Abstract  Nonalcoholic fatty liver disease (NAFLD) is the most common cause of liver dysfunction and is associated with metabolic diseases, including obesity, insulin resistance, and type 2 diabetes. We mapped a quantitative trait locus (QTL) for NAFLD to chromosome 17 in a cross between C57BL/6 (B6) and BTBR mouse strains made genetically obese with the Lept+/ob mutation. We identified Tsc2 as a gene underlying the chromosome 17 NAFLD QTL. Tsc2 functions as an inhibitor of mammalian target of rapamycin, which is involved in many physiological processes, including cell growth, proliferation, and metabolism. We found that Tsc2+/ mice have increased lipogenic gene expression in the liver in an insulin-dependent manner. The coding single nucleotide polymorphism between the B6 and BTBR strains leads to a change in the ability to inhibit the expression of lipogenic genes and de novo lipogenesis in AML12 cells and to promote the proliferation of Ins1 cells. This difference is due to a different affinity of binding to Tsc1, which affects the stability of Tsc2.—Wang, C.-H., D. S. Stapleton, K. L. Schueler, M. E. Rabaglia, A. T. Oler, M. P. Keller, C. M. Kendziorski, K. W. Broman, B. S. Yandell, E. E. Schadt, and A. D. Attie. Tsc2, a positional candidate gene underlying a quantitative trait locus for hepatic steatosis. J. Lipid Res. 2012. 53: 1493–1501.

Supplementary key words  diabetes • fatty acid/metabolism; genetics; triglycerides; cell proliferation; nonalcoholic fatty liver disease

Nonalcoholic fatty liver disease (NAFLD), the accumulation of triglyceride (TG) in hepatocytes, is the most common cause of liver dysfunction and is associated with several metabolic diseases, including obesity, insulin resistance, type 2 diabetes, dyslipidemia, and cardiovascular diseases (1). It affects ~25% of the US population. Twelve to 20% of NAFLD patients develop an inflammatory response and nonalcoholic steatohepatitis, and 5 to 15% progress to fibrosis and cirrhosis as the final stage of the disease, which is a great risk factor for hepatocellular carcinoma (2–4). Ten percent of liver transplants in the USA are done due to cirrhosis related to NAFLD (5).

Genetic background has been shown to play an important role in NAFLD, and the prevalence of NAFLD differs in various racial and ethnic groups, with African-Americans having the lowest incidence (24%), European-Americans having intermediate incidence (33%), and Hispanics having the highest incidence (45%) (6). Genetic approaches, including genome-wide association studies, have been used to identify DNA sequence variations that contribute to NAFLD. Single nucleotide polymorphisms (SNPs) of several genes, including PNPLA3, PPARGC1A, ADIPONECTIN, and CLOCK, have been shown to be involved in NAFLD (7–10). In spite of this progress, the genetic contribution to the regulation of lipid metabolism, which leads to the development and progression of NAFLD, has yet to be elucidated (11).

The repertoire of available inbred mouse strains represents much genetic variance as that found in the human population. This is an important resource for the identification of genes and pathways that confer resistance or susceptibility to diseases, including cancer, diabetes, and infectious diseases (12–17). Although several mouse models have been used to study the roles that specific genes play in lipid metabolism in the liver (18–21), there are only a few published genome-wide scans for this disease phenotype (8, 22).

Our laboratory has carried out genetic studies in two inbred mouse strains that differ in diabetes susceptibility. The C57BL/6 (B6) strain, when made obese, is relatively resistant to diabetes, whereas the BTBR strain develops...
severe diabetes when made obese due to a failure to expand β-cell mass and to a defect in insulin secretion (14, 23, 24). In addition to diabetes, the B6 and BTBR strains differ in their susceptibility to develop hepatic steatosis (25). Based on the differences in liver TG content between the obese B6 and BTBR strains, we sought to map the genetic loci contributing to these differences in a genomewide scan of an F2 cross of ~550 animals. We mapped a quantitative trait locus (QTL) for NAFLD to chromosome 17. We investigated the genes on chromosome 17 from 0 to 60 Mb and identified Tsc2, a candidate gene that regulates NAFLD.

MATERIALS AND METHODS

Animals and breeding strategy

The C57BL/6 (B6) and BTBR T+ tf (BTBR) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred at the University of Wisconsin-Madison as parental lean animals. The $\text{Lept}^{ob}$/ B6 mice as breeders to generate $\text{Lept}^{ob}$/ B6 mice. Tsc2+/– mice in B6 background were gifts from Dr. Avtar Roopra. Wild-type littermates were used as control. All mice were maintained at the Department of Biochemistry, University of Wisconsin-Madison animal care facility on a 12 h dark-light cycle (6 PM to 6 AM). The mice were fed Purina Formulab Chow 5008 and water ad libitum.

Genetic analysis

550 $\text{F}_2^{ob/ob}$ mice were genotyped with the 5K GeneChip (Affymetrix). Traits were mapped across the genome using R/qtl (26). The significance of high logarithm of the odds (LOD) peaks was evaluated using 1,000 permutations (27). Support intervals are genomic regions within 1.5 units of the peak per chromosome (28).

Fasting and refeeding study

Animals were separated into two groups: fasted for 24 h or fasted for 24 h followed by refeeding for 8 h. Liver tissues were collected after euthanization to harvest RNA for quantitative PCR, protein lysates for immune-blotting, and nuclei for detecting nuclear Srebp1c.

Cell culture and retroviral infection

Tsc2+/– mouse embryonic fibroblasts (MEFs) (a gift from Dr. Brendan Manning) were cultured in DMEM containing 25 mM glucose, 100 units/ml of antibiotic-antimycotic, and 10% FBS. The rat β-cell line Ins1 (832/13, a gift from Dr. Chris Newgard, Duke University) was cultured in RPMI 1640 medium containing 11 mM glucose supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 100 units/ml of antibiotic-antimycotic, and 50 mM β-mercaptoethanol. Exogenous Tsc2 were introduced by MMLV-based retroviral infection. A total of 1 × 105 Ins1 cells expressing GFP, B6, or BTBR alleles of Tsc2 were plated into 60 mm dishes. Cells numbers were counted in a TC10 automated cell counter (BIO-RAD) 4, 10, and 14 days after plating to measure the growth rate.

Isolation and quantification of total RNA

RNA from liver and cell lines was extracted using the QIAGEN RNeasy Plus Kit. After extraction, RNA was used for cDNA synthesis (Applied Biosystems). The mRNA abundance was determined by quantitative PCR using FastStart SYBR Green (Roche), and gene expression was represented by comparative delta CT method.

Triglyceride quantification

Triglyceride from 20 to ~40 mg of liver tissue was extracted by chloroform and quantified by a Wako L-Type TG H measurement kit (Wako Chemicals). The amount of TG was normalized to the amount of protein in the sample and presented as μg TG/mg protein.

Immunoprecipitation and immunoblotting

Cells were lysed in 1% Triton-X lysis buffer with a protease and phosphatase inhibitor cocktail (Roche). Immunoprecipitation was performed as described in the Cell Signaling Technology immunoprecipitation protocol with Tsc1 or Tsc2 antibody. Proteins were detected by immunoblotting following the Cell Signaling Technology immunoblotting protocol with desired antibodies. Tsc1, Tsc2, phospho-S6, total Akt, phospho-Akt, and Actin antibodies were purchased from Cell Signaling Technology. Srebp1 antibody was purchased from Santa Cruz Biotechnology.

De novo lipogenesis assay

AML12 cells expressing GFP or different alleles of Tsc2 were starved from insulin for 24 h, followed by a 24-h insulin treatment. Three hours before harvest, cells were incubated in the media with 0.2 mCi/ml $^3$H$_2$O. Cells were harvested in 2.5 M KOH/ EtOH solution and saponified at 85°C for 2 h. Saponifiable lipids were extracted with hexane and separated on TLC plates. The amount of $^3$H incorporation into fatty acid pool was counted as a measurement of de novo lipogenesis.

Measurement of protein turnover

Tsc2+/– MEFs expressing B6 or BTBR alleles of Tsc2 were treated with 100 μg/ml cycloheximide. Cells were harvested in 1% Triton-X lysis buffer as described above 2, 4, and 8 h after treatment. Protein concentration was measured with a Pierce BCA protein assay kit (Thermo Scientific). From each sample, 25 μg of lysate was loaded to perform immunoblotting to determine Tsc2 protein level.

Statistical analysis

Data were expressed as means ± standard error of means. The statistical comparisons were made using Student’s $t$-test at $P < 0.05$.

RESULTS

Strain effect of liver TG content

We measured liver TG in obese (Leptin$^{ob/ob}$) B6 and BTBR mice at 10 weeks of age. The B6 livers contain about three times more TG than livers from obese BTBR mice (Fig. 1A). Obese B6 mice also have higher transcript levels for lipogenic genes, including Srebplc, Fasn, Gpat, and Elovl6 (Fig. 1B), as measured by real-time PCR, indicating that a potential increase in de novo lipogenesis contributes to the higher amount of TG in B6 livers compared with BTBR livers. Based on this large strain difference in liver TG content, we mapped the gene loci contributing to these differences in an F2 cross (sample size ~550) and detected strong linkage on chromosome 17 with a LOD
peak of 15 at \( \sim 42 \text{ Mb} \) (Fig. 2A). F2 mice with the B6/B6 genotype at the peak marker on chromosome 17 had an \( \sim 2 \)-fold higher liver TG content than mice with the BTBR/ BTBR genotype \( (P < 0.0001; \text{Fig. } 2B) \). The heterozygotes had an intermediate phenotype.

**Differential Srebp1c and downstream lipogenic gene expression in WT and Tsc2\(^{+/-}\) mouse livers**

Although positional cloning ultimately identifies specific genes that are causal for a physiological phenotype, this process is slow due to the low resolution inherent in an F2 intercross and the time required to refine QTL intervals by generating congenic mice. We therefore investigated candidate genes on chromosome 17. We focused our attention on the genes reside within 10 to 50 Mb (LOD score greater than 8) with nonsynonymous coding SNPs between B6 and BTBR and identified a promising gene: Tsc2. Tsc2 encodes Tuberin, which functions as a GTPase-activating protein (GAP) toward Rheb to regulate the GTP-binding status of Rheb, which serves as an inhibitor of mammalian target of rapamycin complex (mTORC)1 (29–33).

The role of mTORC1 in regulating metabolism led us to hypothesize that Tsc2 is a gene underlying the chromosome 17 QTL that regulates the accumulation of TG in the liver. Tsc2-null mice are embryonic lethal. However, Tsc2\(^{+/-}\) animals are viable (34). To understand the role of Tsc2 in the regulation of insulin-dependent lipogenesis in vivo, we obtained Tsc2\(^{+/-}\) mice in the B6 background and performed fasting/refeeding studies. We observed a 12.3-fold increase of Srebp1c expression after refeeding in the livers of Tsc2\(^{+/-}\) mice, whereas their wild-type littermates showed only a 2.4-fold induction. The transcript levels of downstream lipogenic genes (Fasn and Elovl6) follow the same trend (Fig. 3A). The higher level of phosphorylated S6 ribosomal protein in Tsc2\(^{+/-}\) mice indicates that it has a higher level of mTORC1 activation compared with wild-type, independent of the phosphorylation status of upstream Akt (Fig. 3B). We also measured the protein level of processed nuclear Srebp1c and found that Tsc2\(^{+/-}\) mice

![Fig. 1. Strain effect of liver TG content. A: TG content in liver tissue was measured in B6 and BTBR mice at 10 weeks of age. The animals were fasted for 4 h before being euthanized. B6 mice had about three times the amount of liver TG as the BTBR mice when they were made genetically obese with Lep ob/ob mutation \((n > 10 \text{ in each group}; P < 0.0001)\). B: Mouse cDNA was made from the livers of previously described animals. The levels of lipogenic gene transcripts were measured by real-time PCR and presented as \( \Delta C_t \) after normalized to \( \beta \) actin as an internal control \((n = 5 \text{ in each group})\).](https://example.com/f1)

![Fig. 2. Liver TG QTL on chromosome 17. A: LOD profile for liver triglyceride on chromosome 17; peak LOD = 15 at \( \sim 42.5 \text{ Mb} \), from a F2 cross of 550 mice. B: Liver TG content according to genotype at the coding SNP of LOD peak marker across the F2 population.](https://example.com/f2)
expressing the BTBR (V552) allele (Fig. 4B). This allelic difference in regulation of lipogenesis provides one mechanism for the difference in hepatic TG accumulation between the two mouse strains.

**Tsc2 allelic differences in regulation of lipogenic gene expression and de novo lipogenesis**

We sequenced the B6 and BTBR alleles of the Tsc2 gene and found a nonsynonymous coding SNP (M552V) located at an unexplored region of the protein. We hypothesized that this coding difference causes the B6 form of Tsc2 to inhibit mTORC1 less efficiently than the BTBR form and therefore causes an increase in lipogenic gene expression in the liver through greater mTORC1 activation. We tested this hypothesis by introducing either the B6 (M552) or the BTBR (V552) forms of Tsc2 into AML12 (a mouse hepatoma cell line) through retroviral infection. The insulin-stimulated expression of lipogenic genes (Srebp1c, Fasn, Gpat, and Elovl6) was greater in cells expressing the B6 (M552) allele of Tsc2 than in cells expressing the BTBR allele (V552) (Fig. 4A). In these experiments, both allelic forms of ectopic Tsc2 were expressed at similar mRNA levels (Fig. 4C).

De novo fatty acid synthesis in these AML12 cells was measured by treating the cells with $^3$H$_2$O for 2 h before harvest and measuring the amount of incorporation of $^3$H into fatty acids. Cells expressing the B6 (M552) allele of Tsc2 have a higher level of de novo lipogenesis than those expressing the BTBR (V552) allele (Fig. 4B). This allelic difference in regulation of lipogenesis provides one mechanism for the difference in hepatic TG accumulation between the two mouse strains.

**Allelic difference in regulation of β-cell proliferation**

Previous work has established Tsc2 as a key regulator of β-cell proliferation (35). Given the fact that BTBR mice are severely diabetic when made obese due to the failure to expand β-cell mass, we hypothesized that the genetic variation of Tsc2 causes insufficient β-cell proliferation through the mTORC1 pathway and contributes to the diabetic phenotype in BTBR animals. To test this hypothesis, we introduced GFP, the B6 (M552), or the BTBR (V552) alleles of Tsc2 into the β-cell line, Ins1 cells, via retroviral infection. Cell proliferation was inhibited by the introduction of either allele of Tsc2 (Fig. 5A). However, the BTBR allele inhibited cell growth to a greater extent than the B6 allele (doubling times 38.5 vs. 29.7 h; $P = 0.0004$; Fig. 5A), whereas the ectopic Tsc2 alleles were expressed at comparable mRNA levels (Fig. 5C). The Tsc2-mediated inhibition of mTORC1 activity was independent of Akt activation, as judged by the phosphorylation status of Akt and S6 ribosomal protein (Fig. 5B). These data confirm prior studies showing that Tsc2 functions as an inhibitor of β-cell proliferation (35, 36) and show that the coding
Tsc2, a gene regulating hepatic steatosis

Cycloheximide to arrest protein synthesis. Protein lysates were harvested at 2, 4, and 8 h after treatment and subjected to immunoblotting to monitor protein degradation. The results indicate that the B6 allelic form of Tsc2 is degraded more rapidly than the BTBR form ($t_{1/2} = 2.3$ vs. 3.7 h; $P < 0.001$). The levels of B6 and BTBR Tsc2 proteins in AML12, Ins1 cells, and the parental strains support these results (Fig. 4C, 5B, and 6C). This difference in turnover of Tsc2 provides a mechanism by which the coding variation of Tsc2 alters the potency of this inhibition, which may contribute to the difference in the proliferation capacity of β cells in B6 versus BTBR islets. We suggest that the polymorphism in the Tsc2 gene affects two distinct functions in two different tissues: TG accumulation in the liver and β-cell proliferation in the pancreas (35).

**Allelic difference in binding Tsc1 and protein turnover**

Binding of Tsc1 to Tsc2 prevents the proteasomal degradation of Tsc2 (37–42). Although the coding variation of Tsc2 between our two mouse strains is located in an unexplored region of the protein, it is near the Tsc1-binding domain (43). We hypothesized that the polymorphism of Tsc2 affects its binding to Tsc1. To test this hypothesis, the B6 (M552) or BTBR (V552) forms of Tsc2 were introduced into Tsc2−/− MEFs, and the Tsc complexes were immunoprecipitated and immunoblotted with Tsc1 or Tsc2 antibodies. When immunoprecipitated with Tsc1, more of the BTBR (V552) form of Tsc2 is coimmunoprecipitated than the B6 (M552) form (Fig. 6A, left panel). In contrast, when the Tsc2 antibody was used to perform the immunoprecipitation, more Tsc1 was pulled down with the BTBR (V552) form than with the B6 (M552) form of Tsc2 (Fig. 6A, right panel). The amounts of Tsc1 and Tsc2 in the complexes were quantified, and the binding efficiencies of Tsc1 and Tsc2 were determined by measuring the amount of binding partner being immunoprecipitated (Fig. 6A). The B6 (M552) form of Tsc2 binds to Tsc1 38% less efficiently than the BTBR (V552) form of Tsc1 on a per-molecule basis.

We asked if the allelic difference in the affinity of Tsc2 for Tsc1 is correlated with a different half-life of the Tsc2 protein. The MEFs described above were treated with cycloheximide to arrest protein synthesis. Protein lysates were harvested at 2, 4, and 8 h after treatment and subjected to immunoblotting to monitor protein degradation. The results indicate that the B6 allelic form of Tsc2 is degraded more rapidly than the BTBR form ($t_{1/2} = 2.3$ vs. 3.7 h; $P < 0.001$). The levels of B6 and BTBR Tsc2 proteins in AML12, Ins1 cells, and the parental strains support these results (Fig. 4C, 5B, and 6C). This difference in turnover of Tsc2 provides a mechanism by which the coding variation
between the B6 and BTBR strains causes differential mTORC1 activation, lipogenesis in the liver, and β-cell proliferation in the pancreas (35).

**DISCUSSION**

* Tsc2 is physically located on chromosome 17 at 24.7 Mb. It encodes Tuberin and was first identified as a tumor suppressor gene that causes Tuberous Sclerosis Complex (TSC) when mutated (44, 45). TSC2 requires heterodimerization with TSC1 to stabilize the protein by inhibiting the interaction between TSC2 and the HERC1 ubiquitin ligase (37). TSC2 functions as a GAP toward Rheb to stimulate the internal GTPase activity of Rheb (29–31, 37). GTP-bound Rheb stimulates the phosphorylation and activation of mTORC (32, 33). mTORC1 signaling is involved in many physiological processes, including cell growth, proliferation, and metabolism (46). Because the TSC1/TSC2 complex is a target of a variety of different signaling molecules, including PI3K, Akt, AMPK, ERK, and IKKβ, to regulate its activity via phosphorylation, it serves as a hub to regulate the physiological outcomes downstream of the mTORC1 pathway (46, 47).

mTORC1 was recently found to be involved in several different aspects of metabolism (48–51). It is required for the stimulation of lipogenesis in rat liver in an insulin-dependent manner. Thus, the insulin induction of lipogenic genes (*Srebp1c*, *Fasn*, and *Scd1*) can be inhibited by the mTORC1 inhibitor rapamycin (50). *Tsc2*−/− MEFs have a rapamycin-sensitive increase in de novo lipogenesis compared with their wild-type counterparts (49). mTORC1 promotes the function of SRE-binding protein by controlling the phosphorylation and localization of Lipin1 (52). These observations led us to hypothesize that *Tsc2* is a gene underlying the chromosome 17 QTL that regulates the accumulation of TG in the liver.

We performed fasting and refeeding studies and observed greater activation of *Srebp1c*, *Fasn*, and *Scd1* can be inhibited by the mTORC1 inhibitor rapamycin (50). *Tsc2*−/− MEFs have a rapamycin-sensitive increase in de novo lipogenesis compared with their wild-type counterparts (49). mTORC1 promotes the function of SRE-binding protein by controlling the phosphorylation and localization of Lipin1 (52). These observations led us to hypothesize that *Tsc2* is a gene underlying the chromosome 17 QTL that regulates the accumulation of TG in the liver.

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We sequenced the B6 and BTBR alleles of the *Tsc2* gene and found a nonsynonymous coding SNP (M552V), located at an unexplored region of the protein. We show that this coding difference causes the B6 form of *Tsc2* to inhibit mTORC1 less efficiently than the BTBR form and therefore causes increased de novo lipogenesis in the liver.

In the presence of insulin, PI3K is recruited to phospho-IRS1 and activated, resulting in the phosphorylation and
activation of Akt. Activated Akt is able to phosphorylate and inactivate Tsc2 (53), which allows Rheb to remain in the GTP-bound form and activate mTORC1 (54), leading to the regulation of downstream pathways, including lipid metabolism. However, surprising results from a recent study show that Akt is able to stimulate lipogenesis through the suppression of Insig2a in an mTORC1-independent manner. This pathway seems to be more pronounced when the regulation of mTORC1 by the TSC complex is absent (55). Other than the canonical Akt-mTORC1 pathway, Akt2 was also recently reported to be an independent signaling molecule regulating postprandial hepatic lipid metabolism (56).

In this study, we have focused on Tsc2’s function in regulating de novo lipogenesis through the mTORC1 pathway. However, decreasing β-oxidation in the liver, increasing free fatty acid uptake from peripheral blood, and decreasing VLDL secretion into circulation can be potentially pathways that also lead to TG accumulation in the liver. Preliminary data from our lab showing that Cpt1 is expressed at a higher level in BTBR livers than in B6 (data not shown) indicates that de novo lipogenesis might not be the only pathway that accounts for the strain difference in hepatic TG accumulation.

In our model, we only observed the strain difference in steatosis susceptibility when the animals were made genetically obese. Leptin-deficiency serves as a challenge for the steatosis to develop by providing excess energy from overeating. It is also possible that the lack of leptin signaling decreases AMPK activity, which has been shown to be an inhibitor of mTORC in the liver (57, 58).

In addition to de novo lipogenesis, Tsc2 has been shown to regulate β-cell proliferation through the mTORC1 pathway. Tissue-specific disruption of Tsc2 induces β-cell proliferation and improved glucose tolerance in a mTORC1-dependent manner (35). Knockdown of Tsc2 increased the number of cells in S/G2-M phase in β-cell lines and was reversed by rapamycin treatment (36). Given that obese BTBR mice develop severe diabetes due to the failure to expand β-cell mass (23), we hypothesized that the genetic variation of Tsc2 causes insufficient β-cell proliferation through the mTORC1 pathway, contributing in part to the diabetic phenotype in BTBR mice. We found that when introduced into Ins1 cells, an insulinoma cell line, the BTBR allele of Tsc2 inhibited cell growth more effectively than the B6 allele. This difference between B6 and BTBR Tsc2 may reflect differential binding to Tsc1, which affects the stability of the Tsc2 protein.

The inhibition of mTORC1 activation by Tsc2 can be regulated on many different levels. The interaction of Tsc2 with Tsc1 affects the half-life of Tsc2. The phosphorylation status of Tsc2 affects its GAP enzymatic activity. We have shown that the coding SNP in Tsc2 affects the interaction between Tsc2 and Tsc1 and results in different half-lives of the Tsc2 protein. The polymorphism between the B6 and BTBR alleles of Tsc2 is located close to its known phosphorylation sites (S540 and S664). Thus, it is possible that the polymorphism affects the phosphorylation status of Tsc2.

NAFLD is a general term used to describe a range of liver disorders, all characterized by the accumulation of excess TG in the liver. In some cases, hepatic steatosis progresses to nonalcoholic steatohepatitis and the development of hepatocellular carcinoma (59–61). Genetic predisposition affects the susceptibility to NAFLD hepatocellular carcinoma (59). Given that Tsc2+/− mice spontaneously develop liver tumors without steatosis (62), the dual function of Tsc2 in regulating lipogenesis and cell proliferation through the mTORC1 pathway leads us to postulate that loss of function of Tsc2 can contribute to hepatic steatosis and hepatocellular carcinoma.

The obese B6 mice are diabetes resistant compared with the obese BTBR mice. Our previous studies showed that the obese B6 mice exhibit greater β-cell mass due to an induction of β-cell proliferation. In contrast, this obesity induction of β-cell mass does not occur in BTBR mice (23). It is likely that multiple genetic loci coordinately regulate β-cell proliferation. The present study suggests that the genetic variation within a single gene, Tsc2, can simultaneously affect hepatic TG and influence β-cell replication.

The authors thank Merck for the DNA extraction and genotyping of the F2 mice, Dr. Avtar Roopra for providing the Tsc2+/− animals, and Dr. Brendan Manning for providing us Tsc2+/− MEFs and for discussion and comments regarding this study.

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