upon the depletion of p66Shc. C, when p66Shc levels are elevated, the recruitment of Grb2 and ARF1 to the EGFR is blocked and the activation of ARF1 is significantly decreased. Additionally, elevated p66Shc levels increased the recruitment of ARF6 to the EGFR resulting in an increase in ARF6 activation. D, the depletion of Grb2 blocks the recruitment of ARF1 to the EGFR and blocks ARF1 activation. In these conditions, p66Shc is no longer recruited to the EGFR. This leads to the attenuation of ARF6 activation and recruitment to the EGFR.

In conclusion, we demonstrate for the first time the importance and engagement of the Ras/ERK1/2 pathway. Additionally, p66Shc acts as a negative regulator of ARF1 activation. Together these two pathways promote breast cancer cell growth and migration. (b) In conditions where levels of p66Shc are reduced, ARF6 is no longer recruited to Grb2 or the EGFR, thus blocking the activation of both ARF6 and Ras/MAPK. In turn, an enhanced recruitment of the Grb2-ARF1 complex to the EGFR is observed promoting ARF1 activation. Together, this leads to a reduction in cell growth and migration. (c) Upon overexpression of p66Shc, Grb2 and ARF1 are no longer recruited to the EGFR leading to a diminished ARF1 activation and a delayed phosphorylation of AKT. Conversely, receptor recruitment and activation of ARF6 are potentiated resulting in a basal increase of Ras/MAPK activation, migration, and the induction of cell death. (d) When Grb2 levels are depleted, ARF1 and the p66Shc-ARF6 complex cannot be recruited to the EGFR and thus neither GTPase are activated.

In conclusion, we demonstrate for the first time the importance of adaptor proteins in the regulation of ARF activity in invasive breast cancer cells. More importantly, we demonstrate that certain adaptors (Grb2) can have similar effects on the activation of different ARF isoforms and others (p66Shc) can have opposing effects. Thus, characterization of the signaling mechanisms leading to breast cancer cell proliferation, migration, and invasion can help discover more specific and effective therapeutic targets.

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