Mammalian Target of Rapamycin Activator RHEB Is Frequently Overexpressed in Human Carcinomas and Is Critical and Sufficient for Skin Epithelial Carcinogenesis

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Abstract
Small GTPase Ras homologue enriched in brain (RHEB) binds and activates the key metabolic regulator mTORC1, which has an important role in cancer cells, but the role of RHEB in cancer pathogenesis has not been shown. By performing a meta-analysis of published cancer cytogenetic and transcriptome databases, we defined a gain of chromosome 7q36.1-q36.3 containing the RHEB locus, an overexpression of RHEB mRNA in several different carcinoma histotypes, and an association between RHEB upregulation and poor prognosis in breast and head and neck cancers. To model gain of function in epithelial malignancy, we targeted Rhee expression to murine basal keratinocytes of transgenic mice at levels similar to those that occur in human squamous cancer cell lines. Juvenile transgenic epidermis displayed constitutive mTORC1 pathway activation, elevated cyclin D1 protein, and diffuse skin hyperplasia. Skin tumors subsequently developed with concomitant stromal angio-inflammatory foci, evidencing induction of an epidermal hypoxia-inducible factor-1 transcriptional program, and paracrine feed-forward activation of the interleukin-6–signal transducer and activator of transcription 3 pathway. Rheb-induced tumor persistence and neoplastic molecular alterations were mTORC1 dependent. Rheb markedly sensitized transgenic epidermis to squamous carcinoma induction following a single dose of Ras-activating carcinogen 7,12-dimethylbenz(a)anthracene. Our findings offer direct evidence that RHEB facilitates multistage carcinogenesis through induction of multiple oncogenic mechanisms, perhaps contributing to the poor prognosis of patients with cancers overexpressing RHEB. Cancer Res; 70(8): 3287–98. ©2010 AACR.

Introduction
The pathway integrating diverse mammalian cell growth signals engages the heterotrimeric protein kinase mTORC1, which, when induced, stimulates growth by promoting ribosomal biogenesis and protein synthesis through effectors p70S6K and eIF-4E-BP (1). Growth pathways converge on the negative regulator of mTORC1, tuberous sclerosis complex 1/2 (TSC1/2). As a GTPase-activating protein toward the small G protein Ras homologue enriched in brain (RHEB), TSC1/2 inhibits RHEB function. GTP-bound RHEB is a direct mTORC1 activator, which is essential for cell growth in Drosophila and mammalian cells. Growth factor receptor activation triggers the phosphatidylinositol 3-kinase (PI3K)/AKT cascade stimulating mTORC1 by repressing TSC1/2, whereas a sufficiency of cellular energy (i.e., high ATP/ADP ratio) suppresses LKB1 and AMP-activated protein kinase signaling, which inhibits mTORC1 by activating TSC1/2. Both growth factor, bioenergetic, and nutrient pathways also regulate mTORC1 through TSC1/2-independent mechanisms (6–8).

mTORC1 hyperactivation occurs in most human cancers via PI3K/AKT signaling, which is induced by either mutation or amplification of genes encoding growth factor receptors, the PI3K catalytic subunit (PIK3CA), or AKT, or loss of function of pathway negative regulators including the tumor suppressors NF1, PTEN, and TSC1/2 (3–5). Dysregulated mTORC1 signaling is present in the tumor-prone Peutz-Jeghers syndrome due to LKB1 inactivating mutations and in human colorectal cancers with APC loss of function (ref. 9 and references therein). Many preclinical studies show that mTORC1 blockade by rapamycin and its derivatives is antineoplastic in lesions induced by a variety of oncogenic signaling inputs, suggesting that mTORC1 is a relevant common effector of cancer development (9–14). RHEB cytogenetic and expression data are limited in human cancers. Unlike other mTORC1 upstream genes, RHEB...
or mammalian target of rapamycin (mTOR) mutations have not been discovered (15, 16). RHEB is located on chromosome 7q36. A recent study showed low-frequency whole chromosome 7 amplification in a cohort of comparative genomic hybridization–analyzed human prostate cancers (17); however, RHEB locus amplification or association between RHEB expression and human cancer prognosis has not been investigated. Recently, two animal studies showed that Rheb gain of function was tumorigenic. Rheb overexpression enhanced Ep–Myc–mediated lymphomagenesis in a mouse bone marrow transplantation model (18) and produced low-grade prostatic intraepithelial hyperplasia (17). However, the lymphoma model did not test Rheb tumorigenicity as a monogenic perturbation, and the nature of a secondary cooperating hit responsible for the long latency and low penetrance of Rheb-mediated prostatic hyperproliferation was not elucidated. Thus, despite progress, important questions remain about RHEB expression and function in tumorigenesis.

Here, we show RHEB overexpression and chromosomal locus amplification in a broad spectrum of human cancers and prognostic association in breast and head and neck squamous cancer. Using a transgenic mouse model of Rheb overexpression and mTORC1 hyperactivation in skin epidermis recapitulating RHEB expression in human cancer cells, we show that Rheb gain of function facilitates cancer progression through multiple mechanisms.

Materials and Methods

Cancer transcriptome database mining. We queried Oncomine and Gene Expression Omnibus (GEO), listing all data sets containing RHEB expression data, and downloaded those trending toward differential expression between cancers and normal tissues or between cancer types/grades. Unpaired two-tailed t tests determined statistical significance associated with differential expression (P < 0.05). This analysis identified the Oncomine Chen_Liver cancer (normal versus cancer), Blaiver_Bladder_2 cancer (low grade versus high grade), Chung_Head and neck cancer (normal versus cancer), and GEO GDS1650 lung adenocarcinoma (normal versus cancer) data sets. The Mann-Whitney U test was used to determine high RHEB–expressing outliers in stage III versus stage I breast cancers from the Oncomine vantVeer_Breast cancer cohort. A second breast cancer data set, Oncomine Minn_Breast_2 cancer, was analyzed as validation for the vantVeer_Breast cancer set. The two breast and one head and neck cancer cohorts were further analyzed for correlation between cancer RHEB expression and clinical outcome. Specifically, each of the three cancer data sets was divided into quartiles based on RHEB mRNA levels, and a survival curve for the upper 25th [RHEB–high (RHEB-Hi)] versus the lower 25th quartiles were compared for the statistical significance using the log-rank (Mantel-Cox) test.

Mouse experiments. Mm-mouse RhoB cDNA was cloned into a 2-kb fragment of human K14 promoter (19). The transgene was used for pronuclear microinjection of FVB/n embryos. A total of eight transgenic founders were generated, and two transmitted the transgene through the germline. In the chemoprevention trial, 8-wk-old transgenic mice were treated with 10 μg/g of RAD001 or vehicle (Novartis Corp.) by gavage once per week for 13 to 15 wk. In the therapeutic trial, tumor-bearing mice between 15 and 25 wk were treated with 7.5 μg/g RAD001 or vehicle thrice per week for 4 wk. For skin carcinogenesis, the back skin of 8-wk-old mice was shaved and topicaly treated with a single dose of 7,12-dimethylbenz(a)anthracene (DMBA; Sigma-Aldrich), 100 μg in acetone. The Animal Studies Committee of Washington University in St. Louis approved all animal work.

Histology, immunofluorescence, and immunohistochemistry. Tissues were obtained from 10% sucrose/10% formaldehyde–perfused mice, and microwave-processed (20), or immersed in zinc fixative (BD Biosciences) for 24 h. Tissue sections were stained with H&E (20). For immunofluorescence, rehydrated sections were blocked with Dako Protein Block (Dako North America). Antigen retrieval was performed in citrate–EDTA-based buffer using a pressure cooker (Biocare Medical). Primary and fluorochrome-conjugated secondary antibodies were diluted in Dako antibody diluent. For immunohistochemical detection, sections were incubated with biotin-conjugated secondary antibodies followed by immunoperoxidase staining using Vectastain ABC Elite kit (Vector Laboratories) and diaminobenzidine (Dako North America). Bromodeoxyuridine (BrdU) mouse pulse labeling and tissue immunostaining were performed as previously described (21). The following antibodies used in immunostaining were from Cell Signaling Technology: rabbit antibodies against phospho-S6 ribosomal protein (S235/236) (1:500), phospho-eIF-4G (S205) (1:100), phospho-RB (1:100), Rheb (1:400), Myc tag (1:500), activated caspase-3 (1:500), phospho-signal transducer and activator of transcription 3 (STAT3) (1:50), survivin (1:100), phospho-extracellular signal-regulated kinase 1/2 (ERK1/2) (1:200), and E-cadherin (1:200). Additional antibodies used were rabbit anti-loricrin, anti–keratin 8 (K8), and anti–keratin 10 (1:500, 1:5,000, and 1:50, respectively; Covance); anti–cyclin D1 (1:100; Abcam); rat anti-CD45, anti–Meca 32, anti–CD4, anti–CD8a, and anti–Gr-1 (1:50, 1:20, 1:20, and 1:20, respectively; BD Biosciences); anti–FoxP3 (1:200, eBioscience); anti–F4/80 (Serotec) antibodies; and biotinylated mouse antibody against keratin 14 (K14; 1:1,000; Lab Vision). For immunofluorescence, slides were incubated with corresponding fluorochrome-conjugated secondary antibodies (1:400; Molecular Probes). BrdU labeling was detected with Alexa Fluor 594–conjugated anti-BrdU antibody (Invitrogen).

Keratinocyte culture. Primary mouse keratinocytes were established from 1-d-old neonatal epidermis as described previously (21). For serum starvation, keratinocytes were cultured previously (21). For serum starvation, keratinocytes were cultured overnight in serum-free medium. For murine interleukin (IL)-6 stimulation, serum-starved keratinocytes were treated with 10 ng/mL recombinant murine IL-6 (eBioscience) for various durations of time.

Immunoblotting. Frozen tissues and pelleted cultured keratinocytes were lysed with ice-cold lysis buffer [1% Triton X-100, 40 mmol/L HEPES (pH 7.5), 120 mmol/mL NaCl, 1 mmol/L EDTA, 10 mmol/L pyrophosphate, 10 mmol/L glycophosphate, 50 mmol/mL NaF, 0.5 mmol/L orthovanadate, and 1:50 diluted protease inhibitor cocktail (Sigma-Aldrich)].

Protein
sample preparation and immunoblotting were according to a standard procedure (Cell Signaling Technology). The following antibodies were used in Western blotting (all at 1:1,000 and all from Cell Signaling Technology): anti-mTOR, anti-phospho-mTOR (S2448), anti-S6K1, anti-phospho-S6K1 (T389), anti-S6 ribosomal protein, anti-phospho-S6 ribosomal protein (S235/236), anti-elf-4G, anti-phospho-elf-4G (S209), anti-AKT, anti-phospho-AKT (T308), anti-phospho-AKT (S473), anti-Janus-activated kinase 2 (JAK2), anti-phospho-JAK2 (Y1007/1008), anti-STAT3, anti-phospho-STAT3 (Tyr 705), anti-phospho-STAT5 (Tyr 657/659), anti-AKT, anti-phospho-AKT (S473), anti-β-catenin, anti-α-SMA, anti-α-actin, anti-E-cadherin, and horseradish peroxidase–conjugated IgG secondary antibodies (1:1,000; Santa Cruz Biotechnology). We also used anti-β-tubulin (1:35,000; Abcam), anti-K14 (1:2,000; Covance), anti-hypoxia-inducible factor-1α (HIF-1α; 1:1,000; Bethyl Laboratories), anti–vascular endothelial growth factor (VEGF; 1:200; Santa Cruz Biotechnology), anti–IL-6 (1:400, Abcam), and horseradish peroxidase–conjugated-goat anti–rabbit secondary antibodies (1:2,000; Santa Cruz Biotechnology).

Quantitative real-time reverse transcription-PCR and ELISA. Total RNA was prepared from mouse back skin using Trizol (Invitrogen). mRNA expression was measured by real-time quantitative PCR using a Taqman Gold PCR kit according to the manufacturer’s instructions (Applied Biosystems). Cytokines in keratinocyte cell culture medium supernatants and/or skin lysates were measured by ELISA according to the manufacturer’s instructions (BD Biosciences).

Tissue microarray analysis. Cancer specimens were collected in the Department of General Surgery and Pathology, Hongqi Hospital, Mudanjiang Medical College. Specimens included (a) stage 1 or II breast cancer (n = 74; 12 cases of ductal carcinoma in situ (DCIS) and 62 cases of invasive ductal cancer; normal breast tissue cores from 13 reduction mammoplasty patients from University of California at San Diego Medical Center were included to the tissue microarray (TMA), (b) Duke’s stage B colorectal cancer (n = 48; 31 also with normal colon from the same patient), and (c) stage T2N0M0 or T3N0M0 gastric cancer (n = 24; 16 also with adjacent normal stomach epithelium) assessed according to the International Union Against Cancer criteria (22). The research was approved by an institutional review board of the Mudanjiang Medical College and University of California at San Diego. TMAs were previously described (23). Typically, two to three cancer cores and one adjacent normal tissue core were obtained from each specimen. Tissue core procurement details are provided in Supplementary Materials and Methods. TMA immunohistochemical analysis was performed as previously described (23). Rheb antibody (AB-2; Calbiochem) or nonimmune rabbit serum was applied at a 1:2,000 dilution (v/v), and a qualitative immunostaining scoring system (immunoscore) was used for RHEB expression quantification (24).

Reverse-phase protein array. RHEB protein expression in a collection of human cancer cell lines was surveyed using high-throughput reverse-phase protein array (RPPA) as previously described (25). Briefly, the conditions of RHEB antibody (AB-2) usage were optimized for RHEB specificity by immunoblotting. Cell lysates prepared for immunoblotting were transferred to SDS sample buffer, incubated for 5 min at 95°C, and printed onto nitrocellulose-coated glass slides with an automated robotic GeneTac arrayer (Genomic Solutions, Inc.). The same stringent conditions for slide blocking, blotting, and antibody incubation, which were used for immunoblotting, were applied.

Statistical methods. Data were presented as the mean ± SD, and statistical significance was determined using the two-tailed unpaired Student’s t test, Mann-Whitney U test, or χ² test (GraphPad Prism) and log-rank (Mantel-Cox) analysis.

Results

RHEB overexpression induces neoplasia via mTORC1

RHEB dysregulation was a novel prognostic factor in human cancer. Interrogation of published human cancer cytogenetic data revealed frequent gain/amplification of a minimally overlapping chromosomal 7q36.1-q36.3 region harboring the RHEB locus in diverse human cancer histotypes (Supplementary Table S1). Of 491 total cancer genomes, 45 (9.2%) contained gain of 7q32-qter or a smaller region. Among these genomes, 16 harbored gain of the 7q36 region. Analysis of cancer transcriptome databases (26, 27) uncovered increased RHEB expression in liver, lung, and bladder cancers (Supplementary Fig. S1A). Differential RHEB protein elevation was evident in TMAs of stomach and colorectal adenocarcinomas compared with respective adjacent histologically normal mucosa (Supplementary Fig. S1B). A striking incremental RHEB induction during breast carcinogenesis with moderate overexpression in DCIS, followed by marked malignant epithelial protein expression in invasive ductal carcinoma (IDC), was detected in a multistage breast TMA (Fig. 1A). Microarray database mining confirmed a statistically significant association between high-level RHEB mRNA upregulation and breast cancer progression, most evident in stage III disease and more frequent in the aggressive estrogen receptor-negative (ER−) and/or progesterone receptor–negative (PR−) cases (Fig. 1B; ref. 28). Quartile analysis of stage III cancers revealed that RHEB-Hi expression exhibited a worse metastasis-free survival compared with RHEB-Low (RHEB-Lo) stage III counterparts (Fig. 1B). Elevated RHEB expression in a second breast cancer cohort was independent of PTEN or HER2 expression and was also significantly associated with either ER or PR negativity and an increased frequency of metastasis (Supplementary Fig. S1C; data not shown). RHEB mRNA overexpression was also detected in head and neck cancer originating in several different sites of the oropharyngeal squamous mucosa (Fig. 1C; ref. 29). Quartile analysis revealed that the RHEB-Hi group possessed a trend for reduced recurrence-free survival versus the RHEB-Lo group (Fig. 1C, middle). These data were independent of epidermal growth factor receptor (EGFR) status (data not shown). Among head and neck cancer site-specific subtypes, RHEB-Hi oropharyngeal carcinomas had a worse disease outcome than RHEB-Lo counterparts (P = 0.029; Fig. 1C, right). Collectively, these data, for the first time, suggested a functional linkage between RHEB overexpression and progression of human cancers originating from several different organ sites in general and squamous cancers in particular.
Next, we surveyed RHEB expression in a collection of human cancer cell lines using high-throughput RPPA (25). RHEB high-expressing subsets were identified among mammary [highest level compared with lowest level (Hi/Lo) = 7.7], lung (Hi/Lo = 4.7), ovarian (Hi/Lo = 6.1), and head and neck cell lines (Hi/Lo = 4.4; Supplementary Fig. S2A). Analysis of human and mouse cancer cell lines by immunoblotting revealed that human SiHa and C33A and mouse C5N squamous carcinomas exhibited the highest RHEB abundance, about 3.1- to 4.7-fold elevated compared with RHEB levels in wild-type (WT) mouse skin epidermis (Supplementary Fig. S2B).

RHEB induced mTORC1 hyperactivation in transgenic epidermis and cultured keratinocytes resistant to serum and amino acid starvation. To develop a general model for studying RHEB overexpression in epithelial carcinogenesis, and a model of squamous carcinogenesis in particular, two independent transgenic mouse lines (K14-Rheb#8 and K14-Rheb#4) were

Figure 1. RHEB overexpression is associated with poor prognosis in several different human epithelial malignancies. A, TMA immunoscores for RHEB protein levels in normal breast tissues, DCIS, and IDC. B, microarray RHEB mRNA level for stage I to III breast cancers and quartile analysis of stage III patients based on RHEB expression ("RHEB-Hi," upper quartile, versus "RHEB-Lo," lower quartile). Distribution of ER+/ER−, PR+/PR− cases (middle) and Kaplan-Meier survival curve (right) in stage III RHEB-Hi versus RHEB-Lo breast cancers. C, RHEB expression in human head and neck cancer subtypes (left), the Kaplan-Meier recurrence-free survival curves for RHEB-Hi versus RHEB-Lo head and neck cancers (middle), and for RHEB-Hi versus RHEB-Lo oropharynx cancers (right). Statistical analyses: two-tailed unpaired t tests (A, left), Mann-Whitney U test (B, left), χ2 tests (B, middle), and log-rank (Mantel-Cox) tests (survival data in B and C).
created targeting murine Rheb overexpression to basal epidermal keratinocytes. Epidermal RHEB expression was elevated 4-fold in K14-Rheb8 mice compared with WT littermate controls, matching expression in SiHa and C33A cell lines (Supplementary Fig. S2B, RHEB versus WT). K14-Rheb8 mice displayed phenotypes at full penetrance and were used for all studies in this report. K14-Rheb4 mice expressed the Rheb transgene at endogenous levels (data not shown), and 10% developed skin pathology identical to line K14-Rheb8 counterparts.

Immunoblotting revealed mTORC1 target activation (phospho-p70S6k1T389 and phospho-eIF-4G S1108) and elevated p70S6K1, S6, and eIF-4G expression in transgenic epidermal extracts compared with WT controls (Supplementary Fig. S3A). The Myc-RHEB fusion protein, phospho-S6S235/236, and phospho-eIF-4GS1108 were markedly upregulated in the epidermal basal, suprabasal, and granular layers (Fig. 2A, Myc tag and RHEB), suggesting RHEB persistence accompanying epidermal differentiation. Neither phospho-AKT S473 (mTORC2 kinase site) nor phospho-AKT T308 levels (PDK1 kinase site) were differentially altered in transgenic epidermis (Supplementary Fig. S3A). Serum-starved keratinocyte cultures derived from neonatal transgenic epidermis revealed persistently elevated mTORC1 signaling (Supplementary Fig. S3B) but similar mTORC2 activity compared with WT keratinocytes (Supplementary Fig. S3C). These data suggested lack of S6K-mediated negative feedback in this model (30).

Figure 2. Rheb induced mTORC1 hyperactivation and produced epidermal neoplasia. A, expression of Myc-RHEB transgene, RHEB, pS6, and eIF-4G. B, histologic analyses of 3-wk-old WT and transgenic back skin (arrowhead: mitotic basal cell). Gr, granular; Sb, suprabasal; Bs, basal layer. C, histology of a large transgenic back skin papilloma. D, analysis of proliferation in WT versus transgenic papilloma (top) and epidermal differentiation in transgenic papillomas (bottom). Bars, 100 μm [A, B (top), and D], 50 μm (B, bottom), and 200 μm (C).
activity was resistant to combined serum and amino acid starvation in transgenic keratinocytes but completely repressed in controls (Supplementary Fig. S3D).

**Juvenile epidermal hyperplasia and universal adult-onset neoplasia in K14-Rheb transgenic mice.** Neonatal transgenic mice exhibited wrinkled back skin, reddened tails and paws, and thickened ears (Supplementary Fig. S4A and B). Histologic analysis of 3-week-old transgenic back skins revealed diffuse epidermal hyperplasia (Supplementary Fig. S4C) and marked keratinocyte hypertrophy in all epidermal layers (Fig. 2B). Spontaneous neoplasia first appeared in transgenic mice at 12 weeks of age, predominantly located on the posterior neck and between the shoulder blades, and reached 100% penetrance by 32 weeks of age (n = 42; Supplementary Fig. S4D). Tumor burden ranged from 1 to 20 (7.8 ± 5.3) per mouse. In contrast, none of the WT littermates (n = 36) evidenced skin pathology. All transgenic tumors were benign, composed of hyperplastic hair follicles and hypertrophic sebaceous glands (Fig. 2C). BrdUrd incorporation was elevated in the basal and immediate suprabasal layers of hyperplasias and neoplasias (Fig. 2D), and both pathologies retained cytokeratin 10 and loricrin expression (Fig. 2D, K10 and Lor). Thus, monogenetic *Rheb* overexpression, although tumorigenic, did not dysregulate epithelial differentiation.

**Figure 3.** Formation/maintenance of transgenic papilloma was mTORC1 dependent. A, time course of papilloma appearance in K14-Rheb mice treated with vehicle or RAD001. B, rapid mTORC1 activity inhibition concomitant with proliferation abrogation and apoptosis induction in RAD001-treated transgenic papillomas for 20 h. C, papilloma regression in 4-wk RAD001-treated transgenic mice. D, histologic (top) and mTORC1 target (bottom) response to 4-wk RAD001 treatment. Bars, 100 μm (B and D, bottom) and 750 μm (D, top).
Formation or maintenance of Rheb-mediated epidermal neoplasia was mTORC1 dependent. To determine whether mTORC1 activity was required for Rheb-induced papilloma formation, 8- to 10-week-old transgenic mice were treated with the mTORC1 inhibitor RAD001 (everolimus), an orally bioavailable derivative of rapamycin, for 13 to 15 weeks. RAD001 completely prevented tumor formation, in contrast to an 88% incidence of neoplasia in vehicle-treated mice (Fig. 3A). Specific inactivation of mTORC1 but not mTORC2 in back skin of RAD001-treated transgenic mice was confirmed by phospho-S6S235/236 (mTORC1 marker) and phospho-AKTS473 (canonical direct mTORC2 site) immunofluorescence, respectively (data not shown). Next, we tested whether established transgenic papillomas could be reversed by RAD001. RAD001 treatment for 20 hours inactivated mTORC1-mediated S6 phosphorylation in tumor basal and suprabasal cells, markedly decreased proliferation (BrdUrd), and induced apoptosis (activated caspase-3; Fig. 3B). Four weeks of inhibitor treatment produced a drastic visual (Fig. 3C, top, RAD001) and histologic (Fig. 3D, RAD001, 4wk) resolution of all papillomas in six transgenic mice. The other six transgenic mice, which exhibited papilloma-associated ulceration, almost completely resolved both wounding and neoplastic pathologies after RAD001 treatment (Fig. 3C, bottom, RAD001). In contrast, papilloma number and size increased in vehicle-treated mice. Abrogation of ribosomal protein S6 phosphorylation consequent to 4-week RAD001 treatment was consistent with potent mTORC1 inhibition in papilloma-bearing transgenic mice (Fig. 3D, bottom; data not shown).

Rheb triggered an mTORC1-mediated angio-inflammatory switch in transgenic papillomas. As histologic analysis revealed markedly increased stromal cellularity in transgenic papillomas, we determined their inflammatory cell repertoire and vasculature. Tumor stroma contained near-confluent

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**Figure 4.** An mTORC1-dependent angio-inflammatory switch in Rheb transgenic skin. A, inflammatory cell stromal and epidermal infiltration and induction of neovascularization in transgenic papillomas, both inhibited by 4-wk RAD001 treatment. B, real-time reverse transcription-PCR (RT-PCR) of mRNAs encoding for angiogenic and inflammatory factors in WT, transgenic hyperplasia, and papilloma, and RAD001-treated transgenic back skin. Data were normalized as Z-score values and presented in a heat map using a blue-white-red (low-to-high expression) scale (left) and the ratio of mean values of hyperplastic, papilloma, and RAD001-treated transgenic back skin samples to the mean value of WT levels (right). C, tissue ELISAs of inflammatory chemokines and cytokines and VEGF in WT, preneoplastic, papilloma, and RAD001-treated transgenic back skin. D, HIF-1α and VEGF protein expression from WT back skin epidermis and transgenic papilloma (left) and ELISAs of conditioned medium from keratinocyte cultures in 8% serum, without serum, or without serum in the presence of rapamycin (Rapa). Columns, mean (n = 4 mice per group); bars, SD. *, P < 0.05, two-tailed Student’s t test (B–D). Bar, 100 μm (A).

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CD45^+ cells and greatly increased blood vessel density and frequency (Fig. 4A). The stromal CD45^+ cell population was composed of Gr-1^+ neutrophils, F4/80^+ macrophages, as well as CD4^+ and CD8^+ T cells (Supplementary Fig. S5). CD4 and CD8 cells additionally infiltrated the neoplastic epidermis (Supplementary Fig. S5). Four-week RAD001 papilloma treatment greatly reduced blood microvessel density and Gr-1^+ and F4/80^+ cells and partially diminished CD4^+ and CD8^+ stromal

Figure 5. STAT3 pathway activation in transgenic epidermis required intact skin. A, STAT3 pathway activation in WT back skin, transgenic papilloma, and transgenic papillomas following RAD001 treatment for either 20 h or 4 wks. B, focal epidermal STAT3 activation (yellow arrow) with concomitant stromal inflammatory cell infiltration in hyperplastic transgenic back skin. C, induction of cyclin D1 expression in hyperplasia (top) to nascent papilloma transition (bottom). D, STAT3 activation in WT and transgenic keratinocytes cultured for 16 h with or without serum. Serum-starved transgenic and nontransgenic keratinocytes stimulated with 10 ng/mL IL-6 for the indicated intervals alone or pretreated with rapamycin 4 h before IL-6 stimulation. Bars, 100 μm (A and C).
infiltration (Fig. 4A; Supplementary Fig. S5). Thus, epidermal \textit{Rheb} dysregulation triggered an mTORC1-dependent "angio-inflammatory switch" in association with neoplastic progression.

We screened lysates from transgenic hyperplastic and neoplastic skin for expression of candidate factors potentially responsible for the angio-inflammatory switch. \textit{IL-1\alpha}, \textit{IL-1\beta} mRNA levels, and mRNA and protein abundance for VEGF, IL-6, tumor necrosis factor-\textit{\alpha} (TNF-\textit{\alpha}), IL-17, CXCL1, CXCL2, transforming growth factor-\textit{\beta} (TGF-\textit{\beta}), and cyclooxygenase 2 (COX2) were increased in hyperplasia, markedly elevated in neoplasia, but greatly reduced by RAD001 (Fig. 4B and C; TGF-\textit{\beta} and COX2 protein data not shown). Consistent with the RAD001-responsive \textit{HIF-1} mRNA signature detected in neoplasia (Fig. 4B), HIF-1\textit{\alpha} and VEGF levels were differentially increased in transgenic hyperplastic epidermis as early as 3 weeks of age (Fig. 4D), suggesting mTORC1-mediated HIF-1\textit{\alpha} stabilization (31). We next determined transgenic keratinocyte paracrine factor expression potentially underlying stromal activation. IL-6, TNF-\textit{\alpha}, CXCL1, and CXCL2 were differentially elevated in rapamycin-sensitive fashion in transgenic keratinocyte-conditioned medium (Fig. 4D).

\textbf{mTORC1-dependent STAT3 activation coincident with induction of the angio-inflammatory switch in transgenic epidermis.} We interrogated activation and consequent downstream target gene induction of the transcription factor STAT3 in \textit{Rheb}-mediated tumorigenesis, as it was a known direct target of both mTORC1 (32) and inflammatory cytokines, and its dysregulation was required for multistage skin carcinogenesis. There was a marked elevated frequency of cells positive for nuclear phospho-STAT3Y705 and increased expression of the STAT3 transcriptional targets \textit{cyclin D1} and \textit{survivin} in transgenic papillomas (Fig. 5A).
To determine a causal role for STAT3 in Rheb-mediated tumorigenesis, precursor skin hyperplasias were analyzed for STAT3 and stromal inflammatory cell expression patterns (Fig. 5B; Supplementary Fig. S6A). Precursor skin hyperplasias seemed to be the site of nascent papillomas, as their epidermis was thicker and the lesions had an undulating appearance suggesting exophytic growth initiation (Fig. 5B and C). Strikingly, phospho-STAT3 Y705 was focally detectable in the epidermis concurrent with subjacent stromal inflammatory cell accumulations in these lesions (Fig. 5B; Supplementary Fig. S6A, yellow arrows). Immunoblotting confirmed that pJAK2 Y1007/1008, phospho-STAT3 Y705, and phospho-STAT3 S727 levels were differentially elevated (Supplementary Fig. S6B), and clustered cyclin D1–positive epithelial cells were present in these nascent neoplastic foci, in contrast to their low frequency in diffuse hyperplasia (Fig. 5C, cyclin D1, bottom versus top). Collectively, these data support a role for progressive STAT3 activation and consequent incremental cyclin D1 dysregulation in the hyperplasia to neoplasia transition.

Next, we determined that phospho-STAT3 Y705 was not activated in keratinocytes cultured in serum-free medium (Fig. 5D) despite the low-level upregulation of a repertoire of cytokines and chemokines (Fig. 4D). In contrast, addition of the STAT3 activator IL-6 to the culture at a papilloma-associated concentration, 10 ng/mL, markedly activated phospho-STAT3 Y705 and phospho-STAT3 S727 in both transgenic and WT keratinocytes. Rapamycin pretreatment did not blunt phosphorylation of either STAT3 site induced by exogenous IL-6 (Fig. 5D). Collectively, these data support the hypothesis that the paracrine signaling from the angio-inflammatory switch was necessary in order for IL-6 to attain a level sufficient to breach the epithelial STAT3 activation threshold.

We next tested the specificity of mTORC1-STAT3 regulation in Rheb neoplasia. Diminution in STAT3 activation occurred as early as 20 hours after RAD001 treatment with coordinate reduction of cyclin D1 and survivin protein (Fig. 5A, RAD001, 20 hours). Four weeks of RAD001 reduced neoplastic epithelial phospho-STAT3 Y705 to near WT levels (Fig. 5A, RAD001, 4 weeks). In contrast, a 6-week trial of the EGFR inhibitor gefitinib had no effect on STAT3 activation or papilloma histology (data not shown). Moreover, 4-week celecoxib, a COX2 inhibitor, therapy also failed to affect papilloma persistence (data not shown). Collectively, these data conclusively show that STAT3 activation in transgenic neoplasia was mTORC1 dependent and subject to feed-forward stromal-epithelial inflammatory amplification (Supplementary Fig. S7).

**Rheb cooperation with DMBA in epidermal squamous carcinogenesis.** To test whether Rheb gain of function could serve as a cooperative event in carcinogenesis, a single topical application of the tobacco carcinogen DMBA was administered to 8-week-old WT and preneoplastic transgenic mice. Rheb-DMBA papillomas developed in both cephalic and caudal back skin (Fig. 6A; Rheb-DMBA papilloma). A total of 9 of 11 transgenic mice between 17 and 30 weeks after DMBA application developed invasive squamous cell carcinomas (Fig. 6B and C). In contrast, six DMBA-treated WT mice, although universally developing sebaceous gland adenomas, did not evidence either papillomas or squamous carcinomas (data not shown). Elevated nuclear phospho-ERK1/2 Y202/Y204 immunoreactivity was uniformly detectable in basal cells of premalignant Rheb-DMBA papillomas (Fig. 6D, top middle), in contrast to its sporadic incidence in terminally differentiated suprabasal cells in vehicle-treated transgenic papillomas (Fig. 6D, top left). There was a further marked increase in nuclear phospho-ERK1/2 Y202/Y204 levels in nests of squamous carcinoma cells at the invasive front of Rheb-DMBA malignancies (Fig. 6D, top right). Although E-cadherin was expressed in all Rheb papillomas, its expression was markedly reduced or absent in the invasive nests of Rheb-DMBA carcinomas (Fig. 6D, bottom middle versus bottom left). Malignant invasive cells also expressed the primitive K8 marker of poorly differentiated squamous carcinoma (Fig. 6D, bottom right).

**Discussion**

Here, we provide evidence for Rheb locus gain/amplification in diverse human cancers and association of Rheb overexpression with poor disease outcome. Transgenic mice overexpressing Rheb in basal epidermal keratinocytes develop multistage epithelial tumorigenesis, with rapamycin-sensitive neoplasias. Systemic RAD001 efficiently inhibited mTORC1 signaling in cultured keratinocytes and neoplasias arising in transgenic mice with lack of mTORC2 negative feedback (30). Rheb gain of function produced mTORC1-mediated overexpression of a collection of growth factors, cytokines, and chemokines triggering a focal stromal angio-inflammatory switch in hyperplasia that was dispersed throughout the stroma in neoplasia. We provided several lines of evidence supporting a pathway wherein mTORC1 induced HIF-1α and HIF-1 downstream angiogenic factors along with cell-autonomous production of cytokines and chemokines.

These factors produced a focal angio-inflammatory switch that in turn elevated the levels of cytokines such as IL-6 to a level sufficient to activate epidermal STAT3 and trigger a feed-forward paracrine stromal-epithelial cross talk culminating in neoplasia (Supplementary Fig. S7). Collectively, these data suggested that multiple mechanisms may be responsible for the selective advantages of Rheb locus amplification in human cancers, including adaptive growth in a suboptimal tumor microenvironment, enhanced tumor nutrient/oxygen perfusion through angiogenesis, stimulation of tumor cell growth through paracrine regulation, and increased sensitivity to carcinogens, such as tobacco.

Chronic inflammation is a risk factor for various forms of human cancer, including breast and head and neck cancers (33, 34). Prolonged nonsteroidal anti-inflammatory drug usage (aspirin or COX2 inhibitors) reduces the incidence of several different types of neoplasia (31, 33). Here, Rheb/mTORC1 hyperactivation in cultured keratinocytes and intact skin upregulated HIF-1α, VEGF, IL-6, TNF-α, CXCL1, CXCL2, and COX2 mRNA and protein expression. Aggregation induction of these direct and indirect downstream targets was a likely...
mechanism for initiation and maintenance of the mTORC1-mediated angio-inflammatory switch. Our result thus extended a previous study showing an mTORC1/HIF-1/VEGF signaling cascade in prostate tumor cells (10), and our recent report on HIF-1 upregulation of TNF-α, CXCL1, and CXCL2 mRNA and protein levels in mouse epithelium (21).

A striking finding in the K14-Rheb transgenic model was that coordinate IL-6 upregulation and STAT3 activation were solely detectable in hyperplasia and neoplasia but absent in cultured keratinocytes, which required exogenous IL-6 to recapitulate in vivo signaling. These experiments showed the necessity of in vivo epithelial-stromal juxtaposition for epithelial RHEB/mTORC1-mediated feed-forward stromal cross talk culminating in tumorigenesis. Consistent with this notion, nascent transgenic papillomatosis was tightly associated with nuclear phospho-STAT3 Y705 activation in squamous basal cells overlying focal inflammatory cell accumulations. Exquisite sensitivity of epidermal STAT3 activity to short-term RAD001 treatment was evidence for potent and rapid stromal inhibition by mTORC1 blockade. Two recent reports showed a requirement for stromal-epithelial IL-6 and STAT3 signaling in carcinogenesis associated with inflammatory bowel disease (35, 36). Moreover, both loss- and gain-of-function mouse models of epidermal STAT3 signaling highlighted a requirement for STAT3 activation in epidermal carcinogenesis (37, 38).

RHEB overexpression in breast and head and neck cancers was a risk factor for cancer progression independent of HER2 amplification or PTEN loss of function in breast cancer or EGFR amplification/overexpression in head and neck cancer. RHEB overexpression could also underlie PI3K/AKT-independent mTORC1 activation in human cancers, previously identified in a subgroup from a large head and neck cancer cohort (39). Carcinogen exposure via smoking is a known risk factor for head and neck cancer (40); however, cooperation between RHEB and smoke carcinogens has not been reported. Here, Rheb potently sensitized transgenic mice to a single dose of the DMBB producing multistage squamous carcinogenesis accompanied by coordinate induction of ERK1/2. These data suggest that these two signaling modules may collaborate in human carcinogenesis. Collectively, the combination of the transgenic, DMBB carcinogen, TMA, and in silico transcriptome data strongly supports a functional contribution of RHEB gene to human carcinogenesis, particularly in malignancies in relation to environmental carcinogens, such as tobacco-associated carcinogens. Our K14-Rheb transgenic mice can be a platform for further dissection of the contribution of mTORC1 to head and neck and breast carcinogenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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