Regulator of G protein signaling 6 is a novel suppressor of breast tumor initiation and progression

Biswanath Maity1, Adele Stewart1, Yunxia O’Malley3, Ryan W.Askeland3, Sonia L.Sugg2 and Rory A.Fisher1,8

1Department of Pharmacology, 2Department of Surgery and 3Department of Pathology, University of Iowa Carver College of Medicine, Iowa City, IA 52242, USA

*To whom correspondence should be addressed. Tel: +1 319 335 8330; Fax: +1 319 335 8930; Email: rory-fisher@uiowa.edu

Breast cancer is a large global health burden and the most frequently diagnosed malignancy in women worldwide. Here, we utilize RGS6−/− mice to interrogate the role of regulator of G protein signaling 6 (RGS6), localized to the ductal epithelium in mouse and human breast, as a novel tumor suppressor in vivo. RGS6−/− mice exhibit accelerated 7,12-dimethylbenz[a]anthracene (DMBA)-induced tumor initiation and progression, as well as decreased overall survival. Analysis of carcinogenic aberrations in the mammary glands of DMBA-treated mice revealed a failure of the DNA damage response concurrent with augmented oncogenesis in RGS6−/− animals. Furthermore, RGS6 suppressed cell growth induced by either human epidermal growth factor receptor 2 or estrogen receptor activation in both MCF-7 breast cancer cells and mammary epithelial cells (MECs). MECs isolated from RGS6−/− mice also showed a deficit in DMBA-induced ATM/p53 activation, reactive oxygen species generation and apoptosis confirming that RGS6 is required for effective activation of the DNA damage response in these cells, a critical countermeasure against carcinogen-mediated genotoxic stress. The ability of RGS6 to simultaneously enhance DNA-damage-induced apoptotic signaling and suppress oncogenic cell growth likely underlie the accelerated tumorigenesis and cellular transformation observed in DMBA-treated RGS6−/− mice and isolated MECs, respectively. Unsurprisingly, spontaneous tumor formation was also seen in old female RGS6−/− but not in wild-type mice. Our finding that RGS6 is downregulated in all human breast cancer subtypes independent of their molecular classification indicates that obtaining a means to restore the growth suppressive and pro-apoptotic actions of RGS6 in breast might be a viable means to treat a large spectrum of breast tumors.

Introduction

Current treatment regimens for breast cancer patients combine surgical tumor resection, chemotherapy, radiation and targeted adjuvants depending on the expression of the hormone receptors for estrogen (ER) and progesterone (PR) and the human epidermal growth factor receptor 2 (HER2). This molecular subclassification often predicts clinical outcome and responsiveness to therapy, but it is becoming abundantly clear that breast cancer is a heterogeneous disease with multiple molecular pathways controlling tumor initiation, growth and metastasis (1). Insufficient understanding of the pathogenic mechanisms underlying breast carcinogenesis remains a critical barrier to its early detection and treatment. Both mutational activation of oncogenic proteins and inactivation of tumor-suppressor genes can occur in breast cancer and often lead to aberrant cell growth and failure of programmed cell death (2,3). Perhaps the best characterized is mutation or loss of the tumor suppressor p53, which occurs in an estimated 26% of breast tumors (4) and correlates with tumor grade and patient outcome (5). Despite the ever increasing number of approved pharmacuetics, breast cancer remains the leading cause of cancer death for women worldwide underscoring the need for novel therapeutics designed to treat or prevent this disease (6).

The regulator of G protein signaling (RGS) family of proteins function as guanosine triphosphatase-accelerating proteins for heterotrimeric G proteins, accelerating termination of G-protein-coupled receptor (GPCR) signaling through stabilization of the transition state in guanosine triphosphate hydrolysis by Gα (7,8). Based on their canonical function as key negative regulators of GPCRs and the numerous studies outlining the pivotal role for GPCRs in cancer development and metastasis (9), it is surprising that little is known about potential roles for RGS proteins in carcinogenesis. RGS6 is a member of the R7 subfamily of RGS proteins, which, in addition to the RGS domain conferring their guanosine triphosphatase-accelerating protein activity, contain two additional semiconserved regions, the DEP (dished, EGL-10, pleckstrin homology) domain and GGL (Gy subunit like) domain, necessary for interaction with R7 family binding protein and the atypical G protein Gβ4, respectively (10,11). Gβ4 is required for R7 family protein stability as knockout of Gβ4 results in systemic ablation of all R7 family members including RGS6 (12). A link between RGS6 and cancer was first suggested when a single-nucleotide polymorphism was identified in the RGS6 gene associated with decreased risk of bladder cancer, especially in smokers (13). RGS6 has also been shown to interact indirectly with the DNA methyltransferase, Dnmt1 (14), which is emerging as a novel oncogene that suppresses the transcription of pro-apoptotic genes (15–17).

Recent studies in our laboratory have demonstrated that RGS6 is expressed in the ductal epithelium of human breast and downregulated in high-grade breast tumors with loss of RGS6 expression correlating with increasing tumor grade. Exogenous expression of RGS6 in breast cancer cell lines resulted in impressive growth arrest and blockade of colony formation in addition to activation of the intrinsic mitochondrial apoptosis pathway. The ability of RGS6 to promote cell death required reactive oxygen species (ROS) generation but was independent of RGS6’s guanosine triphosphatase-accelerating protein activity, suggesting an entirely novel function for an RGS protein in cellular apoptotic signaling (18). In a subsequent study, we found that doxorubicin (Dox), one of the most effective and widely used chemotherapeutic agents in the treatment of breast cancer, induces RGS6 in cancer cells and that RGS6 is absolutely required for the cytotoxic actions of this drug in vitro and in vivo. Cells lacking RGS6 were completely protected from Dox-induced activation of ATM/p53 and subsequent apoptosis. Interestingly, the ability of Dox to promote phosphorylation of ATM and p53 relied primarily on RGS6-mediated ROS generation and not DNA damage (19). Clearly, RGS6-mediated ROS production represents a powerful means to induce cell death and growth arrest in normal and cancerous cells.

Based on these studies, we hypothesized that RGS6 is a previously unrecognized tumor suppressor in breast, where it functions to negatively regulate tumorigenesis by facilitating activation of apoptotic signaling and suppressing cellular growth and transformation in response to genotoxic and oncogenic stress. Here, we provide striking evidence for RGS6’s tumor-suppressor actions in an in vivo mouse model of breast carcinogenesis.
Materials and methods

Antibodies
RGS6-specific antibody was developed in our laboratory. Gβ6, RGS7, RGS11 and RGS9-1 antibodies were generously provided to us by Dr Jason Chen, Virginia Commonwealth University. Antibodies for phospho-Akt (S473), Cyclin D1, p21/waf1 and phospho-extracellular signal-regulated kinase (ERK)1/2, ERK1/2, phospho-p53(S15) and phospho-ATM(S1981) were from Cell Signaling Technology, p53 antibody was from Santa Cruz Biotechnology and β-Actin antibody was from Sigma (St Louis, MO).

Mice
We generated RGS6−/− mice as described previously (20). Wild-type (WT) and RGS6−/− mice were backcrossed onto a C57BL6 background for five generations. All animal experiments were performed in agreement with the Guide for the Use and Care of Laboratory Animals.

7,12-Dimethylbenz(a)anthracene-induced tumor generation
18 WT and 21 RGS6−/− litter-matched virgin female mice were employed to generate mammary tumors using the mammary-specific carcinogen 7,12-dimethylbenz(a)anthracene (DMBA, Sigma). Animals of each genotype received either DMBA (1 mg/20 g body weight) or vehicle control by oral gavage once a week for 5 consecutive weeks beginning at 6 weeks of age. Mice were then maintained continuously to provide an oscillating hormonal environment. The DMBA-treated group was compared to a WT for mammary tumor generation by palpation and the latency for tumor formation and progression to 0.5 cm diameter recorded. DMBA-treated WT and RGS6−/− mice were killed when the mammary tumors reached a 0.5 cm diameter. Tumor laden mammary tissues and normal age-matched controls were excised, fixed using 4% paraformaldehyde and processed into 5 μm paraffin-embedded sections for histological and immunohistochemical studies at the University of Iowa Central Microscopy Research Facility. The remaining tissue was frozen in liquid nitrogen and stored at −80°C.

Cell culture and transfection
MCF-7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified 5% CO2 atmosphere. Cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol essentially as we described previously with control or RGS6-specific small-hairpin interefering RNA (RNAi) subcloned into pSUPER.neo eGFP encoding expression vectors (19). RNAi target sequences are as follows: Control 5′-CCCCACGTAGAAGCTAGAAGTCGTCGTTGCTGATGCTCTGGTTTTTGGAAAG-3′; RGS6-1 5′-CCCCCGCTGGCTGTCCAAG-3′; RGS6-2 5′-CCCCACGCACAGATCGACAGACATTCAAGAGATGTCTGTGGAAA-3′. After incubation, cells were again washed and lysed in PBS with 1% Tween.

Isolation and culture of mammary epithelial cells
Mammary epithelial cells (MECs) from WT and RGS6−/− mice were isolated using collagenase/hyaluronidase solution (StemCell Technologies) according to the manufacturer’s protocol. Briefly, mammary glands were surgically removed from 3-month-old virgin mice, minced with scalps, placed in collagenase/hyaluronidase containing Epicult-B (Stem Cell Technologies) and incubated for 6–8 h at 37°C with occasional pipetting and vortexing. Cells were then centrifuged at 450g and the pellet resuspended in prewarmed trypsin-ethylenediaminetetraacetic acid solution. Cold Hanks’ balanced salt solution containing 2% FBS and ammonium chloride was then added and the suspension centrifuged following a 5 min incubation. Pelleted cells were suspended in a solution containing prewarmed dispase (5 mg/ml) and DNase I (1 mg/ml) followed by addition of FBS/cold Hanks’ balanced salt solution. The cell suspension was filtered through a 40 μm cell strainer resulting in an enriched population of mammary epithelial cells. MECs were seeded and grown in Epicult-B medium containing 5% FBS, 10 ng/ml recombinant human epidermal growth factor, 10ng/ml recombinant human bFGF and 85%). MECs were seeded at a density of 100 cells/well and incubated at 37°C with growth medium replaced every 3 days. After 10 days, colonies were fixed with methanol and stained with 0.5% crystal violet (1:1 methanol to water).

Apoptosis and caspase-3 activity assays
Apoptosis in isolated MECs was quantified using the Cell Death Detection Kit (Roche) according to the manufacturer’s protocol. The activity of caspase-3 in MEC lysates was assayed using Biovision’s caspase-3 activity kit according to the manufacturer’s protocol.

DNA damage estimation
The extent of DNA damage in DMBA-treated MECs was evaluated utilizing the COMET assay (alkaline single-cell gel assay, pH > 13) as we described previously (19). COMET tail length was measured using Comet Assay IV software (Perceptive Instruments).

Measurement of intracellular ROS
Intracellular ROS generation in cultured, DMBA-treated MECs was estimated using the cell-permeable oxidation-sensitive probe, CM-H2DCFDA (BioRad), as described previously (18). Briefly, cells were harvested by centrifugation, washed three times with ice-cold phosphate-buffered saline (PBS), resuspended in PBS and incubated with 5 μM CM-H2DCFDA for 20 min at 37°C. After incubation, cells were again washed and lysed in PBS with 1% TWEEN 20. ROS level was determined at the ratio of dichlorofluorescein excitation at 485 nm to emission at 530 nm.

Immunoblotting
Tumor laden mammary tissue homogenates and cell lysates were prepared in radioimmunoprecipitation assay buffer (1% Tergitol type NP-40, 0.5% sodium deoxycholate and 0.1% sodium deoxycholate in 1x PBS), quantified and probed as we described previously (18,21). Approximately 20 μg of protein per sample was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting.

Immunohistochemistry and Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling staining
Formalin-fixed, paraffin-embedded mammary tumor sections of WT and RGS6−/− mice and human breast cancer tissue specimens obtained from University of Iowa Hospitals were processed to examine expression of different key signaling proteins as we described previously (18). Briefly, sections were dewaxed in xylene, treated with a graded series of alcohol solutions, immersed in 3% hydrogen peroxide to block endogenous peroxide activity, blocked with 5% bovine serum albumin and then incubated overnight at 4°C with specific antibody. Following washing (3 × 10 min) in PBS, sections were incubated for 1 h at room temperature with peroxidase-conjugated secondary antibodies. A positive reaction was detected by exposure to stable diaminobenzidine (General Biosciences Corporation) for 3 min. The sections were counterstained in Harris hematoxylin and observed under the microscope. Apoptotic cells were identified by Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling staining as we described previously (19).

Statistical analysis
Data were analyzed by Student’s t-test and one-way analysis of variance. Results were considered significantly different at P < 0.05. Values are expressed as mean ± standard error of the mean.

Results
RGS6 is one of the primary R7 family RGS proteins expressed in the human and murine mammary ductal epithelium
A majority of breast tumors arise from transformation of cells comprising the mammary duct epithelium. We previously reported that RGS6 expression is localized to ductal epithelial cells of human breast and that this expression is lost in high-grade tumors (18). Expression of Gβ7, requisite RGS6-binding partner necessary for protein stability, mirrors this expression pattern in normal human mammary tissue (Figure 1A). Interestingly, like RGS6, Gβ7 expression is also lost in high-grade tumors isolated from human patients consistent with the co-stabilization relationship described for the RGS6–Gβ7 complex (Supplementary Figure S1, available at Carcinogenesis Online). Immunohistochemical staining revealed expression of R7 family member RGS7 in human
mammary tissue but no detectable expression of the other R7 family member RGS11 or retinal-specific RGS9 splice form RGS9-1 (Figure 1A), indicating that RGS6 is one of the primary R7 family RGS proteins expressed in human breast. As expected based on minimal expression in normal human breast tissue, neither RGS11 nor RGS9-1 were detectable in tumor samples regardless of grade (Supplementary Figure S1, available at Carcinogenesis Online). Although lower in expression compared with RGS6, it also appears that RGS7 expression is downregulated in high-grade human breast tumors (Supplementary Figure S1, available at Carcinogenesis Online), indicating that RGS7 may also play a role in human breast carcinogenesis.

Generation of RGS6−/− mice (20) has afforded us the unique opportunity to study the role of RGS6 in carcinogenesis in vivo. Using an antibody that recognizes all RGS6 splice forms, we were able to

![Image](https://academic.oup.com/carcin/article-abstract/34/8/1747/2463237)

**Fig. 1.** RGS6 is one of the primary R7 family RGS proteins expressed in human and murine mammary ductal epithelium. Immunohistochemical staining for RGS6 and its binding partner Gβ5, as well as R7 family members RGS7, RGS9-1 and RGS11 in the epithelium of human mammary ducts (scale bar = 50 μm) (A). Similar results were obtained in murine tissue samples (B) with RGS6 expression completely absent in the ductal epithelium of RGS6−/− animals (scale bar = 50 μm). (C) Western blotting confirmed expression of RGS6 and RGS7 in breast tissue with loss of Gβ5 expression apparent in RGS6−/− samples. Westerns depicted here are representative of at least three independent experiments with essentially identical results.

![Image](https://academic.oup.com/carcin/article-abstract/34/8/1747/2463237)

**Fig. 2.** DMBA-induced tumorigenesis in WT and RGS6−/− mice. (A) Hematoxylin and eosin staining of representative tumors generated in WT (n = 18) and RGS6−/− mice (n = 21) treated with DMBA (16 weeks; lower panels) compared with breast tissue from untreated controls (upper panels, scale bar = 100 μm). RGS6−/− mice exhibited a decreased latency of tumor formation (B) and growth to a diameter of 0.5 cm (C). Data represent mean ± standard error of the mean. *P < 0.05. (D) Survival curve for DMBA-treated WT and RGS6−/− mice. DMBA treatment led to loss of RGS6 and Gβ5 expression in the mouse mammary epithelium of WT mice by (E) immunohistochemistry (16 weeks post-DMBA treatment; scale bar = 50 μm) or (F) western blot. (G) Virgin female RGS6−/− mice exhibit spontaneous breast tumor formation (WT, n = 36; RGS6−/−, n = 43).
confirm epithelial-specific localization of RGS6 and its binding partner Gβ5 in breast tissue from WT mice (Figure 1B). Recapitulating results in human tissue, no expression of RGS11 or RGS9-1 was observed in mouse breast, but detectable levels of RGS7 were present (Figure 1B and C). Consistent with the co-stabilization relationship between R7 family members and Gβ5 (12), genetic ablation of RGS6 expression decreased Gβ5 levels in the mammary epithelium of mice (Figure 1B). Our immunohistochemical results were confirmed using western blotting showing approximately a 50% reduction in detectable Gβ5 expression in breast tissue isolated from RGS6−/− mice (Figure 1C). It is likely that the remaining Gβ5 protein detected in RGS6−/− mice is stabilized by RGS7 whose expression does not change as a consequence of RGS6 deletion. These results demonstrate that human and murine R7 family protein patterns are identical validating our mouse model for studying the role of RGS6 in mammary carcinogenesis in vivo.

RGS6 loss results in accelerated DMBA-induced tumor initiation and progression and spontaneous breast tumor formation

DMBA is a well-known DNA-damaging carcinogen that induces mammary tumors in nearly 75% of WT mice by 20 weeks postinitiation of drug administration (22). DMBA treatment resulted in ductal hyperplasia and solid tumor formation (Figure 2A, lower panels) in mice of both genotypes (12/21 RGS6−/− and 11/18 WT) not observed in tissues isolated from untreated animals (Figure 2A, upper panels). However, mice lacking RGS6 expression showed vastly accelerated DMBA-induced tumorigenesis including a reduction in the time required for tumor formation (Figure 2B) and tumor growth (Figure 2C). Indeed tumors began to form in RGS6-deficient mice as soon as 13 weeks post-DMBA administration versus 16 weeks in control animals, and tumor growth was accelerated by 60% in RGS6−/− versus control mice (Figure 2C and D). As mice were killed once tumors reached a diameter over 0.5 cm or upon onset of severe symptomology, RGS6−/− mice exposed to DMBA exhibited decreased overall survival compared with their WT counterparts (Figure 2D). Interestingly, like the trend observed in human patient samples, RGS6 expression along with that of its binding partner Gβ5 was lost following 16 weeks of DMBA treatment in mammary tissue (Figure 2F) and the ductal epithelium (Figure 2E) of WT animals. These results suggest that RGS6 downregulation is a requisite step for DMBA-induced tumor formation in normal mice. In addition, approximately 15% of virgin female RGS6−/− mice of age over 1 year develop spontaneous breast malignancies ranging from palpable nodules to large breast tumors (Figure 2G). No such malignancies were observed in age-matched WT controls. Taken together, these results confirm that RGS6 functions as a tumor suppressor in breast in vivo.

RGS6 is required for DMBA-induced pro-apoptotic signaling in the ductal epithelium of mouse mammary tissue

Both oncogenic activation of progrowth signaling and failure of proapoptotic DNA damage signaling have been implicated as causative factors in DMBA-induced mammary carcinogenesis (23). Based on the known role for RGS6 in promoting Dox-induced DNA damage signaling (19), we hypothesized that RGS6 might suppress tumor initiation following DMBA treatment by promoting activation of the DNA damage response (DDR) and facilitating DNA-damage-induced apoptosis. Immunohistochemical staining (Supplementary Figure S2A, available at Carcinogenesis Online) showed that DMBA treatment increased

Fig. 3. Loss of RGS6 leads to deficits in apoptotic signaling and exaggerated oncogenesis in mammary glands of DMBA-treated mice. Quantification of p53 positive nuclei (A), p-ATM positive nuclei (B) and Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling positive cells (C) reveals a failure of DNA-damage-induced pro-apoptotic signaling in the ductal epithelium of RGS6−/− mice (n = 12) following 16 weeks of DMBA treatment compared with WT controls (n = 11). Quantification of cyclin D1 positive nuclei (D), p27kipl expression (H-score) (E) and Dnmt1 positive nuclei (F) reveals a concurrent potentiation of oncogenic signaling in the ductal epithelium of RGS6−/− mice. Data represent mean ± standard error of the mean. *P < 0.01; **P < 0.001.
nuclear p53 expression and ATM phosphorylation in the ductal epithelium of WT mice by more than 7-fold (Figure 3A and B), responses that were severely blunted in animals lacking RGS6 (Supplementary Figure S2A, available at Carcinogenesis Online; Figure 3A and B). Consistent with loss of ATM/p53 activation, DMBA-induced apoptosis also required RGS6 as indicated by a decrease in Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling positive cells in the mammary epithelium of RGS6-deficient animals (Supplementary Figure S2A, available at Carcinogenesis Online, and Figure 3C). Activation of the DDR can induce either growth arrest and cellular senescence to allow for DNA repair or cell death depending on the severity of the genetic damage. Obtaining a means to circumvent the apoptotic arm of this pathway is a hallmark of malignant cells and a key step in tumor initiation. The deficiency in DNA damage signaling following DMBA administration observed in RGS6−/− mice is consistent with the accelerated tumorigenesis observed in these animals.

**RGS6 loss promotes DMBA-induced oncogenic signaling**

DMBA has been shown to increase expression of cell-cycle regulator cyclin D1 with a corresponding decrease in p27kip1, the cyclin-dependent kinase inhibitor in rodent mammary tissue (24). Both of these oncogenic aberrations likely contribute to DMBA-induced mammary tumor growth. Although basal levels of cyclin D1 and p27kip1 in the mammary ductal epithelium did not differ between WT and RGS6−/− mice, increases in cyclin D1 expression and decreases in p27kip1 in response to DMBA treatment were significantly enhanced in the ductal epithelium of RGS6 null animals as shown by immunohistochemistry (Supplementary Figure S2B, available at Carcinogenesis Online) or quantified as the number of immunoreactive nuclei (Figure 3D and E). The DNA methyltransferase Dnmt1, shown to bind RGS6 in a previous study (14), is emerging as an important oncogene that enhances cellular transformation by methylating the promoters of key pro-apoptotic genes and suppressing their transcription (15–17). Interestingly, we show here that RGS6 deficiency resulted in increased Dnmt1 expression following DMBA treatment (Supplementary Figure S2B, available at Carcinogenesis Online and Figure 3F), indicating that RGS6 might block tumorigenesis in part through a Dnmt1-dependent mechanism. Taken together, these results indicate that RGS6 functions as a key cellular countermeasure against aberrant cell growth.

**Silencing of RGS6 enhances HER2- and ER-dependent growth in MCF-7 breast cancer cells**

Given our finding that RGS6 null mice exhibit increased progrowth signaling in the mammary epithelium following DMBA treatment, we undertook studies to elucidate the relevant oncogenic signaling cascades RGS6 functions to inhibit in breast. Between 15 and 30% of breast tumors overexpress the proto-oncogene HER2 with a majority of the remaining tumors relying on ER- or PR-dependent progrowth signals (1). To study the role of RGS6 in suppressing ER

---

**Fig. 4.** RGS6 knockdown potentiates HRG- and E2-induced growth in MCF-7 cells. MCF-7 cells transfected with control or RGS6-1 RNAi were treated with HRG (0.5 nM) or E2 (10 nM) for 0, 6, 12, 24 or 48 h beginning 36 h post-transfection. Proliferation in HRG- (A) and E2- (B) treated cells was quantified using the MTT assay. All experiments were performed in triplicate and data represent mean ± standard error of the mean. *P < 0.05. (C) Loss of RGS6 increased HRG-induced AKT and ERK1/2 phosphorylation and induction of cyclin D1 expression. (D) No induction of AKT or ERK1/2 phosphorylation was seen in E2 treated cells regardless of RNAi transfection.
and HER2 signaling, we first employed well-characterized breast cancer cell lines that harbor expression of both receptors (25). MCF-7 breast cancer cells possess low basal RGS6 levels, but RGS6 can be induced by the chemotherapeutic agent Dox to facilitate activation of ATM/p53 signaling and subsequent apoptosis (19). RGS6 silencing in these cells blocks Dox-induced cytotoxicity (19). Based on these observations and the ability of exogenous RGS6 to induce cell-cycle arrest and suppress growth in this cell line (18), we investigated the effect of RGS6 RNAi on cellular proliferation in these cells. Our RNAi construct was capable of knocking down approximately 50% of RGS6 expression in MCF-7 cells (Figure 4C and D). Interestingly, there was no difference in growth between control and RGS6 RNAi transfected cells (Figure 4A and B). However, RGS6 knockdown potentiated growth induced by heregulin (HRG), an HER2 receptor agonist (Figure 4A) and 17β-estradiol (E2) (Figure 4B). Nearly identical results were obtained using a second RGS6-targeted RNAi construct (Supplementary Figure S3A and S3B, available at Carcinogenesis Online). MCF-7 cells express only low levels of HER2 and so we were surprised to observe a more robust proliferative effect of HRG compared to E2 in these cells. RGS6 also had a more profound effect on blocking HRG-mediated proliferation versus the relatively modest consequence of RGS6 knockdown in E2-treated cells.

Stimulation of heterodimeric receptors containing HER2 facilitates cell growth through induction of multiple signal transduction cascades including ERK1/2 and PI3K/AKT signaling and increases in cyclin D1. RGS6 loss enhanced activation of both networks as shown by increased phosphorylation of ERK 1/2 and AKT and increased cyclin D1 levels in HRG-treated cells transfected with RGS6 RNAi (Figure 4C). Although ER is known to promote activation of these same signaling cascades through transactivation of growth factor receptors, no difference in ERK1/2 or AKT phosphorylation was observed in E2-treated cells or as a consequence of RGS6 knockdown (Figure 4D). RGS6 knockdown with a second RNAi construct yielded similar results (Supplementary Figure S3C, available at Carcinogenesis Online). Our observation that RGS6 knockdown potentiated E2-induced cell growth in the absence of an effect on ERK1/2 or PI3K/AKT signaling suggests that RGS6 blocks the genomic or other non-genomic effects of ER stimulation.

RGS6 suppresses growth, promotes apoptosis and blocks transformation in MECs

As our RNAi was incapable of completely eliminating RGS6 expression in breast cancer cell lines, we then employed primary MECs isolated from WT and RGS6−/− mice to investigate the full consequence of RGS6 loss of ER- and HER2-mediated cellular proliferation in untransformed cells. Both E2- and HRG-induced cellular growth of isolated MECs and the proliferative effect of both these drugs was enhanced in cells lacking RGS6 (Figure 5A and B). Reexpression of RGS6L, the predominant RGS6 isoform expressed in mammary tissue (Figure 1C), suppressed the proliferative capacity of RGS6−/− MECs to that of WT cells (Supplementary Figure S4A, available at Carcinogenesis Online), indicating the phenotype of increased HRG-induced cell growth in RGS6−/− cells was specifically due to RGS6 loss. Increased phosphorylation of ERK1/2 and AKT was also seen in RGS6−/− MECs treated with HRG (Figure 5C) consistent with what we observed in MCF-7 cells. These results suggest that RGS6 normally functions to suppress growth downstream of both ER and HER2 in non-malignant breast cells. In this cell type, RGS6 expression was increased after 48 h of HRG treatment (Figure 5C), indicating that RGS6 can be upregulated in response to both genotoxic (doxorubicin) and oncogenic stimuli in non-cancerous cells.

DMBA has been shown to induce ATM and p53 activation in cells (26). In WT MECs, DMBA treatment induced ATM autophosphorylation (S1981) and phosphorylation of p53 at the ATM phosphorylation site (S15). Consistent with what we observed in vivo, cells lacking RGS6 exhibited reduced levels of both phosphorylated ATM and p53 (Figure 5D). Unlike the robust upregulation in RGS6 levels, we reported in cells treated with the chemotherapeutic agent Dox (19), no increase in RGS6 expression was observed in WT DMBA-treated MECs. It appears that basal RGS6 levels are sufficient in these cells to mediate DDR activation. The loss of ATM/p53...
activation upon DMBA treatment in RGS6−/− MECs was accompanied by a severe impairment in apoptosis as measured by the presence of cytoplasmic histone fragments (Figure 5E) and cleavage of the executioner caspase-3 (Figure 5F). The attenuated DMBA-induced apoptotic response in RGS6−/− MECs was restored by reconstitution of RGS6L expression (Supplementary Figure S4B, available at Carcinogenesis Online). Coincidently, an increase in DNA damage as measured by the COMET assay was seen in cells lacking functional RGS6 expression (Figure 5G, and Supplementary Figure S5, available at Carcinogenesis Online). This is consistent with the impairment of ATM activation in these cells that is necessary for the recruitment of proteins required to repair damaged DNA. The deficiency in this repair process would be expected to further enhance genotoxic stress in RGS6 null, DMBA-treated cells.

We recently showed that the ability of RGS6 to promote activation of the DDR and apoptosis in response to Dox treatment was nearly entirely dependent on ROS generation (19). Loss of RGS6 impaired DMBA-induced apoptosis (Figure 5E) but although the apoptotic response to DMBA treatment in MECs was ameliorated by pretreatment with a general ROS scavenger N-acetyl cysteine, this effect was not completely abolished in cells lacking RGS6 (Figure 5E). This later result suggests that the RGS6-mediated apoptotic response in DMBA-treated MECs does not rely solely on ROS generation. Nevertheless, DMBA-induced production of total ROS was partly impaired in RGS6 null MECs (Figure 5H). Clearly, RGS6 is required for the DMBA-induced apoptotic response but may function to promote apoptosis through ROS-dependent and independent mechanisms.

Long-term exposure to DMBA can induce transformation of isolated, cultured MECs. To assay the effect of RGS6 on cellular transformation, WT and RGS6 null MECs were treated with a continuous dose of DMBA in the culture media for a period of 12 days, replated and stained with crystal violet. The ability of DMBA to induce cellular transformation was potentiated in RGS6−/− MECs as evident by robustly increased colony formation by these cells compared with their WT counterparts (Figure 5I).

**RGS6 loss universally correlates with increasing breast tumor grade independent of ER/PR/HER2 status**

Here, we have shown that RGS6 is capable of suppressing growth downstream of both ER and HER2 receptor activation in MECs and
MCF-7 cells, but the difference in HRG-induced proliferation between WT and RGS6-deficient cells was more robust. Based on these data, we suspected that downregulation of RGS6 expression might be particularly important in HER2 overexpressing tumors. Interestingly, even when subdivided based on molecular classification (i.e. ER/PR/HER2 receptor status), we observed that RGS6 expression was universally lost with increasing breast tumor grade in human patient samples (Figure 6A). Although on average around 80% of RGS6 expression remained in tumors classified as ductal carcinoma in situ, this value dropped to 50% for grade I tumors and plummeted to 20% for all grade III malignancies (Figure 6B). Most striking was our observation that RGS6 expression was lowest in triple negative (ER/PR/HER2-) tumors (Figure 6B), considered the most malignant largely due to a lack of targeted therapeutics. We have shown that RGS6 loss cripples DNA damage signaling in DMBA-treated mammary tissue and isolated MECs including activation of ATM/p53 and subsequent apoptosis. Simultaneously, RGS6 deficiency promotes cellular proliferation both in response to direct HER2 or ER activation or in tissue subjected to continuous genotoxic insult. Like the tumor suppressor p53, RGS6 appears to play a dual role in suppressing carcinogenesis. In response to genotoxic (Dox) or oncogenic stress (HRG), RGS6 can be upregulated and functions to block tumor initiation and progression by curbing cellular proliferation and facilitating apoptosis. The convergence of these two phenomena explains both the enhanced DMBA-induced transformation observed in MECs isolated from RGS6 deficient animals and DMBA-induced cellular proliferation (Supplementary Figure 6, available at Carcinogenesis Online). Although RGS6 can directly induce mitochondrial dysfunction and apoptosis (18), it is also possible that RGS6 promotes apoptotic signaling by inhibiting Dnmt1 expression or activity enhancing the transcription of pro-apoptotic genes normally epigenetically silenced by Dnmt1-mediated methylation. In fact, we recently demonstrated that RGS6 inhibits oncogenic Ras-induced cellular transformation by facilitating destabilization of Dnmt1 and preventing Dnmt1-mediated blockade of cellular apoptosis (Huang et al., in preparation). A similar mechanism likely underlies, at least in part, the ability of RGS6 to suppress HRG-induced cell growth. Although signaling through various GPCRs can stimulate cancer cell proliferation (9), our previous studies suggest that the ability of RGS6 to suppress carcinogenesis is distinct from its canonical function as a G protein regulator and instead requires RGS6-mediated ROS generation (18,19), an observation supported by our findings that DMBA-induced apoptosis in MECs both requires RGS6 and can be blocked by N-acetyl cysteine, an ROS scavenger. Future work will focus on efforts to elucidate the exact mechanism(s) underlying the ability of RGS6 to concurrently suppress oncogenic cellular growth downstream of HER2 and ER and promote cell death.

RGS6 expression is universally downregulated in breast cancer patient samples independent of their molecular classification, suggesting that obtaining a means to circumvent the pro-apoptotic and growth suppressive actions of RGS6 in breast is a major barrier to tumor initiation and progression regardless of the specific oncogenic stimulus. We cannot yet determine whether the loss of RGS6 protein expression we observe is due to genetic deletion, transcriptional repression, or a post translational effect. We are aware of no reports from genome-wide analyses of breast tumor samples identifying specific loss of the RGS6 gene or the segment of chromosome 14 to which it maps. It is possible that loss of βG expression may be causative; however, this is difficult to determine due to the co-stabilization relationship between these two proteins. Identification of the mechanism of RGS6 downregulation would be a critical step in the development of therapeutics designed to restore the apoptotic and growth suppressive actions of RGS6 in breast, which we believe might benefit a large spectrum of cancer patients, especially those with tumors lacking expression of ER, PR or HER2.

Supplementary material
Supplementary Figures 1–6 can be found at http://carcin.oxfordjournals.org/

Funding
National Institutes of Health (CA161882 to R.A.F.).

Acknowledgements
We thank Dr Chantal Allamargot of the University of Iowa Central Microscopy Research Facility for assistance with histology/microscopy experiments and Dr John Koland for providing us with HRG.

References


Received November 21, 2012; revised April 4, 2013; accepted April 11, 2013