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PRINCIPAL INVESTIGATOR: Dr. Vimla Band

CONTRACTING ORGANIZATION: University of Nebraska Medical Center
Omaha, NE 68198

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Dr. Vimla Band

E-Mail: vband@unmc.edu

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14. ABSTRACT
Rho family small GTPases serve as molecular switches in the regulation of diverse cellular functions. Importantly, Rho overexpression is frequently seen in many carcinomas. We demonstrate here, that ectopic expression of wild-type RhoA as well as a constitutively-active RhoA mutant (G14V) in two independent primary hMEC strains led to their immortalization and preneoplastic transformation. These cells have continued to grow over 300 population doublings with no signs of senescence, whereas cells expressing the vector or dominant-negative RhoA mutant (T19N) senesced after 20 population doublings. Significantly, RhoA-T37A mutant, known to be incapable of interacting with many well known Rho-effectors including Rho-kinase, PKN and mDia 1 and 2, was also capable of immortalizing hMECs. Notably, similar to parental normal cells, Rho-immortalized cells have wild-type p53 and intact G1 cell cycle arrest upon adriamycin treatment. Rho-immortalized cells were anchorage-dependent.Microarray expression profiling of Rho-immortalized vs. parental cells showed altered expression of several genes previously implicated in immortalization and breast cancer progression. One of the gene ELF3 elevated in Rho immortal cells also is increased in breast cancer cell lines. Lastly, Rho immortal cells are derived from stem/progenitor cells but lack differentiation ability as compared to parental normal and TERT immortal cells. Taken together, these results demonstrate that RhoA can induce the preneoplastic transformation of hMECs by altering multiple pathways linked cellular transformation and breast cancer

15. SUBJECT TERMS Immortalization, Rho, Signal transduction

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Zhao X, et al Cancer Res. 2009 Jan 15;69(2):483-91

Duan L, et al., J. Biol Chem. 2010 Jan 8;285(2):1555-68.

This report covers the entire funding period including no-cost extension

Title: Human mammary epithelial cell transformation by Rho GTPase through a novel mechanism

INTRODUCTION:

The experiments proposed in this idea grant were designed to test the hypothesis that active Rho, through novel biochemical pathways distinct from those studied previously in the context of Rho function in cell migration, cytoskeleton remodeling, cell cycle progression and oncogenic transformation in fibroblasts, overcomes cellular senescence in human mammary epithelial cells (hMECs) to allow their early neoplastic transformation.

A large body of evidence implicates Ras-like small G-proteins as major players in the regulation of a variety of cellular processes. Rho GTPases cycle between inactive GDP-bound and active GTP-bound states, a transition controlled by guanine nucleotide exchange factor (GEF) proteins which convert the GDP-bound to GTP bound form, and by GTPase activating proteins (GAPs) which stimulates the low intrinsic GTPase activity to convert the active to inactive form (1). It is believed that the multitude of cellular processes regulated by Rho reflects the interaction of the active form with a number of distinct effector molecules and subsequent activation of these effectors (1-3). For example, Rho effectors such as phosphatidylinositol 4-phosphate,5 kinase (PIP5 kinase), Rho-kinase (ROK, and related ROCK kinase), formin homology protein p140-Dia, and rhophilin have been linked to the regulation of actin cytoskeleton organization (1, 4-6), and citron kinase appears to regulate cytokinesis (7,8). Recent evidence suggest a role of Rho effector PKN in cortical actin formation (9) and in G2/M checkpoint regulation (10).

At the cellular level, Rho-family small GTPases have emerged as key regulators of cell adhesion, migration, endocytic trafficking, cytokinesis, gene transcription and cell proliferation, through control of the actin cytoskeleton remodeling and other cellular responses to external stimuli (2, 11, 12). The role of Rho G-proteins in cell proliferation and oncogenesis is emphasized by the fact that most of their exchange factors were originally identified as oncogenes, and by the facilitation of cellular transformation by activated Rho and reversal of various aspects of the transformed phenotype, including invasive behavior, by interrupting Rho function (13-18). Dysfunctional regulation of Rho GTPases has been implicated in certain aspects of cancer development. For instance, overexpression of activated Rho mutants can transform fibroblasts (13). Rho proteins promote cell cycle progression through enhanced CDK activity by regulating the levels of cyclin D1, $p21^{WAF1}$, and $p27^{KIP1}$ (14). Transcriptional upregulation of the levels of particular Rho proteins has been described in many types of human cancers, including cancers of the colon, breast, lung, stomach and pancreas, and were correlated with tumor progression and invasion (15-18). In breast cancer, increased RhoA expression correlated with cancer progression (17,18), and Rho protein overexpression was shown to contribute to breast cancer cell invasion and metastasis (18). However, the role of Rho proteins in the early steps of transformation of primary human epithelial cells, which are normally programmed to undergo replicative senescence, has not been investigated.

When normal human mammary epithelial cells (hMECs) are cultured in vitro, they exhibit a finite life span and then undergo senescence, without any spontaneous transformation (19). Deliberate oncogenic transformation of these cells has provided a practical approach to dissect out the biochemical pathways that mediate early steps in breast cancer. One of the earliest steps in oncogenic transformation of hMECs involves loss of senescence and continuous proliferation, a process referred to as immortalization (19). Delineation of biochemical pathways that mediate MEC immortalization is therefore likely to provide answers to key questions about early breast cancer. At the time of grant submission, we presented preliminary data to demonstrate that overexpression of constitutively-active Rho and more importantly the wild-type Rho, but not a dominant-negative Rho mutant, induces the extension of life span (possible immortalization) of normal hMECs. Significantly, a Rho effector domain mutant incapable of interacting with the previously studied Rho effectors thought to be involved in rodent cell transformation, including Rho-kinase1, Rho-kinase2, PKN and mDia1, retained the hMEC extension of life span (possibly immortalizing) function. Based on these findings, we hypothesized that Rho immortalizes hMECs by a novel biochemical pathway. To test this hypothesis we proposed following aims:

Aim I: **Further examine the interaction of RhoT37A with known Rho effectors expressed in human MECs.**

Aim II. **Identify novel Rho targets relevant to human MEC immortalization.**

Aim III. **Dissect the role of known and/or novel effectors in RhoT37A-mediated immortalization.**

Body:

This DOD grant funding led to publication of **one primary paper** (only abstract included, for details a pdf file is attached) and **one collaborative Rho related paper (**only abstract included, for details pdf file is attached), and **some novel observations presented as unpublished data** (see below, will be pursued further should additional funding becomes available). The reagents and expertise developed during this study also led to other publications unrelated to the specific aims of the project (listed at the end of this report---this grant was acknowledged in these published studies).

Overexpression of RhoA induces preneoplastic transformation of primary mammary epithelial cells. Zhao X et al., Cancer Res. 2009 Jan 15;69(2):483-491.

Preliminary data that was presented in DOD Idea grant was confirmed in this manuscript.
Abstract: Rho family small GTPases serve as molecular switches in the regulation of diverse cellular functions, including actin cytoskeleton remodeling, cell migration, gene transcription, and cell proliferation. Importantly, Rho overexpression is frequently seen in many carcinomas.

However, published studies have almost invariably used immortal or tumorigenic cell lines to study Rho GTPase functions and there are no studies on the potential of Rho small GTPase to overcome senescence checkpoints and induce preneoplastic transformation of human mammary epithelial cells (hMEC). In this study, we showed that ectopic expression of wild-type (WT) RhoA as well as a constitutively active RhoA mutant (G14V) in two independent primary hMEC strains led to their immortalization and preneoplastic transformation. These cells have continued to grow over 300 population doublings (PD) with no signs of senescence, whereas cells expressing the vector or dominant-negative RhoA mutant (T19N) senesced after 20 PDs. Significantly, RhoA-T37A mutant, known to be incapable of interacting with many well-known Rho effectors including Rho kinase, PKN, mDia1, and mDia2, was also capable of immortalizing hMECs. Notably, similar to parental normal cells, Rho-immortalized cells have WT p53 and intact G1 cell cycle arrest on adriamycin treatment. Rho-immortalized cells were anchorage dependent and were unable to form tumors when implanted in nude mice. Lastly, microarray expression profiling of Rho-immortalized versus parental cells showed altered expression of several genes previously implicated in immortalization and breast cancer progression. Taken together, these results show that RhoA can induce the preneoplastic transformation of hMECs by altering multiple pathways linked to cellular transformation and breast cancer.

Distinct Roles for Rho *Versus* Rac/Cdc42 GTPases Downstream of Vav2 in Regulating Mammary Epithelial Acinar Architecture. Duan L, et al., J. Biol Chem. 2010 Jan 8;285(2):1555-1568.

Abstract: Non-malignant mammary epithelial cells (MECs) undergo acinar morphogenesis in three-dimensional Matrigel culture, a trait that is lost upon oncogenic transformation. Rho GTPases are thought to play important roles in regulating epithelial cell-cell junctions, but their contributions to acinar morphogenesis remain unclear. In this study, we showed that the activity of Rho GTPases is down-regulated in non-malignant MECs in three-dimensional culture with particular suppression of Rac1 and Cdc42. Inducible expression of a constitutively active form of Vav2, a Rho GTPase guanine nucleotide exchange factor activated by receptor tyrosine kinases, in three-dimensional MEC culture activated Rac1 and Cdc42; Vav2 induction from early stages of culture impaired acinar morphogenesis, and induction in preformed acini disrupted the pre-established acinar architecture and led to cellular outgrowths. Knockdown studies demonstrated that Rac1 and Cdc42 mediate the constitutively active Vav2 phenotype, whereas in contrast, RhoA knockdown intensified the Vav2-induced disruption of acini, leading to more aggressive cell outgrowth and branching morphogenesis. These results indicate that RhoA plays an antagonistic role to Rac1/Cdc42 in the control of mammary epithelial acinar morphogenesis.

UNPUBLISHED DATA

ELF3 is overexpressed in a subset of breast cancer cell lines. Ets transcription factors comprise a large family of sequence-specific regulators of gene expression with important and diverse roles in development and disease. Ets factors show altered expression in colon cancer, where they regulate pathways relevant to tumor progression. Ets factors also likely act as important modifiers of non-neoplastic intestinal disease by regulating pathways relevant to tissue injury and repair (as reviewed in ref 20). Despite a large body of published work on Ets biology,

much remains to be learned about the precise functions of this large and diverse gene family in normal and cancer mammary cells.

Given the results of our microarray analyses performed between normal and Rho-immortal cells and confirmation by RT-PCR, where we had observed increased expression of ELF3 in RhoA-immortalized hMECs (Zhao et al., Cancer Res 2009), we performed expression analysis of ELF3 in a large set of breast cancer cell lines using Real-time PCR. These results demonstrated high expression levels of ELF3 in several breast cancer cell lines (Fig. 1). These results further implicate ELF3 in breast cancer and suggest potential diagnostic/prognostic role of ELF3 expression in breast cancers. Future studies will be carried out to further extend these findings at a protein level using immunohistochemistry of breast cancer specimens should additional funding becomes available.

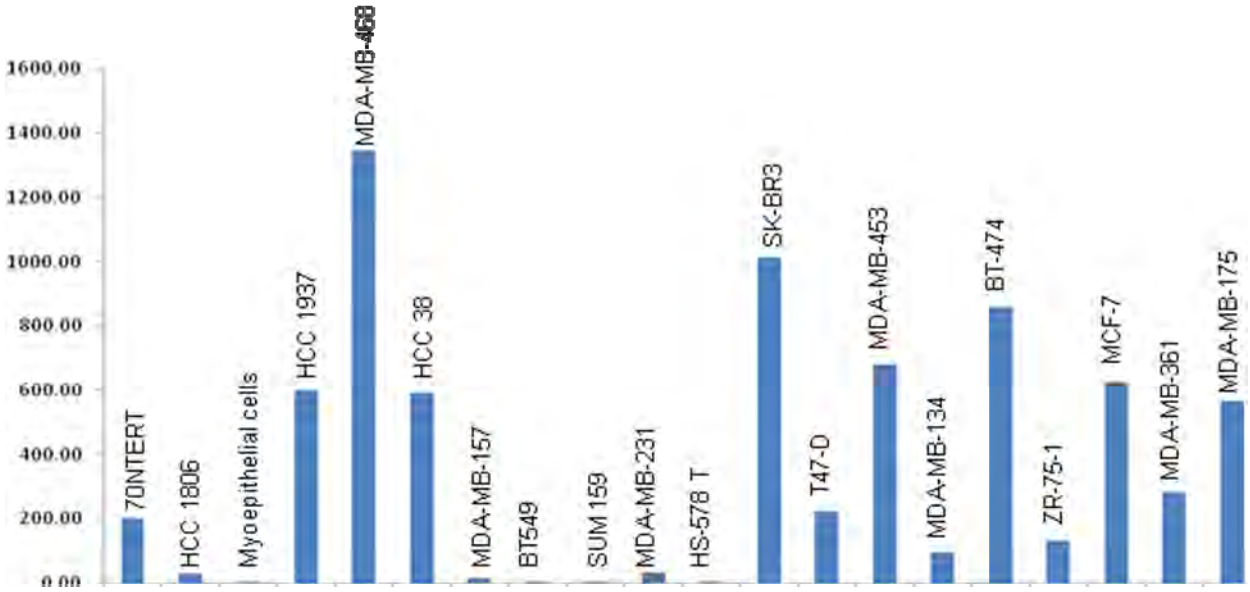

Fig. 1. Real-time PCR analyses of ELF3 expression. mRNA prepared from normal (70NTERT), myoepithelial cells (serve as negative control) and indicated breast cancer cell lines. Y-axis shows arbitrary values. Please note several breast cancer cell lines express high levels of ELF3 mRNA.

RhoA-immortal cells express stem cell markers. We have recently shown that Telomerase-immortalized human mammary stem/progenitor cells express stem cell markers (21). There is increasing evidence that breast and other cancers originate from and are maintained by a small fraction of stem/progenitor cells with self-renewal properties. Whether such cancer stem/progenitor cells originate from normal stem cells based on initiation of a de novo stem cell program, by reprogramming of a more differentiated cell type by oncogenic insults, or both remains unresolved. A major hurdle in addressing these issues is lack of immortal human stem/progenitor cells that can be deliberately manipulated in vitro. We recently showed that

normal and human telomerase reverse transcriptase (hTERT)-immortalized human mammary epithelial cells (hMECs) isolated and maintained in culture conditions that we established earlier (use of DFCI-1 medium) retain a fraction of cells that retain progenitor cell properties. These cells coexpress basal (K5, K14, and vimentin), luminal (E-cadherin, K8, K18, or K19), and stem/progenitor (CD49f, CD29, CD44, and p63) cell markers (21).

Given that RhoA induces immortalization of human mammary cells, we examined selected markers representing various hMEC lineages in normal parental and Rho immortal cells. Notably, we observed similar to hTERT-immortal cells, Rho-immortal cells express basal (K5, K14), luminal (E-cadherin, K18), and stem/progenitor (CD49f, CD44, and p63) cell markers (Fig. 2A and 2B). These results support the notion that Rho-immortal cells are derived from stem/progenitor cells.

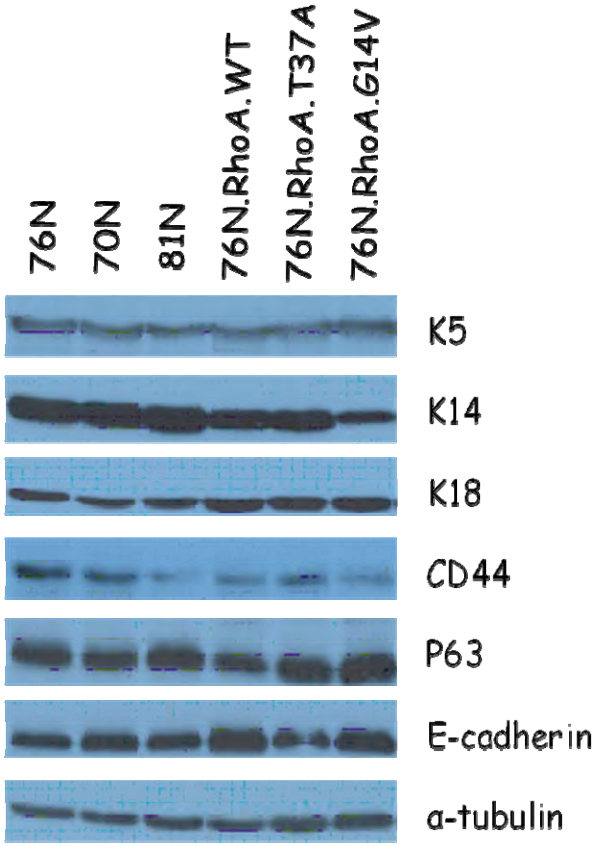

Fig. 2A. Normal Parental and Rho-immortal cells express luminal, myoepithelial and stem cell markers. Indicated cells were cultured in DFCI-1 medium. 50 microgram total protein was run on SDS-PAGE gel, transferred to PVDF membrane and Western blotted with indicated antibodies.

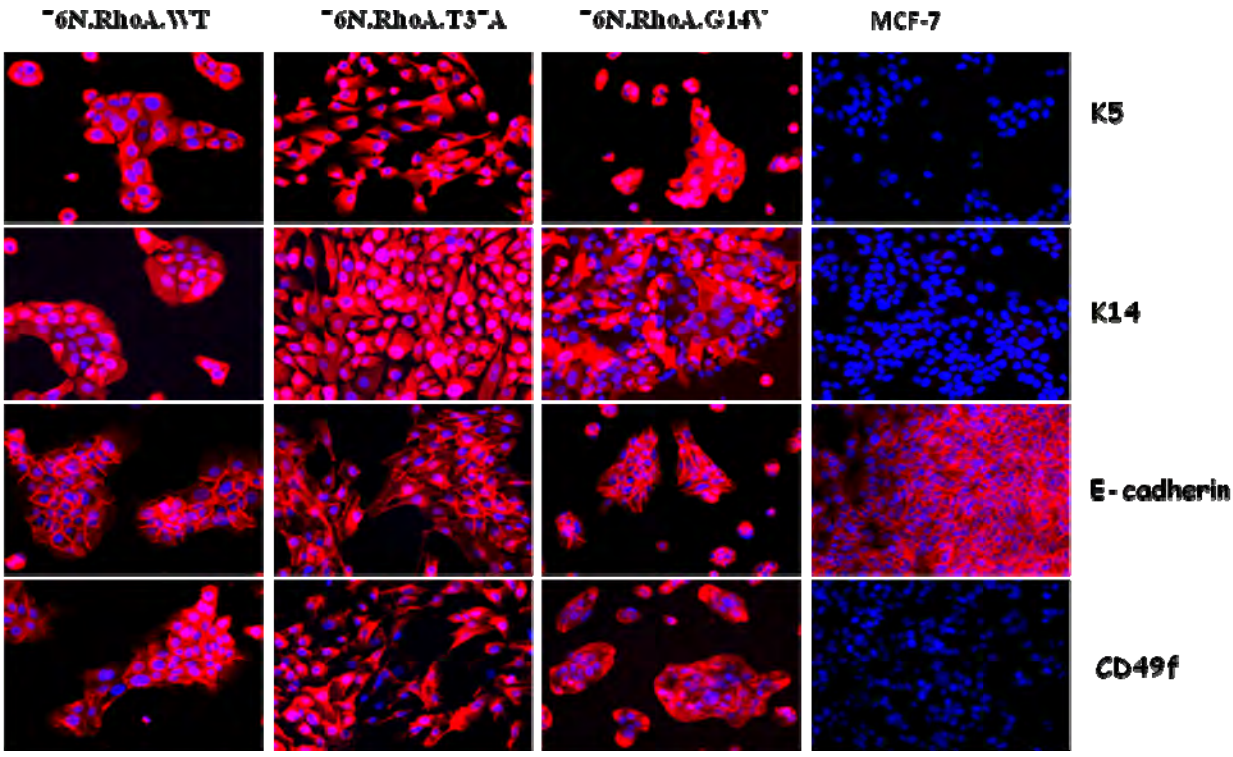

Fig. 2B. Immunofluorenscence staining of Rho immortal cells. Rho immortal cells were stained with various indicated antibodies. Please note all cells express K5, K14, E-cadherin and CD49F proteins. MCF-7 is used as control.

RhoA immortal cells are capable of self renewal but lack differentiation. Defining properties of stem/progenitor cells deduced from studies of embryonic as well as adult stem/progenitor cells include the ability to self renew as well as to produce cells at further stages of differentiation under appropriate conditions. The stable co-expression of basal and stem cell markers on the Rho-immortalized hMECs suggested that these cells may behave similar to our recent observation with TERT immortal cells (21) i.e. ability to self renew and differentiate. For this purpose, we performed similar experiments as we have recently published using TERT-immortal cells. Briefly, When TERT-immortal cells were cultured in MEGM medium on 2D plastic substratum, these cells proliferate as tightly packed epithelial colonies and subsequently, a proportion of cells near the periphery adopted a spindle shaped morphology surrounding the tight epithelial cell colony in the center (21). Notably, while the cells in the center expressed the basal, luminal and stem cell markers like the cells cultured in DFCI-1 medium, the spindle-shaped cells forming the peripheral halo were K5-negative (21) and acquired several well known myoepithelial cell markers (α–SMA, CD10, and Thy-1) that were absent on the parental cells as well as in the central compact part of colonies grown in MEGM (21).

Significantly, when we performed similar experiments with Rho-immortal hMECs, these cells lacked differentiation under the conditions hTERT showed a clear differentiation into myoepithelial cells (Fig. 3). These results demonstrate that expression of Rho in stem/progenitor cells inhibits their ability to differentiate and promote self renewal. These studies are consistent

with the notion that oncogenesis promotes self renewal and prevents differentiation. We are currently asking two questions i) do all breast cancer relevant oncogenes (such as PI3K, PTEN, ErbB2, mutant p53) behave similar to Rho overexpression? ii) what is the biochemical mechanism that favors self renewal vs differentiation? These studies will be pursued if we are successful in obtaining funding for this work.

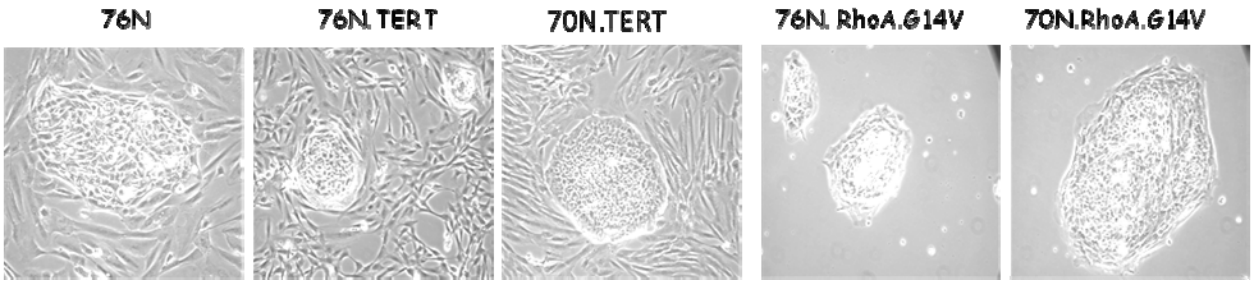

Fig. 3. In vitro self-renewal and myoepithelial cell differentiation of hTERT and Rho-immortal cells in MEGM medium. Morphology of cells during differentiation , loose cells with fibroblastic morphology represent myoepithelial cells and center tight colony represent undifferentiated cells.

Key Research Accomplishments (primary work):

1. We have demonstrated that ectopic expression of wild-type RhoA as well as a constitutively-active RhoA mutant (G14V) in two independent primary hMEC strains led to their immortalization and preneoplastic transformation. These cells have continued to grow over 300 population doublings with no signs of senescence, whereas cells expressing the vector or dominant-negative RhoA mutant (T19N) senesced after 20 population doublings.

2. Importantly, we demonstrate that RhoA-T37A mutant, known to be incapable of interacting with many well known Rho-effectors including Rho-kinase, PKN and mDia 1 and 2, was also capable of immortalizing hMECs.

3. Rho-immortalized cells, similar to parental cells have wild-type p53 and intact G_1 cell cycle arrest upon adriamycin treatment.

4. Rho-immortalized cells are anchorage-dependent.

5. Microarray expression profiling of Rho-immortalized vs. parental cells showed ZNF217, ELF3 and S100P are overexpressed, whereas CLCA2 and DAB2 are down-regulated in RhoA-immortalized hMECs.

6. More importantly, same alteration in expression of these genes was seen breast cancer cells and cancer tissues (Oncomine data).

7. Taken together, these results demonstrate that RhoA can induce the preneoplastic transformation of hMECs by altering multiple pathways linked cellular transformation and breast cancer.

8. ELF3 overexpression seen upon RhoA-induced immortalization is also observed in a large set of breast cancer cell lines.

9. RhoA-immortal cells while maintaining some stem/progenitor cell markers, lack ability to differentiatiate.

Key Research Accomplishments (related work):

10. Three dimensional culture of normal and immortal mammary epithelial cells downregulates Rho GTPases activity, specifically Rac1 and Cdc42.

11. Expression of a constitutively active form of Vav2, a Rho GTPase guanine nucleotide exchange factor activated by receptor tyrosine kinases, activates Rac1 and Cdc42

12. Vav2 induction in hMECs impaires acinar morphogenesis.

13. Knockdown studies demonstrated that Rac1 and Cdc42 mediate the constitutively active Vav2 phenotype, whereas in contrast, RhoA knockdown intensified the Vav2-induced disruption of acini, leading to more aggressive cell outgrowth and branching morphogenesis.

14. These results indicate that RhoA plays an antagonistic role to Rac1/Cdc42 in the control of mammary epithelial acinar morphogenesis.

Reportable Outcomes:

Rho related Publications and a Review (pdf file attached):

Zhao X, Lu L, Pokhriyal N, Ma H, Duan L, Lin S, Jafari N, Band H, Band V. Overexpression of RhoA induces preneoplastic transformation of primary mammary epithelial cells. Cancer Res. 2009 Jan 15;69(2):483-91

Duan L, Chen G, Virmani S, Ying G, Raja SM, Chung BM, Rainey MA, Dimri M, Ortega-Cava CF, Zhao X, Clubb RJ, Tu C, Reddi AL, Naramura M, Band V, Band H. Distinct roles for Rho versus Rac/Cdc42 GTPases downstream of Vav2 in regulating mammary epithelial acinar architecture. J Biol Chem. 2010 Jan 8;285(2):1555-68.

Reagents and expertise developed during this DOD funding period resulted in additional publications (listed below) from the PI's laboratory where this grant was acknowledged.

1. Duan L, Raja SM, Chen G, Virmani S, Williams SH, Clubb RJ, Mukhopadhyay C, Rainey MA, Ying G, Dimri M, Chen J, Reddi AL, Naramura M, Band V, Band H. Negative regulation of EGFR-Vav2 signaling axis by Cbl ubiquitin ligase controls EGF receptor-mediated epithelial cell adherens junction dynamics and cell migration. J Biol Chem. 2011 Jan 7;286(1):620-33.

2. George M, Rainey MA, Naramura M, Ying G, Harms DW, Vitaterna MH, Doglio L, Crawford SE, Hess RA, Band V, Band H. Ehd4 is required to attain normal prepubertal testis size but dispensable for fertility in male mice. Genesis. 2010 May;48(5):328-42.

3. Raja SM, Clubb RJ, Bhattacharyya M, Dimri M, Cheng H, Pan W, Ortega-Cava C, Lakku-Reddi A, Naramura M, Band V, Band H. A combination of Trastuzumab and 17-AAG induces enhanced ubiquitinylation and lysosomal pathway-dependent ErbB2 degradation and cytotoxicity in ErbB2-overexpressing breast cancer cells. Cancer Biol Ther. 2008 Oct;7(10):1630-40. Epub 2008 Oct 9.

Presentations:

X. Zhao, Pokhriyal N, Ma H, Duan L, Band H and Band V. Overexpression of RhoA induces preneoplastic transformation of primary mammary epithelial cells. Department of Defense Era-Of-Hope meeting, Breast Cancer Research Program Meeting, Baltimore, MD

Reagents:

-Generated Rho-immortal MECs and various Rho constructs

Funding applied for based on this work:

Dr. Xiangshan Zhao, first author of the manuscript applied for career development award from Susan Komen Foundation but was not funded.

Dr. Vimla Band, P.I. of this DOD grant applied for Idea expansion award but did not receive funding.

Manuscript included:

Zhao X, Lu L, Pokhriyal N, Ma H, Duan L, Lin S, Jafari N, Band H, Band V. Overexpression of RhoA induces preneoplastic transformation of primary mammary epithelial cells. Cancer Res. 2009 Jan 15;69(2):483-91

Duan L, Chen G, Virmani S, Ying G, Raja SM, Chung BM, Rainey MA, Dimri M, Ortega-Cava CF, Zhao X, Clubb RJ, Tu C, Reddi AL, Naramura M, Band V, Band H. Distinct roles for Rho versus Rac/Cdc42 GTPases downstream of Vav2 in regulating mammary epithelial acinar architecture. J Biol Chem. 2010 Jan 8;285(2):1555-68.

Conclusions:

The present study demonstrates that RhoA, implicated in breast cancer oncogenesis by clinical studies and well known as a critical gatekeeper of receptor signals into multiple cell biological pathways, can induce the immortalization of hMECs. Notably, mammary epithelial cell immortalization by an effector domain mutant of RhoA that is incapable of interacting with well-characterized Rho effectors previously implicated in oncogenic transformation strongly suggest that RhoA-induced early transformation of hMECs proceeds to novel pathways. Microarray analyses between normal and RhoA-immortal cells links several novel pathways to Rho-induced immortalization. Future analyses will focus to uncover the nature of these pathways and to link them to oncogenic pathways in breast cancer. Our novel findings that RhoA-immortalized normal stem/progenitor hMECs loose differentiation ability should provide a model to discover biochemical pathways linked to cancer stem cells.

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DOI:10.1158/0008-5472.CAN-08-2907

Research Article

Overexpression of RhoA Induces Preneoplastic Transformation of Primary Mammary Epithelial Cells

Xiangshan Zhao,[1,4] Lin Lu,[3] Nidhi Pokhriyal,[3,4] Hui Ma,[4] Lei Duan,[2] Simon Lin,[5] Nadereh Jafari,[6] Hamid Band,[2] and Vimla Band[1,3,4]

[1]Department of Genetics, Cell Biology and Anatomy and [2]Eppley Institute for Cancer and Allied Diseases and UNMC-Eppley Cancer Center, University of Nebraska Medical Center, Omaha, Nebraska; [3]New England Medical Center, Tufts University, Boston, Massachusetts; and [4]Evanston Northwestern Healthcare Research Institute, Department of Medicine, [5]Department of Bioinformatics, and [6]Genomic Core, Feinberg School of Medicine, Northwestern University, Chicago, Illinois

Abstract

Rho family small GTPases serve as molecular switches in the regulation of diverse cellular functions, including actin cytoskeleton remodeling, cell migration, gene transcription, and cell proliferation. Importantly, Rho overexpression is frequently seen in many carcinomas. However, published studies have almost invariably used immortal or tumorigenic cell lines to study Rho GTPase functions and there are no studies on the potential of Rho small GTPase to overcome senescence checkpoints and induce preneoplastic transformation of human mammary epithelial cells (hMEC). We show here that ectopic expression of wild-type (WT) RhoA as well as a constitutively active RhoA mutant (G14V) in two independent primary hMEC strains led to their immortalization and preneoplastic transformation. These cells have continued to grow over 300 population doublings (PD) with no signs of senescence, whereas cells expressing the vector or dominant-negative RhoA mutant (T19N) senesced after 20 PDs. Significantly, RhoA-T37A mutant, known to be incapable of interacting with many well-known Rho effectors including Rho kinase, PKN, mDia1, and mDia2, was also capable of immortalizing hMECs. Notably, similar to parental normal cells, Rho-immortalized cells have WT p53 and intact G_1 cell cycle arrest on Adriamycin treatment. Rho-immortalized cells were anchorage dependent and were unable to form tumors when implanted in nude mice. Lastly, microarray expression profiling of Rho-immortalized versus parental cells showed altered expression of several genes previously implicated in immortalization and breast cancer progression. Taken together, these results show that RhoA can induce the preneoplastic transformation of hMECs by altering multiple pathways linked to cellular transformation and breast cancer. [Cancer Res 2009;69(2):483–91]

Introduction

A large body of evidence implicates Ras-like small G proteins as major players in the regulation of a variety of cellular processes. Rho GTPases cycle between inactive GDP-bound and active GTP-bound states, a transition controlled by guanine nucleotide exchange factor proteins, which convert the GDP-bound to GTP-bound form, and by GTPase-activating proteins, which stimulate the low-intrinsic GTPase activity to convert the active to inactive form (1). It is believed that the multitude of cellular processes regulated by Rho reflects the interaction of the active form with several distinct effector molecules and subsequent activation of these effectors (1–3). For example, Rho effectors such as phosphatidylinositol-4-phosphate 5-kinase, Rho kinase (and related ROCK kinase), formin homology protein p140-Dia, and rhophilin have been linked to the regulation of actin cytoskeleton organization (1, 4–6), and citron kinase seems to regulate cytokinesis (7, 8). Recent evidence suggest a role of Rho effector PKN in cortical actin formation (9) and in G_2-M checkpoint regulation (10).

At the cellular level, Rho family small GTPases have emerged as key regulators of cell adhesion, migration, endocytic trafficking, cytokinesis, gene transcription, and cell proliferation through control of the actin cytoskeleton remodeling and other cellular responses to external stimuli (2, 11, 12). The role of Rho G proteins in cell proliferation and oncogenesis is emphasized by the fact that most of their exchange factors were originally identified as oncogenes and by the facilitation of cellular transformation by activated Rho and reversal of various aspects of the transformed phenotype, including invasive behavior, by interrupting Rho function (13–18). Dysfunctional regulation of Rho GTPases has been implicated in certain aspects of cancer development. For instance, overexpression of activated Rho mutants can transform fibroblasts (13). Rho proteins promote cell cycle progression through enhanced cyclin-dependent kinase activity by regulating the levels of cyclin D1, $p21^{WAF1}$, and $p27^{KIP1}$ (14). Transcriptional up-regulation of the levels of particular Rho proteins has been described in many types of human cancers, including cancers of the colon, breast, lung, stomach, and pancreas, and was correlated with tumor progression and invasion (15–18). In breast cancer, increased RhoA expression correlated with cancer progression (17, 18), and Rho protein overexpression was shown to contribute to breast cancer cell invasion and metastasis (18). However, the role of Rho proteins in the early steps of transformation of primary human epithelial cells, which are normally programmed to undergo replicative senescence, has not been investigated.

Here, we report that ectopic overexpression of not only a constitutively active RhoA but also the WT RhoA induces the immortalization of primary human mammary epithelial cells (hMEC). Importantly, a point mutant of RhoA, T37A, previously known not to interact with most well-known Rho effectors, such as Rho kinase, PKN, and mDia, also was capable of immortalizing the hMECs. Rho-immortalized hMECs have an intact G_1 cell cycle checkpoint, do not exhibit anchorage-independent growth, and do

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

X. Zhao and L. Lu contributed equally to this work.

Current address for L. Lu: Research Lab of Cardiovascular Disease, Jiaotong University, Shanghai 200025, China.

Requests for reprints: Vimla Band, Department of Genetics, Cell Biology and Anatomy, University of Nebraska Medical Center, 985805 Nebraska Medical Center, Omaha, NE 68198. Phone: 402-559-8565; Fax: 402-559-7328; E-mail: vband@unmc.edu.

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not form tumors in nude mice. Microarray analyses of Rho-immortalized versus parental MECs revealed altered expression of several genes known to be involved in cellular immortalization and breast cancer progression. These results show that ectopic expression of RhoA can induce the preneoplastic transformation of mammary epithelial cells apparently by dysregulating several biochemical pathways linked to cellular transformation and breast cancer.

Materials and Methods

Cell strains and cell culture. Reduction mammoplasty-derived hMECs, 76N and 70N, were grown in the DFCI-1 medium, as described previously (19). RhoA-immortalized cells were grown in DFCI-1 medium supplemented with 100 μg/mL G418 (Sigma).

Plasmid constructs. Rho constructs were subcloned in pLXSN retroviral vector (Clontech) from pTB701 plasmid (kindly provided by Dr. Yoshitaka Ono, Kobe University, Kobe, Japan). RhoA-T19N was PCR amplified from pcDNA-RhoA.T19N (kindly provided by Dr. Arthur Mercurio, University of Massachusetts Medical School, Worcester, MA) and cloned in pLXSN.

Retroviral infection of mammary epithelial cells. Retrovirus-containing culture supernatants were prepared as described previously (20). 76N or 70N cells (5×10^5 per 100-mm dishes) were exposed to retroviral supernatants containing 4 μg/mL polybrene. Stable cell lines were established by selection in G418 (100 μg/mL).

Western blot analysis and antibodies. Cell lysates were quantitated using the bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.). Denatured proteins were resolved on SDS-PAGE gels, transferred to polyvinylidine difluoride membranes (Millipore), and Western blotted using monoclonal antibodies against anti-RhoA (26C4), anti-p53 (DO-1), and anti-p21 (F-5; Santa Cruz Biotechnology, Inc.) and anti-β-actin (AC-15, Abcam).

Glutathione *S*-transferase pull-down assay. Glutathione *S*-transferase (GST) fusion proteins were expressed in BL21 bacterial cells and purified with glutathione Sepharose 4B beads (Amersham Biosciences). 293T cells were transfected with myc-tagged Prks-ROCK1 or Prks-ROCK2, flag-tagged Prc-PKN-AL, or mDia1 using calcium phosphate method. The transfectants were lysed in lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 100 mmol/L NaCl, 1 mmol/L DTT, 5 mmol/L $MgCl_2$, 50 mmol/L NaF, 1 mmol/L EDTA, 1 mmol/L Na_3VO_4, 10% glycerol, 1% NP40, 1 mmol/L phenylmethylsulfonyl fluoride] and spun at 12,000 rpm, and 1 mg each of these supernatants was incubated with 5 μg of GST or various fusion proteins that were loaded with GTP-γ-S in loading buffer [20 mmol/L Tris-HCl (pH 7.5), 1 mmol/L DTT, 10 mmol/L EDTA, 50 mmol/L NaCl, 5% glycerol, 0.1% Triton X-100, 1 mmol/L $MgCl_2$, 100 μmol/L GTP-γ-S] for 4 h at 4°C. Beads were washed and loaded onto 12.5% SDS-PAGE gel. After electrophoresis, the gel was cut into two parts: the upper part that contained ROCK1, ROCK2, PKN, and mDia1 was transferred into polyvinylidene difluoride (PVDF) membrane and probed with anti-myc or anti-flag antibodies and the lower part that contained GST fusion proteins was stained with Coomassie Blue R-250.

Telomerase assays. Telomerase activity and telomerase length were determined, as described previously (21). Briefly, genomic DNA was isolated from cells using the phenol-chloroform method. Genomic DNA (3–5 μg) was digested with *Hin*fI and *Rsa*I followed by Southern blot analysis using the ^{32}P-labeled TTTAGGG oligonucleotide probe.

DNA damage checkpoint analysis. Cells were treated with 0.5 μg/mL Adriamycin or DMSO for 24 h. For thymidine incorporation, cells were pulsed with [^{3}H]thymidine for 6 h, fixed, and subjected to autoradiography as described previously (21). Labeled nuclei were counted and expressed as % labeled nuclei. Total cell lysates were examined for p53 and p21 protein levels using Western blot analysis.

Anchorage-independent growth in soft agar. A base layer of 0.6% agarose was prepared by diluting a 1.2% sterile stock 1:1 with 2× DMEM or D medium and plating 2 mL per well in six-well plates. The top agarose layer (0.3%; 2 mL) containing 2×10^4 cells was then layered on top of the base layer. The number of colonies was counted after 2 wk; colonies 100 cells or larger were considered positive.

Tumorigenicity assays. Six-week-old female athymic nude (*nu/nu*) mice (Charles River Laboratories) were injected s.c. close to the fourth mammary gland with 10^6 cells in 0.2 mL of 1:1 Matrigel (source) and PBS and observed for any tumor growth. Animals were euthanized and necropsies were performed when tumors reached 1 to 1.5 cm in diameter (in case of positive control cell line) or after 6 mo if no tumors were observed. Each cell line was tested in at least five animals. All animal-related procedures were carried out in accordance with the Institutional Animal Care and Use Committee guidelines.

Microarray analyses. RNA was isolated from parental and Rho immortal 76N cells in three independent experiments. RNA quality check, labeling of cRNA, cRNA fragmentation, hybridization of labeled cRNA to GeneChip, and scanning were performed by Microarray Core Facility, Northwestern University. Affymetrix Human Genome U133 Plus 2.0 chips (containing >47,000 transcripts/chip) were used. After hybridization, the chips were scanned by BeneChip Scanner 3000. Statistical analysis of the microarray data was performed by Bioinformatics Core, Northwestern University. Microarray data were collected and achieved in accordance with the MIAME guideline. The annotation of the HG-U133 Plus 2 microarray was updated using the Entrez gene database at the National Center for Biotechnology Information (NCBI). Raw Affymetrix measurements were normalized with a quantile model and quantified with the RMA algorithm using the Bioconductor package. 5′ to 3′ intensity bias and residuals from the RMA model were used for quality assessment of the microarray results. Unsupervised cluster analysis of the samples, genes with fold changes larger than two, was used to confirm the grouping of different phenotypes and experiment replicates. A linear model with Bayesian adjustment (LIMMA) was used to find differentially expressed genes with a statistical confidence of false discovery rate smaller than 0.01. To visualize results, gene expression was clustered using the TreeView program.

Reverse transcription-PCR and quantitative PCR. Total RNA was isolated using Trizol reagent according to the manufacturer's instructions (Invitrogen). Reverse transcription-PCR (RT-PCR) was performed using SuperScript One-Step RT-PCR kit (Invitrogen). RNA (0.5 μg) was used for each RT-PCR reaction. For quantitative PCR, single-stranded cDNA was produced by reverse transcription using 1 μg RNA in 20 μL reaction (Promega). Quantitative PCR was performed using the SYBR Green reagents on the 7500 Real-Time PCR System (Applied Biosystems).

Results

Overexpression of WT RhoA or activated RhoA-G14V but not RhoA-T19N induces the immortalization of hMECs. The Rho family small GTPases are widely accepted as key regulators of cell adhesion, migration, endocytic trafficking, cytokinesis, gene transcription, and cell proliferation (1, 2, 11, 12). As essentially all of these roles have been assigned based on experiments using immortalized or transformed cell lines that have undergone many genetic alterations, we examined the consequences of RhoA overexpression in primary hMECs. A hMEC strain 76N was infected with retrovirus supernatants generated using the vector, RhoA-WT, RhoA-G14V (constitutively active Rho), or RhoA-T19N (dominant-negative Rho) constructs. Cells were subjected to G418 selection and maintained in G418-containing DFCI-1 medium thereafter. Western blot analysis of lysates after 48 hours of infection showed that all Rho proteins were expressed in transduced cells (Fig. 1*A*). As expected, 76N cells transduced with vector proliferated initially and then senesced ~20 population doublings (PD; Fig. 1*B*). Similarly, 76N cells transduced with dominant-negative RhoA-T19N senesced ~20 PDs (Fig. 1*B*). Both the WT and G14V-expressing cells, however, continued to grow for about a month, followed by about a 2-week "crisis" period where cells stopped growing and eventual emergence of cells that continued to grow with no signs of senescence. These cells have continued to grow beyond 300 PDs without any evidence of senescence, at which time they were

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frozen. Notably, the G14V-immortalized cells reproducibly expressed much lower levels of RhoA protein compared with the WT-immortalized cells (Fig. 1*A*). The reason for the lower protein levels is unclear at present; it may reflect the selection of immortal cells expressing relatively low levels of active G14V protein as high levels of active Rho protein are reported to induce apoptosis (22). These experiments were repeated thrice and similar results were obtained. These results show that overexpression of both the WT and constitutively active RhoA proteins leads to immortalization of primary hMECs. Notably, neither the parental cells nor the vector or T19N transduced cells led to immortal derivatives, indicating that the immortalization process is dependent on the expression of active RhoA.

RhoA-mediated immortalization does not involve Rho effectors, Rho kinase, PKN, and mDia1. The ability of WT and constitutively active RhoA, but not the GDP-binding mutant, to immortalize hMECs suggested that Rho effectors can overcome the senescence checkpoint that limits the life span of normal hMECs. As a large body of literature implicates Rho kinase, PKN, and mDia proteins as major Rho effectors in cell transformation–related phenotypes imparted by active Rho proteins, we wished to examine if RhoA induced hMEC immortalization through these effectors. We used the RhoA-T37A mutant for this purpose as it has been shown in the literature to be incapable of interacting with Rho kinase, PKN, and mDia effectors (23). We first confirmed the reported inability of T37A mutant to interact with specific effector using the well-established pull-down assay using GTP-loaded recombinant GST fusions of Rho proteins (see Materials and Methods). We confirmed that WT and G14V could clearly pull down the Rho kinases ROCK1 and ROCK2 (Fig. 2*A* and *B*) as well as PKN (Fig. 2*C*) and mDia (Fig. 2*D*); in contrast, T37A failed to pull down these effectors under identical conditions. As expected, the T19N protein, used as a negative control, did not interact with any of the effectors tested (Fig. 2).

Next, we used retroviral infection to introduce the T37A protein into hMECs and examined its ability to induce their immortalization. Surprisingly, similar to cells expressing the WT or G14V, cells expressing the T37A mutant continued to grow without any signs of senescence (Fig. 1*B*). These cells have been cultured for >300 PDs without showing any signs of senescence before cryopreservation. Notably, similar to cells immortalized with G14V, cells immortalized with the T37A mutant also express a substantially lower level of this mutant compared with that in the WT-immortalized cells (Fig. 1*A*).

Taken together, these experiments show that the ability of the ectopically overexpressed RhoA-WT, G14V, and T37A to immortalize hMECs indicates that pathways distinct from the well-known effectors of RhoA can mediate RhoA-dependent immortalization of normal hMECs.

Telomerase activity increases with RhoA-induced immortalization of hMECs. An essentially invariant feature of human cells undergoing immortalization is the induction of telomerase activity (21, 24–27). We therefore assessed the level of telomerase activity in hMECs transduced with WT, G14V, or T37A at different passages using the TRAP assay. As expected, the parental hMECs as well as the vector-transduced cells showed barely detectable levels of telomerase activity (Fig. 3*A lanes 2* and *3*), whereas the TERT-immortalized 76N cells (positive control) exhibit high telomerase activity (Fig. 3*A*, *lane 1*). Notably, telomerase activity increased with increasing PDs in cell lines where immortalization was eventually achieved (Fig. 3*A*).

Induction of telomerase activity is thought to play a key role in negating the telomere attrition associated with replicative senescence by maintaining and/or elongating the telomeres (27). To examine if the induction of telomerase activity during RhoA-induced immortalization contributes toward stabilization and/or elongation of telomeres, we measured telomere length in these cells using the TRF assay. Initially, hMECs transduced with the WT, G14V, or T37A RhoA proteins showed an average telomere length of 6 to 9 kb, similar to that of parental 76N cells; however, with increasing PDs, hMECs immortalized as a result of the overexpression of Rho proteins showed telomeres of ~2.5 kb (Fig. 3*B*). These cells have maintained the same telomere lengths in subsequent passages (data not shown). These data suggest that telomerase activity in Rho-expressing cells does not result in a net increase in telomere length but seems to maintain telomeres. Collectively, these results are consistent with the idea that ectopic overexpression of RhoA proteins induces the immortalization of hMECs via a telomerase-dependent pathway.

Rho-immortalized cells maintain an intact cell cycle checkpoint. We have previously shown that immortalization of hMECs with viral oncogenes, such as human papillomavirus (HPV) E6 or E7, or overexpression of mutant cellular genes, such as

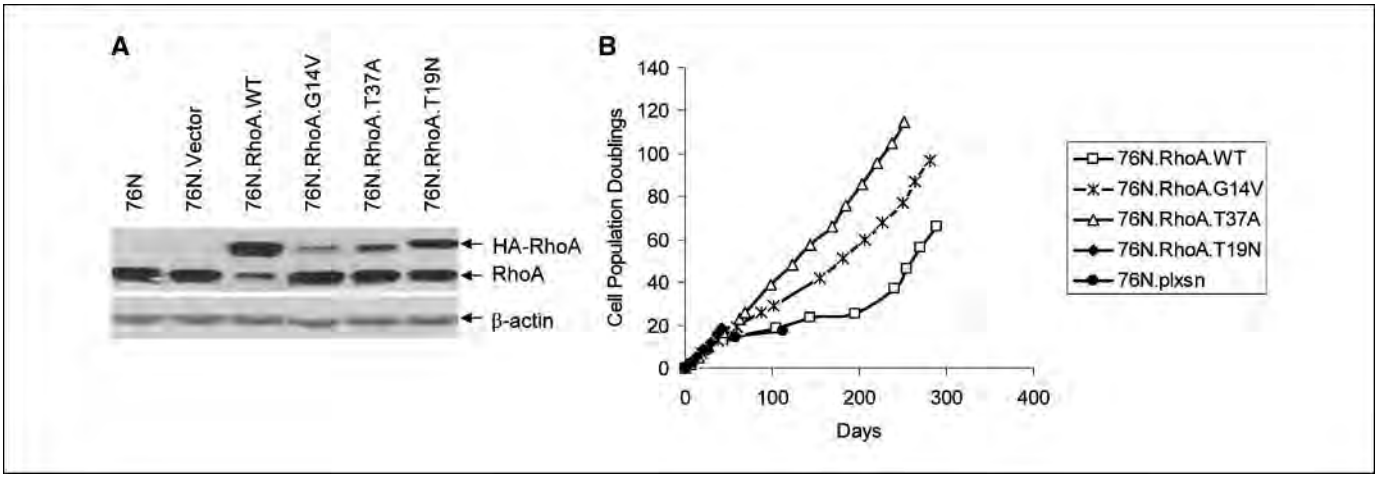

Figure 1. RhoA overexpression induces hMEC immortalization. *A,* cell lysates from indicated cells were analyzed for RhoA expression using anti-RhoA or β-actin (loading control) antibodies by Western blotting. *B,* cumulative PDs of cells expressing vector or various Rho mutants.

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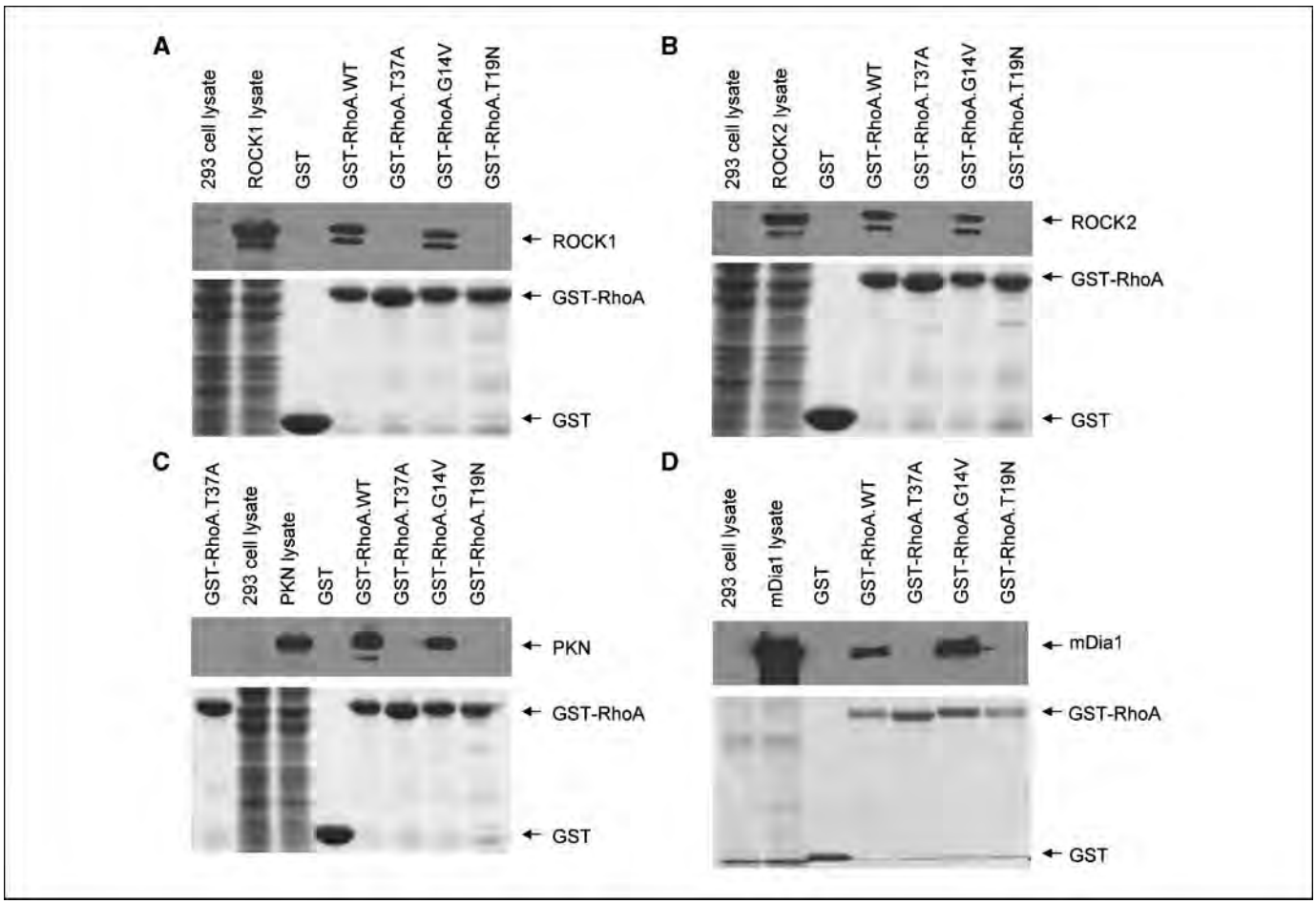

Figure 2. Mutant RhoA-T37A is incapable of interacting with well-known Rho effectors. Various plasmids, Prks-ROCK1, Prks-ROCK2, Prc-PKN-AL, and pFL-mDia1, were transfected into 293T cells, and cell lysates were incubated with GTP-γ-S–loaded GST, or various GST fusion proteins, and loaded into SDS-PAGE gel. After separation of proteins, the gels were cut into two parts: the upper part was transferred to PVDF membrane and probed with anti-myc and anti-flag antibodies to detect myc-tagged ROCK1 or ROCK2 and flag-tagged PKN or mDia1 and the lower part that contains GST or GST fusion proteins was stained with Coomassie Blue R-250.

mutant p53, causes the abrogation of the DNA damage checkpoint (28–31). In contrast, we have shown that overexpression of another cellular gene, *Bmi-1*, led to immortalization without abrogating the DNA damage checkpoint (21). To assess the effect of Rho-induced immortalization on DNA damage cell cycle checkpoint, Rho-immortalized cells and normal parental cells as well as the HPV E6–immortalized hMECs (used as positive control) were treated with Adriamycin for 24 hours and assessed for their ability to incorporate [^{3}H]thymidine (an indication of DNA synthesis). As expected, the parental 76N cells failed to incorporate [^{3}H]thymidine after Adriamycin treatment, indicating an intact DNA damage–induced cell cycle arrest. In contrast, the HPV E6–immortalized MECs continued to incorporate [^{3}H]thymidine after Adriamycin treatment, indicating an abrogation of the DNA damage cell cycle checkpoint (Fig. 4*A*). Importantly, hMECs immortalized by the ectopic expression of each of the RhoA proteins behaved similar to normal parental cells, showing that expression of RhoA does not affect the DNA damage cell cycle checkpoint (Fig. 4*A*). Consistent with [^{3}H]thymidine incorporation, p53 levels increased dramatically after Adriamycin treatment of 76N as well as RhoA-immortalized cells but not in E6-immortalized cells (Fig. 4*B*), indicating that p53 expression and function are intact in RhoA-immortalized cells.

RhoA-induced immortalization is a generalized phenomenon in hMECs. Considering that RhoA expression in one hMEC strain, 76N, reproducibly induced their immortalization, we wished to assess if this is a generalized phenomenon in hMECs. For this purpose, we retrovirally infected an independent hMEC strain 70N with RhoA constructs, as above. Similar to the results obtained with 76N cells, 70N cells expressing RhoA-WT, G14V, or T37A, but not the vector- or T19N-transduced cells, exhibited immortalization (Fig. 5*A*). We repeated these experiments twice and obtained immortal cells in both cases. 70N cells immortalized with Rho are in continuous passage for >200 PDs with no signs of senescence. Similar to 76N cells, these cells show an intact DNA damage–induced p53 induction response (Fig. 5*B*).

RhoA-immortalized cells are anchorage dependent and are unable to form tumors in nude mice. To assess if the immortalization of hMECs initiated by RhoA protein overexpression represents a preneoplastic transformation or a more advanced stage of oncogenic transformation as would be suggested by prior studies of Rho protein overexpression in model cell system (13), we examined their ability to grow in soft agar. Although human tumor cell lines do exhibit anchorage independence for growth, most immortal cells do not exhibit anchorage independence (20). Similar to parental cells, Rho-immortalized cells failed to form colonies in

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soft agar, whereas Hs578T, a metastatic breast cancer cell line used as a positive control, formed large soft agar colonies (Supplementary Fig. S1). Thus, Rho expression does not confer anchorage independence in hMECs.

To determine whether anchorage-dependent growth of Rho-immortalized cells reflected their incomplete neoplastic transformation, we examined their ability to grow as xenogeneic transplants in nude mice, a trait that correlates well with advanced malignant behavior of human breast cells. For this purpose, we injected 2×10^6 cells mixed with Matrigel into the mammary gland area of nude mouse, as Matrigel has been reported to enhance the tumorigenic potential of human cells (32). As expected, five of five mice injected with MDA-MB-231 cells, a breast tumor cell line known to form tumors in nude mice and used as positive control, formed large tumors. In contrast, none of the RhoA-immortalized cells exhibited any tumor growth (Supplementary Table S1) even when maintained for up to 6 months before euthanasia. Taken together, these experiments clearly show that ectopic overexpression of RhoA induces preneoplastic transformation/immortalization but not full transformation.

Microarray analyses. In view of our results that not only the WT and constitutively active RhoA but also a mutant (T37A) that failed to interact with major oncogenic transformation–relevant effectors could induce the immortalization of primary hMECs, we carried out gene expression profiling analyses to identify the potential pathways that could contribute to RhoA-induced immortalization. Therefore, we compared the gene expression profiles of normal hMECs with those of cells immortalized using RhoA-WT, G14V, or T37A using the Affymetrix Human Genome U133 Plus 2.0 chips with >47,000 transcripts for microarray analysis. The microarray data showed that the expression of ~30 genes was increased, whereas that of a set of ~100 genes was reduced in cells immortalized with RhoA proteins (NCBI Gene Expression Omnibus accession number, GSE 12917; Supplementary Table S2). Based on published links of the candidate genes to cell transformation, we selected a subset of genes, *ZNF217, ELF3, S100P, CLCA2*, and *DAB2*, and confirmed altered expression in immortalized cells using RT-PCR, Western blotting, and real-time PCR. Our results show that *ZNF217, ELF3*, and *S100P* are overexpressed (Fig. 6*A*; Supplementary Fig. S2), whereas *CLCA2* and *DAB2* are down-regulated in RhoA-immortalized hMECs (Fig. 6*A* and *B*; Supplementary Fig. S2). Importantly, the altered expression levels of these genes were also observed in several breast cancer cell lines (Fig. 6*C* and *D*), implying that these genes may in fact be relevant to Rho-induced immortalization of hMECs and that these genes may be linked to oncogenic transformation in breast cancer.

Discussion

A large number of studies have implicated the crucial role of Rho family GTPases in several cell biological processes linked to oncogenesis: they regulate cell migration through actin cytoskeleton reorganization, participate in transcriptional regulation, and are linked to cell cycle control. Consistent with these functions, Rho proteins have been linked to human cancer (15–18). Rho

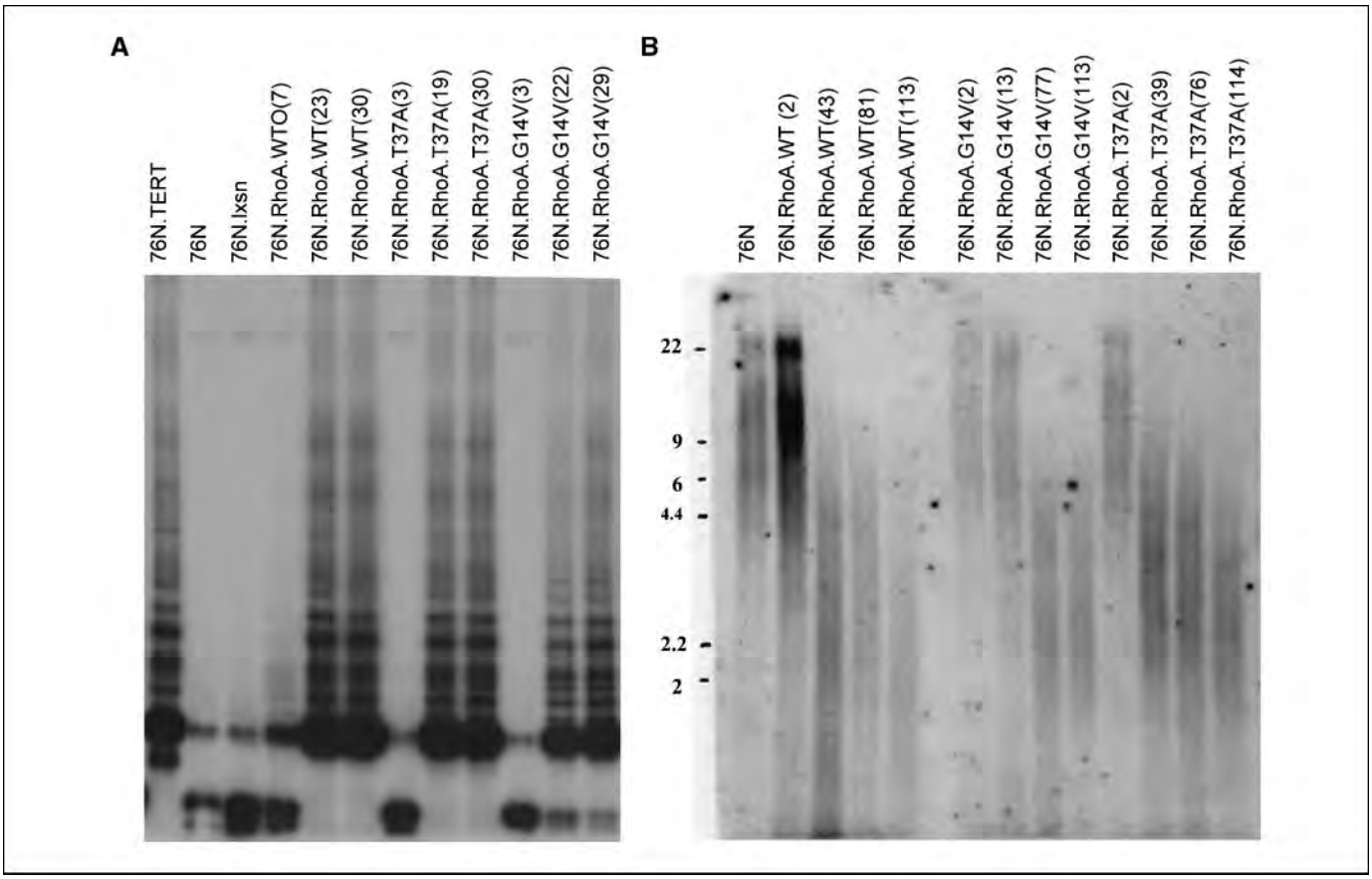

Figure 3. Telomerase activity is induced in immortal cells and the telomere length is maintained. *A,* telomerase activity at indicated passages (shown in parenthesis) was measured with extracts of 76N.TERT (positive control), 76N, 76N transduced with pLXSN vector (negative control), RhoA-WT, or various Rho mutants. *B,* the telomere length was determined by digesting genomic DNA from cells. The digested DNA was hybridized with a telomeric probe as described in Materials and Methods.

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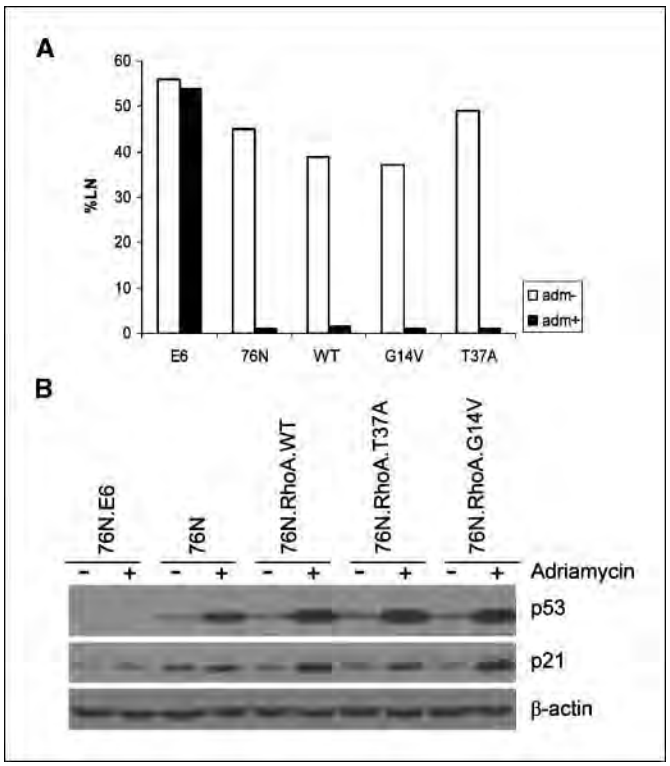

Figure 4. Rho-immortalized cells express normal p53 and maintain intact cell cycle checkpoint. *A,* 76N (used as positive control), 76N-E6 (used as negative control), and RhoA-immortalized cells were assessed for their ability to synthesize DNA [% labeled nuclei (*%LN*)] using [^{3}H]thymidine incorporation after Adriamycin treatment. *B,* immunoblotting of cell lysates with antibodies against p53, p21, or β-actin (as control) after treatment with Adriamycin.

proteins have been implicated in breast tumor progression: for example, elevated RhoA expression is seen in breast tumors compared with adjacent normal breast tissue, and migration and invasion properties of breast cancer cells were blocked by inhibiting Rho activity (17, 18). In addition, RhoC has been linked to inflammatory breast cancer and overexpression of RhoC in immortalized hMECs induces their transformation (33). Importantly, given the linkage of Rho proteins to integrin receptor signaling and cell migration, essentially all of the previous studies have examined the role of Rho proteins in the context of late events in tumor progression, often with metastatic and invasive behaviors (15–18, 34, 35). In contrast, there have been no studies to date to assess the potential role of Rho proteins in very early events in oncogenic transformation of hMECs.

Here, we have carried out studies to examine the ability of RhoA protein to overcome senescence in normal hMECs. We show using two independent hMEC strains that RhoA overexpression led to their escape from senescence and continuous proliferation. Notably, not only the constitutively active RhoA but also the WT protein overexpression induced the immortalization of normal hMECs. An active Rho GTPase that was needed for immortalization was shown by the inability of a GDP-locked Rho protein to immortalize hMECs. The ability of WT RhoA to immortalize hMECs is significant because activating RhoA mutations are not reported in human cancers but overexpression of WT Rho is a frequent phenomenon in human cancers, including breast cancers. Thus, our results are consistent with the clinical data showing increased RhoA expression with breast tumor progression (17, 18).

Consistent with other models of mammary epithelial cell immortalization, RhoA-immortalized cells exhibit increased telomerase activity and stabilization of telomeres as they overcome the senescence checkpoint. However, we observed increase in telomerase activity in RhoA-immortalized cells after several passages of overexpression of RhoA, suggesting that it may not be a direct effect of RhoA overexpression. Thus, it is difficult to ascertain that increase in telomerase activity is a cause or effect of immortalization.

Unlike other models of deliberate hMEC immortalization, such as the expression of HPV E6 or SV40 large T (19, 20, 28, 31, 36), RhoA-immortalized cells maintained a functional p53 protein and an intact DNA damage cell cycle checkpoint. Thus, in contrast to observations made by us and others that abrogation of p53 function is a crucial event in hMEC immortalization by viral oncogenes, γ-radiation, RhoA-induced immortalization seems to proceed without a requirement to abrogate p53 function. Thus,

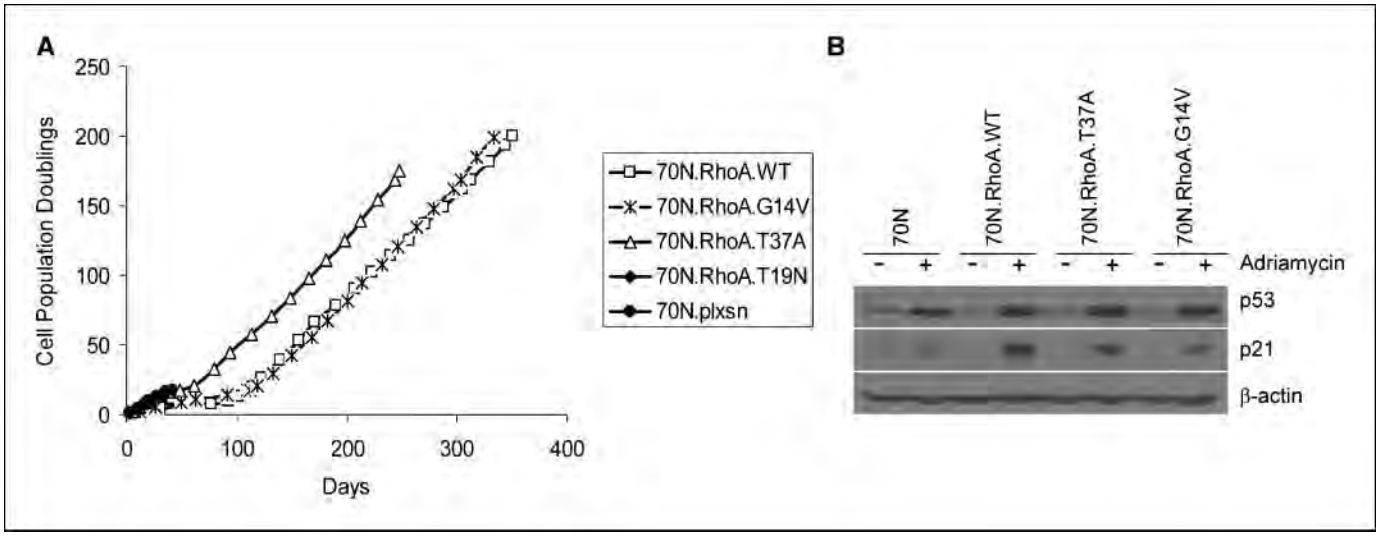

Figure 5. RhoA-induced immortalization is a generalized phenomenon. *A,* cumulative PDs of 70N cells infected with vector, WT RhoA, constitutively active RhoA (*G14V*), mutation in effector binding region (*T37A*), and dominant-negative RhoA (*T19N*). *B,* immunoblotting of cell lysates with antibodies against p53, p21, or β-actin (as control) after treatment with Adriamycin.

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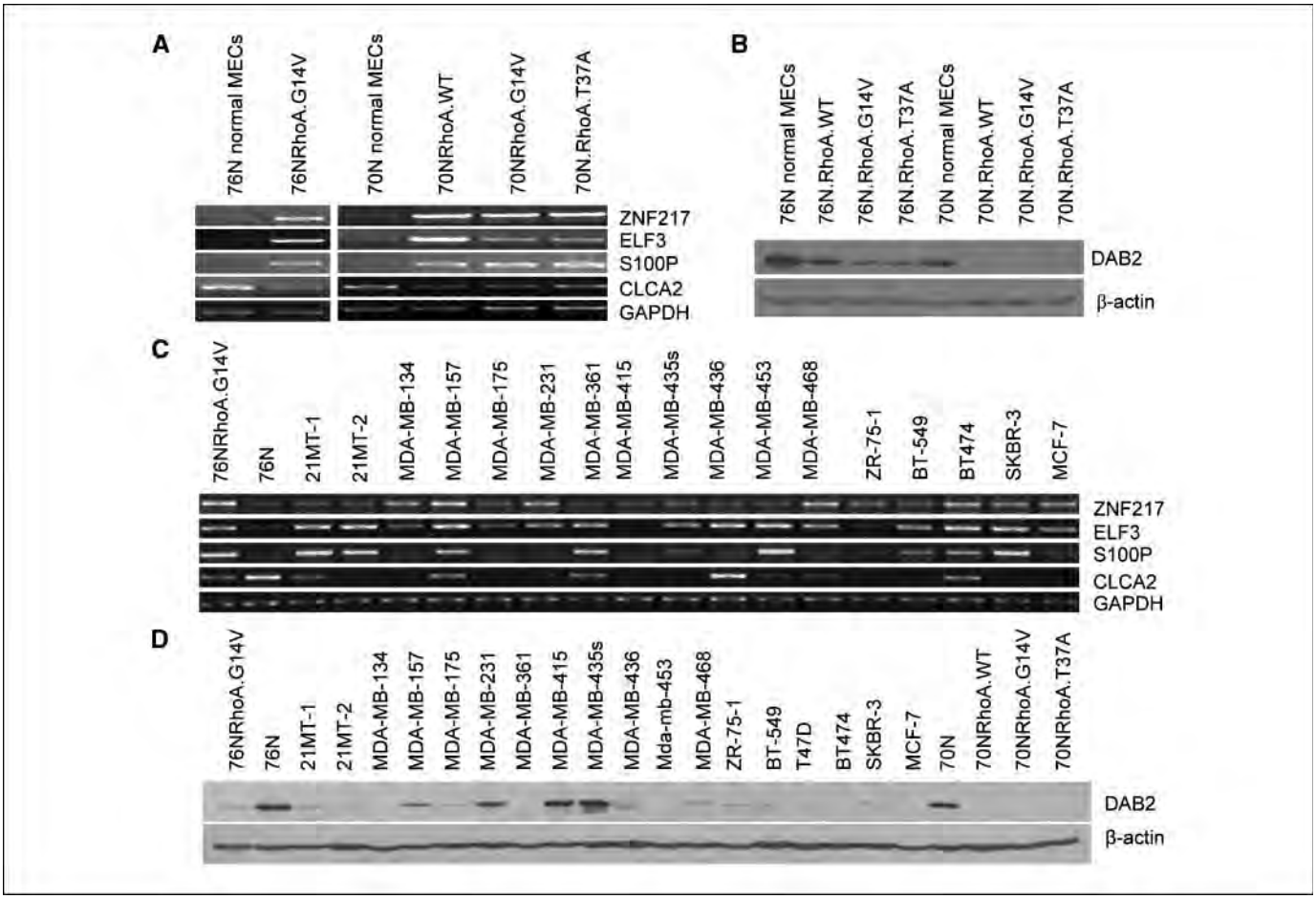

Figure 6. Microarray comparison of Rho-immortalized cells with parental cells identified several differentially expressed genes. Confirmation by RT-PCR and Western blotting. *A,* RT-PCR analyses showed that ZNF-217, ELF3, and S100P mRNAs were overexpressed in RhoA-immortalized cells, whereas CLCA2 mRNA expression was lower in RhoA-immortalized cells compared with parental 76N or 70N cells. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a PCR control. *B,* Western blotting of indicated cell lysates showed that DAB2 protein is decreased in RhoA-immortalized cells compared with parental 76N or 70N cells. β-Actin was used as a loading control. *C,* RT-PCR analysis of ZNF217, ELF3, S100P, and CLCA2 mRNA expression in breast cancer cell lines. Similar to RhoA-immortalized cells, several breast cancer cell lines showed increased mRNA expression for ZNF217, ELF3, and S100P and decreased mRNA expression for CLCA2 compared with normal 76N cells. *D,* Western blotting of cell lysates from breast cancer cell lines showed that, similar to Rho-immortalized cells, several breast cancer cell lines express lower levels of DAB2 protein compared with normal 76N and 70N cells.

active RhoA-dependent signals either by themselves or in conjunction with other events that occur in hMECs in culture seem to be sufficient to induce the immortalization of hMECs, without abrogating p53 function. In this regard, it will be of significant interest in the future to explore the role of p16 hypermethylation and loss of expression, and the ensuing loss of Rb function, which characterizes hMEC cultures during their initial selection process *in vitro* (37) cooperation with Rho to induce immortalization.

In addition to preservation of the p53-dependent G_1 cell cycle checkpoint, RhoA-immortalized cells exhibit an inability to grow in an anchorage-independent manner and do not form tumors when implanted in immune-incompetent mice, suggesting that overexpression of RhoA induces a state of preneoplastic transformation of hMECs rather than full transformation. In this regard, the RhoA overexpression model of hMEC immortalization resembles other models that we and others have investigated using viral oncogenes, mutant cellular genes, radiation, or carcinogen treatment; all of these manipulations induce immortalization but not full transformation (19–21, 28–31, 36–38). Thus, the hMEC model described here provides a relatively unique system driven by a breast cancer–relevant cellular gene overexpression with a functional p53 and preneoplastic transformation for biological studies to understand the further genetic alterations that can collaborate with Rho signaling pathways to induce the full transformation of hMECs.

Several downstream effectors have been linked to Rho GTPase functions in normal cells as well as their oncogenic activity measured in rodent fibroblasts. Our initial analyses suggest that the mechanisms by which RhoA overexpression induces the early neoplastic transformation of hMECs are likely to be distinct from traditionally explored pathways. In our studies, we made the unexpected observation that an effector domain mutant of RhoA, T37A, retained the ability to immortalize hMECs. As previously suggested, we found that RhoA-T37A is unable to bind to key effectors of RhoA, Rho kinase (ROCK1 and ROCK2), PKN, and mDia1, which have been linked to RhoA-dependent oncogenic transformation (15–18, 34). These results suggest that hMEC immortalization is unlikely to be through the activation of the well-characterized Rho effectors previously linked to oncogenic transformation.

Our microarray data provide an initial hint about the pathways that might be relevant to RhoA-induced immortalization of hMECs.

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Our analyses showed ~30 genes whose expression was up-regulated and ~100 genes whose expression was down-regulated in Rho-immortalized (as well as RhoA-T37A immortalized) cells compared with the normal parental cells (Supplementary Table S2). In our initial work, we used RT-PCR, real-time PCR, and Western blotting to confirm our microarray-based expression changes for a subset of five genes as these are altered in breast cancers. These studies confirmed that RhoA-immortalized cells have a reduced expression of CLCA2 and DAB2, whereas ELF3, S100P, and ZNF217 mRNA expression was up-regulated (Fig. 6*A* and *B*; Supplementary Fig. S2). Importantly, several breast cancer cell lines showed that the expression of these genes was altered in the same direction as in RhoA-immortalized hMECs (Fig. 6*C* and *D*), consistent with their potential involvement in breast cell transformation.

Prior studies have shown that ELF3/ESE1, an ETS family transcription factor, is up-regulated in a subset of breast tumors as well as during tumorigenic progression of MCF-12A hMEC line (39, 40). Similarly, several studies have implicated S100P in cellular immortalization (26, 41) and overexpression of S100P contributes to tumorigenesis as it promotes tumor growth, invasion, and cell survival (42). ZNF217 is frequently amplified in breast cancer (43), and its overexpression has been shown to induce mammary epithelial cell immortalization (38). CLCA2 (chloride channel, calcium activated, family member 2) is reportedly lost during tumor progression in human breast cancer; CLCA2 was found to be expressed in normal breast epithelium but not in breast cancer (44). Another study showed that expression of CLCA2 in CLCA2-negative MDA-MB-231 and MDA-MB-435 cells reduced the Matrigel invasion *in vitro* and metastatic tumor formation of MDA-MB-231 cells in nude mice (45). DAB2 (disabled 2) or DOC-2 (differentially expressed in ovarian carcinoma 2), originally isolated as a potential tumor suppressor gene from human ovarian carcinoma, is involved in modulating multiple signaling pathways and protein trafficking (46). Decreased expression of DOC-2/DAB2 has been observed in several cancers, including prostate, mammary, colon, and choriocarcinoma (46, 47). DOC-2/hDab-2 expression in breast cancer cells resulted in sensitivity to suspension-induced cell death (anoikis; ref. 48). Significantly, our analyses of Oncomine database[7] showed that S100P overexpression in breast cancers is correlated with high tumor grade in two breast cancer data sets, and its expression is higher in invasive breast cancers compared with breast ductal carcinoma *in situ* (Supplementary Fig. S3). Similarly, DAB2 expression is down-regulated in breast cancers in one data set and its down-regulation is correlated with lymphocytic infiltration and tumor grade in another two data sets (Supplementary Fig. S4). Thus, future studies to perturb the expression of these candidate genes in RhoA-immortalized hMEC system as well as analyses of how their expression is controlled by Rho-dependent signaling pathways should add significantly to our understanding of early oncogenic transformation of hMECs with direct relevance to human breast cancer.

In conclusion, the present study shows that RhoA, implicated in breast cancer oncogenesis by clinical studies and well known as a critical gatekeeper of receptor signals into multiple cell biological pathways, can induce the immortalization of hMECs. Notably, mammary epithelial cell immortalization by an effector domain mutant of RhoA that is incapable of interacting with well-characterized Rho effectors previously implicated in oncogenic transformation strongly suggests that RhoA-induced early transformation of hMECs proceeds to novel pathways. The system described here should prove suitable for future analyses to uncover the nature of these pathways and to link them to oncogenic pathways in breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Distinct Roles for Rho *Versus* Rac/Cdc42 GTPases Downstream of Vav2 in Regulating Mammary Epithelial Acinar Architecture*[S]

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Lei Duan[‡§1], Gengsheng Chen[§2], Sumeet Virmani[§3], GuoGuang Ying[§4], Srikumar M. Raja[‡§], Byung Min Chung[‡§], Mark A. Rainey[‡§], Manjari Dimri[§], Cesar F. Ortega-Cava[‡§], Xiangshan Zhao[§¶], Robert J. Clubb[‡§], Chun Tu[‡§], Alagarsamy L. Reddi[§5], Mayumi Naramura[‡§‖], Vimla Band[‡§¶6], and Hamid Band[‡§¶‖**7]**

*From the [‡]Eppley Institute for Cancer and Allied Diseases, the [¶]Department of Genetics, Cell Biology, and Anatomy, the [‖]Department of Biochemistry and Molecular Biology, College of Medicine, and the **University of Nebraska Medical Center-Eppley Cancer Center, University of Nebraska Medical Center, Omaha, Nebraska 68198, the [§]Evanston Northwestern Healthcare (now NorthShore University HealthSystem) Research Institute, Department of Medicine, Feinberg School of Medicine, Northwestern University, Evanston, Illinois 60201*

Non-malignant mammary epithelial cells (MECs) undergo acinar morphogenesis in three-dimensional Matrigel culture, a trait that is lost upon oncogenic transformation. Rho GTPases are thought to play important roles in regulating epithelial cell-cell junctions, but their contributions to acinar morphogenesis remain unclear. Here we report that the activity of Rho GTPases is down-regulated in non-malignant MECs in three-dimensional culture with particular suppression of Rac1 and Cdc42. Inducible expression of a constitutively active form of Vav2, a Rho GTPase guanine nucleotide exchange factor activated by receptor tyrosine kinases, in three-dimensional MEC culture activated Rac1 and Cdc42; Vav2 induction from early stages of culture impaired acinar morphogenesis, and induction in preformed acini disrupted the pre-established acinar architecture and led to cellular outgrowths. Knockdown studies demonstrated that Rac1 and Cdc42 mediate the constitutively active Vav2 phenotype, whereas in contrast, RhoA knockdown intensified the Vav2-induced disruption of acini, leading to more aggressive cell outgrowth and branching morphogenesis. These results indicate that RhoA plays an antagonistic role to Rac1/Cdc42 in the control of mammary epithelial acinar morphogenesis.

Differentiated epithelia display a polarized architecture that is essential for their functional role as protective barriers and secretory or absorptive surfaces. The polarized epithelial cells associate with each other through lateral cell-cell junctions, which functionally and biochemically segregate the apical surface from the extracellular matrix-contacting basal surface (1, 2). The cell-cell junctions and cell-extracellular matrix interactions stabilize the epithelial structure and ensure appropriate signaling (1, 2). Loss of apical and basolateral polarity is an invariant feature of tumors arising from epithelial cells, also known as carcinomas, which account for most human cancers (3).

In vitro polarity and morphogenesis of epithelia are typically studied using model cell lines, such as Madin-Darby canine kidney (MDCK)[8] cells as monolayers or in three-dimensional extracellular matrix gels, where cells form a hollow cyst with apicobasal polarity (4). However, linkage of polarity and morphogenesis to oncogenicity has increasingly led to the use of immortalized, non-tumorigenic human epithelial cells. For example, immortalized, non-tumorigenic human mammary epithelial cells (MECs) form basolaterally polarized acinar structures in three-dimensional culture on reconstituted matrices, such as Matrigel (5, 6). These acini consist of a monolayer of cells surrounding a hollow lumen, which is formed during morphogenesis through the elimination of central cells (6, 7). MECs in mature acini exhibit basolateral polarity with an integrin-enriched basal surface contacting the extracellular matrix, basolateral E-cadherin-enriched adherens junctions (AJs), and an apical surface enriched in proteins, such as GM130 or Muc1

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[1] To whom correspondence may be addressed: Dept. of Radiation Oncology, Rush University Medical Center, 1750 W. Harrison St., Jelke Bldg. Rm. 1306A, Chicago, IL 60612. E-mail: lei_duan@rush.edu.

[2] Present address: Coskata Energy, Warrenville, IL 60555.

[3] Dept. of Radiology, Northwestern University Feinberg School of Medicine, Chicago, IL 60611.

[4] Tianjin Cancer Hospital Cancer Institute, Tianjin Medical University, W. HuanHu Rd., HeXi District, Tianjin 30074, China.

[5] Dept. of Medicine, Northwestern University Feinberg School of Medicine, Chicago, IL 60611.

[6] To whom correspondence may be addressed: University of Nebraska Medical Center, 986805 Nebraska Medical Center, Omaha, NE 68198-5805. Tel.: 402-559-8565; Fax: 402-557-7238; E-mail: vband@unmc.edu.

[7] To whom correspondence may be addressed: University of Nebraska Medical Center, 986805 Nebraska Medical Center, Omaha, NE 68198-6805. Tel.: 402-559-8572; Fax: 402-559-4651; E-mail: hband@unmc.edu.

[8] The abbreviations used are: MDCK, Madin-Darby canine kidney; RTK, receptor tyrosine kinase; AJ, adherens junction; MEC, mammary epithelial cell; GEF, guanine nucleotide exchange factor; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; PI3K, phosphatidylinositol 3-kinase; DOX, doxycycline; Tet-On, tetracycline-inducible; shRNA, short hairpin RNA; RBD, Rho-binding domain; PBD, p21-binding domain; DAPI, 4′,6-diamidino-2-phenylindole; YFP, yellow fluorescent protein.

(7–9). Although the available immortalized and non-tumorigenic MEC lines, such as MCF10A, do not exhibit clear tight junctions, the ease of visualizing MEC architecture in three-dimensional culture has led to their extensive use in analyzing mechanisms of MEC morphogenesis and alterations of these processes during oncogenic transformation. When grown on Matrigel, non-tumorigenic MECs usually cease to proliferate by approximately day 14 to form quiescent, regular acinar structures (10, 11). In contrast, both oncogenically transformed MECs and breast cancer cells fail to form monolayer structures in Matrigel but proliferate continuously to form larger, irregular structures without hollow lumina (5, 12). The transition from acinar to irregular structures provides a relatively easy means of visualizing perturbations in polarity and morphogenesis as a result of alterations in specific biochemical pathways (6, 13, 14).

Receptor tyrosine kinases (RTKs) of the epidermal growth factor receptor (EGFR) family play critical roles in breast cancer tumorigenesis. EGFR overexpression is found in a significant proportion of breast cancers and correlates with increased aggressiveness and poor prognosis (15–17). When overexpressed in immortalized MECs, EGFR causes disruption of acinar structures (18), implying that EGFR levels need to be tightly controlled to maintain MEC homeostasis. Notably, EGFR levels are down-regulated during MEC acinar morphogenesis (19). Another EGFR family receptor, ErbB2, also induces irregular acinar structures when overexpressed in MECs (10).

Rho, Rac1, and Cdc42 are small GTPases that cycle between the GTP-bound active form and the GDP-bound inactive form, which are regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins, respectively (20). These GTPases control epithelial cell polarity, as demonstrated in both two- and three-dimensional cell culture systems (1, 21, 22). Previous work has shown that RhoA, Rac1, and Cdc42 are required for the establishment of AJs and participate in tight junction formation in model epithelial cells (1, 23). In three-dimensional culture, Rac1 and Cdc42 play essential roles in the establishment of apicobasal polarity of MDCK cells (24, 25). Paradoxically, these GTPases also disrupt cell-cell junctions and cell polarity when their constitutively active forms are expressed (26–29). Activation of these GTPases by RTKs is also known to regulate cell-cell junctions (30–33). For example, hepatocyte growth factor stimulation of the c-Met receptor activates Rho and Rac1, which in turn are critical for hepatocyte growth factor-induced loss of cell-cell adhesion and disruption of polarity in MDCK cells (34–37). In addition, transforming growth factor-β can induce loss of epithelial cell polarity through the ubiquitination and degradation of RhoA (38), and overexpression of constitutively active Rac1 can disrupt AJs (26). Notably, Rho, Rac1, and Cdc42 have been found to be overexpressed in breast cancer tissues, and their overexpression correlates with breast cancer progression (39). Understanding how Rho GTPases are regulated and how they function in controlling mammary epithelial architecture and morphogenesis, especially downstream of RTKs, is of considerable biological importance.

Rac1 and Cdc42 activation is associated with the disruption of epithelial polarity downstream of PI3K (27, 28). RTKs, such as EGFR, activate PI3K in MECs and are often overexpressed in breast cancer cells (40–42). Taken together, these results imply a role for Rac1- and Cdc42-directed GEFs in the loss of cell polarity in epithelial cells. Among the known Rac1 and Cdc42 GEFs, the Vav family of proteins (Vav1 to -3) are unique in that they directly couple to EGFR and undergo activation through phosphorylation of tyrosine residues located in their N-terminal acidic region (43). Vav2 is a ubiquitously expressed member of the Vav family. Studies in transfected cells have shown that Vav2 interacts with phosphorylated tyrosine residues on EGFR and is subsequently activated by tyrosine phosphorylation and interaction with PIP3, which is generated by PI3K (44). Although Vav2 was initially characterized as a GEF for Rho, Rac1, and Cdc42 (45, 46), recent findings suggest that it may predominantly activate Rac1 (47). Therefore, Vav2 is a likely candidate to be the Rac1-directed GEF downstream of EGFR and PI3K and a potential participant in the disruption of cell polarity in MECs and breast cancer cells.

Although stimulation of EGFR in model cells activates Vav2 and Rho GTPases (46, 48), our previous studies show that MEC lines, such as 16A5 and MCF10A (18), when grown in three-dimensional Matrigel cultures, retain their polarity despite the use of EGF-containing media. Because of this, we considered the possibility that alterations in Rho GTPase signaling machinery during three-dimensional culture may preserve MEC polarity. Because our preliminary data showed that Vav2 is the only Vav family member detected in a number of MECs, we investigated how Vav2 functions within MECs during three-dimensional culture. We found that growing 16A5 MECs in three-dimensional culture results in EGFR down-regulation. The three-dimensional cultured cells showed reduced Vav2 phosphorylation and a decrease in RhoA, Rac1, and Cdc42 activation in response to EGF stimulation. To test whether or not Vav2 and its downstream Rho GTPases affect MEC morphogenesis and architecture, we created a tetracycline-inducible (Tet-On) 16A5 MEC line in which the expression of a constitutively active Vav2 mutant (Y172F) is under the control of doxycycline (DOX). By inducing active Vav2 expression in combination with shRNA-mediated knockdown of RhoA, Rac1, or Cdc42 at different stages of acinar morphogenesis, we show that RhoA and Rac1/Cdc42 play distinct and seemingly opposite roles in the regulation of MEC polarity and morphogenesis.

EXPERIMENTAL PROCEDURES

Antibodies and Other Reagents—Rabbit anti-Vav2 peptide sera were generated against Vav2 amino acids 208–222 (QETEAKYYRTLEDIE) through a commercial vendor (Animal Pharma Inc.). The monoclonal anti-EGFR (528, ATCC) and anti-E-cadherin (clone E4.6) (49) (provided by Drs. Michael Brenner and Jonathan Higgins, Brigham and Women's Hospital, Boston, MA) antibodies were purified from mouse hybridoma supernatants. Purified anti-phosphotyrosine antibody 4G10 (50) was provided by Dr. Brian Druker (Oregon Health Sciences University, Portland, OR). The following antibodies were commercially obtained: monoclonal anti-RhoA, anti-Rac1, and anti-GM130 (BD Biosciences); anti-Rac1 (Cytoskeleton); anti-β-actin (Sigma); anti-α6 integrin

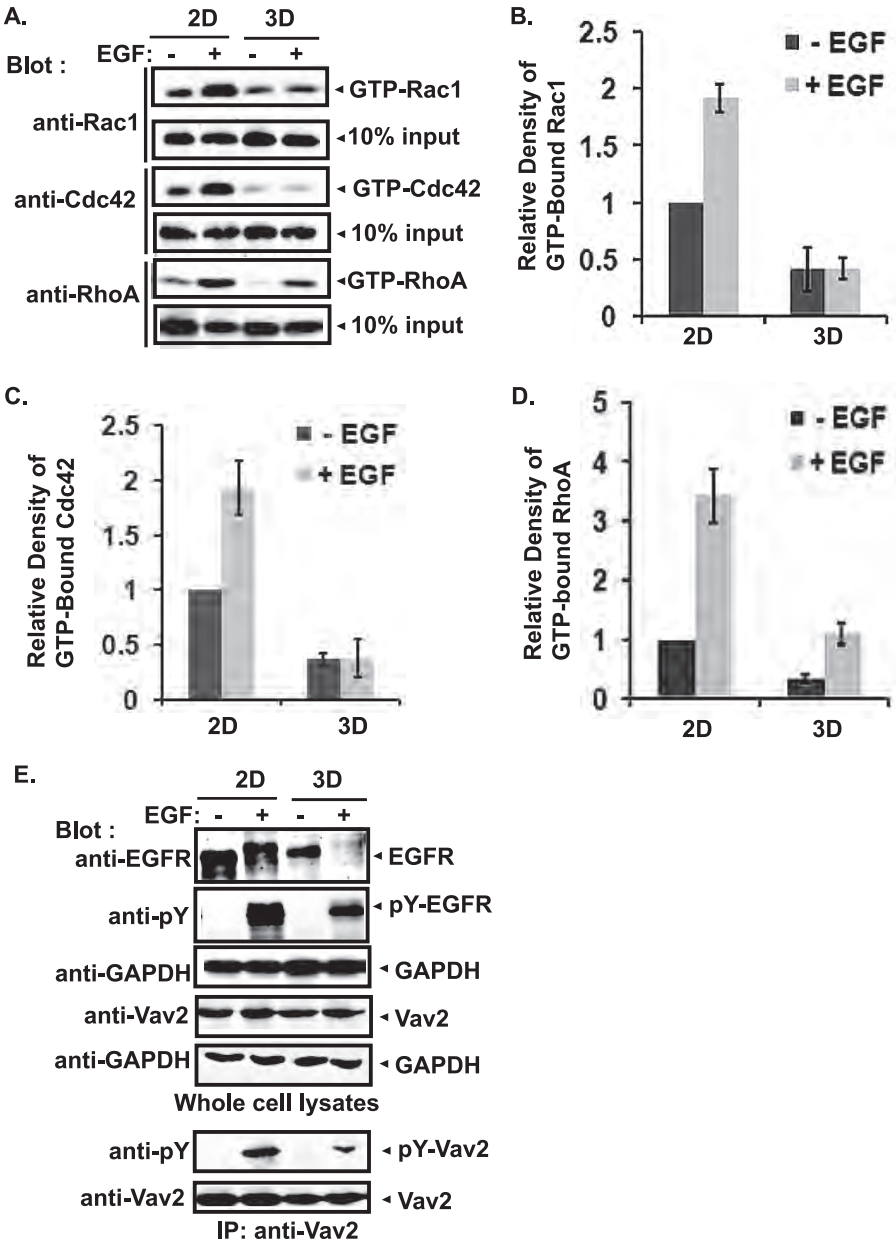

FIGURE 1. **Down-regulation of EGFR levels and phosphorylation, Vav2 phosphorylation, and activities of RhoA, Rac1, and Cdc42 in mammary epithelial cells grown in three-dimensional culture together with selective blunting of EGF-stimulated Rac1 and Cdc42 but not RhoA activation.** *A*, 16A5 cells were grown either in two- or three-dimensional culture (*2D* or *3D*, respectively) for 10 days and then EGF-starved for 3 days. The cells were either left unstimulated or stimulated with EGF (100 ng/ml) for 10 min prior to lysis. Whole-cell lysates were subjected to GST-RBD or GST-PBD pull-down and immunoblotted for RhoA, Rac1, or Cdc42 (representative image of three independent experiments is shown). *B–D*, the GTP-bound forms of Rac1, Cdc42, and RhoA were normalized to the loading control, and the average relative density in resting *versus* EGF stimulated cells and two-dimensional *versus* three-dimensional culture-grown cells were calculated (*error bars* represent S.D.). *E*, whole-cell lysates were immunoblotted for EGFR, phosphotyrosine (*pY*), or Vav2 and reprobed for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (loading control). Anti-Vav2 immunoprecipitates (*IP*) were immunoblotted for phosphotyrosine and reprobed for Vav2.

(Chemicon Inc.); and anti-Cdc42 (Cell Signaling). Alexa Fluor 594-conjugated phalloidin used to stain polymerized actin was from Invitrogen.

shRNA Constructs and cDNAs—The shRNA sequences specific for the genes of interest were identified using the online S-fold software, subjected to a BLAST search against the NCBI data base to minimize off-target possibilities, and cloned into the pSuper.retro vector (OligoEngine Inc.). The scrambled control nucleotide sequence is AAGAGCATCTCCACCTCTA. The shRNA sequences are as follows: for RhoA, GCAGGTAGAGTTGGCTTTG (sequence 1) and CGACAGCCCTGATAGTTTA (sequence 2); for Rac1, GACACGATCGAGAAACTGA (sequence 1) and GTGAAGAAGAGGAAGAGAA (sequence 2); and for Cdc42, GATAACTCACCACTGTCCA (sequence 3) and ACACAGAAAGGCCTAAAGA (sequence 5). The C-terminally YFP-tagged murine Vav2 cDNA was obtained from the Signaling Consortium (The Signaling Gateway) and subcloned into the pRevTRE vector (Invitrogen) through an engineered SalI site.

Human MEC Lines and Cell Culture—The 16A5 cell line is an HPV E6/E7-immortalized derivative of the primary MEC line 76N obtained from a normal human mammoplasty specimen (51). These cells were routinely maintained in two-dimensional culture as described (51). Standard procedures were used to introduce pSuper.retro-based retroviruses into 16A5 cells to generate stable shRNA-expressing lines (18). The transductants were selected and maintained in DFCI-1 medium (51) supplemented with puromycin (0.5 μg/ml) or G418 (500 μg/ml) and used as polyclonal cell lines. The 16A5-Tet-On cell line is a clone of the 16A5 cell line infected with pRev-Tet-On vector (Invitrogen) and selected in G418 for maximal induction of transfected genes.

For three-dimensional Matrigel culture, 2.5×10^3 cells in 0.4 ml of 2% reduced growth factor Matrigel (BD Biosciences) were added to DFCI-1 medium containing 3 ng/ml EGF and plated in a 60-mm plate onto a polymerized layer of 100% Matrigel, as described previously (11). Fresh medium was added to the cells every 2 days. Phase-contrast images were obtained at the indicated time points.

EGF Stimulation and GST Pull-down of Activated RhoA, Rac1, and Cdc42—For EGF stimulation, cells were grown for 3 days in DFCI-1 medium without EGF; EGF was then added at 100 ng/ml at the indicated time points before cell lysis. Bacterially expressed and purified GST-RBD (Rho-binding

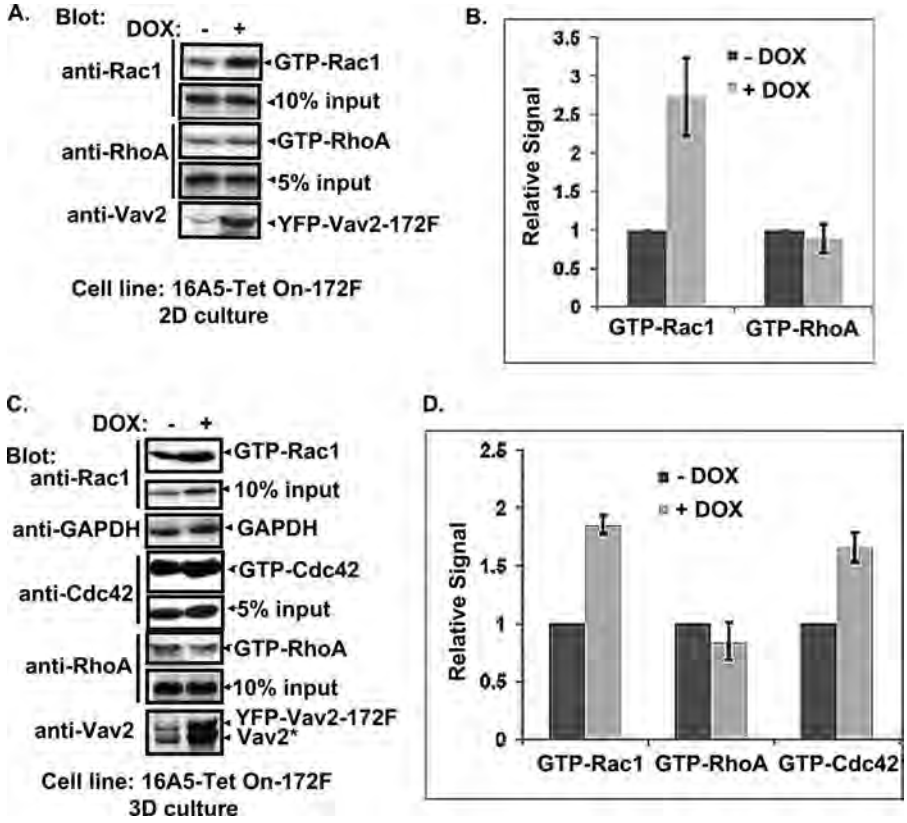

FIGURE 2. **Induced overexpression of constitutively active Vav2-Y172F mutant activates Rac1 (and Cdc42) but not RhoA in two-dimensional (*2D*) as well as three-dimensional (*3D*) culture.** 16A5-Tet-On-Vav2-Y172F cells were grown in two-dimensional culture in DFCI medium without or with DOX induction (2 μg/ml) for 3 days (*A* and *B*). Alternatively, cells were grown in three-dimensional culture for 14 days and then treated with vehicle or DOX (2 μg/ml) for 3 days (*C* and *D*). Rac1, Cdc42, and RhoA activation was assessed using pull-down assays (*A* and *C*) and quantified (*C* and *D*) as in Fig. 1 (representative image of three independent experiments is shown).

domain of rhotekin; interacts with activated GTP-bound RhoA) or GST-PBD (p21-binding domain of Pak1; interacts with activated GTP-bound Rac1 and Cdc42) was used to pull-down GTP-bound forms of RhoA or Rac1 and Cdc42, respectively, as described previously (52, 53). Two-dimensional cultured 16A5 MECs were washed with ice-cold Tris-buffered saline and lysed in radioimmune precipitation buffer (50 mM Tris, pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10 μg each of leupeptin and aprotinin, and 0.1 mM phenylmethylsulfonyl fluoride). Three-dimensional cultured cells were lysed in the same buffer by scraping the Matrigel-cell mixture from the plates. Cell lysates were rocked at 4 °C for 10 min and cleared by centrifugation at 16,000 $\times$ *g* at 4 °C for 10 min to remove the insoluble fraction and Matrigel. Equal aliquots of lysates were incubated with 20–30 μg of purified GST-RBD or GST-PBD immobilized on glutathione-Sepharose beads at 4 °C for 45 min. The beads were washed four times with wash buffer (50 mM Tris (pH 7.5), 1% Triton X-100, 150 mM NaCl, 10 mM $MgCl_2$, and 1 mM phenylmethylsulfonyl fluoride). Bound RhoA, Rac1, or Cdc42 protein was detected by Western blotting for RhoA (catalog number 26C4, Santa Cruz Biotechnology, Inc. (Santa Cruz, CA)), Rac1 (catalog number ARC03, Cytoskeleton), or Cdc42 (catalog number 2462, Cell Signaling).

Confocal Immunofluorescence Microscopy—For immunofluorescence analysis, the three-dimensional cultures were prepared in 8-well chamber slides (BD Biosciences). The acinar structures were fixed in 4% formaldehyde, phosphate-buffered saline on the indicated days, permeabilized with 0.5% Triton X-100 for 5 min, and stained with anti-E-cadherin, anti-GM130, or anti-α6 integrin primary antibodies. This was followed either with Alexa Fluor 488- or Alexa Fluor 594-conjugated secondary antibodies and DAPI or Topro-3 (Molecular Probes) or with Alexa Fluor 594-conjugated phalloidin. The slides were mounted with Vectashield mounting medium (Vector Laboratories). Images were acquired with a Nikon C1 or a Zeiss LSM 510 confocal microscope under $\times$400 or $\times$600 magnification. All images represent the central plane of the acini using 0.5-μm-thick optical sections.

Statistical Analysis—One-way analysis of variance and Student's *t* test were used to determine the statistical significance of differences in loss of polarity between 16A5-Tet-On-172F cells expressing control shRNA *versus* RhoA, Rac1, or Cdc42 shRNAs.

RESULTS

Down-regulation of EGFR, Reduction in Vav2 Phosphorylation, and Activation of Rho GTPases in Mature Mammary Epithelial Acini—We have previously shown that the immortalized, non-malignant 16A5 MEC line resembles the MCF10A MEC line in forming basolaterally polarized acini in three-dimensional Matrigel culture (18) with relatively nondescript tight junctions, as observed with zona occludens-1 staining (data not shown). However, these structures are sensitive to the levels of EGFR; overexpression of EGFR induced abnormal structures in a significant proportion of acini (18). We therefore hypothesized that controlled EGFR signaling via downstream activation of Rho GTPases may be critical for the maintenance of acinar integrity. EGF stimulation indeed activated Rac1, Cdc42, and RhoA in two-dimensional culture, but surprisingly we found that EGF stimulation did not result in Rac1 and Cdc42 activation, whereas RhoA was weakly activated in three-dimensional as compared with two-dimensional cultures (Fig. 1, *A–D*). This result was confirmed in three independent experiments.

We reasoned that differential EGFR levels in two-dimensional *versus* three-dimensional may account for this difference. To test if EGFR was indeed down-regulated in three-dimensional cultures of 16A5 cells, we compared EGFR protein

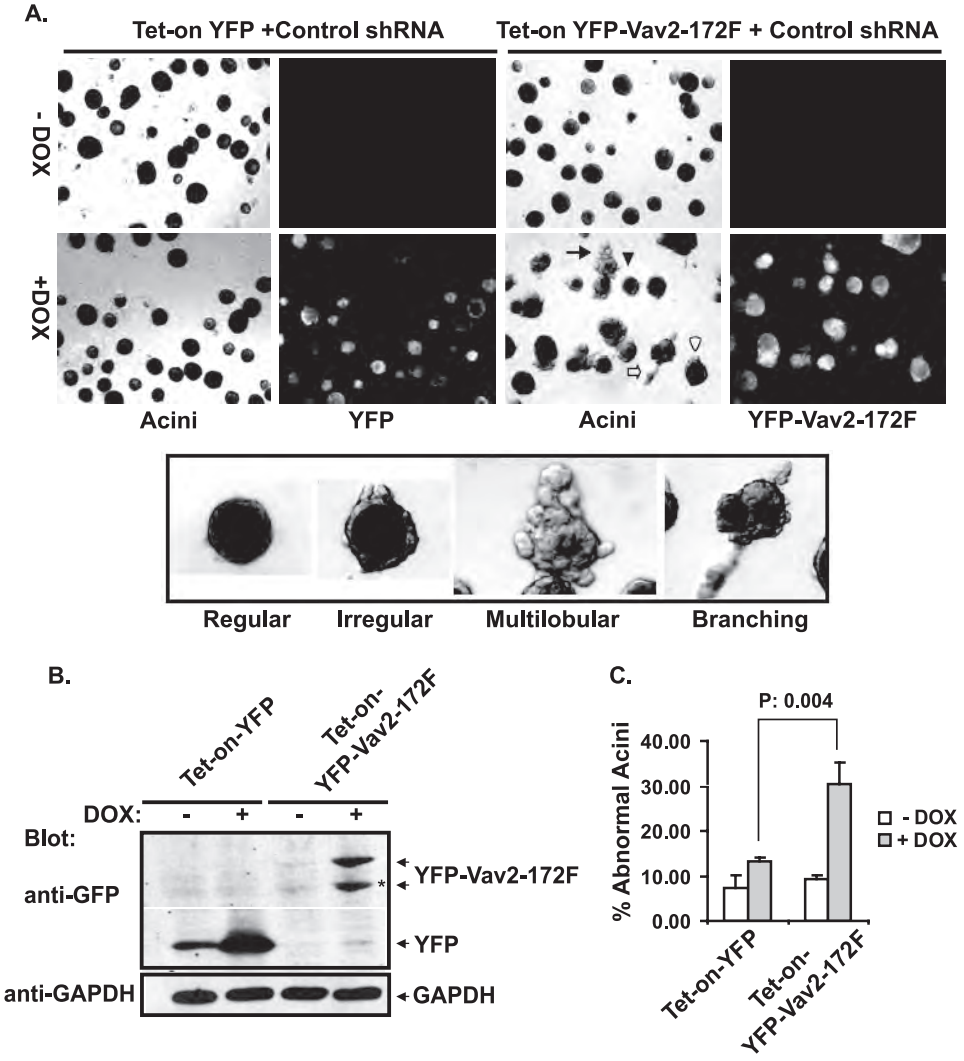

FIGURE 3. **Inducible expression of constitutively active Vav2-Y172F in preformed mammary epithelial cell acini induces abnormal structures.** *A*, 16A5-Tet-On-YFP or 16A5-Tet-On-Vav2-Y172F cells were grown in Matrigel for 14 days to generate acini and then induced with DOX (2 μg/ml) for 6 days. Cells were analyzed using a phase-contrast microscope under transmitted light for acinar morphology (Acini) and under fluorescence light for YFP-tagged Vav2 proteins. *B*, whole-cell lysates of acini prepared as in *A* were immunoblotted with an anti-green fluorescent protein antibody to detect YFP-tagged Vav2 protein (*, a postlysis cleavage product of YFP-Vav2-Y172F). *C*, irregular (see *A*, *open arrowhead*, *enlarged* in the *lower panel*), multilobular (see *A*, *black arrow*, *enlarged* in the *lower panel*), or branched (see *A*, *open arrow*, enlarged in the *lower panel*) acini were counted in three replicates of each cell line, and the mean percentage of abnormal acini are presented with S.D. as *error bars*. The *p* values of the difference between DOX-induced 16A5-Tet-On-YFP and 16A5-Tet-On-Vav2-Y172F cell lines (two-tailed *t* test) are indicated *above* the *error bars*. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

levels in 16A5 MECs grown in two-dimensional *versus* three-dimensional culture. Western blotting (Fig. 1*E*, *top*) and fluorescence-activated cell sorting analysis (25–30% lower mean fluorescence intensity; data not shown) showed that EGFR was indeed substantially down-regulated in three-dimensional culture (the latter was assessed when acini were fully formed; day 14). Furthermore, EGF-induced phosphorylation of EGFR was markedly decreased in three-dimensional compared with two-dimensional cultured cells (Fig. 1*E*, *middle*). These results reveal that the EGFR-signaling pathway is differentially regulated in two-dimensional *versus* three-dimensional cultures (19).

Because EGFR is known to activate Rac1 and Cdc42 through Vav2 (44, 46), which is the only detectable Vav family member in several MEC and breast cancer cell lines (data not shown), and Rac1 activation can disrupt breast cancer cell polarity (27), we examined Vav2 phosphorylation in response to EGF stimulation in two-dimensional *versus* three-dimensional cultured cells. Consistent with reduced EGFR activation, Vav2 phosphorylation in response to EGF was significantly lower in three-dimensional *versus* two-dimensional cultured cells despite similar protein levels (Fig. 1*E*). Thus, attainment of polarity in three-dimensional MEC culture is associated with EGFR down-regulation and reduced Vav2 activation, which is accompanied by a relatively selective blunting of EGF-induced Rac/Cdc42 but not RhoA activation.

Inducible Expression of Vav2-Y172F Mutant Activates Rac1 and Cdc42 but Not RhoA in MEC Acini and Induces the Disruption of Preformed Acinar Structure and Formation of Abnormal Structures in Three-dimensional Matrigel Culture—The correlative findings above suggested that Vav2, whose phosphorylation was reduced in three-dimensional culture in conjunction with reduced Rac/Cdc42 activity, could serve as an important driver of Rac1 and Cdc42 activation in MECs. To assess if this was indeed the case, we generated a Tet-On 16A5 cell line (16A5-Tet-On-172F) in which the expression of YFP-tagged, constitutively active Vav2 mutant (Vav2-Y172F) is DOX-inducible. DOX-induced expression of Vav2-Y172F in 16A5-Tet-On-172F cells grown in two-dimensional culture for 3 days led to increased levels of active Rac1 and Cdc42 but not of RhoA (Figs. 2, *A* and *B*, and 10*C*). Similarly, inducible expression of Vav2-Y172F in preformed 16A5 MEC acini (cultured in Matrigel for 14 days prior to induction) for 3 days activated Rac1 and Cdc42 but not RhoA (Fig. 2, *C* and *D*). In fact, expression of Vav2-Y172F in acini decreased the GTP-bound form of RhoA (Fig. 2, *C* and *D*). These results suggest that Vav2 is primarily a Rac1 and Cdc42 GEF in 16A5 MECs, a result consistent with recent results where Vav2 activated Rac1 but not RhoA in pancreatic cancer cells (47).

Given the recently identified role for Rac1 in the disruption of mammary epithelial cell polarity downstream of PI3K (54), we surmised that activation of Rac1/Cdc42 by active Vav2 could disrupt MEC architecture and morphogenesis in three-dimensional culture. To test this hypothesis, 16A5-Tet-On-

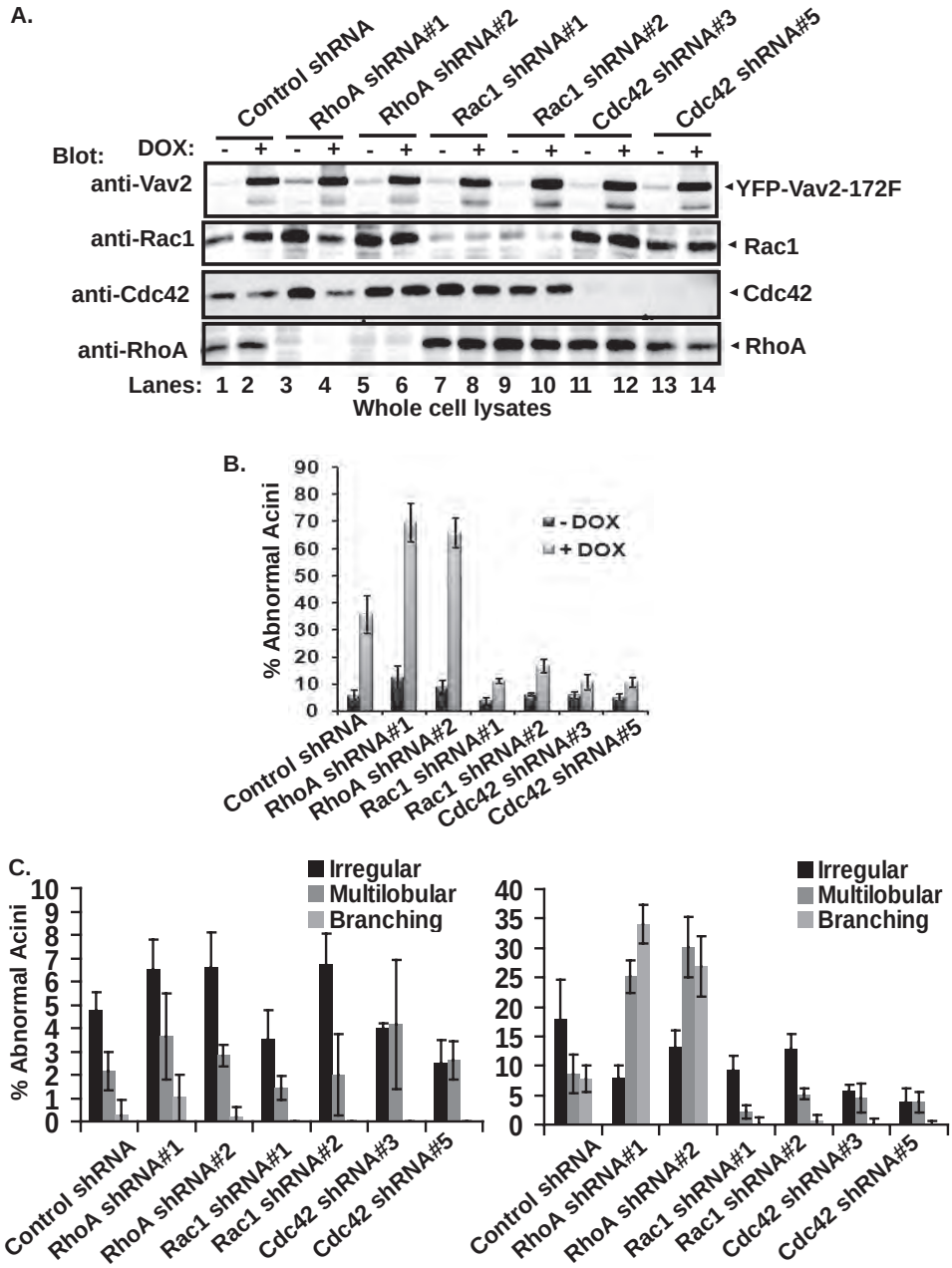

FIGURE 4. **Opposite effects of RhoA *versus* Cdc42/Rac1 knockdown on Vav2-Y172F-induced abnormalities of mammary epithelial acinar structures.** *A*, 16A5-Tet-On-Vav2-Y172F cells with stable expression of control shRNA, RhoA shRNAs, Rac1 shRNAs, or Cdc42 shRNAs were grown in Matrigel for 14 days to generate acini and then induced with DOX for 6 days. The cells were lysed, and whole-cell lysates were immunoblotted for Vav2, Rac1, Cdc42, and RhoA. *B*, the percentage of abnormal acini with characteristics of irregular, multilobular, or branching structures were counted in four replicates and are presented as mean percentages of abnormal acini with S.D. values shown as *error bars*. The significance of differences between the control and DOX-induced experimental groups was determined using one-way analysis of variance followed by the two-tailed *t* test. The following sets show a statistically significant difference in the mean percentage of abnormal acini between control and specific shRNA: RhoA shRNA 1, $p = 0.0009$; RhoA shRNA 2, $p = 0.0016$; Rac1 shRNA 1, $p = 0.0078$; Rac1 shRNA 2, $p = 0.0143$; Cdc42 shRNA 3, $p = 0.0057$; Cdc42 shRNA 5, $p = 0.0086$. *C*, the percentages of irregular, multilobular, and branching structures (based on data shown in *B*) are shown with S.D. values shown as *error bars*. *Left panel*, −Dox, *right panel*, +Dox.

172F cells were grown in Matrigel for 14 days, and constitutively active Vav2 was then induced with DOX. Phase-contrast microscopy demonstrated that, similar to parental cells, 16A5-Tet-On-172F cells formed regular acini by day 14 when grown on Matrigel. Analysis after 6 days of DOX induction, using a phase-contrast microscope with both direct and fluorescence light, indicated that a substantial proportion of preformed acini showed disruptions of acinar architecture (Fig. 3*A*). Although parts of the original acinar structure were still discernible (Fig. 3*A*), other regions showed abnormal architecture, which we categorized as irregular (regional irregular outlining; Fig. 3*A*, *open arrowhead* and *enlarged* in the *lower panel*), multilobular (two or more irregular cellular masses; Fig. 3*A*, *black arrowhead* and *enlarged* in the *lower panel*), and branching (Fig. 3*A*, *open arrow* and *enlarged* in the *lower panel*).

In contrast, vehicle-treated 16A5-Tet-On-172F cells displayed structures comparable with those of uninduced cells (Fig. 3*A*) or DOX-induced 16A5-Tet-On-YFP control cells (Fig. 3, *A*–*C*). These results indicate that activation of Vav2 in mature 16A5 MEC acini disrupts the acinar architecture and results in regionally unregulated cell proliferation. Notably, overexpression of the Vav2-Y172F mutant did not affect EGFR degradation (supplemental Fig. 1*E*), excluding the possibility that this phenotype is a result of altered EGFR down-regulation.

Given our previous findings that EGFR overexpression in 16A5 cells leads to disruption of cell polarity (18), we asked whether Vav2 participates in the EGFR-mediated disruption of acinar architecture. 16A5-Tet-On-YFP, YFP-WT Vav2, and YFP-Vav2-Y172F cells grown in Matrigel for 14 days were induced with DOX in the presence or absence of EGF for 7 days. The abnormal acinar structures (as defined above) were quantified at day 21 of Matrigel culture. Interestingly, an increase in abnormal acinar structure was observed upon induced expression of WT Vav2 only in the presence of EGF (supplemental Fig. 1). On the other hand, Vav2-Y172F expression was associated with a significant increase in abnormal acinar structures in the absence of EGF, with a further increase in the presence of EGF (supplemental Fig. 1). These results suggest that Vav2 plays an important part in EGFR-dependent disruption of three-dimensional MEC architecture.

RhoA and Rac1/Cdc42 Play Opposite Roles in Regulating MEC Acinar Architecture and Morphogenesis—The differential down-regulation of EGF-induced Rac1/Cdc42, compared with

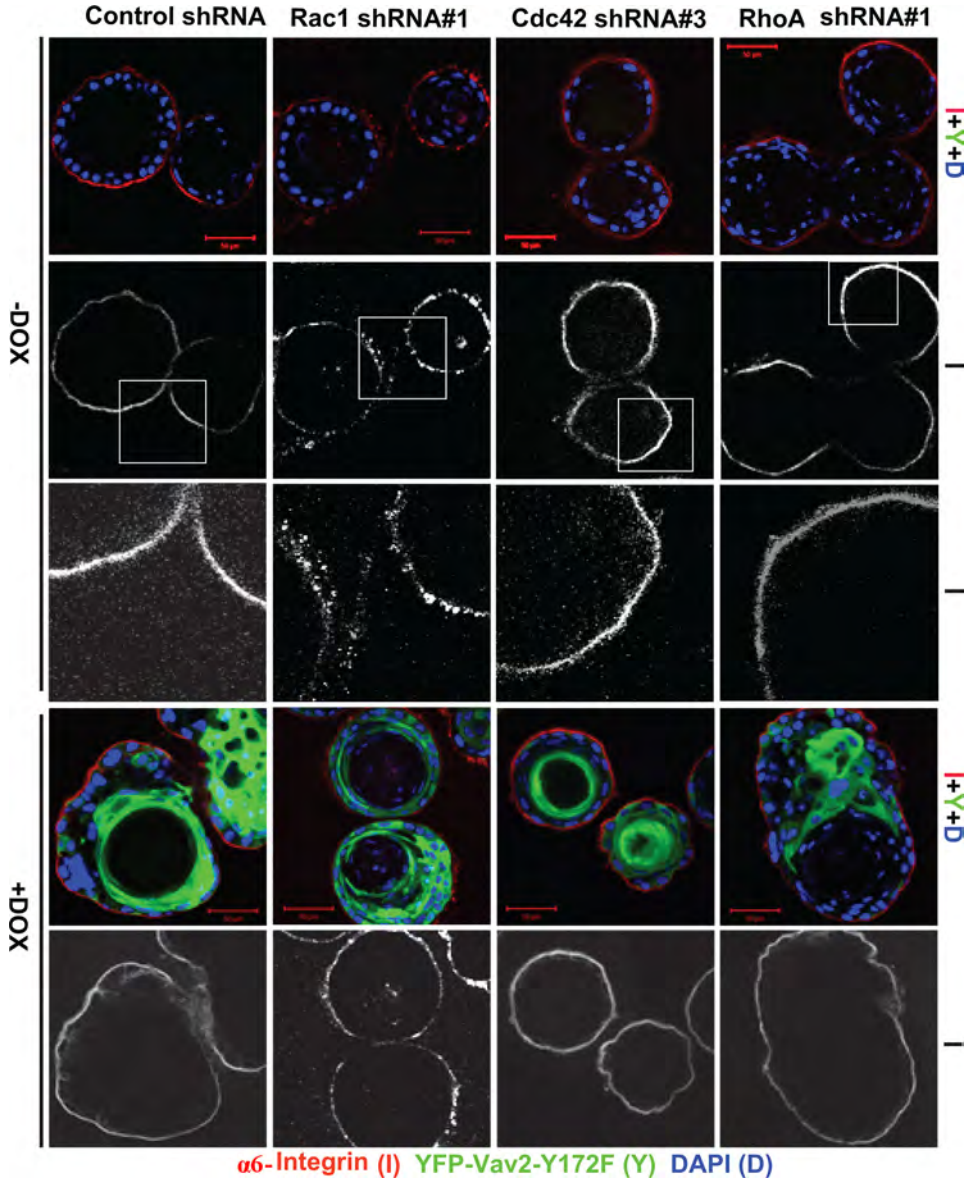

FIGURE 5. **Confocal image analysis of the role for RhoA, Cdc42, and Rac1 in Vav2-Y172F-induced alterations in established MEC acini; analysis using the basal polarity marker α6 integrin.** The 16A5-Tet-On-Vav2-Y172F cells with stable expression of control shRNA, RhoA shRNA, Rac1 shRNA, or Cdc42 shRNA were grown in Matrigel for 14 days to generate acini and then induced with DOX (2 μg/ml) for 6 days. The cells were immunostained with an anti-α6 integrin (*I*, *red*) antibody together with DAPI (*D*, *blue*) to visualize nuclei, and confocal images were acquired at the central plane of the acinar structures (*scale bar*, 50 μM). *Yellow fluorescence* (*Y*) represents induced Vav2 proteins. *Black and white images* are presented in the *lower panels* (*white*, α6 integrin; *gray*, nuclei).

RhoA activation, in three-dimensional MEC culture, together with the correlation of activated Vav2-induced disruption of MEC acini with Rac1/Cdc42 activation (without concomitant RhoA activation), suggests that RhoA activation is compatible with retention of normal acinar architecture, whereas Rac/Cdc42 activation is not. When combined, these data point to potentially distinct roles for these Rho family GTPases.

To address this possibility, we evaluated the effects of Rho GTPase knockdown on activated Vav2-induced disruption of MEC acinar architecture and morphogenesis. Western blot analyses demonstrate the selective knockdown of RhoA (*lanes 3–6* in *panel 4*), Rac1 (*lanes 7–10* in *panel 2*), and Cdc42 (*lanes 11–14* in *panel 3*) when cognate shRNAs were stably expressed in 16A5-Tet-On-Vav2-Y172F cells (Fig. 4*A*).

The control shRNA or individual Rac1/Cdc42/RhoA shRNA-expressing 16A5-Tet-On-Vav2-Y172F cell lines were plated in Matrigel. Vav2-Y172F expression was induced starting either on day 3 (before the control cells attained polarity; see Fig. 6) or on Day 14 (after the control cells exhibited polarity; Figs. 4 and 5). Cells induced from day 3 onward were harvested at day 14, whereas those induced from day 14 onward were harvested on day 21. The proportion of cells with abnormal structures was quantified, and the cells were subsequently immunostained with polarity markers and visualized with a confocal microscope at the central plane of the acinar structures. When grown without Vav2 induction, cells with Rac1 or Cdc42 knockdown formed regular sized acini by day 20, whereas a relatively small proportion of acini in RhoA knockdown cells were irregular (Fig. 4*B*). When 16A5-Tet-On-Vav2-Y172F cells with control shRNA were induced from day 14 to day 20, they showed regional disruption of acinar architecture and formation of irregular, multilobular, or branching structures, as anticipated from previous results (Figs. 4–8).

Immunostaining showed that α6 integrin (basal surface marker; Fig. 5) was confined to the basal surface in all of the cell lines. However, the α6 integrin staining showed an intermittent pattern (Fig. 5, *box*) in Rac1 knockdown cells as opposed to continuous staining in all other cell lines (Fig. 5). Quantification of cells with different staining patterns in three independent experiments indicated that more than 90% of the control shRNA-, Cdc42 shRNA-, or RhoA shRNA-expressing acini showed a continuous integrin staining pattern, whereas an average of 67% of Rac1 shRNA-expressing acini exhibited an intermittent integrin staining pattern (data not shown).

Staining for GM130 (apical marker) revealed that it was oriented to the apical side of the nuclei in a majority of the control shRNA-expressing cells in the absence of DOX (Fig. 6*A*, *white arrows* indicate apical orientation, and *open arrows* indicate irregular orientation). However, the apical orientation of GM130 appeared to be diminished in RhoA, Cdc42, and Rac1 shRNA-expressing acini (Fig. 6*A*). Visual inspection and quantification showed a remarkable reduction in the proportion of

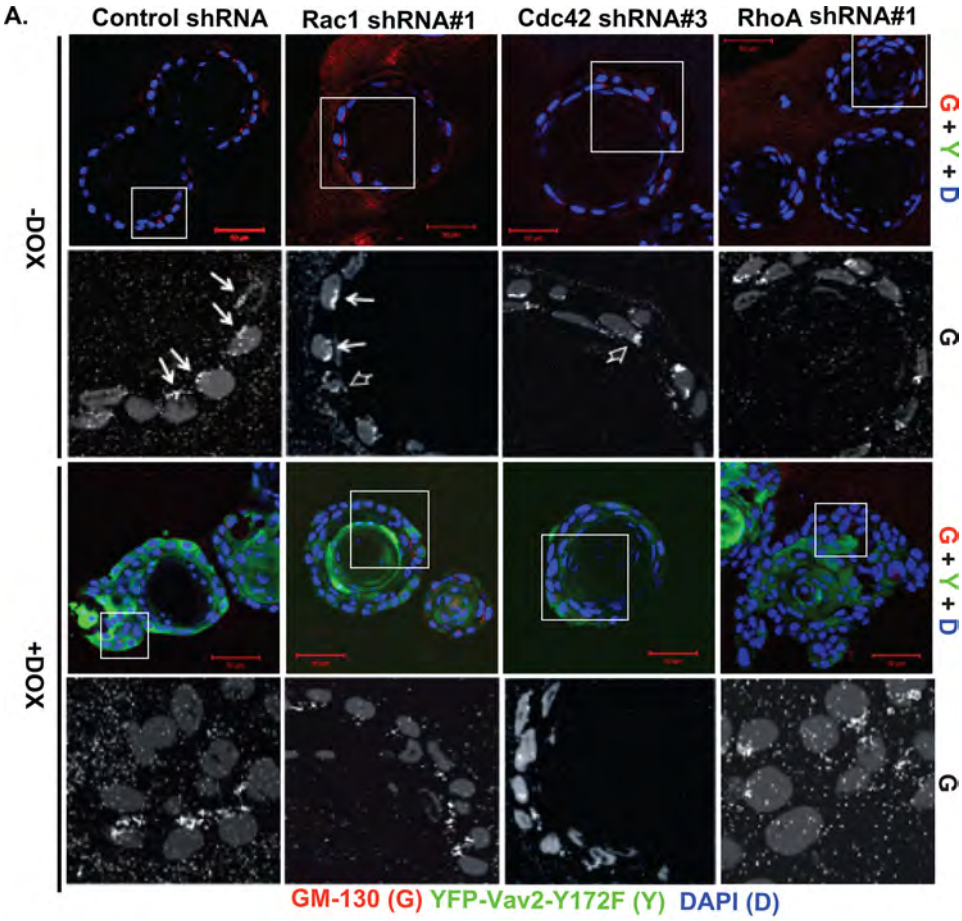

FIGURE 6. **Confocal image analysis of the role for RhoA, Cdc42, and Rac1 in Vav2-Y172F-induced alterations in established MEC acini; analysis using the apical polarity marker GM130.** *A*, the acini were prepared as in Fig. 5 and immunostained with anti-GM130 (*G*, *red*) antibody together with DAPI (*D*, *blue*) to visualize nuclei, and confocal images were acquired at the central plane of the acinar structures (*scale bar*, 50 μM). *Green fluorescence* (*Y*) represents induced Vav2 proteins. *Black and white images* are presented in the *lower panels* (*white*, GM-130; *gray*, nuclei). *B*, the number of cells (from a total of 10 acini for each condition) exhibiting apical orientation of GM130 upon visual inspection were counted and presented as a percentage of total cells; average values from three experiments are presented with S.D. shown as *error bars*.

cells with apical orientation of GM130 in acini of RhoA, Cdc42, or Rac1 shRNA-expressing cells (Fig. 6*B*). Upon DOX induction, the polarized GM130 orientation was lost in cells that formed abnormal structures, and the proportion of cells with apical orientation of GM130 was significantly reduced (Fig. 6*B*).

Immunostaining for E-cadherin or F-actin revealed the basolateral distribution of E-cadherin (Fig. 7) and apicolateral distribution of F-actin (Fig. 8) in all of the cell lines. Notably, in cells that formed multilayered abnormal structures upon DOX induction, there was a diminution of staining for AJs (Fig. 7, *white arrowhead*) and junctional actin cables (Fig. 8, *white arrowheads*).

The formation of abnormal structures upon induction of Vav2-Y172F expression was significantly enhanced in RhoA knockdown cells, whereas it was reduced in Rac1 or Cdc42 knockdown cells (Fig. 4*B*). Further quantification of the abnormal acini as irregular, multilobular, and branching structures revealed that Vav2-Y172F expression primarily led to irregular acini in control shRNA-expressing cells (Fig. 4*C*, *right*). However, Vav2-Y172F expression in combination with RhoA knockdown led to a more pronounced abnormality, as shown by predominantly multilobular and branching acini (Fig. 4*C*, *right*). In contrast, the Vav2-Y172F-induced branching phenotype was not seen in Rac1 or Cdc42 knockdown cells (Fig. 4*C*, *right*).

Induction of Vav2-Y172F expression from day 3 onward in control shRNA-expressing cells resulted in primarily multilobular acini lacking lumina (Fig. 9). Vav2-Y172F expression in RhoA knockdown cells resulted in tubule-like branching in addition to multilobular acini (Fig. 9, *arrowheads*). On the other hand, Rac1 knockdown cells largely formed regular acinar structures even when Vav2-Y172F expression was induced (Fig. 9).

Reorganization of Cell-Cell Junctional Actin Cytoskeleton and Disruption of AJs upon Inducible Expression of Activated Vav2; the Role of Rac1 and RhoA—Since Rac1 activation has been shown to disrupt AJs in epithelial cells (26) and Vav2 regulates the actin cytoskeleton in model cell systems (46), our findings of reduced E-cadherin and junctional actin staining in activated Vav2-induced abnormal acini prompted us to assess whether Vav2 activation is able to reorganize the junctional actin cytoskeleton and disrupt AJs in MECs.

Because the visualization of AJs and associated junctional actin structures in epithelial cells is optimal for cells grown as monolayers, 16A5-Tet-On-172F cells were grown in two-dimensional cultures in EGF-deprived DFCI medium until cell-

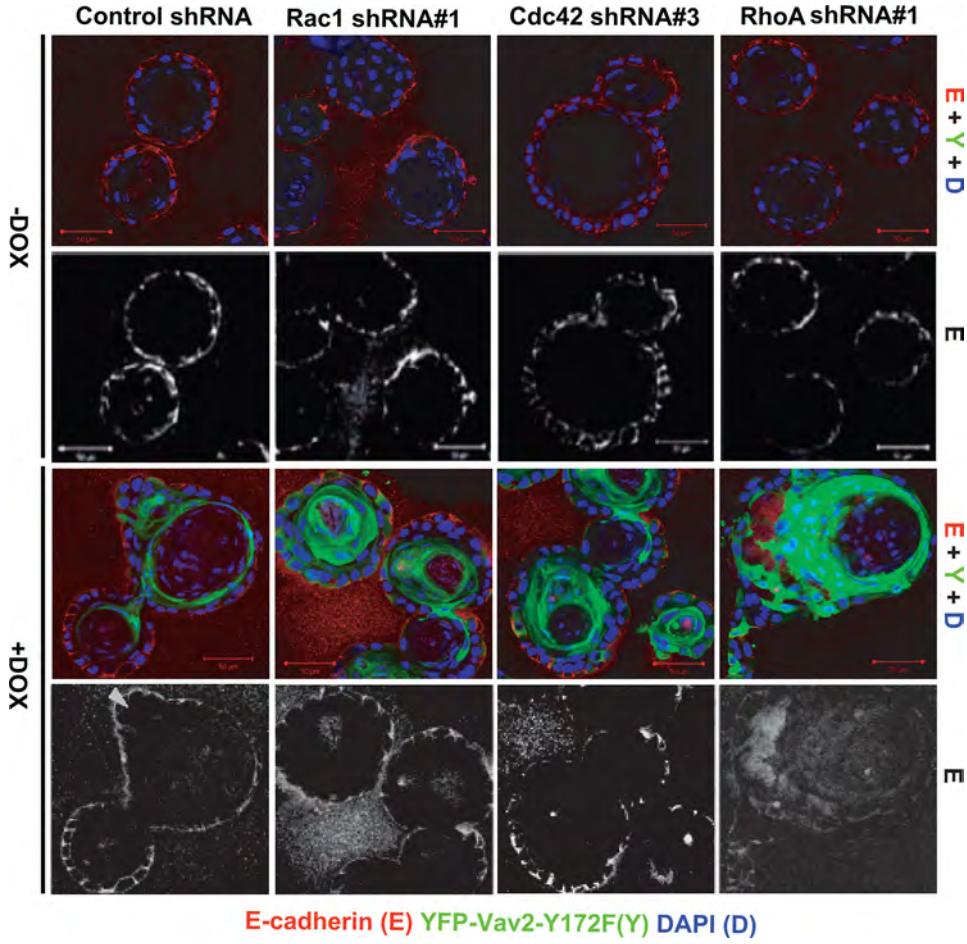

FIGURE 7. **Confocal image analysis of the role for RhoA, Cdc42 and Rac1 in Vav2-Y172F-induced alterations in established MEC acini; analysis using the basolateral polarity marker E-cadherin.** The acini were prepared as in Fig. 5 and immunostained with an anti-E-cadherin (*E*, *red*) antibody together with DAPI (*D*, *blue*) to visualize nuclei, and confocal images were acquired at the central plane of the acinar structures (*scale bar*, 50 μM). *Green fluorescence* (*Y*) represents induced Vav2 proteins. *Black and white images* are presented in the *lower panels* (*white*, E-cadherin; *gray*, nuclei).

cell adhesions formed. The cells were then treated with vehicle or DOX for 3 days to induce Vav2-Y172F expression; confocal imaging confirmed the YFP-Vav2-Y172F expression in DOX-treated but not in untreated cells (Fig. 10, *green*, *top*). Compared with control cells, Vav2-Y172F-expressing cells spread out more and became flatter as they increased in size. The induced expression of Vav2-Y172F was accompanied by a loss of circumferential actin cables and the formation of thin perijunctional actin bundles. In addition, E-cadherin staining at the cell-cell junctions was discontinuous, with more diffuse cytoplasmic staining in Vav2-Y172F-expressing cells (Fig. 10).

Similar to three-dimensional cultures, the phenotype induced by the overexpression of Vav2-Y172F was significantly blocked by Rac1 knockdown but not by RhoA knockdown (Fig. 10). Instead, RhoA knockdown exacerbated the Vav2-Y172F-induced disruption of AJs, with reduced E-cadherin staining at cell-cell interfaces. In addition, E-cadherin colocalized with the reorganized actin cytoskeleton, and more cytoplasmic E-cadherin staining was observed (Fig. 10). These results suggest that Rac1 but not RhoA is required for Vav2-mediated reorganization of the junctional actin cytoskeleton and disruption of AJs.

Knockdown of RhoA did not affect the Vav2-induced Rac1 activation (supplemental Fig. 3, *A* and *B*), thereby excluding the possibility that functional antagonism, with RhoA inhibiting the activation of Rac1 by Vav2, is involved. Furthermore, knockdown of Rac1 did not affect the Vav2-induced activation of Cdc42, and knockdown of Cdc42 did not change Rac1 activation by Vav2 (supplemental Fig. 3, *C* and *D*). These results suggest that Vav2 activates Rac1 and Cdc42 simultaneously and that the combined activity of Rac1 and Cdc42 action probably mediates the disruption of AJs and acinar architecture downstream of Vav2.

To test whether RhoA regulates AJs downstream of EGFR, WT RhoA was stably overexpressed in the telomerase (hTERT)-immortalized MEC line 81N-Tert (supplemental Fig. 2*A*) because RhoA could not be stably overexpressed in 16A5 cells. EGF-induced activation of RhoA was enhanced in RhoA-overexpressing 81N-Tert cells (supplemental Fig. 2*A*). Compared with control cells, RhoA-overexpressing 81N-Tert cells showed very little reorganization of the junctional actin cytoskeleton and AJs upon EGF stimulation. In contrast, circumferential actin cables were more intense in RhoA-overexpressing cells upon EGF stimulation (supplemental Fig. 2*B*). Altogether, these results strongly suggest a functional role for RhoA in maintaining epithelial AJs in the presence of activated EGFR and Vav2.

DISCUSSION

Rho GTPases have been implicated in mammary tumorigenesis based on their overexpression in breast cancer cells (39) and the functional roles they play in controlling cell proliferation, survival, migration, and polarity. An invariant feature of oncogenic transformation of mammary and other epithelial cells is a loss of polarity and an inability to undergo acinar morphogenesis in three-dimensional culture (13, 14). Thus, understanding the relative importance of distinct Rho GTPases in regulating mammary epithelial acinar morphogenesis and polarity is of substantial physiological as well as cancer-related importance.

Here, we show that three-dimensional acinar morphogenesis is associated with the down-regulation of Rac1/Cdc42 activation, whereas RhoA activation remains intact. By utilizing a DOX-inducible activated Vav2 expression system together with shRNA knockdown of Rac1, Cdc42, and RhoA, we show that different Rho GTPases play functionally opposite roles in

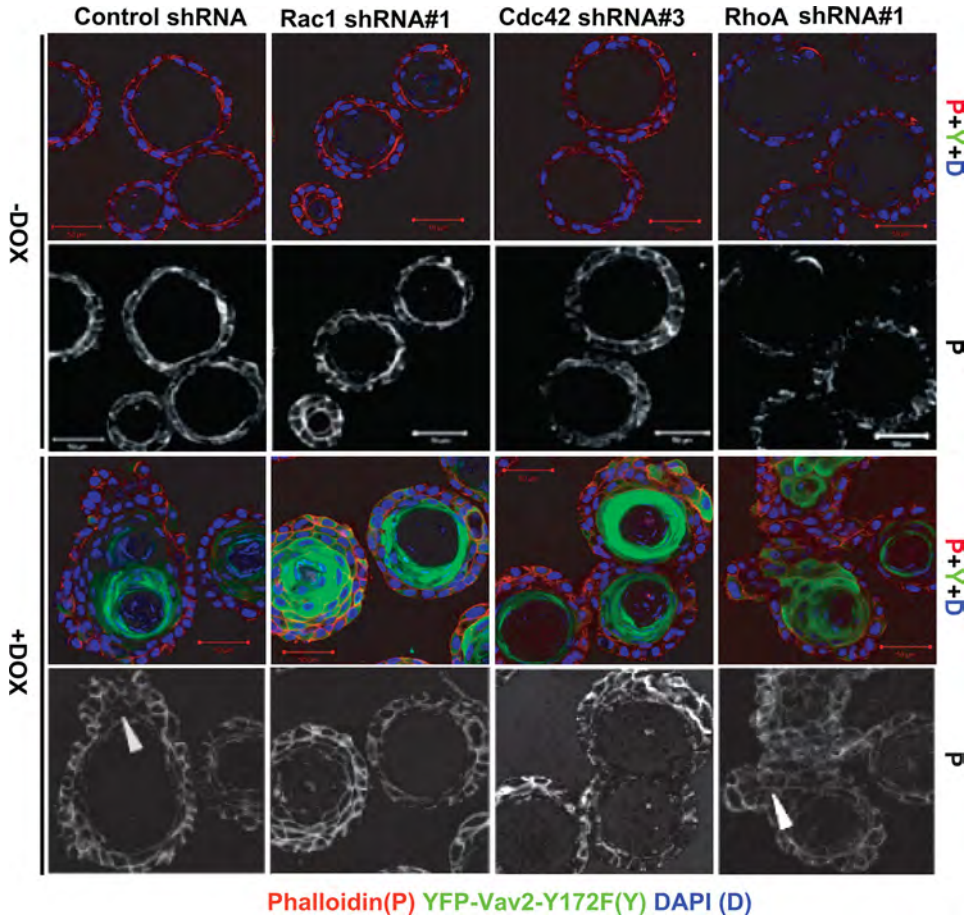

FIGURE 8. **Confocal image analysis of the role for RhoA, Cdc42, and Rac1 in Vav2-Y172F-induced alterations in established MEC acini; analysis of apicolateral actin staining with phalloidin.** The acini were prepared as in Fig. 5 and stained with phalloidin (*P*, *red*) together with DAPI (*D*, *blue*) to visualize nuclei, and confocal images were acquired at the central plane of the acinar structures (*scale bar*, 50 μM). *Green fluorescence* (*Y*) represents induced Vav2 proteins. *Black and white images* are presented in the *lower panels* (*white*, actin; *gray*, nuclei).

the regulation of mammary epithelial cell polarity and acinar morphogenesis; the activation of Rac1/Cdc42 promotes disruption of polarity and abnormal acinar morphogenesis, whereas Rho activity appears to preserve polarity and acinar structure.

In the context of the regulation of polarity, Rac1 is required for the appropriate orientation of apical and basal polarity of MDCK cells (25) and is aberrantly activated downstream of PI3K within invasive breast cancer cells and perturbs their polarity (27). Cdc42 is also essential for the establishment of apical polarity in MDCK cells (24). However, less is known about the role of RhoA activation in acinar morphogenesis. Interestingly, RhoA activity was reported to be up-regulated in response to changes in extracellular matrix rigidity within three-dimensional culture, and this increase in RhoA activation induced the distortion of acinar structures (29). In our studies, EGFR stimulation is associated with RhoA activation both in two-dimensional and three-dimensional MEC culture. However, because Rac1/Cdc42 activation was not observed in three-dimensional culture, RhoA activity did not promote the disruption of acinar architecture.

Activation of Rho GTPases is associated with the stimulation of a number of cell surface receptors. In the context of epithelial cells, stimulation through RTKs, such as EGFR, is a potent means of controlling Rho GTPases (55). Consistent with studies in model cell lines, the Vav family of proteins represent some of the key Rho GTPase-directed GEFs (43). We found that several MEC lines, including the 16A5 cells utilized in this study, only express one member of this protein family, Vav2. This allowed us to drive Rho GTPase activation in MECs to assess the impact on cell polarity and acinar morphogenesis. Furthermore, down-regulation of EGFR, which is associated with acinar morphogenesis in three-dimensional culture, was observed in conjunction with reduced Vav2 phosphorylation and reduced Rac/Cdc42 activity, providing further support for the decision to use activated Vav2 to assess the role of downstream Rho GTPases.

DOX-inducible expression of a constitutively active form of Vav2 (Vav2-Y172F) in three-dimensional culture of 16A5 MECs led to the activation of Rac1 and Cdc42 but not RhoA. More importantly, controlled expression of activated Vav2 at different stages of MEC acinar morphogenesis demonstrated not only that Vav2 could block the completion of acinar morphogenesis but also that it could disrupt established acinar structures and lead to the formation of abnormal structures reminiscent of cancerous cellular growths. These alterations in acini were associated with a local loss of polarity as assessed with markers of apicobasal polarity.

The phenotype induced by Vav2-Y172F is largely dependent on the activation of Rac1 and Cdc42 because knockdown of Rac1 or Cdc42 significantly blocked the phenotypic characteristics associated with Vav2 induction. In contrast, knockdown of RhoA led to a partial distortion of acinar architecture by itself and further promoted the phenotype induced by activated Vav2. Moreover, compared with mainly irregular structures induced by Vav2-Y172F alone (a milder phenotype), overexpression of Vav2-Y172F in combination with RhoA knockdown resulted in the formation of predominantly multilobular and branching structures, a more severe phenotype associated with characteristics of cancerous cells (6). Thus, Rac1/Cdc42 and RhoA appear to play opposite roles in regulating three-dimensional acinar structure, with RhoA functioning to stabilize acinar structures, while Rac1/Cdc42 acts to promote acinar disruption.

Interestingly, knockdown of Rac1 resulted in an intermittent α6 integrin staining pattern at the basal surface and reduced the

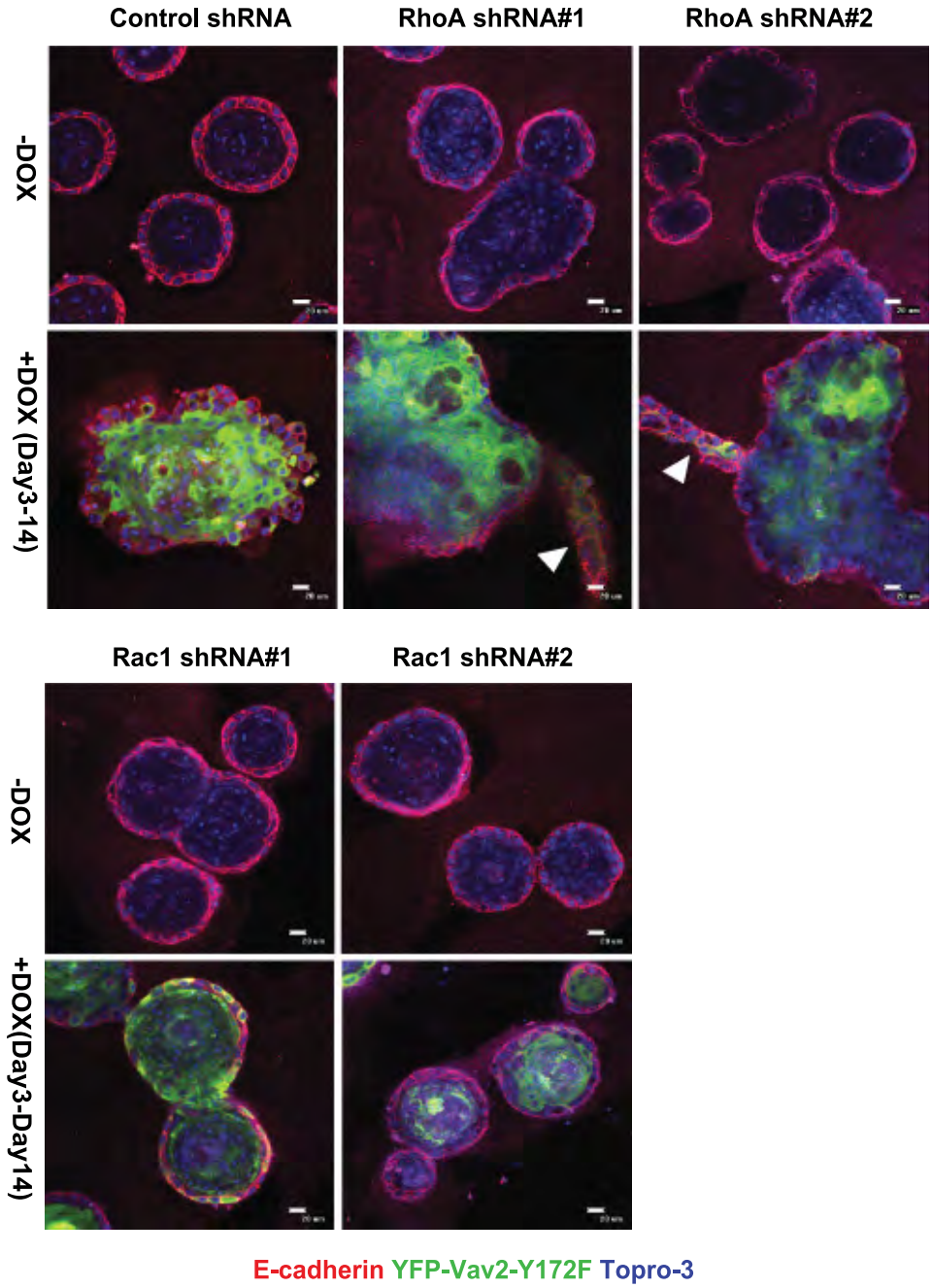

FIGURE 9. **Overexpression of Vav2-Y172F during the early phase of three-dimensional Matrigel culture blocks acinar morphogenesis; role of Rac1 and RhoA.** 16A5-Tet-On-Vav2-Y172F cells with stable expression of control shRNA, RhoA shRNAs, or Rac1 shRNAs were seeded in Matrigel, and Vav2-Y172F expression was induced with DOX starting on day 3. On day 14 (when control cultures show regular acini), the cells were immunostained with an anti-E-cadherin antibody (*red*) together with Topro-3 (*blue*) to visualize nuclei, and confocal images were acquired at the central plane of the acinar structures (*scale bar*, 50 μM). *Green fluorescence* represents induced Vav2 proteins. The *arrowheads* indicate branching structures; these were observed in a majority of acini formed by RhoA knockdown cells.

apical orientation of GM130 without remarkably affecting basolateral E-cadherin staining. Rac1 is required for the orientation of apical polarity in MDCK cells through the mediation of laminin assembly under the basal surface (25). Under our experimental conditions, the presence of laminin in Matrigel is seemingly sufficient for the proper orientation of α6 integrin to the basal surface. However, the distribution of integrin was different from the control cells, implying that Rac1 knockdown affected basal polarity, which may account for the diminished apical orientation of GM130. Cdc42 knockdown significantly reduced the apical orientation of GM130 in the cells, consistent with the finding that Cdc42 is essential for the establishment of apical polarity in MDCK cells (24).

Knockdown of RhoA also impaired the apical orientation of GM130. In MDCK cells, RhoA is essential for the formation of tight junctions (30, 56), which segregates apical and lateral membrane domains (57). Although 16A5 cells did not form tight junctions (based on ZO1 staining; data not shown) and less than 75% of cells had an apical orientation for GM130 (Fig. 6*B*), the significant reduction in the apical orientation of GM130 in RhoA knockdown cells suggests that RhoA plays a role in the apical orientation of these cells. The lack of intact junctional actin rings and AJs in RhoA knockdown cells, in conjunction with the maintenance of junctional actin rings and AJs by RhoA overexpression in response to EGF stimulation in two-dimensional culture, implies that RhoA, by promoting the formation of cell-cell junctions, is critical for the orientation of apical polarity. The inability to maintain cell-cell junctions is likely to contribute to the exaggerated phenotype induced by Vav2-Y172F expression in RhoA knockdown cells. More detailed analyses of how these Rho GTPases and their effectors regulate apicobasolateral polarity in the cell system described here should help reveal the coordinated mechanisms that control MEC morphogenesis and tumorigenic phenotypes.

Notably, knockdown of Rac1 or Cdc42 antagonized the effects of Vav2-Y172F expression but did not affect the activity of the other GTPase in Vav2-Y172F-expressing MECs (Fig. 10*C*), indicating that each one of these proteins plays a role in Vav2-induced disruption of acinar morphogenesis. Combined knockdown studies will be required to assess if they play an additive/synergistic role or if they influence each other's function in the context of acinar morphogenesis.

It is instructive to view the present observations of the down-modulation for the EGFR and Rac1/Cdc42 signaling axis during

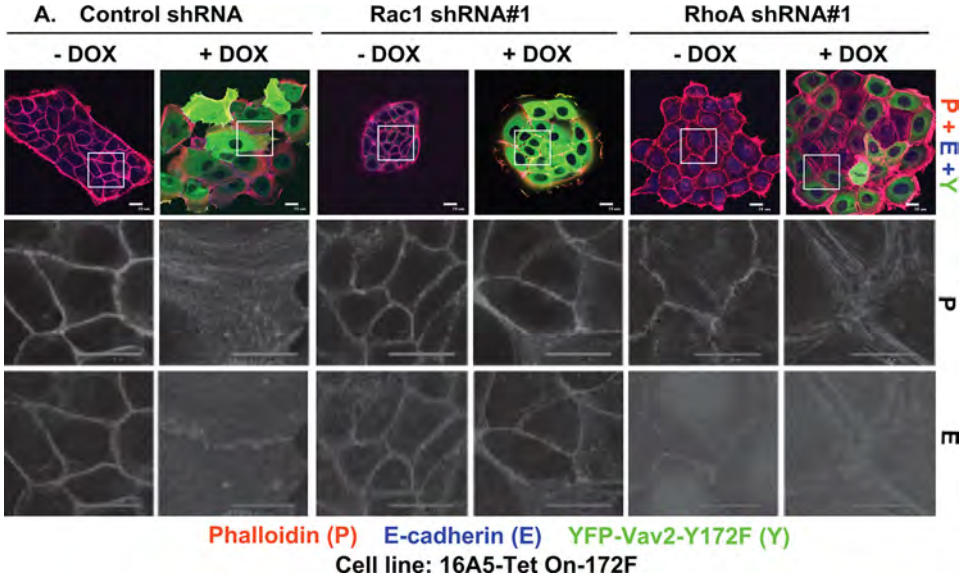

FIGURE 10. **Overexpression of Vav2 induces remodeling of cell-cell junctional actin structures; role of Rac1 and RhoA.** 16A5-Tet-On-Vav2-Y172F cells were grown in two-dimensional culture and EGF-starved together with treatment of vehicle (−*DOX*) or DOX (2 μg/ml, +*DOX*) for 3 days (expression of YFP-Vav2-Y172F (*Y*) in induced cells is seen in *green*). The cells were immunostained for E-cadherin (*E*, *blue*) and F-actin (phalloidin (*P*), *red*), and confocal images were acquired at the subapical plane. The *top panels* show images of whole-cell colonies, and selected areas are shown at higher magnification in the *lower panels* (*middle panels*, F-actin; *bottom panels*, E-cadherin).

acinar morphogenesis of MECs in the context of 1) our previous results, where EGFR overexpression in 16A5 and MCF10A MECs, especially when combined with clinically observed c-Src overexpression, induced a loss of polarity and acinar architecture as well as invasive behavior in three-dimensional cultures (18); 2) the findings that EGFR is often overexpressed in breast cancer cells and associated with invasion (41, 42); and 3) the observation that abnormal Rac1 and Cdc42 activation contributes to polarity disruption in epithelial cells and breast cancer cells (28, 54). Collectively, it appears reasonable to suggest that activation of Rac1/Cdc42 plays an important role in the disruption of MEC architecture and morphogenesis and that aberration in this signaling axis may contribute to oncogenesis during the development of breast cancer. Interestingly, overexpression of the Rac-GEF Tiam-1 has been reported in breast cancer cell lines and patient samples (58). We have detected Vav2 protein expression in a number of breast cancer cell lines that express EGFR or overexpress ErbB2 and, in some cases, express constitutively active PI3K mutants (data not shown). Because ErbB receptors and PI3K are known to activate Vav2 (48), it is likely that Vav2 may play a role downstream of ErbB receptors, in collaboration with PI3K mutations, to activate Rac/Cdc42 signaling. Indeed, induced overexpression of WT Vav2 resulted in EGF-dependent disruption of acinar architecture in 16A5 cells. Future studies should help elucidate a role for Vav2 in the regulation of cellular architecture and oncogenesis in cancer cells.

Our findings that Vav2 activates Rac1 and Cdc42 but not RhoA in MECs are supported by similar recent observations in pancreatic cancer cells (47). In conjunction with the differential down-regulation of Rac1/Cdc42/RhoA activation during MEC acinar morphogenesis, these results suggest that Vav2 may play a more important role in activating Rac1/Cdc42 in MECs, whereas RhoA activation downstream of EGFR probably involves other GEFs. However, Vav2 has been previously characterized as a GEF for RhoA, Rac1, and Cdc42; therefore, other factors, such as induction of Rho-family member-specific inhibitors, may also need to be considered in future studies.

Consistent with our results, a recent study of mammary branching morphogenesis using primary, organotypic three-dimensional culture of mouse mammary epithelia has found that Rac mediates duct initiation, whereas activation of Rock, a RhoA effector, restores epithelial architecture (59). Moreover, c-MET-induced scattering of MDCK cells was blocked by RhoA activation (35, 37), whereas spatially restricted degradation of RhoA was shown to facilitate transforming growth factor-β-mediated disruption of tight junctions in MECs (38), further supporting a general role for RhoA in the maintenance of normal epithelial cell architecture. In the 16A5 MEC system used here, RhoA does not directly affect Vav2 activity because Vav2-induced activation of Rac1 is unaffected by knockdown of RhoA, suggesting that RhoA signaling antagonizes Rac1/Cdc42 at a step after their initial activation. One potential mechanism by which RhoA maintains epithelial cell polarity may be through the inactivation of cofilin, an actin-severing protein (60), which could possibly occur through the activation of both Rho kinase and LIM-kinase, the latter an inactivator of cofilin (61). Future studies will be necessary to explore the role of cofilin and other factors that could act as molecular switches between acinar and branching morphogenesis orchestrated by RhoA and Rac1/Cdc42.

When analyzing the DOX-inducible MEC system, we noted that the YFP-Vav2-Y172F protein accumulated and displayed higher fluorescence intensity near the apical side and within the lumina of a large proportion of acini after induction for 7 days. Because the lumen had already formed within acini when Vav2 expression was induced at day 14 (supplemental Fig. 1*D*), it is likely that Vav2-Y172F expression caused inward cell growth and partial refilling of the lumen. Staining of the acini for activated caspase-3 showed that although the vehicle-treated cells had positive caspase-3 staining in the lumen, the filled lumen in acini with YFP-Vav2-Y172F induction did not (supplemental Fig. 1*C*), implying that Vav2 may mediate antiapoptotic signaling. Some of the acini accumulated green YFP-Vav2 proteins in the lumen without positive nuclear staining. Whether or not Vav2 is toxic to the cells and caused non-apoptotic cell death with nuclear disappearance and exudation of Vav2 proteins to the lumen requires further investigation. Future studies will also focus on which antiapoptotic signaling pathways are regulated by Vav2 and whether such signaling pathways are

an integral part of the Rac1/Cdc42 signaling axis in acinar morphogenesis.

In conclusion, our studies, utilizing a three-dimensional culture system of mammary epithelial acini together with inducible gene expression and knockdown strategies, reveal that the RhoA and Rac1/Cdc42 signaling pathways play disparate and apparently antagonistic roles in the remodeling of epithelial cell architecture and morphogenesis. These results suggest a potential role for the Vav2-Rac1/Cdc42 signaling pathway in RTK-mediated disruption of MEC architecture and breast tumorigenesis. Further biochemical and cell biological analyses of MECs and other epithelial cell systems should facilitate a better molecular understanding of the biological roles of Vav2 and the counterbalancing roles of the Rho family GTPases reported here.

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