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**Rap1 integrates tissue polarity, lumen formation, and tumorigenic potential in
human breast epithelial cells**

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Key words: ECM; mammary gland

Abbreviations: 3D, three-dimension(al); DA-Rap1, dominant-active Rap1;

DN-Rap1, dominant-negative Rap1; ECM, extracellular matrix;

EGFR, epidermal growth factor receptor; GAP, GTPase activating protein;

GEF, guanine nucleotide exchange factor; lrECM, laminin-rich ECM;

MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase;

S6RP, S6 ribosomal protein

Abstract

Maintenance of apico-basal polarity in normal breast epithelial acini requires a balance between cell proliferation, cell death, and proper cell-cell and cell-extracellular matrix signaling. Aberrations in any of these processes can disrupt tissue architecture and initiate tumor formation. Here we show that the small GTPase Rap1 is a crucial element in organizing acinar structure and inducing lumen formation. Rap1 activity in malignant HMT-3522 T4-2 cells is appreciably higher than in S1 cells, their non-malignant counterparts. Expression of dominant-negative Rap1 resulted in phenotypic reversion of T4-2 cells, led to formation of acinar structures with correct apico-basal polarity, and dramatically reduced tumor incidence despite the persistence of genomic abnormalities. The resulting acini contained prominent central lumina not observed when other reverting agents were used. Conversely, expression of dominant-active Rap1 in T4-2 cells inhibited phenotypic reversion and led to increased invasiveness and tumorigenicity. Thus, Rap1 acts as a central regulator of breast architecture, with normal levels of activation instructing apical polarity during acinar morphogenesis, and increased activation inducing tumor formation and progression to malignancy.

Introduction

Rap1, a member of the Ras family of small GTPases, is activated in response to a number of extracellular stimuli, including growth factors, cytokines, and cell-cell and cell-extracellular matrix (ECM) adhesion (1, 2). Like all G proteins, activation of Rap1 is mediated by specific guanine nucleotide exchange factors (GEFs), and in turn disrupted by GTPase activating proteins (GAPs). Active GTP-bound Rap1 functions through its many effectors, including the Rho GTPase family member, Rac1 (3, 4), to regulate inside-out signaling to integrins (5, 6) and cadherins (7-9) and to control cytoskeletal structure (10), endothelial cell polarity (11, 12), and differentiation (9, 13). Despite its original discovery as an inhibitor of Ras-mediated transformation (14), Rap1 and its GEFs and GAPs have been found to be dysregulated in a variety of mouse and human cancers (15-19). Deregulating Rap1 activity by knocking out its GAP Spa1 in mice leads to the development of myeloproliferative disorders mimicking human chronic myeloid leukemia (20), and overexpression of Rap1 induces oncogenic transformation in cultured fibroblasts (21). Additionally, the E6 protein of oncogenic human papillomavirus transforms cells in part by degrading the Rap1-GAP E6TP1 (22, 23). Because it both responds to and regulates cell-cell and cell-ECM adhesions, Rap1 is emerging as a key regulator of morphogenesis (24, 25).

During normal development, integration of signals from the microenvironment, including cell-cell and cell-ECM adhesion, leads to establishment of tissue structure and apico-basal polarity (26, 27). Loss of normal tissue structure and polarity are hallmarks of tumor progression (27, 28). To delineate the mechanisms regulating tissue polarity

and its loss in breast cancer, we have used an assay in which normal and malignant human breast epithelial cells are cultured within a physiologically relevant three-dimensional (3D) laminin-rich ECM (lrECM). Phenotypically normal nonmalignant S1 cells from the HMT-3522 tumor progression series form polarized and growth-arrested acini when cultured in 3D lrECM, resembling the structures formed by primary breast epithelial cells taken from reduction mammoplasty (29). In contrast, tumorigenic T4-2 cells form highly proliferative disorganized apolar structures reminiscent of malignant tumors in vivo. Expression levels of EGFR, β 1-integrin, and their downstream effectors, including MAPK and PI3K, are increased in T4-2 compared to S1 cells, and downregulation of any of these signaling pathways in T4-2 cells cultured in 3D lrECM leads to growth arrest and reversion to a phenotype resembling normal polarized acini in vivo (30-32).

In the 3D assay, proliferation and tissue polarity appear phenotypically coupled, yet they were shown to be controlled by distinct signaling pathways, with high levels of Akt and the small GTPase Rac1 correlating with loss of growth control and tissue polarity, respectively; downregulation of Rac1 activity was necessary for restoration of basal polarity (32). However, while apical polarity as demonstrated by ZO-1 localization was partially restored, the acini failed to form lumina. Since Rap1 is an upstream activator of Rac1 (3, 4) and regulates a number of pathways (24, 25), we postulated that it may integrate microenvironmental signaling in these cells in 3D. We measured Rap1 activity and showed that it was much higher in malignant cells, which led us to hypothesize that Rap1 activation may play a role in loss of apical polarity and lumen

formation during tumor progression. Here we show that this is indeed the case, and that exogenous expression of dominant-active Rap1 in T4-2 cells cultured in 3D IrECM interferes with reversion of tissue structure and malignant phenotype and establishment of tissue polarity. We show also that a lower level of Rap1 activity is required for lumen formation in breast acini. Surprisingly, decreasing Rap1 activity had no effect on cell proliferation, even though PI3K signaling through Akt and PTEN were normalized. These data underscore that Rap1 functions as an organizer of breast acinar apical polarity, and demonstrate that its dysregulation causes destruction of tissue architecture and leads to tumor progression.

Methods

Reagents and antibodies. Growth factor-reduced Matrigel™ (BD Biosciences) with protein concentration of ~10 mg/ml was used for 3D lrECM assays. Rat tail collagen I (~3 mg/ml; Vitrogen 100, Celtrix Laboratories) was used to thinly coat the surfaces of culture dishes as described(30). Inhibitors and antibodies used for reversion assays were as follows: EGFR inhibitor tyrphostin AG1478 (Calbiochem), MEK inhibitor PD98059 (Calbiochem), PI3K inhibitor LY294002 (Cell Signaling Technologies), human EGFR function-blocking antibody mAb225 (Oncogene Research). Antibodies used for biochemical analyses and immunostaining were as follows: anti-total and phospho-Erk1/2 (Thr202/Tyr204), anti-total and phospho-p38 MAPK (Thr180/Tyr182), anti-total and phospho-Akt (Ser473), anti-total and phospho-p90RSK (Ser380), anti-total and phospho-GSK3β (Ser9), anti-cleaved caspase-3 (Asp175), anti-PTEN, and anti-FOXO1 from Cell Signaling Technologies; anti-β1-integrin, anti-GM130, and anti-total and phospho EGFR from BD Transduction; anti-α6-integrin from Chemicon; anti-β-catenin and anti-Rap1 from Santa Cruz; anti-Bim from Calbiochem; anti-Ki67 from Zymed; anti-β-actin from Sigma; rhodamine-phalloidin from Molecular Probes.

Cell culture. HMT-3522 human mammary epithelial cells were maintained as described previously (30). Three-dimensional (3D) cultures on top of lrECM were prepared by growing cells as monolayers, followed by trypsinization and plating (3×10^5 cells/ml) on polymerized lrECM. Culture medium containing 5% lrECM was added and replaced every 2 days. For reversion assays, antibodies and inhibitors were added to culture

medium. At days 3, 5, and 8 of culture, colony size and Ki67 positive indices were measured. Colonies which contained Ki67-positive cells were scored as positive.

Plasmid constructs and transfections. The cDNAs encoding human Rap1-V12 (dominant-active) and Rap1-N17 (dominant-negative) were PCR-amplified using pcDNA-Rap1-V12 and pcDNA-Rap1-N17 (gift of Dr. Kinashi T., Kansai Medical School) as templates, respectively. Amplified fragments were digested with EcoRI and EcoRV, and subcloned into pEYFP-C1 (BD Biosciences Clontech). HMT-3522 mammary epithelial cells were transfected with pEYFP-Rap1V12, pEYFP-Rap1N17, or pEYFP-C1 using Lipofectamine 2000 (Invitrogen). Stably transfected cells were selected with 100- μ g/ml G418 and positive populations were enriched by EPICS Cell Sorting System (Beckman Coulter).

Immunoblotting and immunostaining. Cells cultured in 3D IrECM were first isolated as colonies in ice-cold PBS containing 5mM EDTA, as previously described (30), and lysed thereafter in RIPA buffer (1% Nonidet P-40, 0.5% deoxycholate, 0.2% sodium dodecyl sulfate, 150 mM sodium chloride, and 50 mM Tris-HCl pH 7.4 containing protease inhibitor cocktail (Calbiochem)). Equal amounts of protein lysates were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane. Membranes were blocked with TBS containing 3% skim-milk or 5% BSA and incubated with primary antibodies (1:1000 dilution), followed by incubation with HRP-labeled secondary antibodies (1:1000 dilution). Positive bands were detected with SuperSignal West Dura (Pierce). For immunostaining of 3D IrECM-

cultured samples, cells were incubated with 18% sucrose in PBS for 15min, and then with 30% sucrose in PBS for 15min. Cells were smeared on frosted glass slides (VWR) and air-dried briefly for further processing. After fixation with 3% formaldehyde in PBS for 15 min or methanol/acetone (1:1) for 10 min, cells were permeabilized with 0.3% Triton X-100 in PBS, washed twice with PBS, and blocked with 1% BSA in PBS. Samples were incubated with primary antibody diluted 1:100-300 in blocking buffer for 1 h at room temperature, followed by incubating with FITC- or Cy3-conjugated secondary antibody, and processed following the same protocol as monolayer-cultured samples. Nuclei were counterstained with DAPI (Sigma) and actin-filaments were stained with rhodamine-phalloidin. Samples were mounted with Prolong antifade reagent (Molecular Probes) and observed with Nikon DIAPHOT 300 or Zeiss LSM 410 confocal microscope. Images for figures were pseudo-colored and resized using Adobe Photoshop.

Pull-down assays. Rap1 activity was measured using Rap1 Assay Reagent (Upstate). Cells were lysed in TLB (50mM Tris-HCl, pH 7.4, 500mM NaCl, 1% NP40, 2.5mM MgCl₂, and 10% glycerol) at 4 °C for 30 min. Lysates were cleared by centrifugation at 15,000g at 4 °C for 15 min. Supernatants were incubated with 30 µg of RalGDS-RBD fusion protein coupled with agarose beads for 1 h at 4 °C. Agarose beads were washed three times in TLB and resuspended in Laemmli buffer. Samples were analysed by SDS-PAGE, followed by transfer to nitrocellulose membranes. Affinity-purified activated Rap1 was detected by immunoblotting using an anti-Rap1 antibody.

Invasion assay. The migration and invasion capacity of cells were evaluated in 24-well chambers with filter inserts (8- μ m pores) or filter inserts coated with 30 μ l of Matrigel protein at 6-8 mg/ml, respectively. Approximately 1×10^5 cells were plated into the upper chamber in 300 μ L of culture medium. The lower chamber was filled with 350 μ L of medium containing 5% fetal bovine serum. After culture for 48hr, cells were fixed with 5% glutaraldehyde in PBS and stained with 0.5% toluidine blue (Sigma) in 2% Na_2CO_3 . Cells on the upper side of the filter, including those in the Matrigel, were removed with a cotton swab, and cells on the lower side of the filter were visualized and counted. Each experiment was repeated four times in duplicate, and one representative experiment is shown.

Tumor formation in vivo. Tumor formation ability in vivo was examined by injecting 1×10^7 cells subcutaneously into the rear flanks of Balb/c female athymic nude mice (Simonsen Laboratories) at 7 weeks of age. Tumors were measured weekly for 4-10 weeks and tumor size was recorded at the time of sacrifice. Tissue from injection sites was paraffin embedded, sectioned, and stained with Hematoxylin and Eosin for histology.

Results

Lumen formation and acinar polarity are disrupted by Rap1 activation.

We had found previously that malignant T4-2 cells (which form disorganized and apolar colonies when grown in 3D IrECM; Fig. 1A) showed appreciably higher levels of Rac1 activity relative to their nonmalignant S1 counterparts, and that down-modulation of Rac1 caused T4-2 cells to form polarized acinar structures without lumina (32). Since Rac1 functions downstream of Rap1 (3, 4), we examined Rap1 activity in S1 and T4-2 cells using pull-down assays (33). Whereas the total level of Rap1 was similar in these two cell lines, the level of active GTP-bound Rap1 was appreciably higher in T4-2 than in S1 cells when cultured in 3D IrECM (Fig. 1B). As shown previously for a number of other signaling molecules in our system (reviewed in (27)), this difference was observed only in 3D cultures and not in cells grown on tissue culture plastic, underscoring the importance of tissue context in regulation of signaling pathways.

Blocking Rap1 activity restores tissue polarity and induces lumen formation.

We asked whether down-modulating Rap1 could restore normal tissue architecture in malignant T4-2 cells. We established stable T4-2 transfectants that exogenously expressed dominant-negative Rap1 (T4-DN-Rap1) or vector only (T4-vector) as a control. T4-vector cells cultured in 3D IrECM behaved in a manner similar to untransfected cells, forming large disorganized colonies that could be induced to undergo phenotypic reversion with the EGFR inhibitor AG1478 (Fig. 1C). In sharp contrast, T4-DN-Rap1 cells had markedly different morphology in 3D IrECM, forming organized acinar structures similar to nonmalignant S1 cells even in the absence of the

reverting agents (Fig. 1C). Indirect immunofluorescence of T4-DN-Rap1 and AG1478-treated acini showed correct localization of the basal marker $\alpha 6$ -integrin, the basolateral marker β -catenin, and the apical marker GM130 (a Golgi component that distributes to the apical side of the nucleus in polarized cells). In T4-vector colonies, $\alpha 6$ -integrin, β -catenin, and GM130 were all randomly distributed, confirming that polarity was impaired. Therefore, expression of DN-Rap1 was sufficient to induce polarized acinar architecture in T4-2 cells in 3D IrECM.

Despite the fact that $\alpha 6$ -integrin, β -catenin, and GM130 were correctly localized by down-modulating either EGFR or Rap1, we observed distinct differences between the resulting reverted acini. At each day of culture, T4-DN-Rap1 acini were twice as large as AG1478-treated T4-vector acini (Fig. 1D). Whereas inhibition of EGFR led to growth arrest by day 5, T4-DN-Rap1 cells continued to proliferate as assessed by a high percentage (~45%) of Ki67-positive acini remaining at day 8 of culture (Fig. 1D). The architectural differences between AG1478-treated vector acini and T4-DN-Rap1 acini became more evident by day 15 in 3D IrECM, at which time there were prominent lumina in greater than 60% of T4-DN-Rap1 acini but in fewer than 2% of AG1478-treated vector acini (Fig. 2A). Establishment of apical polarity involves creation of apical membrane domains associated with the presence of filamentous actin (34, 35). We detected apically localized actin in T4-DN-Rap1 acini but not in AG1478-treated T4-vector acini (Fig. 2A), confirming that DN-Rap1 leads to the development of both apical and basal polarity in 3D IrECM.

Lumen formation is accompanied by apoptotic cell death within acinar structures (36-38). Bim, a pro-apoptotic BH3-only Bcl-2 family protein, was identified as having a role in this process (39). We examined apoptotic cell death *in situ* by indirect immunofluorescence for activated caspase-3; as expected (40), apoptosis was restricted to those cells not in contact with basement membrane within T4-DN-Rap1 acini (Fig. 2A). Furthermore, western blotting of lysates demonstrated that Bim was upregulated at day 10 of culture in T4-DN-Rap1 acini (Fig. 2B). In contrast, we detected neither activated caspase-3 nor upregulation of Bim in AG1478-treated T4-vector acini. Thus normalization of Rap1 activity caused reversion of T4-2 cells with a resulting architecture comparable to that of non-malignant mammary epithelial cells, such as MCF-10A, which form lumina in 3D IrECM. The resulting acini form prominent lumina despite persistent proliferation, extending our previous observation that normal tissue polarity can be uncoupled from growth control (32). These results suggest that formation of organized acinar structure by expressing DN-Rap1 in malignant T4-2 cells is achieved by increased apoptotic signaling within the center of the colonies.

Dominant-active Rap1 desensitizes T4-2 cells to reversion by treatment with EGFR inhibitors.

Whereas expression of DN-Rap1 reverted cells to a normal tissue polarity when cultured in 3D IrECM, stable T4-2 transfectants that exogenously expressed dominant-active Rap1 (T4-DA-Rap1) formed disorganized clusters indistinguishable from controls (compare Fig. 1A to Fig. 3A). However, in the presence of AG1478, T4-DA-Rap1 cells failed to revert, forming large colonies with improperly localized $\alpha 6$ -integrin (Fig. 3A).

Reverting T4-vector cells by treatment with AG1478 resulted in down-regulation of both EGFR and β 1-integrin, as reported previously for wild-type T4-2 cells (31). However, expression of these molecules was unaffected in AG1478-treated T4-DA-Rap1 cells (Fig. 3B). Expression of DA-Rap1 likewise blocked restoration of tissue polarity by treatment with standard inhibitory concentrations of the function-blocking EGFR antibody mAb225 (2 μ g/ml; Fig. 3C) or the MAPK pathway inhibitor PD98059 (10 μ M; Fig. 3C). To determine if the resistance to inhibitors of the EGFR pathway was dose-dependent or absolute, we applied higher doses of AG1478, mAb225, or PD98059 and found that a 3-4-fold higher concentration of each antagonist was required to revert T4-DA-Rap1 cells than to revert the controls (Fig. 3C). In contrast, T4-DA-Rap1 cells were reverted successfully by standard treatment with the PI3K inhibitor LY294002 (Fig. 3C). These data suggest that expression of DA-Rap1 prevents T4-2 cells from sensing the 3D IrECM microenvironment, and results in an uncoupling of EGFR and β 1-integrin signaling pathways analogous to the effects of growing T4-2 cells on tissue culture plastic (31).

Rap1 activity affects invasive phenotype and tumorigenesis of malignant T4-2 cells.

To explore whether these findings had relevance to tumor formation in vivo, we investigated two aspects of malignant behavior: invasion in Matrigel-coated transwell chambers and frequency of tumor formation. We found that invasiveness correlated with Rap1 activity: invasion of T4-DN-Rap1 and T4-DA-Rap1 cells were 50% and 400% that of controls, respectively (Fig. 4A). To determine the effect of Rap1 on the tumorigenic potential of T4-2 cells, xenograft tumors were formed by injecting T4-vector, T4-DN-Rap1, and T4-DA-Rap1 cells subcutaneously into athymic nude mice. Tumor growth

correlated with Rap1 activity: the percentage of mice bearing large ($>10 \text{ mm}^3$) tumors was enhanced by DA-Rap1 and abrogated by DN-Rap1 (Fig. 4B). Pathological examination revealed that tumors derived from T4-DA-Rap1 cells were of a higher grade of malignancy than controls. At week 10 after injection, the tumors formed by control T4-vector cells were no longer proliferative, as determined by the absence of Ki67 staining, while T4-DA-Rap1 cells were still dividing (data not shown). In addition, blood vessel infiltration was frequently observed in xenografts derived from T4-DA-Rap1 cells (Fig. 4C).

MAPK and PI3K signaling are modulated by Rap1.

To dissect the molecular mediators of Rap1 signaling in 3D IrECM, we examined the expression and phosphorylation levels of downstream signaling molecules. Although T4-DA-Rap1 cells were resistant to reversion by AG1478, and T4-DN-Rap1 cells adopted normal tissue structure and polarity in the absence of EGFR inhibitors, we could detect no differences in the levels of active or total EGFR under these two conditions (Fig. 5A). This is in contrast to cells reverted by small molecule inhibitors of EGFR or PI3K as well as β 1-integrin or EGFR inhibitory antibodies (30-32). However, Erk1/2 and its target molecule p90RSK were more highly phosphorylated in T4-DA-Rap1 than in T4-DN-Rap1 cells or in controls, consistent with studies reporting activation of Erk1/2 by Rap1 (13, 19), and consistent with our inability to revert them using standard concentrations of MEK inhibitor (Fig. 4C). Another MAPK family protein, p38, which was shown to be activated by Rap1 in neuronal cells (41), was unaffected, suggesting that Rap1 acts downstream of EGFR and upstream of Erk1/2 specifically in T4-2 cells.

To delineate the connection between Rap1 and apoptotic mechanisms carving the lumen, we measured the level of phosphorylated active Akt, which plays a central role in activating survival signals and suppressing death signals (42). We found that its level was greatly decreased in T4-DN-Rap1 cells (Fig. 5B). Phosphorylation of GSK3 β , a target of Akt, was similarly downregulated, whereas the PI3K antagonist PTEN was upregulated in T4-DN-Rap1 cells, indicating that PI3K signaling through Akt was decreased by expression of DN-Rap1. Unsurprisingly, we did not detect significant differences in the expression of phospho-Akt, phospho-GSK3 β , or PTEN in T4-DA-Rap1 cells compared to controls, consistent with our ability to revert them with equal doses of the PI3K inhibitor LY294002. We found that expression of the pro-apoptotic factor FOXO1 was reduced in T4-DA-Rap1 and increased in T4-DN-Rap1 cells at both the mRNA and protein levels (Fig. 5C), providing further evidence that cell death pathways were affected by Rap1 activity, and implicating FOXO1 in lumen formation in mammary epithelial cells. Thus, upregulated activity of Rap1, as is found in tumorigenic T4-2 cells, uncouples normal microenvironmental cues from apoptotic signaling, thereby inhibiting establishment of tissue polarity, lumen formation, and acinar morphogenesis.

Discussion

The functional unit of the mammary gland is the acinus, the establishment and maintenance of which depend on integration of cues from the surrounding microenvironment (43). A defining feature of the acinus is that its constituent cells are polarized, with distinct basolateral and apical membrane domains, surrounding a central lumen, which is required for secretion and storage of milk during lactation. One of the early events in breast cancer progression (44) is the loss of the cues that maintain the lumen (28, 36, 37, 39, 45), i.e., aberrations in both apoptosis and autophagy. Here, we identify Rap1 as a central modulator of lumen formation in breast epithelial cells, functioning upstream of the previously identified regulator Bim as well as several other pro-apoptotic molecules, including PTEN and FOXO-1. The level of Rap1 activity correlates with the architecture of the acinus: if the level is appropriate, acini are formed; if it is too high, apoptosis is impaired, cells lose polarity, become motile, and acini are filled.

Whereas inhibiting Rap1 activity restored tissue polarity and reduced tumorigenicity of T4-2 cells, high levels of Rap1 activity rendered cells resistant to reverting agents, and resulted in formation of high grade tumors. These data underscore the notion that tissue polarity and malignancy are inversely related (43), but that tissue polarity and growth suppression are regulated by distinct pathways: here, cells expressing DN-Rap1 formed correctly polarized acini yet continued to proliferate more than their vector-transfected AG1478-reverted counterparts, and xenografts derived from these cells essentially failed to generate tumors. These data, and our previously published work

delineating the roles of Akt and Rac1 in proliferation and polarity downstream of PI3K (32), suggest that reversion of the malignant phenotype need not necessarily target deregulated proliferation if the treatment restores tissue architecture. Moreover, a polarized epithelial architecture has a protective effect, preventing malignancy even in cells with underlying genomic abnormalities (46-48). Intriguingly, down-modulating Rap1 activity also down-modulated Akt signaling – as evaluated by levels of phospho-Akt, phospho-GSK3 β , and PTEN (Fig. 5B) – but had no effect on cell proliferation. These data are in contrast to reversion by treatment with the PI3K inhibitor LY294002, which also down-modulates signaling through both Akt and Rac1, but which leads to restoration of tissue polarity accompanied by growth modulation (32). One obvious difference between these two treatments is that PI3K inhibition leads to feedback modulation on both EGFR and β 1-integrin levels (32, 49), whereas DN-Rap1 does not, suggesting either that PI3K and Rap1 are parallel pathways downstream of EGFR, or that Rap1 is activated independently of EGFR in tumorigenic T4-2 cells. In fact, DN-Rap1 is thus far the only reverting agent found that fails to normalize the activities of the other untreated pathways.

The data demonstrating that dysregulation of Rap1 correlates with destruction of tissue architecture and increased grade of malignancy are supported by several other studies showing a role for Rap1 in tumor progression (15, 16, 18, 20, 50). In the case of T4-2 cells, active Rap1 down-modulates pro-apoptotic pathways and upregulates MEK. T4-DA-Rap1 cells have a molecular signature that suggests a more aggressive malignant phenotype compared to vector controls. That T4-DA-Rap1 cells formed larger and more

aggressive tumors suggests that hyperactivation of Rap1 either directly or indirectly stimulates the production of cytokines or pro-angiogenic molecules. Indeed, activation of Rap1 was previously found to stimulate production of vascular endothelial growth factor (VEGF) in transformed prostate cells (51), and T4-DA-Rap1 cells show enhanced expression of VEGF by cDNA microarray analysis (not shown).

The integration of signals from cell-cell and cell-ECM adhesions is crucial for organizing acinar architecture and maintaining mammary tissue homeostasis. Decreased β 1-integrin signaling and lack of ECM contact induce apoptosis of mammary epithelial cells resulting in the induction of acinar morphogenesis and lumen formation (37, 40); these signals are aberrant in tumorigenic cells such as T4-2. Appropriate Rap1 signaling appears to redress the balance of these elements. Further studies on the effects of Rap1 on integrins, cadherins, Rac1, and FOXO1 in our culture models will yield insight into the molecular mechanisms organizing acinar architecture and breast tumor progression.

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Figure Legends

Figure 1. Downregulation of Rap1 activity in malignant T4-2 cells restores tissue polarity in 3D IrECM culture. (A) Phase-contrast images of S1 and T4-2 cells cultured in 3D IrECM. (B) Rap1 expression and activity levels in S1 and T4-2 cells. Bar, 10 μm . (C) Morphology of T4-vector treated with and without AG1478 and T4-DN-Rap1 colonies in 3D IrECM. Cell polarity was examined by staining markers for basal ($\alpha 6$ -integrin), basolateral (β -catenin), and apical (GM130) membrane domains. Bar, 5 μm . (D) Cell proliferation in 3D IrECM was determined by measuring colony size (left) and Ki-67 positive colonies (right). Shown are averages and standard deviation across three independent experiments. (*) $p < 0.05$.

Figure 2. Bim-mediated lumen formation is induced in T4-DN-Rap1 cells but not in AG1478-treated control cells. (A) Phase contrast images after 15 days of culture in 3D IrECM, showing prominent lumina in T4-DN-Rap1 acini but not in AG1478-treated T4-vector acini. The localization of actin filaments in apical membrane domains confirmed lumen formation and establishment of apical polarity in T4-DN-Rap1 acini. Apoptotic cell death was examined by staining for active-caspase3 at day 10 of culture. (B) Expression level of Bim in T4-vector, AG1478-treated T4-vector, and T4-DN-Rap1 cells at day 10 of culture in 3D IrECM. Bar, 5 μm .

Figure 3. T4-DA-Rap1 cells show dose-dependent resistance to reversion by inhibitors of EGFR and MAPK. (A) Phase-contrast images and immunofluorescence localization of

α 6-integrin in T4-DA-Rap1 cells with or without 70nM AG1478 in 3D IrECM. (B) Levels of β 1-integrin, EGFR, and phospho-EGFR in 3D IrECM cultured T4-vector cells, and T4-DA-Rap1 cells in the presence and absence of 70nM AG1478. (C) Dose-dependent effect of reverting agents on T4-DA-Rap1 cells in 3D IrECM. Cells were treated with different doses of inhibitors against EGFR (AG1478 and mAb225), MEK (PD98059) or PI3K (LY294002). Bar, 5 μ m.

Figure 4. Level of Rap1 activity affects invasiveness and tumorigenic potential of T4-2 cells. (A) Invasiveness of T4-vector, T4-DA-Rap1, and T4-DN-Rap1 cells was examined using matrigel-coated transwell filters. Quantification at 48 hr after plating. (B) The transfectants were subcutaneously injected into nude mice to examine tumorigenic potential. Tumor size measured at 8 weeks after injection and mice bearing tumors larger than 10mm³ were scored. (C) Histology of tumors derived from T4-vector and T4-DA-Rap1 cells.

Figure 5. Phenotype modulation caused by Rap1 occurs through different signaling pathways from those targeted by EGFR or β 1-integrin modulation. Activity and expression of the components of EGFR-MAPK pathway (A) and PTEN/Akt pathway (B) were examined by western blotting.

(C) The levels of FOXO1 protein and mRNA were determined by western blotting and RT-PCR analysis, respectively.

Figure 1: Down-regulation of Rap1 activity in malignant T4-2 cells restores tissue polarity in 3D IrECM culture

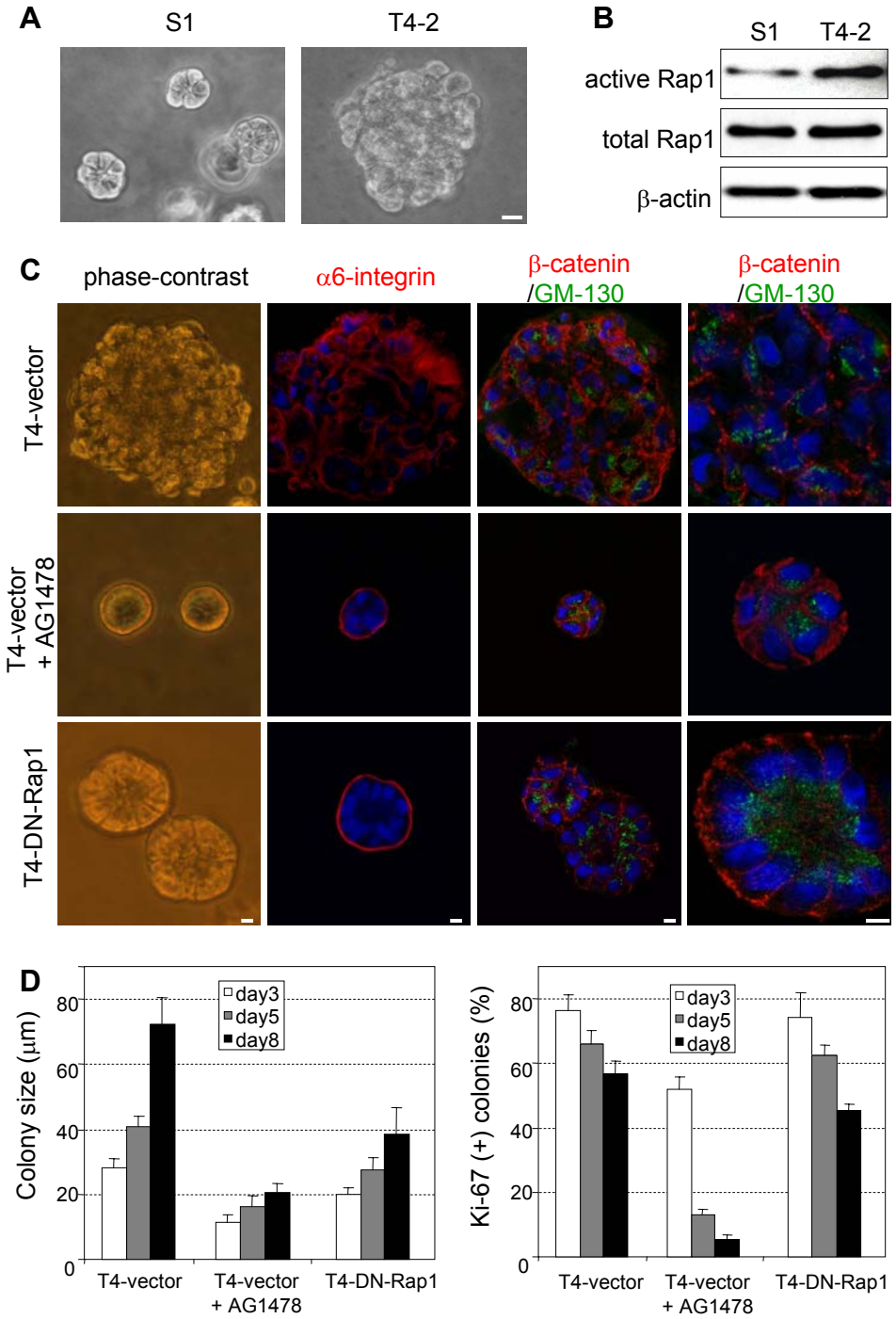


Figure 2: Bim-mediated lumen formation is induced in T4-DN-Rap1 cells but not in EGFR inhibitor-treated control cells

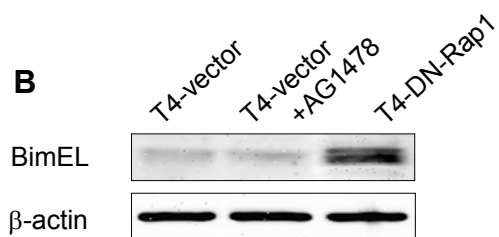
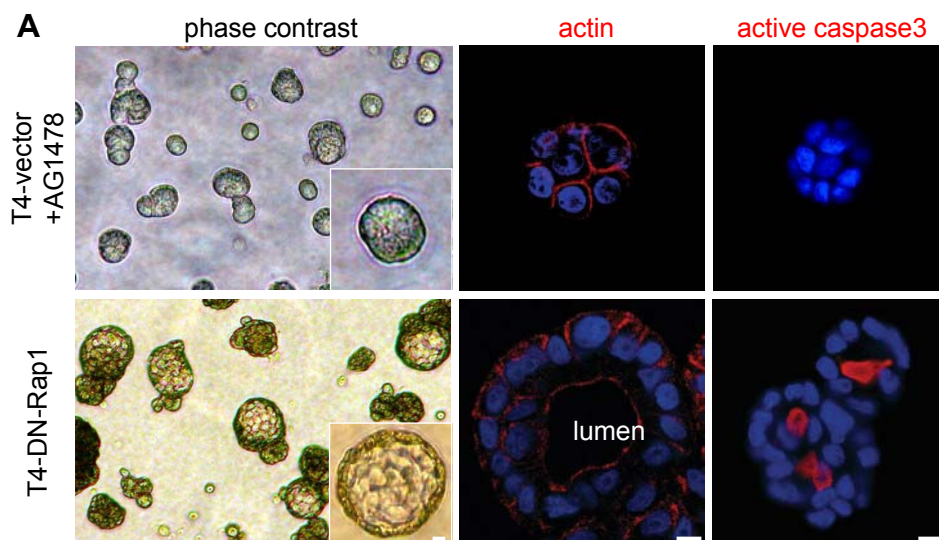


Figure 3: T4-DA-Rap1 cells show dose-dependent resistance to reversion by inhibitors against EGFR and MAPK

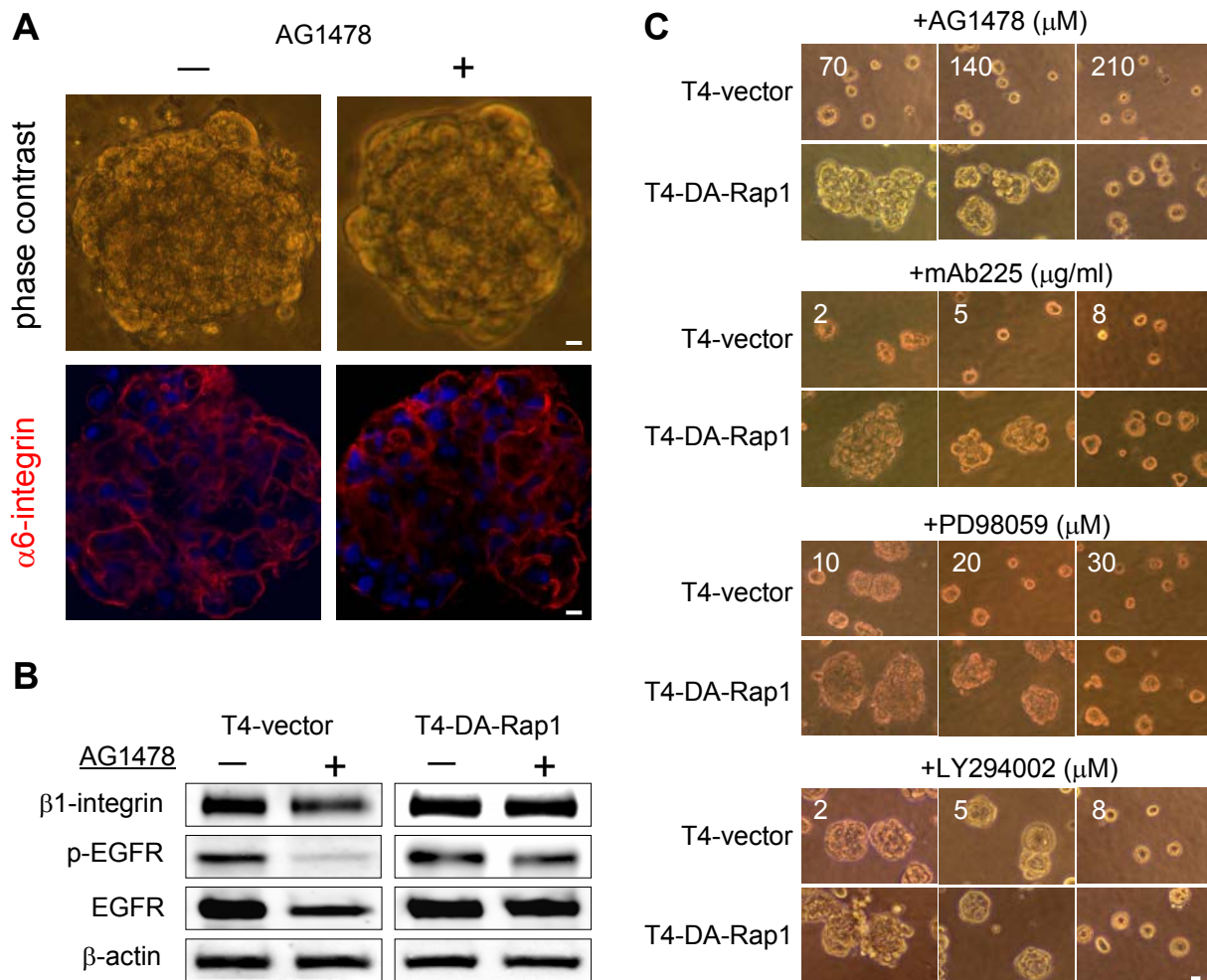


Figure 4: Level of Rap1 activity affects invasiveness and tumorigenic potential of T4-2 cells

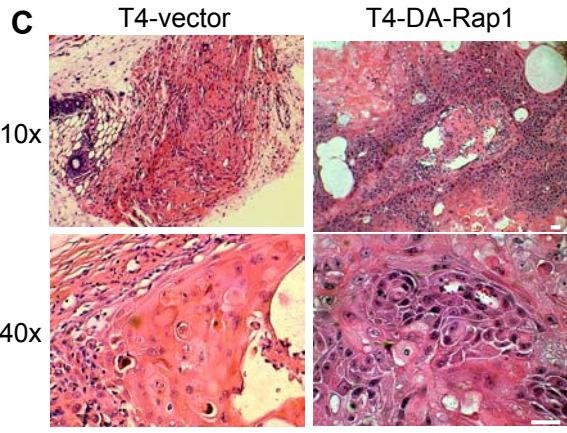
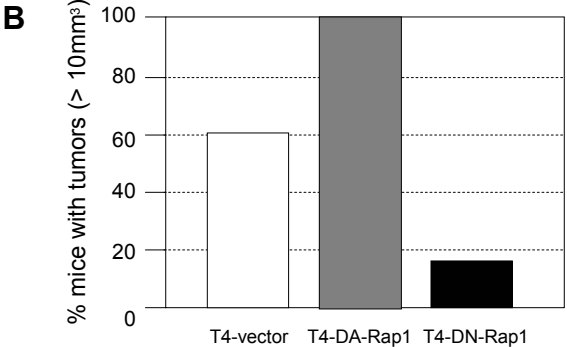
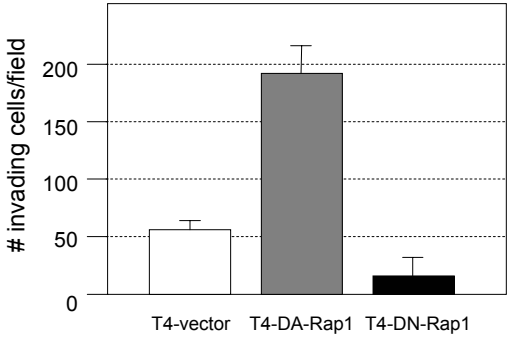
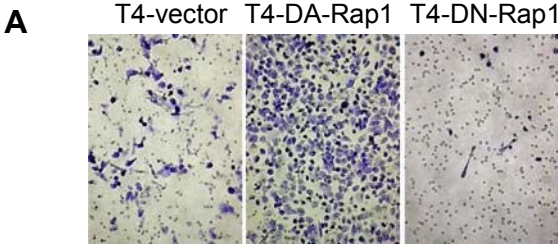


Figure 5: Phenotype modulation by Rap1 occurs through different signaling pathways than those targeted by EGFR or β 1-integrin modulation

