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Tuberous sclerosis complex 2 loss-of-function mutation regulates reactive oxygen species production through Rac1 activation

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Abstract

The products of the TSC1 (hamartin) and TCS2 (tuberin) tumor suppressor genes negatively regulate cell growth by inhibiting mTOR signaling. Recent research has led to the postulation that tuberin and/or hamartin are involved in tumor migration, presumably through Rho activation. Here we show that LEF-8 cells, which contain a Y1571 missense mutation in tuberin, express higher Rac1 activity than tuberin negative and positive cells. We also provide evidence of obvious lamellipodia formation in LEF-8 cells. Since the production of $TSC2^{Y1571H}$ cannot form a hetero-complex with hamartin, we further analyzed another mutant, $TSC2^{R611Q}$, which also lacks the ability to form a complex with hamartin. Introducing both forms of mutated TSC2 into COS-1 cells increased Rac1 activity as well as cell motility. We also found these two mutants interacted with Rac1. We further demonstrated that the introduction of mutated TSC2 into COS-1 cells can generate higher reactive oxygen species (ROS). These results indicate that loss-of-function mutated tuberin can activate Rac1 and thereby increase ROS production.

Keywords: Tuberous sclerosis complex (TSC); Tuberin; Hamartin; Rac1; Reactive oxygen species (ROS); Migration

Tuberous sclerosis complex (TSC) is an autosomal dominant disorder affecting 1 in 6000 live births (1). Clinical presentation of TSC is variable and affects multiple organs, including the brain, eye, skin, kidney, heart, and lungs, and is characterized by the presence of benign tumors termed hamartomas [1]. More than half of cases are due to sporadic mutations. Two tumor suppressor genes, TSC1 and TSC2, have been associated with occurrence of the disease. TSC1 on chromosome 9q34 encodes the 150 kDa protein hamartin, while TSC2 on chromosome 16p13.3 encodes the 200 kDa tuberin protein [2]. Loss of heterozygosity (LOH) in the TSC1 or TSC2 region leads to hamartomas [3]. Recent studies have shown that tuberin and hamartin can form a physical and functional complex that negatively regulates a small GTPase, Ras homologue enriched in brain (Rheb), through the GTPase-activating (GAP) domain on tuberin [4]. Therefore, downstream of Rheb, the mammalian target of the Rapamycin (mTOR) signaling network, which plays central role in the regulation of protein synthesis and cell growth, is inhibited [5]. Moreover, the microsomal fraction containing hamartin and tuberin was found to be insoluble in nonionic detergent and distributed, in part, in a low-density fraction coincident with caveolin-1 [6].

Lymphangioleiomyomatosis (LAM) occurs in 34–42% of women with TSC [7], and is characterized pathologically

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by a diffuse, bilateral proliferation of abnormal smooth muscle cells, accompanied by extensive reactive epithelial hyperplasia [8] and cystic degeneration of lung parenchyma. LAM can occur as an isolated disorder, referred to as sporadic LAM, or in patients with TSC [9]. Angiomyolipoma pulmonary LAM cells from some sporadic LAM patients contain somatic mutations in the TSC2 gene [9]. Furthermore, LAM is a potentially metastatic disease [9], suggesting a role for TSC1 and TSC2 in cell motility. Effects of TSC gene mutations might disrupt normal neuronal migration and cerebral cortical lamination, indicating a direct connection of TSC2 to cell migration [10].

Cell migration involves dynamic and regulated changes to the cytoskeleton and cell adhesion. The Rho GTPase family, which includes RhoA, Rac1, and Cdc42, plays important roles in actin cytoskeletal remodeling [11]. RhoA promotes the formation of stress fibers that are linked to focal adhesions, Rac1 induces the formation of membrane ruffles and lamellipodia, and Cdc42 induces filopodia formation. Activation of RhoA, Rac1, and Cdc42 is critical for the regulation of cell adhesion and motility. Deregulation of this balance promotes cell transformation and metastasis [12].

In this study, we used a GTP-Rac1 pull-down assay to show that Rac1 activity is higher in LEF-8 cells, which contain a missense mutant of TSC2 ($TSC2^{Y1571H}$), than in CACL-I-III ($TSC1^{-/-}$), EEF-4 ($TSC2^{+/+}$) or EEF-8 ($TSC2^{-/-}$) cells. Furthermore, overexpression of TSC2 loss-of-function mutants ($TSC2^{Y1571H}$ and $TSC2^{R611Q}$) promotes Rac1 activation and cell motility. These two mutants also interacted with Rac1 and promoted ROS generation upon introduction into cells. These data indicate that tuberin regulates Rac1 activity and subsequently controls ROS production.

Materials and methods

Antibodies and plasmids. Anti-tuberin (C-20) antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA); anti-Rac1 antibody from Upstate Biotechnology (Lake Placid, NY); anti-hamartin antibody from ZYMED Laboratories (San Francisco, CA); and anti-HA antibody from Sigma (St. Louis, MO). pcDNA3-TSC1 was kindly provided by E. Henske (Fox Chase Cancer Center, Philadelphia, PA) and GFP-pcDNA3 was a kind gift from Y. Mitsuuchi (Temple University, Philadelphia, PA).

Cell culture. Transfection–CACL-I-III (renal carcinoma cell line derived from $TSC1^{+/-}$ mouse) [13], EEF-4 (embryonic fibroblast cell line derived from Eker rat), EEF-8 (embryonic fibroblast cell line derived from Eker rat) and LEF-8 (renal carcinoma cell derived from Eker rat) [14] cells were maintained in Dulbecco's modified Eagle's medium nutrient mixture F-12 HAM (Sigma) supplemented with 10% fetal bovine serum. HEK293T and COS-1 cells were obtained from ATCC and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Transfections were performed using Lipofectamine 2000 Transfection Reagent (Invitrogen) following the manufacturer's instructions.

Rac1 activity assays. Transfected cells were detected using EZ-Detect Rac1 Activation Kit (PIERCE, Rockford, IL), according to the manufacturer's instructions, and Rac1 antibody.

Actin staining. Cells plated on 4-well chamber slides (Falcon, San Jose, CA) were washed twice with PBS, fixed with 4% paraformaldehyde-PBS for 15 min at RT, and treated with 1% Triton X-100 for 25 min at RT. The slide was then stained with Alexa Fluor 568 Phalloidin (Invitrogen) in PBS for 20 min at RT, washed twice with PBS, and mounted. Images were obtained using a confocal microscope (OLYMPUS).

Immunoprecipitation and Western blotting. Cells were washed twice with ice-cold PBS and placed in lysis buffer (20 mM Hepes [pH 7.4], 100 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100, protease inhibitor cocktail, and phosphatase inhibitor cocktail). Lysates were incubated with a specific antibody in the presence of protein A-agarose beads. The beads were washed with IP buffer (10 mM Tris–HCl [pH 7.6], 150 mM NaCl, 1% Triton X-100) and the immunoprecipitates were resolved using SDS–PAGE. Western blotting was carried out using HRP-conjugated anti-rabbit/mouse IgG antibodies as a secondary antibody and the GE Healthcare ECL System (Piscataway, NJ) for detection.

Wound healing assays. Transfected cells were wounded by pushing a 10-μl pipette tip through the cell monolayer, after which they were incubated with fresh medium supplemented with 10% fetal bovine serum for 9 h. Images were obtained with a confocal microscope (OLYMPUS). For ROS production inhibition assay, NADPH oxidase inhibitor, apocynin (100 μM) was added to the medium and incubated for 9 h before observation.

Measurement of intracellular ROS production. Cells transfected with TSC2 or mutant TSC2 using Lipofectamine 2000 Transfection Regent (Invitrogen) were washed with pre-warmed PBS and incubated with DCF-DA (Invitrogen). DCF-DA fluorescence was detected at excitation and emission wavelengths of 488 and 520 nm, and measured with a Shimadzu RF-5000 fluorescence spectrophotometer (SHIMADZU).

Results

High Rac1 activity in TSC2 mutant cells

A possible link between tuberin and the Rac1 signaling pathway was investigated using a pull-down assay with PAK1-PBD beads to analyze the effects of mutated tuberin on Rac1 activity. Rac1 activity levels were measured in cell lines derived from TSC model animals: EEF-4 (hamartin and tuberin-positive), EEF-8 (tuberin negative), CACL-1-111 (hamartin negative) and LEF-8 (carries the Y1571H mutant on tuberin). Although an equal amount of total Rac1 expression was found in each cell line, Rac1 activity was highest in LEF-8 cells (Fig. 1A).

We then investigated whether the high Rac1 activity in LEF8 cells affects cell morphology. It is well known that localization of actin filaments is regulated by the Rho family of proteins. In particular, lamellipodia are a product of activated Rac1-induced localization of actin filaments in the plasma membrane. Each cell line was stained with rhodamine phalloidin, a fluorescent probe that is selective for fibrous actin, and observed by confocal microscopy (Fig. 1B). Much more lamellipodia formation was observed in LEF-8 cells than in other cell lines, indicating that, indeed, mutation in TSC2 can cause high Rac1 activity.

Mutant tuberin increases Rac1 activity

To elucidate whether the high activity of Rac1 in LEF-8 cells is specific to LEF-8 cells or is due to mutation of the TSC2 gene, we investigated Rac1 activity in COS-1 cells transfected with TSC1 and wild type TSC2 or TSC1 and mutant TSC2 derived from LEF-8 cells ($TSC2^{Y1571H}$).

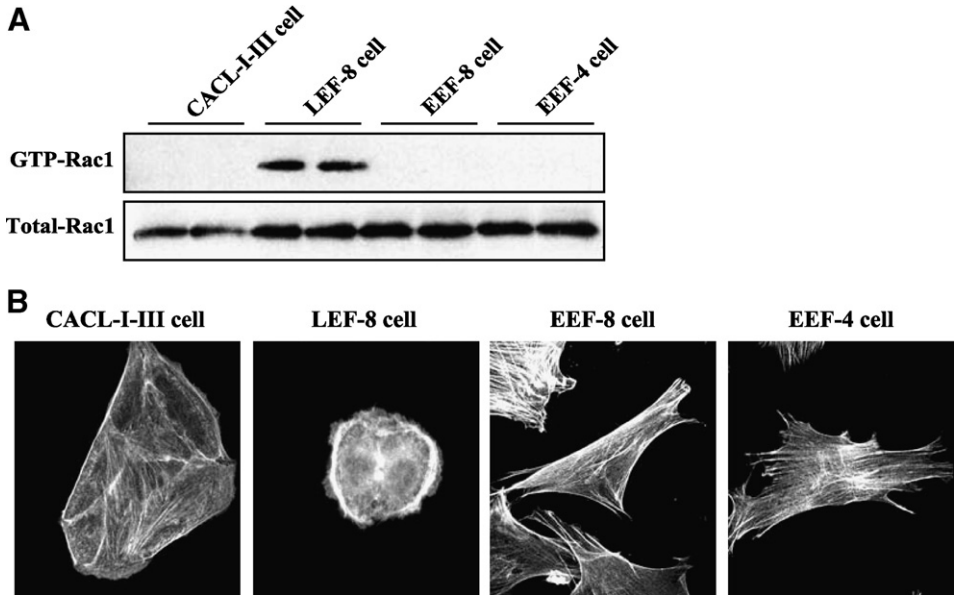

Fig. 1. Rac1 is activated in LEF-8 cells. (A) Western blot analysis with anti-Rac1 antibody to detect GTP-Rac1 using a pull-down assay with PAK1 PBD agarose. The procedure was repeated in CACL-I-III, EEF-4, EEF-8 and LEF-8 cells. (B) Alexa phalloidin staining of F-actin in the CACL-I-III, EEF-4, EEF-8, and LEF-8 cell lines.

A disease-derived mutant [15] of TSC2 ($TSC2^{R611Q}$) was also used. Rac1 activity was measured using pull-down assays with PAK1-PBD beads (Fig. 2A). Although total expression of hamartin, tuberin/mutated tuberin and Rac1 was not affected, Rac1 activity was higher in cells transfected with mutant TSC2 than in cells transfected with wild type TSC2. Thus, we suggest that the high Rac1 activity observed in LEF-8 cells is due to mutation of the TSC2 gene.

We next investigated whether the high Rac1 activity caused by mutated tuberin affects cell motility, a typical feature of Rac1 activation. A wound healing assay was conducted using COS-1 cells transfected with either GFP-tagged wild type TSC2 or TSC1 with GFP-tagged mutant

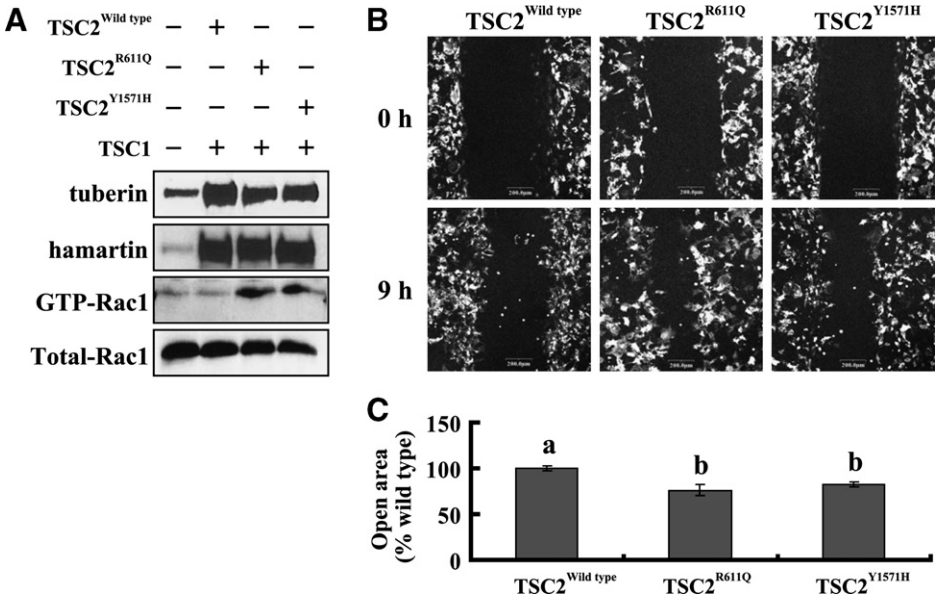

Fig. 2. Rac1 activity and cell migration is increased in cells transfected with $TSC2^{R611Q}$ and $TSC2^{Y1571H}$. (A) COS-1 cells transfected with TSC1 and wild type TSC2 or TSC1 and mutant TSC2, were assayed for Rac1 activity: Western blot analysis with anti-Rac1 antibody to detect GTP-Rac1 using a pull-down assay with PAK1 PBD agarose. (B) Analysis of cell motility using a wound healing assay: COS-1 cells transfected with GFP-tagged wild type TSC2 and TSC1, GFP-tagged $TSC2^{R611Q}$ and TSC1, or GFP-tagged $TSC2^{Y1571H}$ and TSC1 were scratched in a line with a pipette tip. After 9 h incubation, images were obtained using a confocal microscope. (C) Cell migration is evaluated by measuring the open area. Values are the means ± SE ($n = 5$). Bars not sharing a letter differ significantly, $p < 0.05$ (one-way ANOVA).

TSC2 (Fig. 2B). GFP positive cell motility was significantly higher in cells transfected with either TSC2 mutant than in cells transfected with wild type TSC2 (Fig. 2C).

Rac1 co-immunoprecipitates with mutated tuberin

To clarify how mutated tuberin activates Rac1, we conducted an immunoprecipitation assay using anti-HA. After transfecting mutated TSC2 with TSC1 into COS-1 cells, cell lysate were subjected to immunoprecipitation assay. As shown in Fig. 3A, Rac1 co-immunoprecipitated with tuberin mutants (Y1571H and R611Q) and their interaction to Rac1was increased approximately 2- and 1.5-folds, respectively. To further confirm the interaction of tuberin and Rac1 interaction, endogenous tuberin and Rac1 binding was examine in COS-1 cells. As indicated in Fig. 3B, we found out that endogenous Rac1 can bind with tuberin.

Generation of reactive oxygen species is increased by mutant tuberin

A recent study postulates the involvement of reactive oxygen species (ROS) in tumor metastasis, a complicated process that includes cell migration [16]. ROS are generated by NADPH oxidase, which consists of a membrane-localized cytochrome b558 comprised of two subunits, gp91phox (or Nox 2) and p22phox, as well as the cytosolic components p47phox and p67phox. Rac1 is necessary for full NADPH oxidase activity [17]. The effect of a mutant TSC2 and, thereby, high Rac1 activation on ROS generation was evaluated by transfecting mutant TSC2 into COS-1 cells. ROS production was measured with DCF-DA. We found a significantly increase in Rac-1 activity (Fig. 2A) as well as ROS production in cells introducing $TSC2^{R611Q}$ or $TSC2^{Y1571H}$ into COS-1 cells (Fig. 4A). We then were interested whether if there is causal relationship of high ROS production and migration. By inhibiting the ROS production by NADPH oxidase inhibiter, apocynin (Fig. 4B), and the cell migration rate was not affected. Thus, we concluded that the ROS production result from mutated tuberin, does not participate in cell migration.

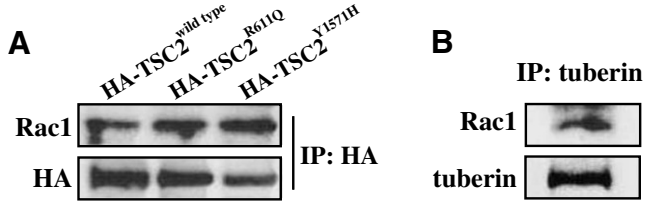

Fig. 3. Tuberin mutants interact with Rac1. (A) COS-1 cells were transfected with wild type HA-TSC2 or mutant HA-TSC2. The cell lysates were immunoprecipitated with anti-HA antibody followed by Western blotting analysis with anti-HA and anti-Rac1 antibodies. (B) Non-transfected COS-1 cells lysates were immunoprecipitated with anti-tuberin antibody followed by Western blotting analysis with anti-tuberin and anti-Rac1 antibodies.

Discussion

It is well established that tuberin and hamartin negatively regulate cell growth by inhibiting mTOR signaling. However, the complexity of TSC cannot be explained by this singular pathway. Analysis of tumors from either TSC patients or model animals has revealed that TSC1 and TSC2 contribute to cell development, cell migration and cell cycle control [18].

Two groups have postulated the involvement of TSC2 in cell migration during metastasis. However, the molecular mechanism remains unsolved. Goncharova et al. developed a model of Rac1 activation by tuberin and hamartin. In their work, they present data indicating that activation of Rho by free hamartin could cause inactivation of Rac1 [19]. In contrast, our data show that hamartin-negative cells did not have constitutively high Rac1 activity. Rather, we found that the introduction of mutant tuberin ($TSC2^{R611Q}$ and $TSC2^{Y1571H}$) into cells results in high Rac1 activity. Thus, we conclude that Rac1 activity is not regulated through hamartin, but through tuberin itself. TSC common feature of these tuberin mutants is that they had lost the ability to form a hetero-complex with hamartin [14,20] and we were also able to conform it (data not shown). Thus, we first postulated that a free form of tuberin might cause induction of Rac1 activation. However, since little activation was observed in $TSC1^{-/-}$ cells (CACL-1-111). It is likely that neither free tuberin nor hamartin is the only cause of Rac1 activation. Therefore, we postulate that Rac1 interacts with tuberin and theses interaction is enhanced when they are mutated.

We then found data indicating that ROS production was increased in the cells with mutated tuberin. Previous data showed TSC2 lacking the C-terminus increases ROS production in NIH3T3 cells [21]. Therefore we assume that the C-terminus of TSC2 which contains the GAP domain [4] may contribute to either activate and/or maintain the activation of Rac1.

ROS are a key factor in cell migration and promote cell proliferation. However, when ROS activity was inhibited by NADPH oxidase inhibitor apocynin in COS-1 cells expressing mutant TSC2, we did not observe obvious migration defect. These results imply that cell migration is not affected by the high ROS production. Therefore, the high ROS production may rather play a role in the cell signaling pathway and cause abnormal cell signaling [16]. Moreover, Rac1-dependent activity may contribute to migration defects observed in cortical tubers.

In this study, we show that ROS generation and Rac1 activity are promoted by mutant tuberin proteins. Since a recent study showed that ROS can act as an extracellular signal [16], our data may provide some evidence of ROS involvement during tumor cell development. Especially demonstration using in primary mouse fibroblast cells show that activity of Rac1 leads to a premature senescence through modulating cellular ROS and genomic stability [22]. Thus, high cellular ROS production may be one of

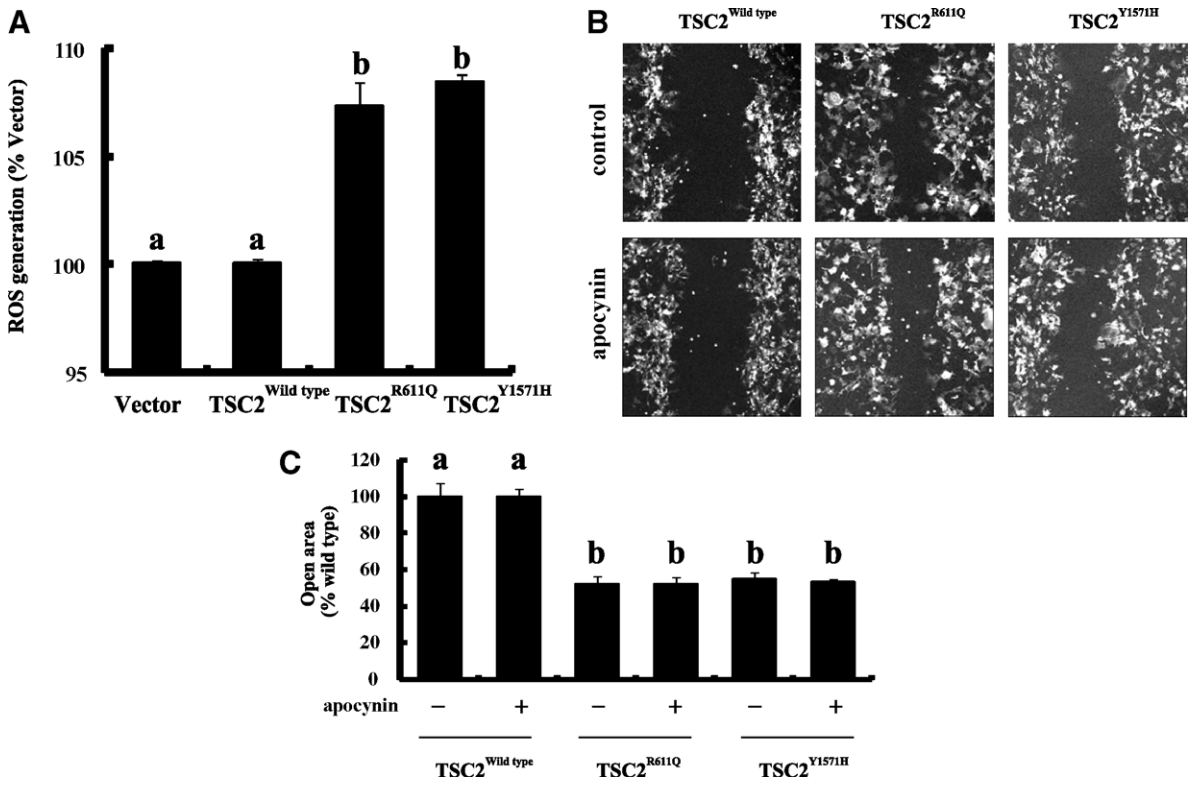

Fig. 4. Increase ROS production in presence of tuberin mutants. (A) COS-1 cells were transfected with wild type TSC2 or mutant TSC2. ROS production was measured using DCF-DA. Values are the means ± SE ($n = 5$). Bars not sharing a letter differ significantly, $p < 0.05$ (one-way ANOVA). (B) Effect of apocynin on wound healing assay. COS-1 cells transfected with GFP-tagged wild type TSC2 and TSC1, GFP-tagged $TSC2^{R611Q}$ and TSC1, or GFP-tagged $TSC2^{Y1571H}$ and TSC1 were treated by apocynin or DMSO (control), and scratched in a line with a pipette tip. After 9 h incubation, images were obtained using a confocal microscope. Data are shown of control (upper) and apocynin (lower) treated cells after 9 h. (C) Cell migration is evaluated by measuring the open area. Values are the means ± SE ($n = 3$). Bars not sharing a letter differ significantly, $p < 0.05$ (ANOVA).

the causes of tumor formation in TSC patients. More interesting is the possibility that $TSC2^{R611Q}$ and $TSC2^{Y1571H}$ serve as loss-of-function mutations with respect to the mTOR pathway and gain-of-function mutations in regards to Rac1 activity. Finally, our study may suggest good reason for the use of anti-oxidant products when treating TSC patients.

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