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SHORT COMMUNICATION

The G1556S-type tuberin variant suppresses tumor formation in tuberous sclerosis 2 mutant (Eker) rats despite its deficiency in mTOR inhibition

M Shiono[1], T Kobayashi[1], R Takahashi[2], G Sun[1], M Abe[1], D Zhang[1], L Wang[1], X Piao[1], Y Takagi[1], R Mineki[3], H Taka[3], N Tada[4], S Sonobe[5], S Momose[6], M Ueda[2] and O Hino[1]

[1]*Department of Pathology and Oncology, Juntendo University School of Medicine, Tokyo, Japan;* [2]*PhoenixBio Inc., Tochigi, Japan;* [3]*Division of Proteomics and BioMolecular Science, Juntendo University School of Medicine, Tokyo, Japan;* [4]*Atopy Research Center, Juntendo University School of Medicine, Tokyo, Japan;* [5]*Department of General Thoracic Surgery, Juntendo University School of Medicine, Tokyo, Japan and* [6]*Department of Pathology, Saitama Medical Center, Saitama Medical University, Saitama, Japan*

Tuberin, a tumor-suppressor protein produced by the tuberous sclerosis gene *TSC2*, downregulates the Rheb-mTOR-S6K pathway (mTOR axis). Comparison of the effects of human tuberin mutations, such as G1556S, suggests that pathways other than the mTOR axis might also be involved in the pathogenesis of tuberous sclerosis. Here we test this possibility using the rat G1556S-type mutation (GSM) and a transgenic Eker (*Tsc2* mutant) rat system. Cells expressing GSM-tuberin failed to downregulate the mTOR axis. GSM-tuberin had an altered localization, which underlie its reduced ability to form a complex with hamartin, and a site-specific alteration in phosphorylation status indicating diverse regulation by Akt. GSM-transgenic (GSM-Tg) rats exhibited suppression of macroscopic renal tumors following *N*-ethyl-*N*-nitrosourea treatment. Intriguingly, rats with weaker GSM-Tg expression showed microscopic cystic and pre-tumorous lesions that were restricted in size and expansion, although they had hyper-phosphorylation of ribosomal protein S6. These results highlight a novel pathway involving tuberin that regulates tumor suppression independently of the mTOR inhibitory function. Identification of such a novel pathway will provide clear implications for generation of new therapeutic targets in the treatment of these tumors.
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Tuberous sclerosis (TSC) is an autosomal, dominantly inherited disorder with an estimated prevalence of 1/6000 (Gomez *et al*., 1999). Clinical symptoms range widely from central nervous system abnormalities (cerebral tubera, seizures, mental retardation) to multiple hamartomas of various organs (kidney or liver angiomyolipoma, heart rhabdomyoma, lung lymphangioleiomyomatosis, retinal hamartoma, angiofibroma). Mutations at two genes, *TSC1* (hamartin; 9q34) and *TSC2* (tuberin; 16p13.3), are associated with these phenotypes, and these genes have been labeled as tumor suppressor genes.

Eker rats are heterozygous for a mutation of *Tsc2,* the homolog of human *TSC2*, and develop hereditary renal cancer by 1 year of age (Eker and Mossige, 1961; Hino *et al*., 1993a, 1994; Yeung *et al*., 1994; Kobayashi *et al*., 1995). The homozygous mutation is embryo lethal. Earlier, we generated transgenic Eker rats carrying the cDNA and promoter of wild-type (WT) rat *Tsc2*, and demonstrated that the transgene (Tg) inhibited both renal carcinogenesis in Eker heterozygotes and embryonic lethality in homozygotes (Kobayashi *et al*., 1997). Studies using *Drosophila melanogaster* and mammals have revealed that a complex of tuberin and hamartin downregulates the Rheb-mTOR-S6K1 pathway (mTOR axis) (Manning and Cantley, 2003; Li *et al*., 2004; Martin and Hall, 2005). Lesions in both animal models and TSC patients display hyper-phosphorylation of ribosomal protein S6 (rpS6), the substrate for S6K1 (Kenerson *et al*., 2002; Kwiatkowski *et al*., 2002). On the basis of observations made in these studies, the mTOR axis was assumed to have a key function in tumorigenesis resulting from *Tsc1*/*Tsc2* mutations, and thus the possibility of therapy based on the inhibition of this axis was considered. mTOR was originally identified as the target molecule of rapamycin, suggesting that this compound might prove effective. We found that the tumorigenicity of $Tsc2^{-/-}$ cells derived from mice was effectively inhibited by rapamycin treatment (Kobayashi *et al*., 2003). Other groups reported a similar effect when Eker rats or knockout mice were treated with rapamycin, although they had some residual tumors (Kenerson *et al*., 2005; Lee *et al*., 2005, 2006). These results suggest that rapamycin has the potential for successful therapy, and clinical trials of rapamycin in TSC patients have been initiated. Although the outcomes of these trials are not yet available, there are concerns regarding the efficacy of rapamycin monotherapy and the possible immunosuppressive effect of high doses of the drug

Correspondence: Dr O Hino, Department of Pathology and Oncology, Juntendo University School of Medicine, 2-1-1, Hongo, Bunkyo-ku, Tokyo 113-8421, Japan.
E-mail: ohino@juntendo.ac.jp
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(Easton and Houghton, 2006; Faivre *et al*., 2006; Franz *et al*., 2006; Granville *et al*., 2006; Bissler *et al*., 2008; Davies *et al*., 2008). Both concerns might be circumvented by the development of drugs that act synergistically with rapamycin and could be used in a combined therapy, or of alternative drugs with different targets to rapamycin. Hence, exploration of pathways independent of the rapamycin-sensitive mTOR axis is desirable to identify novel molecular targets.

Analyses of TSC patients showed that the severity of symptoms varied with particular mutations of human tuberin (Niida *et al*., 1999). Thus patients with the N525S mutation of tuberin display a distinctive phenotype (cortical tubers, subependymal giant cell astrocytomas, renal angiomyolipomas, cardiac rhabdomyomas) despite the fact that the mutated protein retains its Rheb-GAP activity and inhibitory function in the mTOR axis (Nellist *et al*., 2001, 2005; Shah and Hunter, 2004). In contrast, the G1556S mutation, located in the Rheb-GAP-related domain, severely affects Rheb-GAP activity but causes relatively mild clinical symptoms in patients who show normal brain imaging, have lesions affecting the skin only, and occasionally display other hamartomas (Mayer *et al*., 2004; Nellist *et al*., 2005). These variants provide potential clues for unmasking different aspects of the mTOR axis and/or other unknown pathways in the pathogenesis of TSC. Here, we used the Tg-Eker rat system to analyze the characteristic effects of the G1556S-type mutation (GSM) *in vivo* and identified a novel property of tuberin that was independent of its known regulatory function in the mTOR axis.

We examined whether rat GSM-tuberin showed altered complex formation with hamartin in HEK293T cells. As reported earlier (Mayer *et al*., 2004; Nellist *et al*., 2005), the expression level of GSM-tuberin in cell lysates was slightly lower than that of WT-tuberin. Hence, we also transfected a larger amount (1.5-fold) of the GSM-tuberin expression plasmid to increase the level of expression of the protein. We found that GSM-tuberin exhibited a markedly reduced interaction with hamartin, even at the higher level of expression (Figure 1a). As reported earlier, Y1571H-type-tuberin also exhibited a reduced interaction (Aicher *et al*., 2001). In contrast, rat tuberin with an N525S-type mutation (NSM-tuberin) had the same interaction activity as normal tuberin (Figure 1a). Immunocytochemical analysis using a laser confocal microscope revealed novel qualitative differences between WT- and GSM-tuberin. In HEK293T cells expressing WT- or NSM-tuberin, the protein was characteristically distributed in the cytosol as a few perinuclear-associated dense bodies (PNDBs; Supplementary Figure S1a and b). However, GSM-tuberin rarely formed PNDBs. Instead, the dominant pattern was a homogeneous distribution in the cytosol without PNDBs (Supplementary Figure S1c). In the cells in which hamartin was expressed, the protein was present in dense bodies scattered throughout the cytosol (Supplementary Figure S1d). When hamartin was coexpressed with WT- or NSM-tuberin, both proteins were restricted to the PNDBs (Figure 1b and Supplementary Figure S1e). In contrast, hamartin was diffusely distributed in the cytosol of cells that coexpressed GSM- or Y1571H-type-tuberin (Figure 1c and Supplementary Figure S1f). It appeared that neither GSM- nor Y1571H-type-tuberin could stabilize hamartin at the PNDBs. Overall, our results suggest that the reduced interaction of GSM-tuberin with hamartin primarily resulted from defects in localization of GSM-tuberin.

GSM-tuberin showed faster gel mobility than WT-tuberin (Figure 2). Human G1556S-tuberin, which shows a similar gel mobility shift, has been suggested to be hypo-phosphorylated (Mayer *et al*., 2004). Hence, we performed a mass spectrometry (LC-ESI-MS/MS) analysis to examine the precise phosphorylation status of each tuberin variant. We found that a serine residue, corresponding to Ser939 of human tuberin (hereafter, phosphorylated residues are described using the corresponding numbers of human tuberin for simplicity), showed hypo-phosphorylation in GSM- but not NSM-tuberin (Supplementary Figure S2a and Table S1). This residue is one of the major phosphorylation target sites (Ser939 and Thr1462) of Akt (Inoki *et al*., 2002; Manning *et al*., 2002). Next, we carried out an immunoblotting analysis using a phosphorylation-specific antibody for Ser939 of human tuberin that also cross-reacts with rat tuberin. Consistent with the results from the MS analysis, the immunoblots indicated a lower phosphorylation level in GSM-tuberin (Figure 2). The amount of phospho-Akt (Ser473) was not significantly different, suggesting that the altered phosphorylation state resulted from a change in tuberin (such as conformation) and not the kinase activity of Akt. The MS and immunoblot analyses indicated that the various tuberin types did not differ significantly in their phosphorylation of Thr1462 (data not shown). The MS analysis also suggested that Ser1132, which belongs to the category of minor Akt-dependent phosphorylation sites (Inoki *et al*., 2002; Manning *et al*., 2002), was hyper-phosphorylated in GSM-tuberin compared with WT- and NSM-tuberin (Supplementary Figure S2b and Table S1). Unfortunately, no phosphorylation-specific antibody is available for this site, precluding the possibility of examining its phosphorylation status by an immunoblot. Our results suggest that GSM differentially influences susceptibility to Akt-dependent phosphorylation at several different residues. This is consistent with and seems to be the explanation for the observation that human G1556S-tuberin is not totally resistant to Akt-dependent phosphorylation, as occurs in R611Q-mutant tuberin, but displays a reduced susceptibility (Mayer *et al*., 2004), that is hypo-phosphorylation at a major site and hyper-phosphorylation at a minor site gives reduced, but not abolished, phosphorylation. The sites corresponding to Ser939 and Thr1462 are conserved in *Drosophila melanogaster* as phosphorylation targets. In contrast, the residue corresponding to Ser1132 is conserved in vertebrates (mouse, rat and *Fugu*) but not in *Drosophila*. Phosphorylation at the two major Akt-target sites negatively regulate the tuberin/hamartin interaction, and positively regulates the mTOR axis (Dan *et al*., 2002; Inoki *et al*., 2002;

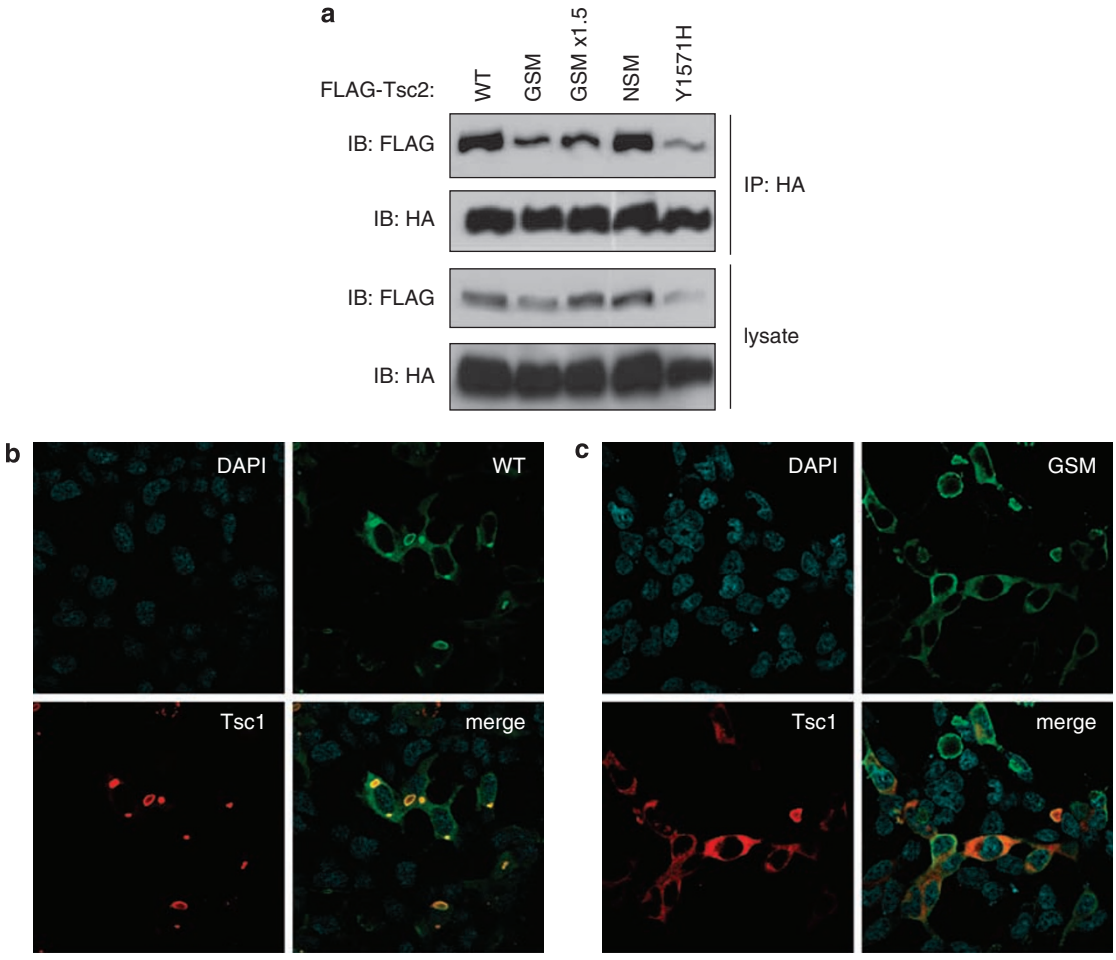

Figure 1 Detection of interaction between hamartin and tuberin variants. (**a**) Co-immunoprecipitation analysis. HEK293T cells were plated in collagen-coated 6-well plates at a density of 5×10^4 cells/well. After 1 day of plating, the cells were co-transfected with plasmids for HA-tagged hamartin and FLAG-tagged WT-tuberin or variants (GSM-, NSM- and Y1571H-type) using FuGENE6 (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. After 2 days, the cells were lyzed in NP40 lysis buffer (10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5 mM EDTA, 10 mM NaF, 1% NP40, 2 μg/ml aprotinin, 5 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 mM Na_3VO_4, 10 mM β-glycerophosphate, 10 mM $Na_4P_2O_3$) and incubated for 30 min with gentle agitation at 4 °C for extraction. Whole cell extracts were then centrifuged at 12 000 g for 20 min at 4 °C and the supernatant was harvested as the lysate. Immunoprecipitation was performed using a monoclonal anti-HA agarose conjugate (Sigma, St Louis, MO, USA) according to the manufacturer's protocol. Then immunoblotting (IB) was performed as described earlier (Sun *et al*., 2007) using the appropriate antibodies against each tag (Sigma). In the case of GSM-tuberin, a transfection with a 1.5-fold greater amount of plasmid was also performed (lane GSMx1.5). For plasmid construction, see Supplementary materials and methods. (**b** and **c**) Laser-confocal imaging of transiently expressed WT- (**b**) or GSM-tuberin (**c**) with hamartin in HEK293T cells. Cells were plated in a glass-bottomed dish (Matsunami, Osaka, Japan) at a density of 1×10^4 cells/well, transfected as in (**a**). 48 h after transfection, cells were fixed and permeabilized in 2% paraformaldehyde and 0.1% Triton X-100 for 30 min at 4 °C. They were then incubated with primary antibodies overnight at 4 °C, secondary antibodies (Alexa Fluor 488-labeled anti-mouse IgG; Alexa Fluor 568-labeled anti-rabbit IgG, Invitrogen, Carlsbad, CA, USA) and the DNA stain 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, Invitrogen) for 1 h at room temperature, and examined using an LSM510 META laser confocal microscope (Carl Zeiss, Oberkochen, Germany). The separate panels show the following images: upper left, DAPI (blue); upper right, anti-FLAG (tuberin, green); lower left, anti-HA (hamartin, red); lower right, merged image.

Manning *et al*., 2002). Relatively little attention has been paid to the function that minor Akt-target sites may have in mTOR inhibition of tuberin. However, this may need to be reconsidered if these minor Akt target sites regulate tuberin function in a different manner to the major Akt target sites. Interestingly, the Y1571H-type-tuberin exhibited a phosphorylation status similar to that of GSM-tuberin, suggesting that a common mechanism may be involved in these mutation-induced functional alterations in hamartin binding and/or inhibition of the mTOR axis (Supplementary Table S1). Overall, the regulation of tuberin by Akt-dependent phosphorylation seems to be more complicated than that considered earlier and might be the key to elucidating the novel tuberin-mediated pathway discussed below.

We next investigated the effect of the tuberin variants on the mTOR axis. WT- and NSM-tuberin showed suppression of S6K and S6 phosphorylation, but cells with GSM-tuberin did not inhibit phosphorylation to the same extent as cells without exogenous tuberin (Figure 3a). Even when the cells were transfected with a

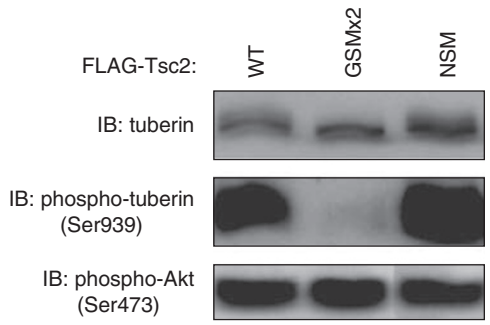

Figure 2 Phosphorylation status of tuberin variants. HEK293 cells were transfected with plasmids for FLAG-tagged WT-tuberin or variants (GSM or NSM) using FuGENE6. After 48 h of transfection, cell lysates were obtained as described in Figure 1 and immunoprecipitation was performed using an anti-FLAG M2 affinity gel (Sigma) according to the manufacturer's instructions. Immunoblotting (IB) was performed using anti-tuberin (C18; Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho(S939)-tuberin (Epitomics, Burlingame, CA, USA) or anti-phospho(Ser473)-Akt antibody (D9E; Cell Signaling Technology, Beverly, MA, USA). In the case of GSM-tuberin, a transfection with a twofold greater amount of the expression plasmid was also performed (lane GSMx2).

twofold larger amount of GSM-tuberin than of WT- or NSM-tuberin, S6K and S6 phosphorylation were not inhibited (Figure 3a; lane, GSM × 2). In another experiment using MKOC1-277 ($Tsc2^{-/-}$) cells (Kobayashi *et al*., 2003), phospho-rpS6 was also completely suppressed in cells expressing WT- or NSM-tuberin, compared with other $Tsc2^{-/-}$ cells without exogenous tuberin (Figure 3b and c). However, cells expressing GSM-tuberin retained the upregulated phospho-rpS6 state (Figure 3d). In this experiment, the inability of GSM-tuberin to form PNDBs was again observed (Figures 3b–d). From these analyses, we conclude that rat GSM-tuberin showed the same properties as its human counterpart. Of particular importance was the loss of mTOR-inhibitory activity by GSM, which could not be rescued by augmented expression. These results, together with those of the immunocytochemical analyses, suggest that the mTOR-inhibiting complex might be formed at PNDBs.

To investigate the function of GSM-tuberin *in vivo* using our transgenic Eker rat system (Kobayashi *et al*., 1997; Momose *et al*., 2002), we constructed Tgs for the expression of GSM-tuberin (GSM-Tg) (Supplementary Figure S3a). We obtained five transgenic founder rats following the introduction of GSM-Tg and another five that carried the WT-Tg (Supplementary Figure S3b and data not shown). The animals were used to establish two independent lines that showed stable germ-line transmission (Figures 4a, b and Supplementary Figure S3c). We compared the tumor suppressive function of GSM-Tg and WT-Tg in $Tsc2^{+/Eker}$ rats using transplacental ENU-treatment (Hino *et al*., 1993b; Kobayashi *et al*., 1997; Momose *et al*., 2002). Non-Tg-carrying $Tsc2^{+/Eker}$ rats were used as a control and found to develop multiple renal carcinomas bilaterally at either 6 or 10 weeks of age (Figure 4c and Supplementary Table S2, Tg(−); $n = 14$). As reported earlier, the introduction of WT-Tg completely suppressed ENU-induced renal carcinomas (Figure 4c and Supplementary Table S2, WT; $n = 6$) (Kobayashi *et al*., 1997; Momose *et al*., 2002). Surprisingly, renal carcinomas were completely suppressed in a line carrying GSM-Tg (Figure 4c and Supplementary Table S2, GSM; $n = 6$). These results suggest that tuberin can function as a tumor suppressor even when its inhibitory activity in the mTOR axis has been lost. Further analysis revealed that another GSM-Tg line that seemed to have a weaker expression level, judging from northern blot analysis (Figure 4b, Weaker-GSM; $n = 8$), exhibited an intriguing phenotype. Although tumor suppression was evident in this transgenic line (Figure 4c and Supplementary Table S2), the rats had multiple small cystic and pre-tumorous lesions with hyper-phosphorylation of rpS6 (Figure 4d). Nevertheless, these lesions were minimal compared with those in Tg-negative rats. It was of particular interest that, even at a weak level of expression, GSM-Tg could exert an apparent restrictive effect against cystogenesis and tumor-expansion despite the hyper-activated state of rpS6. The tumor suppressive function of GSM-tuberin is of interest in terms of the mild TSC symptoms in patients with G1556S mutation. One plausible hypothesis is that, in addition to mTOR inhibition, tuberin has another function that is not affected by GSM and is important for tumor suppression in the transgenic rats. This presumptive function may be conserved and related to pathogenesis in humans. Another possible explanation is that the residual mTOR-inhibitory activity of GSM-tuberin is sufficient to suppress tumorigenesis. However, this is unlikely because mTOR inhibition could not be achieved by increasing the expression level of GSM-tuberin *in vitro*, though the possibility cannot be excluded completely.

Crosses of rats heterozygous for *Tsc2* and carrying the WT-Tg produced homozygous *Tsc2* mutant offspring ($Tsc2^{Eker/Eker}$) at a rate consistent with the expected Mendelian ratio (7/64 = 10.9%). In contrast, we have only managed to obtain 2 homozygotes among 88 offspring (2.3%) in a similar cross but with parents carrying the GSM-Tg. Hence, the ability of GSM-Tg to rescue homozygous $Tsc2^{Eker/Eker}$ mutant embryos seems to be markedly reduced compared with that of WT-Tg. We also cultured fibroblasts from each of the Eker homozygotes rescued by GSM-Tg and confirmed that the characteristic features of GSM-tuberin, in terms of mTOR inhibition and phosphorylation of S939, were also present *in vivo* (Supplementary Figure S4). Although GSM-tuberin retains its tumor-suppressive function, it may have difficulty in suppressing embryonic lethality in *Tsc2* mutant homozygotes. As mTOR has a pivotal function in the coordination of cell metabolism, cell growth and cell proliferation (Astrinidis and Henske, 2005; Guertin and Sabatini, 2007), its dysregulation may have a more severe disruptive effect on various aspects of embryonic development. Possibly, an unknown feedback system is active in rescued pups to circumvent lethal defects induced by *Tsc2*-deficiency during development. If such a feedback mechanism exists, then its elucidation will also be important to our

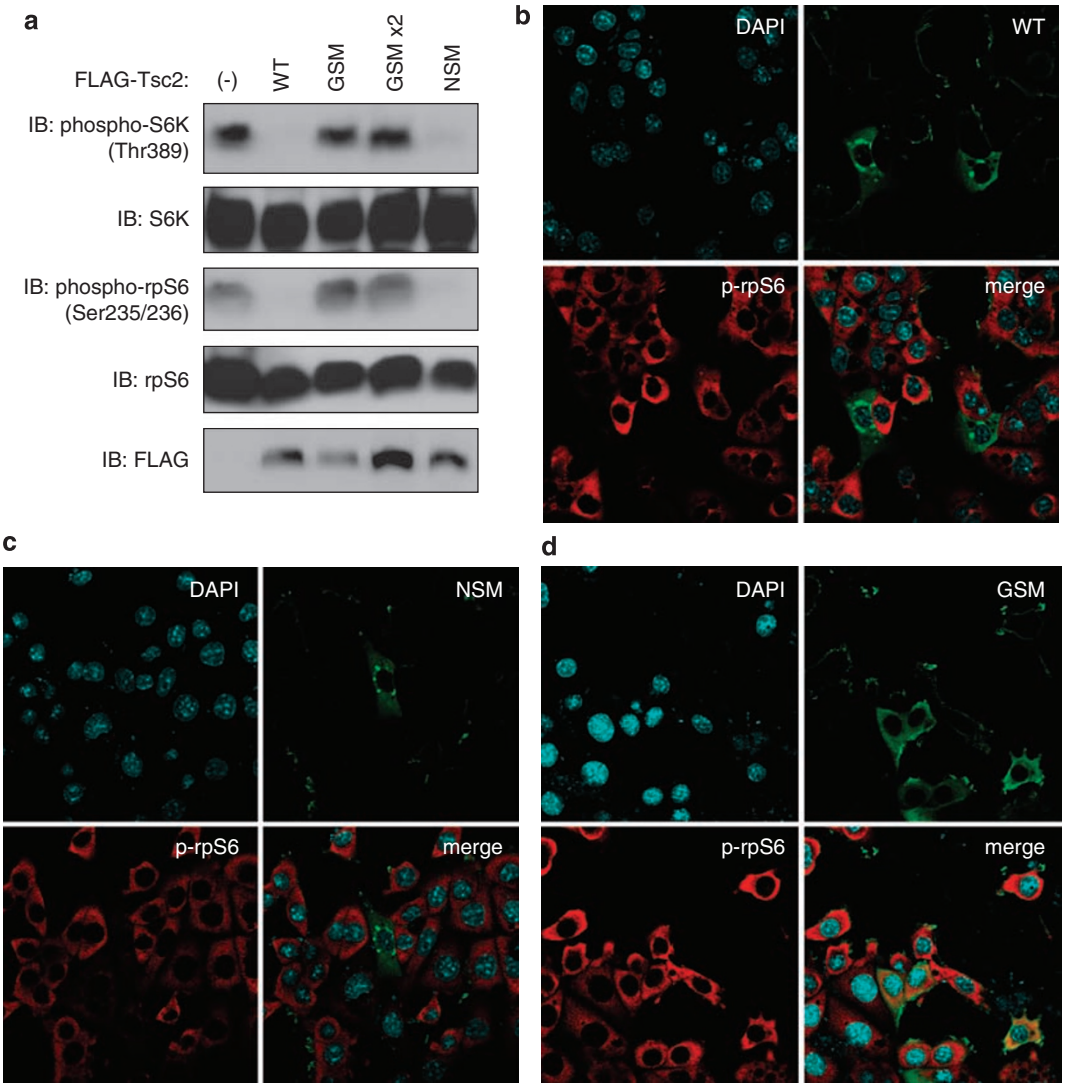

Figure 3 Effects of tuberin variants on mTOR axis. (**a**) Rheb-induced S6K and S6 phosphorylation in cells expressing each tuberin variant. HEK293 cells were co-transfected with plasmids for HA-hamartin, HA-Rheb (see Supplementary materials and methods) and each of the FLAG-tuberin variants as described in Figure 1. After 48 h of transfection, cells were lyzed in the SDS-PAGE sample buffer and the protein concentration was determined with a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Lysates were first analyzed for expression of transfected plasmids by immunoblotting (IB) with tag antibodies. Only the result for FLAG (tuberin) is shown. The status of the mTOR axis was examined using an anti-phospho(Thr389)-p70 S6Kinase (108D2, Cell Signaling Tech.), anti-p70 S6Kinase (C-18, Santa Cruz Biotech.), anti-phospho(Ser235/236)-S6 ribosomal protein (Cell Signaling Tech.) or anti-S6 ribosomal protein antibody (Cell Signaling Tech.). In the case of GSM-tuberin, a transfection with a twofold greater amount of plasmid was also performed (lane GSMx2). Lane (−) indicates the empty plasmid control for tuberin-expression. (**b**–**d**) S6 phosphorylation in *Tsc2*-deficient cells expressing each tuberin variant. MKOC1-277 cells were cultured in RPMI1640 medium supplemented with 10% FBS and antibiotics (Kobayashi *et al*., 2003). FLAG-tagged WT- (**b**), NSM- (**c**), and GSM-tuberin (**d**) were transiently expressed in MKOC1-277 ($Tsc2^{-/-}$) cells. Indirect immunofluorescent staining was performed as described in Figure 1 using primary antibodies against the FLAG tag and phospho-ribosomal protein S6 (Ser 235/236). DAPI was used for nuclear staining. The panels show the following images: upper left, DAPI (blue); upper right, anti-FLAG (tuberin, green); lower left, anti-phospho-rpS6 (red); lower right, merged image.

understanding of the mechanism of tumor suppression. It is notable that one of the $Tsc2^{Eker/Eker}$ mutants carrying GSM-Tg died suddenly with no obvious cause. Possibly, the presumptive feedback mechanism might not be sufficient to maintain normal health during the later stages of adult life of $Tsc2^{Eker/Eker}$ mutant rats.

The results obtained in this study seem to support the existence of another pathway, in addition to the mTOR axis, which has a tumor suppressive function. It has been reported that monotherapeutic administration of rapamycin to *Tsc2*-deficient animal models (knockout mice and Eker rats) with an established tumor, produced tumor regression but either with a residual tumor or failure to eradicate microscopic pre-tumorous lesions (Kenerson *et al*., 2005; Lee *et al*., 2005, 2006). These results also suggest the existence of a second pathway involving tuberin, in addition to the mTOR axis, although there is concern regarding drug resistance

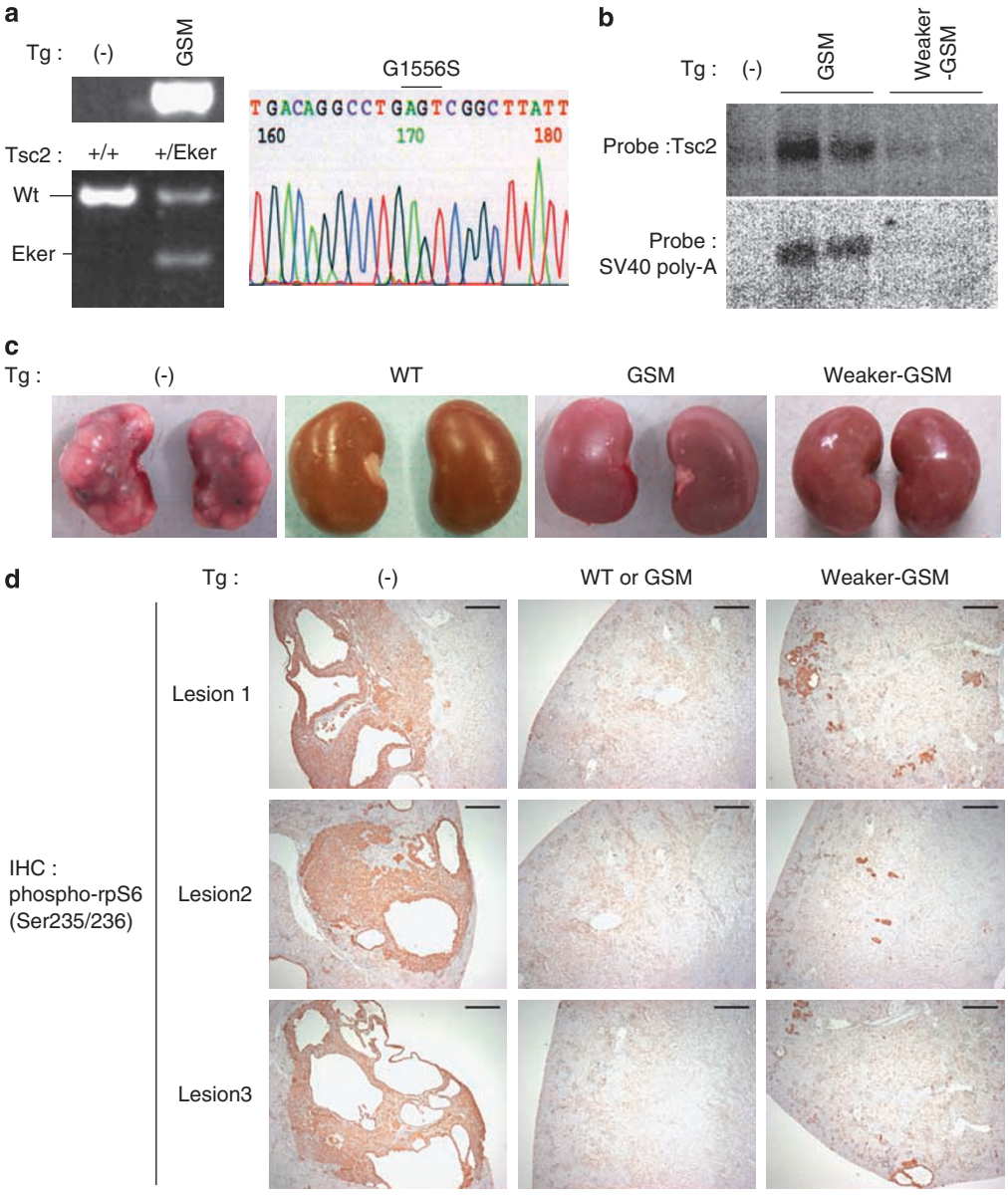

Figure 4 *In vivo* analyses of GSM-Tg. (**a**) Confirmation of genotype. PCR genotyping of endogenous *Tsc2* was performed as described earlier (Fukuda *et al*., 1998) except for the use of 3MFJ2 primer (5′-CTATGGCCACATGTGACCAA-3′) in place of the 3MFJ primer. For PCR genotyping of Tgs, RTSC68 (5′-TGAGTCCAATAAGCCCATCC-3′, forward, exon34) and RTSC10 (5′-CAAAGTCATTGCCCAGGTG-3′, reverse, exon 37) primers were used. Representative results for Tg (left upper) and endogenous *Tsc2* (left lower) of a control rat [(−) and +/+] and a GSM-Tg rat (GSM and +/Eker) are shown. Band position of the endogenous wild-type *Tsc2* allele (Wt) and of the germ-line mutant *Tsc2* allele of an Eker rat (Eker) are indicated. Right panel shows the result of sequence analysis of the amplified Tg-specific PCR fragment. The position of the G1556S-type mutation is marked by a bar. The primers and the PCR conditions are described in the Supplementary materials and methods. (**b**) Northern blot analysis of GSM-Tg expression level**s**. Total RNAs were isolated from kidneys using TRIZOL reagent according to the manufacturer's protocol (Invitrogen). Ten microgram of total RNA was subjected to northern blotting. Hybridization was performed with either a *Tsc2* cDNA probe or a probe containing the SV40 poly-A additional signal for Tg-specific transcripts. Lanes: (−), non-transgenic control; GSM, two pups from the first line; Weaker-GSM, two pups from the second line. (**c**) Suppression of ENU-induced renal carcinogenesis by Tg. ENU was administered transplacentally at embryonic day 15 (80 mg/kg body weight of pregnant rat) and offspring were sacrificed at 10 weeks of age (Momose *et al*., 2002). Representative macroscopic views of kidneys from non-transgenic (non-Tg) and transgenic (WT-Tg, GSM-Tg, and GSM-Tg with weak expression level) Eker heterozygotes. (**d**) Immunohistochemistry (IHC) for phospho-S6 protein. Kidney tissues were fixed in 10% buffered-formalin, embedded in paraffin and sectioned. Specimens were deparaffinized, preheated in 10 mM sodium citrate buffer (pH 6.0) in a microwave oven, and pretreated with 3% H_2O_2 solution for 10 min. Blocking was performed using 5% normal goat serum in wash buffer (1% BSA, Tris-buffered saline, 0.1% Tween 20, 0.07% NaN_3). Specimens were then incubated with the primary antibody against phospho(Ser235/236)-S6 ribosomal protein (1/200 dilution in wash buffer) overnight at 4 °C. Antibody binding was detected using the Envision system (DAKO, Glostrup, Denmark) and 3,3′-diaminobenzidine tetrahydrochloride (DAB; Dojindo, Kumamoto, Japan) as the substrate for peroxidase, followed by counterstaining with hematoxylin. Three representative and independent kidney specimens from each group are shown. Scale bar 500 μm.

against rapamycin. The transgenic system exploited here allowed us to investigate this putative pathway without the confounding effects of possible drug resistance. The mTOR pathway has an important function in *Tsc2* tumor progression, but its contribution to early tumorigenic events is less certain (Kenerson *et al*., 2005). The fact that rapamycin diminished but spared some lesions, and was ineffective in suppressing precursor lesions, might indicate that the rapamycin-sensitive pathway has a more significant function at later stages. The new pathway proposed here might restrict expansion of tumors despite upregulation of the rpS6 status, and might possibly be important in earlier stages of tumorigenesis. Taken together, the evidence suggests that the pathways might show stage specificity and division and/or coordination of labor in *Tsc2*-deficient tumors. Reviews of clinical trials of rapamycin for cancer therapy indicate that most tumor types do not respond to single-agent therapy with rapamycin derivatives (Easton and Houghton, 2006; Faivre *et al*., 2006; Granville *et al*., 2006). In this regard, there is an urgent need to elucidate the mechanism of this new pathway as a further target in a combined therapy with rapamycin and/or other available compounds (Fabian *et al*., 2005; Dancey and Chen, 2006; O'Reilly *et al*., 2006).

Through our use here of the transgenic Eker rat system, we have obtained novel *in vivo* data that indicate the existence of an earlier unknown pathway and of regulatory behavior in which tuberin is involved. The new pathway displayed a tuberin-mediated tumor-suppressor function in our system and will be the focus of our next studies; this pathway may prove to be a candidate target for TSC and cancer therapies. Other human *TSC2* mutations that are associated with milder phenotypes have been reported (Jansen *et al*., 2006). These mutations may also provide clues to aid the unraveling of the pathogenesis of TSC. Our transgenic Eker rat system could also be utilized for *in vivo* functional analysis of tuberin affected by such mutations.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)