

# Tuberin activates and controls the distribution of Rac1 via association with p62 and ubiquitin through the mTORC1 signaling pathway

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**Abstract.** Recent studies indicated that the tuberous sclerosis 2 (*TSC2*) gene product, tuberin, regulates Rac1 activity. However, the underlying mechanism by which tuberin regulates Rac1 activity has not been clearly elucidated to date. To better understand the molecular link between tuberin function and Rac1, we characterized the activity and distribution of Rac1 in mouse *Tsc2*-deficient renal tumor cells using restoration experiments with wild-type tuberin. Rac1 activity was significantly higher in tuberin-expressing cells compared with control *Tsc2*-deficient cells. Further, Rac1 activation was induced by rapamycin treatment or knockdown of raptor, but not rictor, in *Tsc2*-deficient cells, indicating that mTORC1 is an upstream negative regulator of Rac1. Intriguingly, Rac1 appeared to form cytoplasmic dots in *Tsc2*-deficient cells, but not in tuberin-expressing and since rapamycin treatment dispersed these dots, involvement of aberrant mTOR complex 1 (mTORC1) activation in the dot formation was suspected. Moreover, the dots were co-localized with p62/sequestosome-1 and ubiquitin. These findings imply that Rac1 distribution and/or its degradation may be regulated by tuberin through the mTORC1 signaling pathway.

## Introduction

The tumor suppressor genes, tuberous sclerosis complex 1 (*TSC1*) and *TSC2*, encode hamartin and tuberin, respectively, which are key upstream negative regulators of the rapamycin-sensitive

mTOR complex 1 (mTORC1). Hamartin and tuberin form a complex and function as a GTPase-activating protein (GAP) for the small G protein Rheb, a direct activator of mTORC1 (1,2). Mutations of *TSC1* or *TSC2* induce abnormal activation of mTORC1 that contributes to the development of tuberous sclerosis complex (TSC) including seizure, mental retardation, and formation of benign tumors in multiple organs such as brain, kidney, heart, lung and skin. In animal models, *Tsc1*- or *Tsc2*-deficiency causes renal tumor development in heterozygous mutants. In TSC patients, neurological manifestations vary from patient to patient including subependymal giant-cell astrocytoma (SEGA), severe and refractory epilepsy, psychiatric disease and mental retardation. Standard therapy for SEGA, which develops in up to 20% of young patients (3,4), has been surgical resection. Currently, administration of rapamycin has therapeutic potential for TSC including SEGA (5,6), however, there is limited efficacy since it cannot completely eliminate all of the symptoms. Therefore, better understanding of the mTOR signaling pathway and downstream molecules of hamartin/tuberin will lead to development of novel, more efficient treatments for TSC.

mTOR is a member of the phosphoinositide-3 kinase (PI3K) family and is a highly conserved serine/threonine kinase that integrates signals from growth factors, nutrients and stresses. It regulates multiple processes such as mRNA translation, cell cycle progression, autophagy, cell proliferation, growth and survival (7,8). mTOR forms two structurally and functionally distinct complexes referred as mTORC1 and mTORC2 (7). mTORC1 consists of mTOR, GβL/mLST8 and raptor, and regulates cell growth and proliferation by phosphorylating its downstream targets, S6 kinase (S6K) and 4E-binding protein 1 (4E-BP1), key regulators of protein synthesis. By contrast, mTORC2 is composed of mTOR, GβL/mLST8 and rictor, and directly activates Akt by phosphorylating it at Ser473 (7). mTORC1, but not mTORC2, is directly inhibited by rapamycin (9). As compared to mTORC1, the function of mTORC2 is less understood, although some contribution to the regulation of cytoskeleton has been reported (10).

mTORC1 negatively regulates autophagy by phosphorylating ATG1 and ATG13 (11). Autophagy is thought to be a fundamental process by which cytoplasmic proteins and organelles are degraded. Autophagy is induced by starvation or stress,

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**Abbreviations:** mTOR, mammalian target of rapamycin; mTORC1, mTOR complex 1; mTORC2, mTOR complex 2; TSC, tuberous sclerosis complex

**Key words:** tuberin, Rac1, mTOR complex 1, p62, ubiquitin

whereby double membrane vesicles (autophagosomes) engulf damaged proteins or organelles before fusion with lysosomes to form autolysosomes which degrade their contents to regenerate nutrients (12). Impairment of autophagy is tightly associated with the development of various pathologic conditions including tumorigenesis and neurodegenerative disease (13,14). By contrast, there are reports demonstrating that autophagy is required for the development and progression of tumors, possibly to support their aggressive growth (15). Similarly, in TSC-associated lesions treated with rapamycin, re-activation of autophagy may play some cyto-protective role (16). As recently demonstrated using model animals, the inhibition of autophagy could be a novel treatment option for TSC-related pathology, although side-effects are a concern (17).

Several recent studies have reported the involvement of hamartin and/or tuberin in cell motility control through the regulation of the Rho family of small GTPases, including RhoA, Rac and Cdc42, which are key regulators of actin cytoskeleton remodeling, cell adhesion and migration. RhoA regulates the formation of stress fibers that promotes focal adhesions, while Rac and Cdc42 promote actin polymerization, which induce formation of lamellipodia and filopodia formation, respectively (18,19). Overexpression of tuberin in *Tsc2*-null rat leiomyoma cells activates Rac1 and inhibits Rho in an mTOR-independent manner (20). An antagonistic role of tuberin on Rac1-inhibitory function of hamartin was suggested (20). A later study showed that knockdown of *TSC2* in colon cancer cells resulted in decreased cell motility accompanied by reduced activity of Rac1 (21). A contribution of mTORC1-induced negative feedback regulation of PI3K pathway in Rac1 inhibition was suggested (21). On the other hand, it has been shown that mTORC2 controls the actin cytoskeleton through regulation of Rac1 and RhoA in fibroblast cells (10). Another study indicated that mTORC2 bound to PIP3-dependent Rac exchange factor (P-Rex1) activates Rac1 and cell migration (22). P-Rex1 and Rac1 are known to be involved in neural differentiation and migration, which may be relevant to TSC-associated neural disorders (23,24). However, the precise signal transduction system regulated by hamartin and/or tuberin in Rac1 activation is controversial.

Here, we report that tuberin regulates Rac1 activity and distribution in an mTORC1-dependent manner in *Tsc2*-deficient renal tumor cells. We also report that the punctate cytoplasmic Rac1 distribution might be related to a degradation system involving p62/sequestosome1 (SQSTM1) and ubiquitin.

## Materials and methods

**Plasmid construction.** To establish stable cell lines, plasmid vectors in the pRevTet-Off system (Clontech, Mountain View, CA) were used. pRevTRE-rTsc2 for expression of wild-type tuberin was previously described (25). pRevTRE-LacZ was generated by inserting a *LacZ* coding DNA fragment into the pRevTRE vector. The expression vector for the flag-tagged wild-type tuberin was previously described (26). For construction of the Rac1 expression vector, rat *Rac1* cDNA was amplified by reverse transcription-polymerase chain reaction (RT-PCR) using primers, RACF1 (5'-CCGAATTCCAGGCCATCAAG TGTGTGGT-3') and RACMYCR (5'-CCCCTCGAGTTACA ACAGCAGGCATTTTCTCT-3'). After digestion with *EcoRI* and *XhoI*, the Rac1 cDNA fragment was subcloned into the

pCAG-GS vector (26) that had been modified to introduce an N-terminal Myc tag.

**Generation of cell lines and culture conditions.** Transfection of pTA-IRES-Puro, a tetracycline-controlled transactivator (rTA) expression plasmid, into *Tsc2*-deficient mouse renal tumor (MKOC1-277) cells and selection of rTA-expressing cells were as previously described (25). Subsequently, retro-viruses derived from pRevTRE-rTsc2 or pRevTRE-LacZ and generated by Ecopack 293 producer cells (Clontech) were used to infect the rTA-expressing cells, and tuberin-(WT) or  $\beta$ -galactosidase-expressing (LacZ) cell lines were selected using 200  $\mu$ g/ml hygromycin B (Clontech) and 100 ng/ml doxycycline (DOX) (Sigma-Aldrich, St. Louis, MO). MKOC1-277 cells were maintained in RPMI-1640 medium (Sigma-Aldrich) containing 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin. LacZ and WT cells, as well as previously established E8 and T2-5 cell lines (25), were maintained in RPMI-1640 medium containing 10% FCS, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 200  $\mu$ g/ml hygromycin B (Clontech), 0.6  $\mu$ g/ml puromycin (Sigma-Aldrich) and 100 ng/ml DOX. Analyses were performed with maximal induction of tuberin in the absence of doxycycline as described below. For rapamycin treatment, the cells were additionally incubated for 4 h in the medium containing 20 nM rapamycin.

**Western blot analysis.** Protein concentrations were determined by DC-protein assay (Bio-Rad, Hercules, CA). Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to Immobilon membranes (Millipore, Billerica, MA), blocked in 1% skim milk in Tris-buffered saline containing 0.05% Tween-20, and probed with the appropriate antibodies. The following primary antibodies were used: mouse monoclonal antibodies (mAb) against Rac1 (Abcam, Cambridge, MA) and  $\beta$ -galactosidase (Promega, Madison, WI); rabbit polyclonal antibodies from Santa Cruz Biotechnology (Santa Cruz, CA) against S6K, tuberin, and Rac1; and rabbit mAbs from Cell Signaling Technology (Beverly, MA) against phospho-S6K (Thr389), phospho-Akt (Ser473),  $\beta$ -actin, Akt, raptor and rictor. To probe with both rabbit and mouse antibodies, EnVision System (Dako, Carpinteria, CA) was used as previously described (27). ECL reagents (GE Healthcare) were used for detection and visualization of the bands.

**Rac1 activation assay.** To assess the amount of activated Rac1, GST pulldown assay was performed using the GST-tagged p21 binding domain of PAK1 (PAK-PBD) according to the manufacturer's instructions (Cytoskeleton, Denver, CO). Briefly, cells were seeded at a density of  $1.0 \times 10^6$  cells in 10-cm dishes, cultured overnight in the presence of DOX, and then incubated in the absence of DOX for 48 h. Subsequently, cells were lysed in cell lysis buffer containing protease inhibitors (provided by the kit). Protein concentrations were determined and adjusted with ice-cold cell lysis buffer for equal protein loading. To equal volume of each sample, 20  $\mu$ l of PAK-PBD beads were added and incubated for 60 min at 4°C with rotation. Beads were washed once with 500  $\mu$ l of wash buffer (provided by the kit), resuspended in 20  $\mu$ l of 2X SDS-PAGE sample buffer, boiled, and loaded onto a 12% gel for SDS-PAGE. The amount of

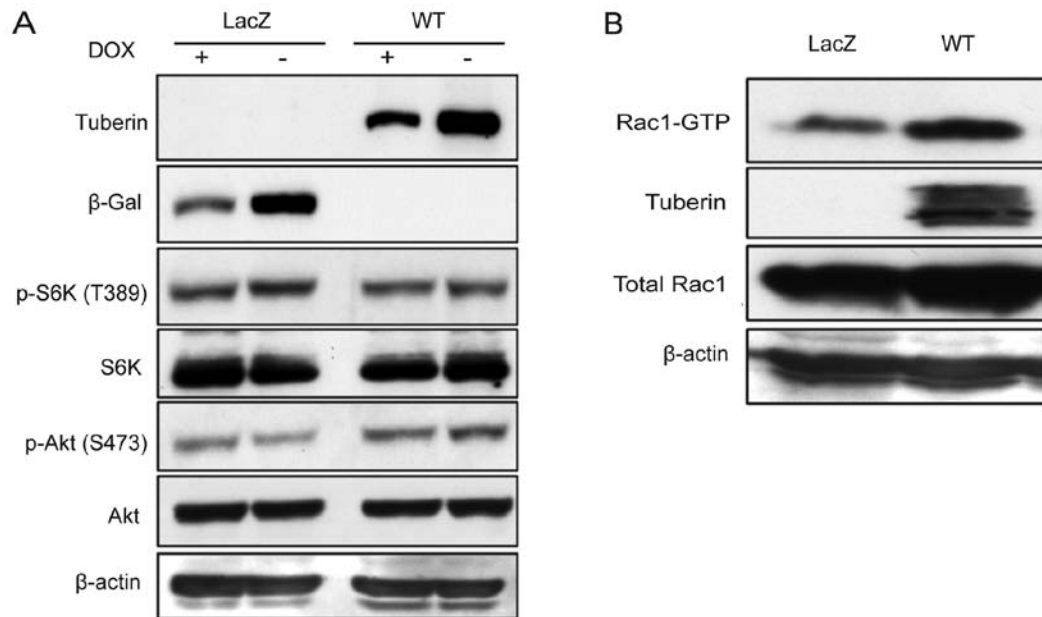


Figure 1. Wild-type tuberin expression activates Rac1 in Tsc2-deficient cells. (A) Restoration of tuberin in Tsc2-deficient cells. LacZ (negative control, lanes LacZ) and WT (tuberin-expressing, lanes WT) cells were seeded and cultured in standard growth medium in the presence (+) of DOX (100 ng/ml) for 24 h. Cells were then incubated in the absence (-) of DOX for 48 h. Subsequently, total cellular proteins were separated through SDS-polyacrylamide gels. Western blot analysis was performed for  $\beta$ -galactosidase ( $\beta$ -gal), phospho-S6K (Thr389) [p-S6K (T389)], total S6K (S6K), phospho-Akt (Ser473) [p-Akt (S473)], total Akt and tuberin (TSC2). (B) Rac1 activation assay. LacZ and WT cells were grown in standard growth medium without DOX for 48 h and analyzed for Rac1 activation using the GST-pulldown assay. Proteins bound to beads (upper panel) and in cell lysates (lower panel) were analyzed for active Rac1 (GTP-Rac1) and total Rac1, respectively, by immunoblot analysis with anti-Rac1 antibody.

activated and total Rac1 in each sample was analyzed using the mouse monoclonal anti-Rac1 antibody by western blot analysis.

**RNA interference.** For gene silencing of raptor and rictor, the cells were incubated with the appropriate siRNA for 48 h in the absence of DOX. Transfection of 25 nM siRNA was performed with Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) and OPTI-MEM (Gibco/Invitrogen, Carlsbad, CA) according to the manufacturer's protocols, and 48 h after transfection, cells were lysed for further analysis. The following siRNA sequences, which reflect sense strands without 3'-overhang, were used: raptor, 5'-GCGUCCUUCUGUGGUCAA-3'; rictor, 5'-GGUUGGAAUGAUGGGCUU-3'; and control, 5'-GCUGCAAUCG AUUGAUAGC-3'.

**Immunofluorescence and confocal microscopy.** Cells were seeded at a density of  $2 \times 10^4$  cells in glassbottom dishes (Greiner Bio-One International AG, Frickenhausen, Germany) and cultured as described for Rac1 activation assay. Cells were fixed and permeabilized with 4% paraformaldehyde and 0.25% Triton X-100 for 30 min at 4°C. This was followed by incubation with the appropriate primary antibody for 1 h at room temperature and secondary antibody (Alexa-Fluor 488-labeled IgG; Alexa-Fluor 568-labeled IgG, Invitrogen) together with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Invitrogen) for 1 h at room temperature. Actin was stained using phalloidin (Invitrogen). The following primary antibodies were used: mouse mAbs against ubiquitin (Millipore), early endosome antigen 1 (BD Transduction Laboratories, Franklin Lakes, NJ), transferrin receptor (Invitrogen), and Flag (Sigma);

rabbit polyclonal antibody against Myc (Sigma); rat mAb against lysosomal-associated membrane protein 1 (Santa Cruz Biotechnology); and guinea pig polyclonal antibody against p62 (ProGen, Toowong, Australia). The secondary antibody used for p62 was Alexa-Fluor 594-labeled goat anti-guinea pig IgG (Invitrogen). Stainings were examined using TCS-SP5 laser confocal microscopy (Leica Microsystems, Wetzlar, Germany).

**Transient transfection.** MKOC1-277 cells were seeded at a density of  $2 \times 10^4$  cells in glass bottom dishes (Greiner Bio-One International AG) and cultured overnight. Plasmids were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol, and 48 h after transfection, cells were fixed and permeabilized for confocal microscopy as described above.

**Electron microscopy and immunoelectron microscopy using ultrathin cryosections.** Cells were fixed in 2% glutaraldehyde-2% paraformaldehyde (PA) buffered with 0.1 M phosphate buffer (PB) (pH 7.2) for ordinary electron microscopy, and in 4% PA buffered with PB (pH 7.2) for immunoelectron microscopy (28). In the former procedure, the cells were postfixed with 1% OsO<sub>4</sub> and embedded in Epon 812 after dehydration. Ultrathin sections, 60 nm, were cut with an ultramicrotome (UC6, Leica) and stained with uranium acetate and lead citrate. In the latter procedure, fixed cells were rotated in 2.3 M sucrose in PB overnight and plunged into liquid nitrogen. Sections approximately 60 nm thick were cut with a cryo-ultramicrotome (UC7/FC7, Leica) and reacted overnight at 4°C with rabbit anti-Rac1 (1:10) or p62 (Wako; 1:10) and mouse anti-ubiquitin (clone: FK2) (Enzo

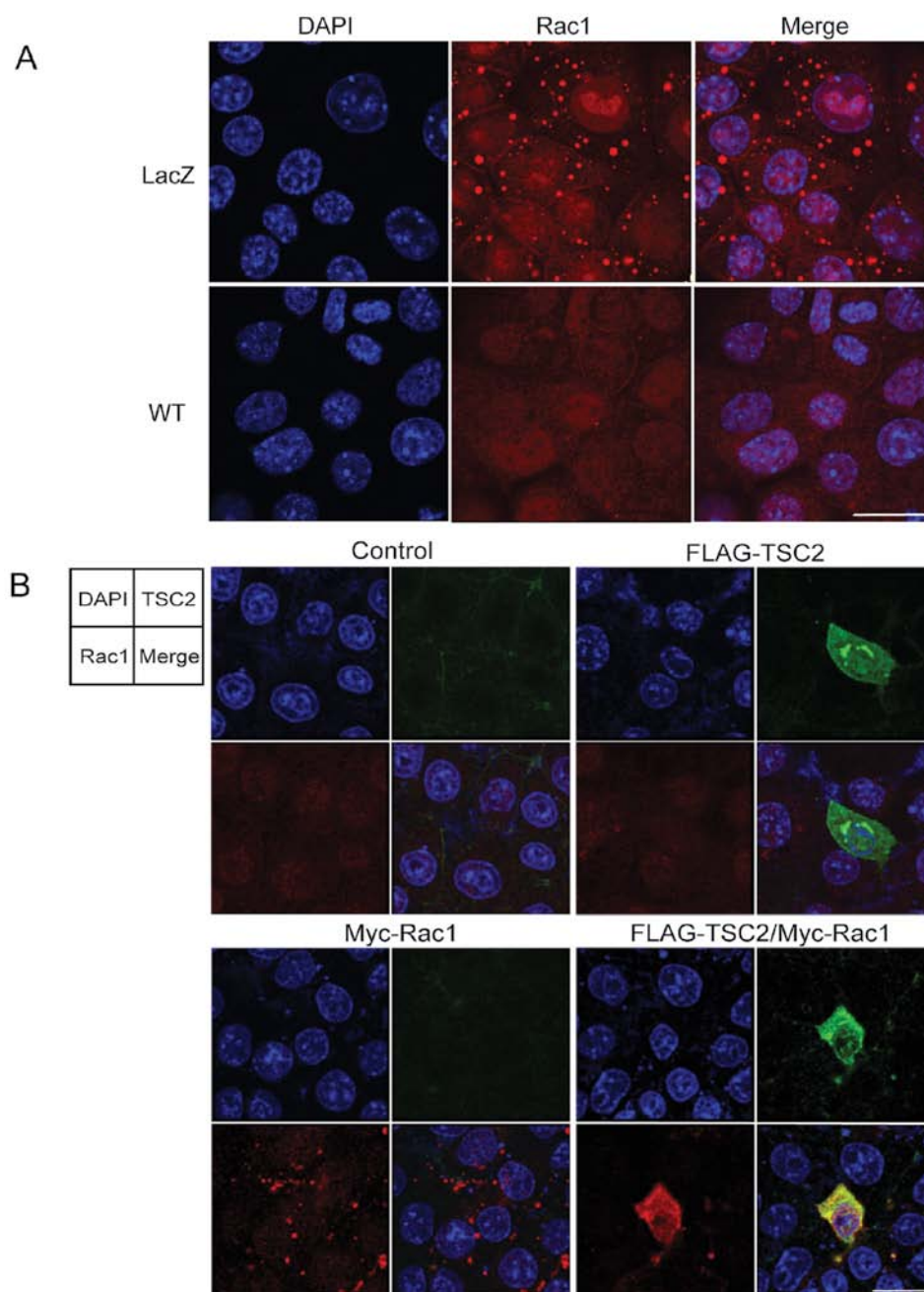


Figure 2. Wild-type tuberin expression modulates the distribution of Rac1 in Tsc2-deficient cells. (A) Immunocytological analysis of Rac1. LacZ and WT cells were grown in standard growth medium without DOX for 48 h, then fixed and subjected to immunofluorescence with anti-Rac1 antibody. Results of DAPI (blue, left images), Rac1 staining (middle panels, red), and merged images (right images) are shown. Bar, 20  $\mu$ m. (B) Transient expression of tuberin in Tsc2-deficient cells. MKOC1-277 cells were transfected with expression plasmids for FLAG-tagged tuberin and/or Myc-tagged Rac1, and 48 h after transfection, cells were fixed and examined for localization of FLAG-tuberin and Myc-Rac1 by double immunofluorescence. The panels show the results of FLAG-tuberin (upper right), Myc-Rac1 (lower left), both FLAG-tuberin and Myc-Rac1 expression (lower right), and control empty vectors (upper left). The arrangement of images in each panel is indicated: DAPI (blue); FLAG-tuberin (green); Myc-Rac1 (red). Bar, 20  $\mu$ m.

Life Sciences; 1:10) and then for 1 h with goat anti-rabbit and mouse IgG conjugated with 10- and 5-nm colloidal gold particles (British Biocell International). The specimens were examined with a Hitachi H-7100 or HT7700 electron microscope.

## Results

*Restoration of tuberin activates Rac1 in Tsc2-deficient cells.* To explore novel functions of tuberin, we established Tsc2-deficient

mouse renal tumor cells in which expression of wild-type tuberin was restored (WT cells, Fig. 1A) (25). Although we utilized the conventional Tet-off system, suppression of expression by DOX in established cells was limited and tuberin was still expressed even in the presence of 1  $\mu$ g/ml DOX (data not shown). Thus, we established and used  $\beta$ -galactosidase-expressing cells as tuberin-negative control cells (Fig. 1A). The levels of phosphorylation on p70 S6K and Akt were decreased and increased in WT cells, respectively (Fig. 1A). To test whether tuberin

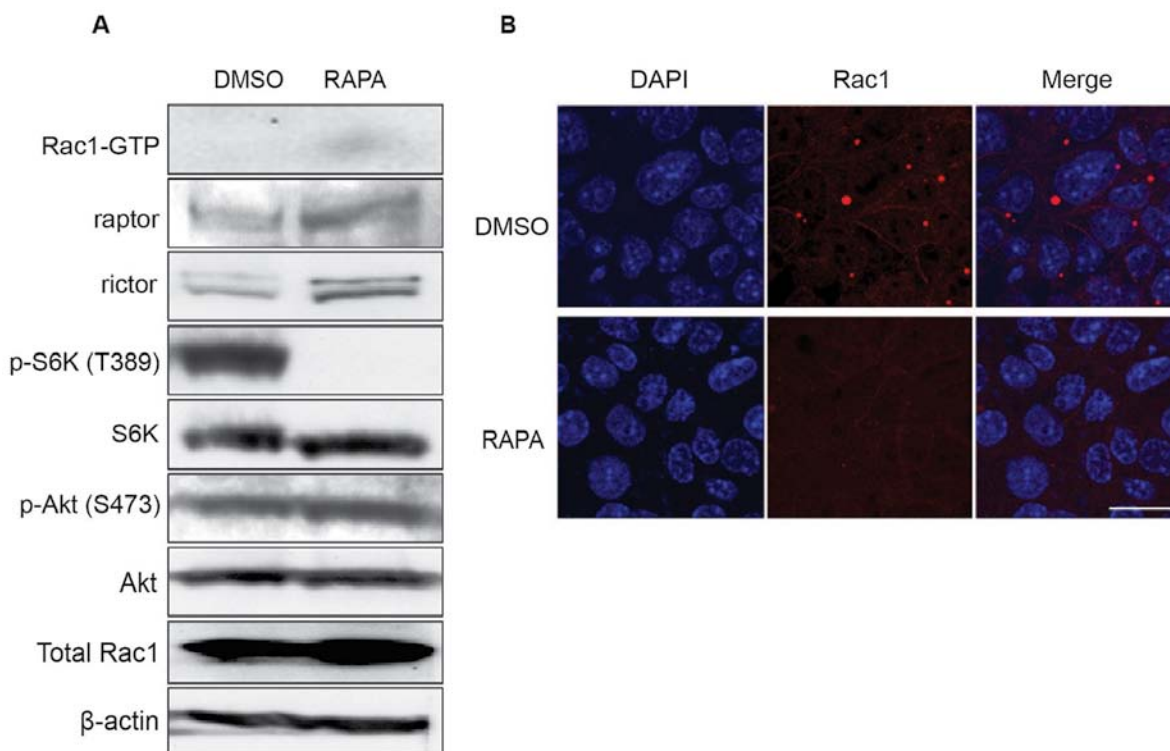


Figure 3. Rapamycin restores the activation and distribution of Rac1 in *Tsc2*-deficient cells. (A) Rac1 activity following rapamycin treatment. MKOC1-277 cells were grown in the absence of DOX for 48 h, then treated with vehicle (DMSO) or rapamycin (RAPA, 20 nM) for 4 h. The amount of activated Rac1 was analyzed using the pulldown assay. Intriguingly, when *Tsc2*-deficient cells were treated with rapamycin, the amount of activated Rac1 increased (upper panel). (B) Distribution of Rac1 following rapamycin treatment. Cells were treated with drugs as in (A), then fixed and subjected to immunofluorescence as described in Fig. 2A. Results of DAPI (blue, left images), Rac1 staining (red, middle images), and merged images (right images) are shown. DMSO, vehicle only; RAPA, rapamycin treatment. Bar, 20 μm.

regulates Rac1 activity, LacZ and WT cells were analyzed using GST-pulldown assay. Consistent with previous findings (20,21), the amount of activated Rac1 was significantly increased in WT cells compared with LacZ cells (Fig. 1B). Similar results were obtained using previously established tuberin-restored (T2-5) and control (E8) cells (data not shown) (25). These results suggest that tuberin regulates Rac1 activity as well as the mTORC1 pathway when restored in *Tsc2*-deficient cells.

**Tuberin affects Rac1 distribution.** We also investigated Rac1 distribution in LacZ and WT cells by confocal microscopy. Intriguingly, Rac1 appeared to form large dots within the cytoplasm in LacZ cells, whereas WT cells showed diffusely distributed Rac1 (Fig. 2A). We then performed transient co-transfection with expression plasmids for FLAG-tuberin and Myc-Rac1 in parental *Tsc2*-null cells (MKOC1-277). As expected, Rac1 appeared to form dots in the cytoplasm in the absence of co-expressed tuberin. By contrast, Rac1 was diffusely distributed in the presence of tuberin (Fig. 2B). Taken together, these results suggest that the regulatory mechanism of Rac1 distribution is controlled by tuberin.

**Rac1 activity as well as distribution are regulated by mTORC1.** We examined whether the regulation of Rac1 by tuberin is functionally related to the mTORC1 signaling pathway. First, MKOC1-277 cells were treated with vehicle (DMSO) or rapamycin (20 nM) for 4 h and analyzed for Rac1 activity and distribution. By this treatment, phosphorylation of S6K was

completely suppressed while Rac1 activation was increased (Fig. 3A) and its distribution became diffuse (Fig. 3B). To further test the contribution of mTORC1 as well as mTORC2 in Rac1 regulation, we employed gene silencing technology to knock-down raptor or rictor in both LacZ and WT cells. Since S6K and Akt are downstream targets of mTORC1 and mTORC2, respectively, phosphorylation of these proteins was decreased following knockdown of raptor and rictor, respectively. Consistent with the results of rapamycin treatment, increased activation of Rac1 was observed in raptor siRNA-treated LacZ and WT cells compared with control siRNA-treated cells (Fig. 4A and data not shown). While the amount of activated Rac1 following rictor siRNA treatment was similar to control siRNA-treated WT cells, on the other hand, rictor knockdown in LacZ cells showed a slight decrease in Rac1 activation as compared to control siRNA treatment (Fig. 4A), which may be partly due to the elevated raptor expression in these cells. These results suggest that Rac1 activity is negatively regulated by mTORC1. We also obtained similar results from E8 and T2-5 cells (data not shown). Distribution of Rac1 became diffuse after raptor, but not rictor or control, siRNA treatment in LacZ cells (Fig. 4B-D). In WT cells, Rac1 was diffusely distributed under all conditions regardless of siRNA treatment (data not shown). Taken together, these results indicate that Rac1 activity correlates with its distribution and is regulated by mTORC1.

**Actin stress fiber organization is correlated with Rac1 activity and distribution.** Furthermore, actin stress fiber formation and



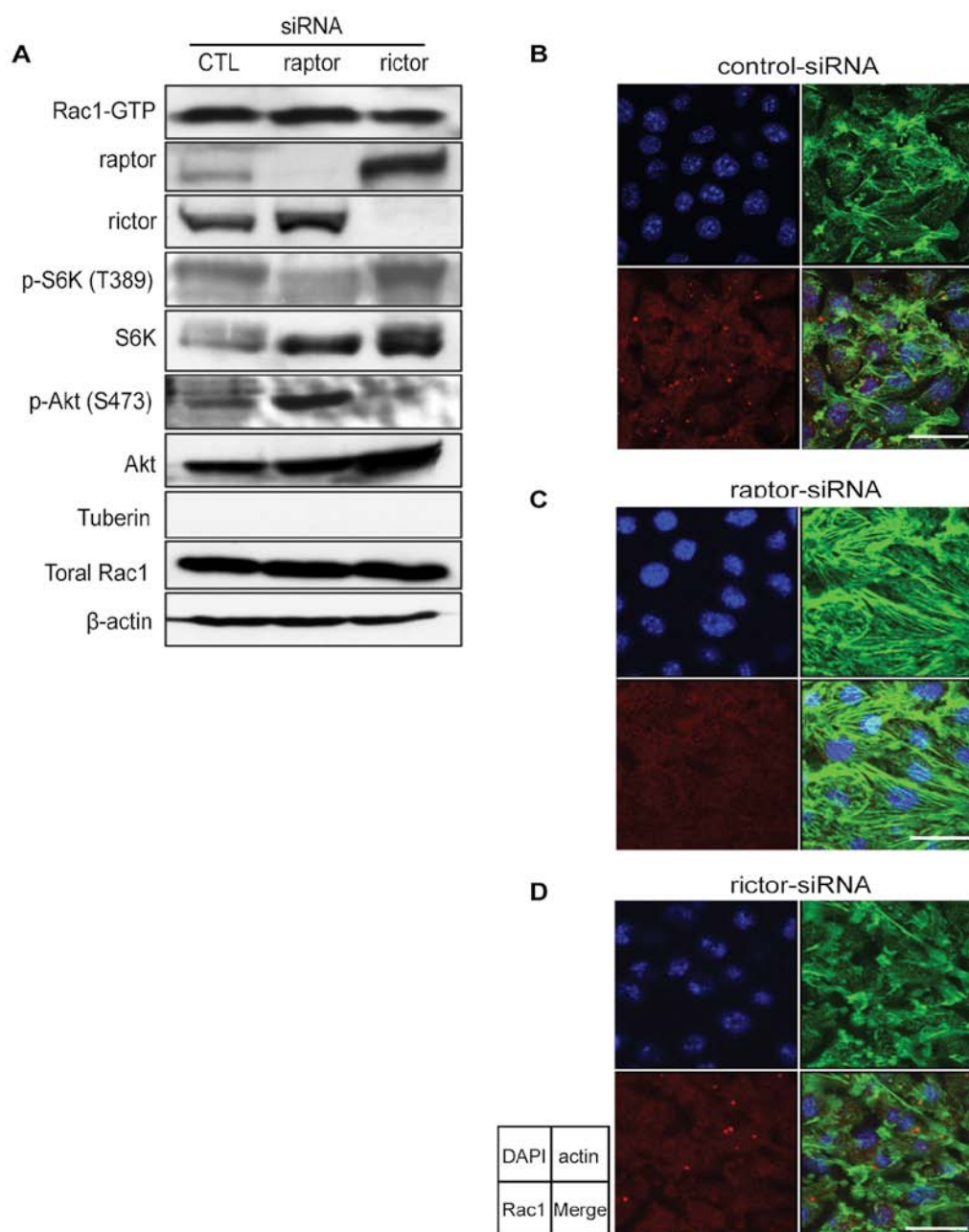


Figure 4. Knockdown of raptor enhances Rac1 activity and disperses dots in LacZ cells. (A) Rac activation assay. LacZ cells were incubated with raptor, rictor or control (CTL) siRNA in the absence of DOX for 48 h. The amount of activated Rac1 was analyzed using the GST-pulldown assay as described in Fig. 1B. In addition, total lysates were analyzed by immunoblot analysis for raptor and rictor to monitor knockdown. (B-D) Double immunofluorescence of LacZ cells treated with (B) control, (C) raptor, or (D) rictor siRNA. Cells were cultured and treated with siRNA as in (A), then subjected to immunofluorescence for Rac1 and phalloidin to detect F-actin. The separated panels show the following images: left upper, DAPI (blue); right upper, phalloidin (actin, green); left lower, Rac1 (red); right lower, merged image. Bars, 20  $\mu$ m.

organization, which are downstream effects of Rac1 (29,30), were assessed following raptor or rictor knockdown in LacZ and WT cells (Fig. 4B-D and data not shown). Aberrantly distributed actin fibers became well-ordered when LacZ cells were treated with raptor siRNA (Fig. 4B and C). In the case of rictor siRNA treatment, the intensity of actin staining seemed to become weaker without apparent changes in fiber organization (Fig. 4B and D). In WT cells, distribution of actin fibers was still well-ordered in all siRNA treatments (data not shown). Similar results were obtained using E8 and T2-5 cells (data not shown). These results suggest that mTORC1 inhibits stress fiber

formation, at least in part, by inhibiting Rac1 in *Tsc2*-deficient renal tumor cells.

*Rac1 dots were colocalized with p62 and ubiquitin, but not LAMP1, EEA1 and TR.* From the observations described above, dot formation seemed to be connected with inactivation of Rac1. Thus, we focused our investigation on protein degradation and intracellular membrane compartment systems (31). First, to elucidate which system is involved in Rac1 dot formation, MKOC1-277 cells were co-stained for Rac1 and membrane compartment markers such as lysosomal-associated membrane

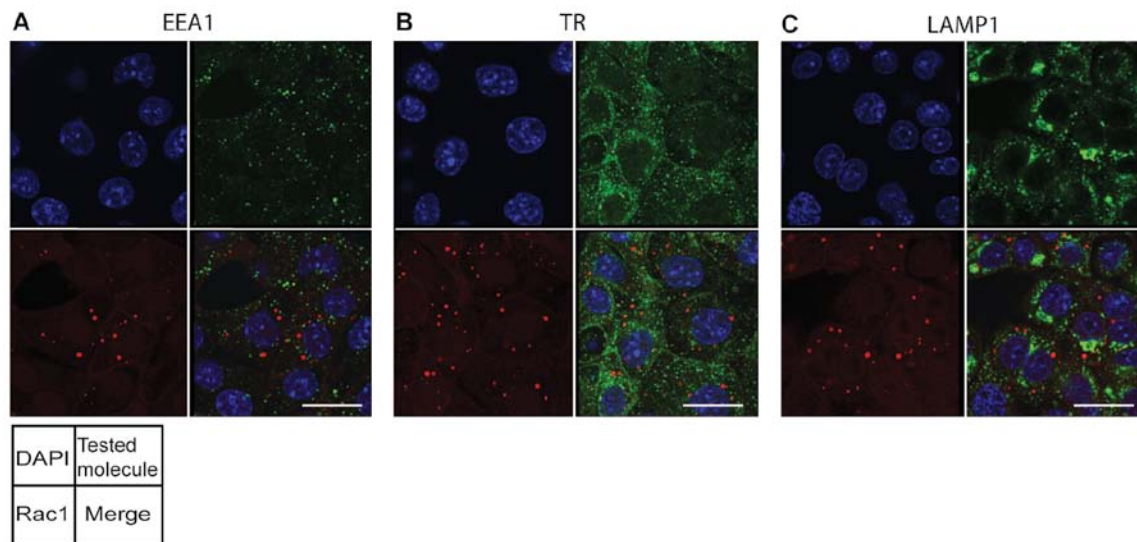


Figure 5. Rac1 dots are not co-localized with EEA1, transferrin receptor, and LAMP1 in Tsc2-deficient cells. (A-C) MKOC1-277 cells were grown in DOX-free medium for 48 h, then fixed and double immunofluorescence of Rac1 with (A) EEA1, (B) transferrin receptor (TR), and (C) LAMP1 was performed. The separated panels show the following images: left upper, DAPI (blue); left lower, Rac1 (red); right upper, tested molecule (green); right lower, merged image. Bars, 20  $\mu$ m.

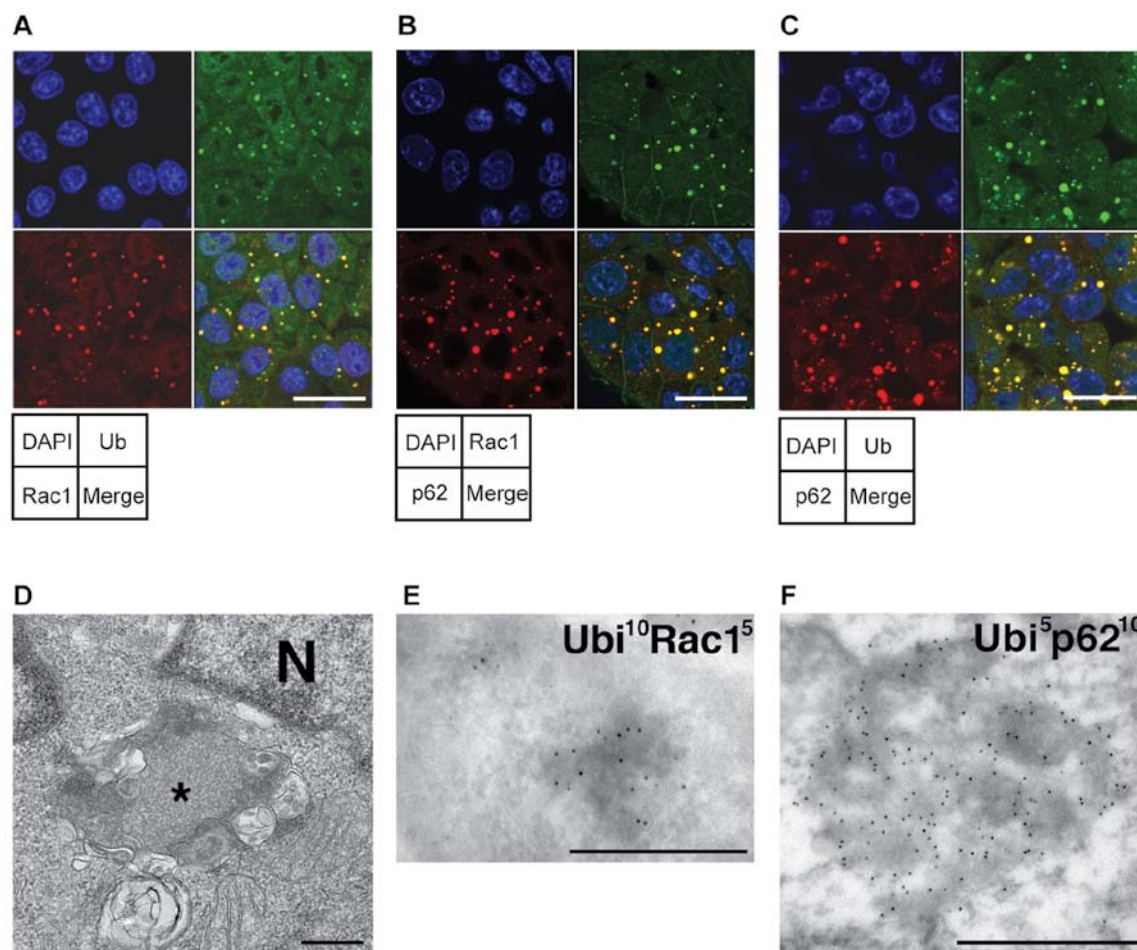


Figure 6. Ubiquitin and p62 are colocalized with Rac1 dots in Tsc2-deficient cells. (A and B) Double immunofluorescence of Rac1 with (A) ubiquitin or (B) p62. Double immunofluorescence was performed as described in Fig. 5. The separated panels show the following images: left upper, DAPI (blue); left lower, Rac1 (red); right upper (green); (A) ubiquitin or (B) p62; right lower, merged image. Bars, 20  $\mu$ m. (C) Co-localization of ubiquitin (Ub, green, upper right) with p62 (red, lower left). The upper left and lower right images show DAPI staining (blue) and merged images, respectively. Bar, 20  $\mu$ m. (D-F) Ultrastructural characterization of the aggregate structure in Tsc2-deficient cells. (D) Ordinary electron microscopy. Asterisk, aggregate. N, nucleus. (E and F) Immunoelectron microscopy using ultrathin cryosections. In Tsc2-deficient cells gold particles indicating (E) ubiquitin (10 nm) and Rac1 (5 nm) or (F) ubiquitin (5 nm) and p62 (10 nm) specifically co-localize in the aggregates. Bars, 0.5  $\mu$ m.

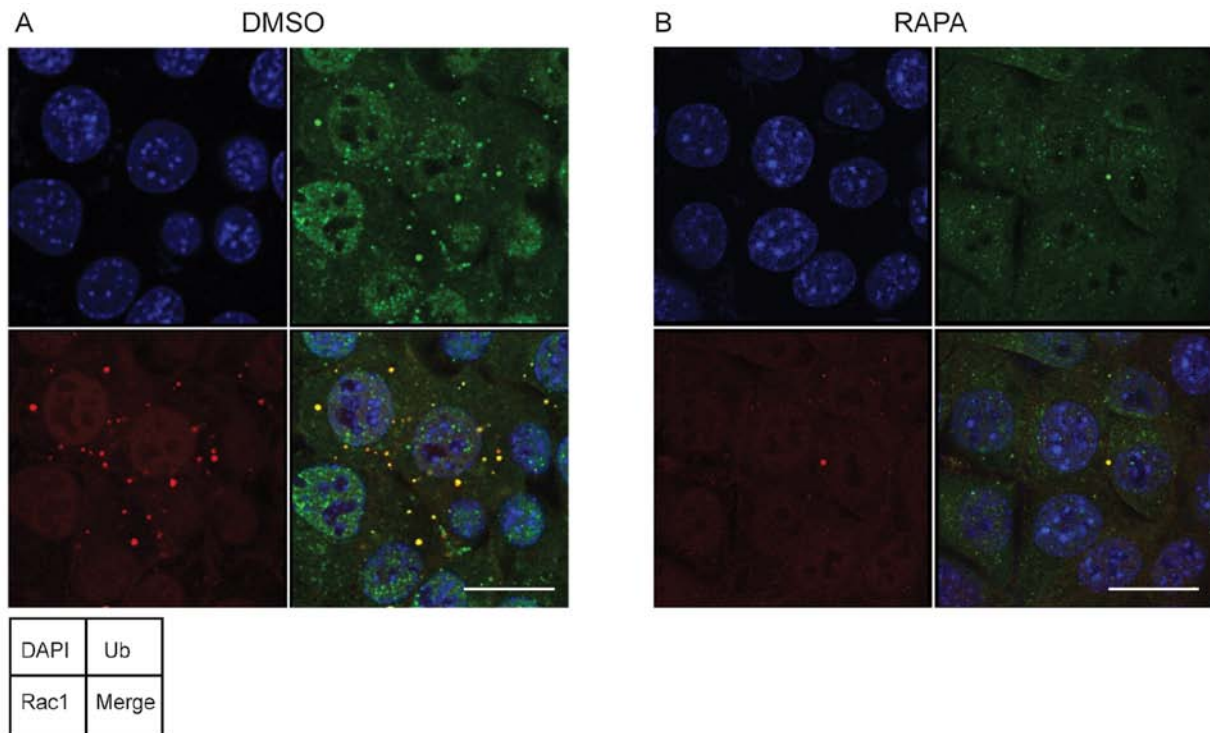


Figure 7. Rapamycin disperses the ubiquitin-positive Rac1 dots. (A and B) Double immunofluorescence of MKOC1-277 treated with rapamycin. MKOC1-277 cells were cultured in DOX free medium for 48 h, then treated with (A) vehicle (DMSO) or (B) 20 nM rapamycin (RAPA) for 4 h. Cells were fixed and stained with anti-Rac1 (lower left panels) and anti-ubiquitin (upper right panels) antibodies. The upper left and lower right images show DAPI staining (blue) and merged image, respectively. Bars, 20  $\mu$ m.

protein 1 (LAMP1, lysosome), early endosomal antigen 1 (EEA1, early endosome), and transferrin receptor (late endosome) (Fig. 5A-C). Colocalization of ubiquitin and Rac1 was also examined. As shown in Fig. 6A, ubiquitin colocalized with Rac1 dots, whereas EEA1, transferrin receptor and LAMP1 did not. We then performed staining of MKOC1-277 cells for p62, which is generally degraded by autophagy (32,33), a process that has been shown to be inhibited by mTORC1 and downregulated in *Tsc2*-null cells (16,34). Interestingly, p62 colocalized with Rac1 in MKOC1-277 cells (Fig. 6B). Ubiquitin also colocalized with p62, consistent with a previous report that found that these two proteins interact through the ubiquitin associated domain of p62 (Fig. 6C) (32). By conventional electron microscopy, we found aggregate structures in the cytoplasm of *Tsc2*-deficient cells (Fig. 6D). By immunoelectron microscopy we also confirmed aggregates doubly positive for ubiquitin and Rac1 or p62 in *Tsc2*-deficient cells (Fig. 6E and F). We observed that the number and size of ubiquitin dots in MKOC1-277 cells were significantly reduced by rapamycin treatment, similarly to those of Rac1 dots (Fig. 7). These findings suggest that in *Tsc2*-null cells Rac1 is incorporated into ubiquitin- and p62-positive aggregate, resulting in the inactivation and degradation of Rac1.

## Discussion

In this study, we aimed to elucidate the downstream signals of tuberlin that regulate Rac1 activity. Rac1 plays an important role in cytoskeletal dynamics by regulating actin remodeling, thereby promoting cell migration, invasion and metastasis (35,36). Regulation of Rac1 activity is also known to be

important for the neuronal cell differentiation process, such as neurite development (19,37). Thus, we believe that the signal transduction pathway involving tuberlin and Rac1 has relevance to not only clinical manifestation of TS, but also cancer development and progression including nervous system tumors.

Previous reports have illuminated various aspects of tuberlin's role on Rac1 regulation. Goncharova *et al* reported that hamartin inhibits Rac1 activity, whereas tuberlin counteracts the effects of hamartin to re-activate Rac1 and subsequently inhibit Rho (20). Rac1 and Rho activities may be regulated by hamartin and tuberlin in a coordinated fashion in an mTOR-independent manner (20). However, so far, the precise mechanisms of Rac1 regulation involving the downstream pathways of tuberlin have not been elucidated. Consistent with previous reports, we showed here that tuberlin positively regulated Rac1 activity in our experimental cell system, which was further demonstrated to be dependent on mTORC1. Our findings agree with a recent study which found that mTORC1-dependent regulation of cell migration, polarity and activation of Rac1 and/or CDC42 was through tuberlin signaling (21). This link between mTORC1 and Rac1 may provide important clues into the evaluation of the efficacy of rapamycin and side-effects on TS and cancers.

We show for the first time that Rac1 forms cytoplasmic dots in *Tsc2*-null cells. In addition, Rac1 dots co-localized with ubiquitin and p62. Furthermore, the number and size of the ubiquitin dot structures were significantly decreased and reduced in *Tsc2*-null cells treated with rapamycin. The amount of activated Rac1 was increased and stress fiber



formation was restored by knockdown of raptor, but not rictor, in *Tsc2*-null cells. These observations suggest that Rac1 activity is correlated with its distribution and is regulated by protein degradation in an mTORC1-dependent manner.

Impairment of the protein degradation systems, including the autophagy-lysosome and ubiquitin-proteasome pathways, is involved in various pathogenesis (12-14,38). Autophagy targets cellular proteins, protein aggregates and organelles for degradation in lysosomes (12). Protein complexes or organelles are engulfed by autophagosomes and bind to lysosomes that digest target proteins (12). Autophagy is thought to be important for cellular response to starvation as well as normal turnover of cytoplasmic constituents; starvation induces and mTORC1 suppresses autophagy (34). By contrast, the ubiquitin-proteasome pathway is another process that degrades misfolded proteins in the nucleus and cytoplasm via the E1-E2-E3 ubiquitin conjugation system (39). Recent studies suggested that, like many other proteins, the Rho family of GTPases are degraded by ubiquitinylation (40). However, there is no clear consensus as to the relationship between mTOR and regulation of ubiquitinylation.

The adaptor protein p62 (also known as sequestosome 1) is thought to be a scaffold protein initially identified as the atypical protein kinase C (aPKC)-binding protein (41). p62 binds to LC3 on the autophagosome membrane to carry ubiquitylated target proteins for degradation through its ubiquitin associated domain (32). We demonstrated that Rac1 co-localized with both p62 and ubiquitin in *Tsc2*-null cells, which suggest that Rac1 may be degraded through either the autophagy-lysosome or ubiquitin-proteasome pathway. Autophagosomes generally require fusion with lysosomes to form autolysosomes for degradation of proteins not required. In our study, the Rac1 dots did not co-localize with EEA1, transferrin receptor or LAMP1. This result may be explained by the increased mTOR activity in *Tsc2*-null cells, which may have blocked fusion of autophagosomes and lysosomes. The proposed ubiquitin- and p62-mediated Rac1 degradation system may be arrested by the above mechanism and not due to downregulation of Rac1 in *Tsc2*-deficient cells. Further studies are needed to clarify the presence of such degradation system and the links between distribution, degradation and activity of Rac1.

In summary, our study revealed that tuberin positively regulates Rac1 activity and controls its intracellular distribution through the mTORC1 signaling pathway. In addition, Rac1 dots in *Tsc2*-null cells might reflect the presence of as yet unidentified ubiquitin- and p62-dependent Rac1 degradation system.

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