Tuberous sclerosis complex (TSC) is an autosomal dominant tumor-suppressor gene syndrome caused by inactivating mutations in either TSC1 or TSC2, and the TSC protein complex is an essential regulator of mTOR complex 1 (mTORC1). Patients with TSC develop hypomelanotic macules (white spots), but the molecular mechanisms underlying their formation are not fully characterized. Using human primary melanocytes and a highly pigmented melanoma cell line, we demonstrate that reduced expression of either TSC1 or TSC2 causes reduced pigmentation through mTORC1 activation, which results in hyperactivation of glycogen synthase kinase 3β (GSK3β), followed by phosphorylation of and loss of β-catenin from the nucleus, thereby reducing expression of microphthalmia-associated transcription factor (MITF), and subsequent reductions in tyrosinase and other genes required for melanogenesis. Genetic suppression or pharmacological inhibition of this signaling cascade at multiple levels restored pigmentation. Importantly, primary melanocytes isolated from hypomelanotic macules from 6 patients with TSC all exhibited reduced TSC2 protein expression, and 1 culture showed biallelic mutation in TSC2, one of which was germline and the second acquired in the melanocytes of the hypomelanotic macule. These findings indicate that the TSC/mTORC1/AKT/GSK3β/β-catenin/MITF axis plays a central role in regulating melanogenesis. Interventions that enhance or diminish mTORC1 activity or other nodes in this pathway in melanocytes could potentially modulate pigment production.

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Tuberous sclerosis complex inactivation disrupts melanogenesis via mTORC1 activation

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Tuberous sclerosis complex (TSC) is an autosomal dominant tumor-suppressor gene syndrome caused by inactivating mutations in either TSC1 or TSC2, and the TSC protein complex is an essential regulator of mTOR complex 1 (mTORC1). Patients with TSC develop hypomelanotic macules (white spots), but the molecular mechanisms underlying their formation are not fully characterized. Using human primary melanocytes and a highly pigmented melanoma cell line, we demonstrate that reduced expression of either TSC1 or TSC2 causes reduced pigmentation through mTORC1 activation, which results in hyperactivation of glycogen synthase kinase 3β (GSK3β), followed by phosphorylation of and loss of β-catenin from the nucleus, thereby reducing expression of microphthalmia-associated transcription factor (MITF), and subsequent reductions in tyrosinase and other genes required for melanogenesis. Genetic suppression or pharmacological inhibition of this signaling cascade at multiple levels restored pigmentation. Importantly, primary melanocytes isolated from hypomelanotic macules from 6 patients with TSC all exhibited reduced TSC2 protein expression, and 1 culture showed biallelic mutation in TSC2, one of which was germline and the second acquired in the melanocytes of the hypomelanotic macule. These findings indicate that the TSC/mTORC1/AKT/GSK3β/β-catenin/MITF axis plays a central role in regulating melanogenesis. Interventions that enhance or diminish mTORC1 activity or other nodes in this pathway in melanocytes could potentially modulate pigment production.

Introduction

Tuberous sclerosis complex (TSC) is an autosomal dominant disorder characterized by the presence of hamartomas (distinctive tumors) in multiple organ systems including the brain, skin, eyes, heart, lungs, and kidneys, as well as by refractory epilepsy and a variety of neuropsychiatric manifestations (1, 2). TSC is caused by inactivating mutations in either TSC1 or TSC2 (3). TSC1 and TSC2, together with TBCD17, form the TSC protein complex that functions as a GAP toward the GTPase Ras homolog enriched in brain (RHEB) (4). RHEB is a critical upstream regulator of mTOR complex 1 (mTORC1), which coordinates multiple cellular pathways that control cell size and anabolic metabolism as a prerequisite for cell division (4–11). In cells lacking either TSC1 or TSC2, RHEB-GTP levels are high, leading to the constitutive activation of mTORC1 (9, 12). mTORC1 activation leads to a complex set of feedback or counterregulatory effects that inhibit PI3K/AKT signaling, which is demonstrated through reduced levels of phosphorylated AKT (p-AKT) (Ser473) and AKT kinase activity. These counterregulatory effects include phosphorylation and degradation of insulin receptor substrate 1 (IRS-1) by S6K1 (13–15); reduced expression of IRS-2 (13, 14); reduced expression of PDGFR (16); reduced activity of mTORC2, which phosphorylates AKT at Ser473 (17); and phosphorylation and inactivation of GRB10 by mTORC1 (18, 19).

Several types of skin lesions are common in TSC: hypopigmented macules (also called ash-leaf or white spots), facial angiofibroma, cephalic plaque, shagreen patches, and ungual fibromas (2, 20). We have recently shown that facial angiofibroma and other TSC cutaneous tumors are due to 2-hit mutational inactivation of both alleles of either TSC1 or TSC2 and that lesions on the face often sustain UV-induced CC>TT mutations in the second allele (21). Hypopigmented macules typically appear similar to an ash leaf in configuration, with relative hypopigmentation in comparison with surrounding skin, and are most commonly seen on the limbs and trunk (22, 23). Since these macules can be detected at birth, they were proposed as one of the earliest diagnostic markers of TSC 50 years ago (24). Ultrastructural studies have shown that melanocyte content in TSC hypomelanotic macules is normal, while intracellular pigment granules are reduced in size and number (22, 25). Despite these morphologic and histologic studies, however, the mechanism of TSC-associated hypopigmentation has remained unknown.

Melanin is produced by skin melanocytes and other anatomically restricted pigment cells in the eye and brain (26–31). In the pigment biosynthesis pathway, tyrosinase (encoded by TYR) catalyzes the initial and rate-limiting step in melanin production by converting L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) (32, 33). The melanocyte-specific isoform of the microphthalmia-associated transcription factor M-MITM controls the transcription of TYR and other genes required for melanogenesis and melanosome formation (e.g., premelanosome protein [PMEL], dopachrome tautomerase [DCT], MelanA [MLANA])...
through binding to conserved E-boxes [CAC(G/A)TG] or M-boxes [TCAC(G/A)TG] in their promoter regions. Mutations in MITF cause the autosomal dominant disorder Waardenburg syndrome type 2A (WS2A), in which aberrant pigmentation and other features are seen (34–36). Melanocyte lineage expression of MITF is conferred by the context-dependent activity of several transcription factors: paired box 3 (PAX3), CAMP-responsive element–binding protein (CREB), and β-catenin, in concert with T cell transcription factor/lymphoid enhancer–binding factor 1 (TCF/LEF1) (37, 38). WNT/β-catenin signaling is required for the developmental pathway that leads to melanocyte derivation from the neural crest (39–41). Wnt ligand leads to phosphorylation and inhibition of glycogen synthase kinase 3β (GSK3β), disassembly of the destruction complex, an increase in β-catenin levels, and β-catenin translocation to the nucleus, where it binds to the TCF/LEF transcription factors to enhance the transcription of MITF and other targets (42–46). AKT signaling also negatively regulates both GSK3β and GSK3α (47, 48). Insulin and other growth factors lead to activation of AKT, which phosphorylates GSK3 at Ser9 (GSK3α) or Ser21 (GSK3β), thereby inhibiting GSK kinase activity.

Connecting the earlier understanding of the migration of melanocyte precursors during development (49) with previous studies on the role of mTOR in the regulation of pigment (43, 50), we hypothesized that TSC hypopigmented macules occurred because of a second-hit mutation and loss of TSC1/TSC2 occurring at the melanoblast stage of development, leading to clonal skin patches in which melanocytes have mTORC1 activation and reduced pigmentation. We show here that, in both melanocytes and melanoma cells, reduced expression of either TSC1 or TSC2 leads to inhibition of AKT, followed by enhanced, activated GSK3β, which in turn leads to phosphorylation of β-catenin and a marked reduction in nuclear β-catenin levels, followed by reduced LEF1 transcriptional activity, reduced MITF transcription and expression, and, ultimately, markedly reduced melanogenesis. Importantly, we found that melanocyte cultures from hypomelanotic macules from several TSC patients had reduced TSC2 and activated mTORC1 — with biallelic mutation in TSC2 in 1 patient — and were deficient in melanin production, which was consistent with our hypothesis.

Results

The TSC protein complex controls melanogenesis. To examine the role of the TSC protein complex in melanogenesis regulation, we studied immortalized human primary melanocytes (iHPMs) (51) and the relatively highly pigmented human melanoma cell line SK-MEL-30. Suppression of either TSC1 or TSC2 expression, using lentiviral shRNA, led to a marked reduction in visible pigmentation in iHPMs (Figure 1, B and F), with a reduction in melanin content (Figure 1, C and G) and TYR activity (Figure 1, D and H). Similar effects were seen in the SK-MEL-30 melanoma cell line (Supplemental Figure 1, B–D and F–H; supplemental material available online with this article; doi:10.1172/JCI84262DS1. See complete unedited blots in the supplemental material). The pigmentation reduction was proportional to the degree of TSC1 or TSC2 suppression seen across the 3 different shRNA constructs used for each gene (Figure 1, A–H and Supplemental Figure 1, A–H). As expected, the knockdown cells showed elevated levels of p-S6 (Ser235/236); p-S6 (Ser240/244); p-70S6K1 (Thr389); p-4E-BP1 (Thr37/46), as well as reduced p-AKT (Thr308) and p-AKT (Ser473), all of which were consistent with mTORC1 activation and feedback inhibition of AKT signaling due to reduced expression of the TSC protein complex (Figure 1, A and E, and Supplemental Figure 1, A and E). TSC2 expression was reduced in the cell lines when TSC1 was reduced, as has been seen previously (10).

Because the transcription factor MITF controls expression of TYR and other enzymes in pigment biosynthesis, including PMEL, DCT, and TYRP1 (52), we asked whether suppression of TSC1/TSC2 affected its expression. MITF-M and its downstream target genes PGC-1α, TYR, TYRP1, PMEL, and DCT (53) showed reduced expression at the protein (Figure 1, A and E, and Supplemental Figure 1, A and E) and mRNA levels (Figure 1M and Supplemental Figure 1M). Note that mRNA experiments were performed only for the shRNAs with the strongest effects: shTSC1-3 and shTSC2-2 in both iHPMs and SK-MEL-30 cells.

We sought to confirm that the loss of pigmentation was due to the loss of TSC1-TSC2 protein complex GAP activity toward RHEB. We expressed WT TSC2 or a TSC2 mutant (P419S) derived from a TSC patient with defective GAP activity in the TSC2-knockdown cell lines. WT, but not mutant TSC2 reduced mTORC1 activity, restored the expression of MITF, PGC-1α, and TYR, and restored TYR activity, melanin production, and pigmentation in iHPMs and SK-MEL-30 cells (Figure 1, I–L and Supplemental Figure 1, I–L, respectively).

To confirm that the reduced pigmentation was due to effects on MITF expression in the TSC1/TSC2-knockdown cells, we expressed MITF-M in the cells expressing the most efficient TSC1 and TSC2 shRNAs. Ectopic MITF expression at a level similar to that in control cells restored both pigmentation and TYR protein expression to levels seen in the control cells (Figure 1, O and P, and Supplemental Figure 1, O and P).

TSC protein complex controls melanogenesis through inhibition of mTORC1 activity. To confirm that the loss of pigmentation seen in these cell lines with shRNA against TSC1 or TSC2 was due to enhanced mTORC1 activity, we treated the cells with the mTORC1 allosteric inhibitor rapamycin. Rapamycin treatment (20 nM) rapidly blocked mTORC1 activity in the knockdown cells (data not shown), as seen with other cell types. After 4 days of rapamycin treatment, MITF expression was restored, as was that of its target genes PGC-1α and TYR in both TSC1- and TSC2-knockdown cells (Figure 2, A and E, and Supplemental Figure 2, A and E). Visual assessment of pigmentation, TYR activity, and melanin content of the rapamycin-treated cells confirmed that all were restored to baseline levels (Figure 2, B–D and F–H; and Supplemental Figure 2, B–D, and F–H). We also noted that rapamycin-treated control cells increased their pigmentation to a minor extent (Figure 2, C and G, and Supplemental Figure 2, C and G). Previous studies have shown that rapamycin treatment elevates the levels of MITF and its target genes TYR, TRYPI, and PMEL in mouse melanoma cells (54, 55), human primary melanocytes, and human melanoma cells (43), though to a modest extent (<2-fold). Combined with our results, this suggested that it was specifically mTORC1 activity due to TSC1/TSC2 loss that was leading to reduced MITF expression and thus reduced pigmentation. To confirm our results, we also
Figure 1. Disruption of the TSC protein complex induces loss of pigmentation in melanocytes. (A–D) Highly pigmented iHPMs were infected with lentiviruses expressing TSC1 shRNA or scrambled control (Scr) shRNA and selected with puromycin. (A) Immunoblot analysis shows that TSC1 depletion led to reduced MITF-M and its downstream targets PGC-1α and TYR, as well as mTORC1 activation. (B) Cell pellets from TSC1-depleted cells had reduced pigmentation. (C and D) TSC1-depleted cells had reduced melanin and TYR activity. (E–H) Entirely similar to the data shown in A–D, except that TSC2 shRNAs were expressed. (I–L) TSC2-depleted iHPMs were reconstituted with lentiviruses containing WT TSC2 or patient-derived TSC2-mutant (P419S) or empty vector control viruses (EV) and selected with puromycin. (I) Immunoblot analysis shows that WT TSC2, but not the TSC2 mutant, rescued mTORC1 activation, MITF and PGC-1α expression, and TYR protein levels. (J) Pigmentation, (K) melanin content, and (L) TYR activity were also all rescued by WT TSC2. Data in C, D, G, H, K, L, and M represent the mean ± SD from at least 3 independent experiments. *P < 0.05, by 2-tailed Student’s t test. (M) Expression of the indicated genes was measured by qPCR in control or shRNA-expressing cells. GAPDH was used as a control. (N) Immunoblot analysis of lysates from TSC1- or TSC2-depleted cells treated with MG132 (25 μM) for 6 hours. MITF, PGC-1α, and TYR were not rescued by MG132 treatment. (O and P) TSC1- or TSC2-depleted cells were infected with lentiviruses containing MITF-M or empty vector control (Scr) and selected with puromycin. (O) Immunoblot analysis shows increased MITF and TYR expression in cells infected with MITF-M lentivirus. (P) Ectopic expression of MITF rescued the pigmentation loss in TSC1- and TSC2-depleted cells. The immunoblots presented in the figure panels include replicate samples run on parallel gels.
Figure 2. Rapamycin, but not Torin-1, restores the loss of pigmentation in TSC protein complex–deficient melanocytes. (A–D) iHPMs stably expressing shTSC1 or control (Scr) were treated with rapamycin (Rapa) (20 nM) or vehicle control (DMSO) for 72 hours. (A) Immunoblot analysis shows recovery of MITF expression, suppression of mTORC1, and recovery of p-AKT levels in cells treated with rapamycin. (B) Pigmentation, (C) melanin content, and (D) TYR activity were all rescued by rapamycin. (E–H) Entirely similar to the data shown in A–D, except using iHPMs with shTSC2 knockdown. (I–L) iHPMs stably expressing shTSC1, shTSC2, or control (Scr) were treated with Torin-1 (250 nM) or vehicle control (DMSO) for 72 hours. (I) Immunoblot analysis shows complete suppression of mTORC1 and mTORC2. (J) Pigmentation, (K) melanin content, and (L) TYR activity, none of which were rescued by Torin-1 treatment. (M–P) iHPMs were infected with control (Scr) or shRaptor or shRictor lentiviruses and selected with puromycin for 6 days. (M) Immunoblot analysis shows that Rictor knockdown reduced MITF, PGC-1α, and TYR expression. (N) Pigmentation, (O) melanin content, and (P) TYR activity, all of which show reduced pigmentation in Rictor-knockdown cells. Data in C, D, G, H, K, L, O, and P represent the mean ± SD from at least 3 independent experiments. *P < 0.05, by Student’s t test. The immunoblots presented in the figure panels include replicate samples run on parallel gels.
treated the cells with the catalytic mTORC1/2 inhibitor Torin-1 (56). Interestingly, in contrast to rapamycin, Torin-1 was not able to restore MITF expression, its target genes PGC-1α or TYR, or pigmentation in either TSC1- or TSC2-knockdown cells (Figure 2, F–I, and Supplemental Figure 2, I–L), even though it completely inhibited mTORC1 as assessed by loss of phosphorylation of S6, S6K1, and 4E-BP1 (Figure 2I and Supplemental Figure 2I). We suspected that inhibition of mTORC2 by Torin-1, indicated by the loss of phosphorylation of AKT (Figure 2I and Supplemental Figure 2I), accounted for this difference. To examine that possibility further, we knocked down Raptor and Rictor, which are essential and unique components of mTORC1 and mTORC2, respectively, using shRNA in both iHPMs and SK-MEL-30 cells. Depletion of Raptor had the expected effects on Raptor expression and reduced levels of the marker of mTORC1 activity, p-S6 (Ser235/236). Similarly, shRictor had the expected effects on Rictor levels and reduced p-AKT (Ser473) levels, indicating blockade of mTORC2 activity. Inhibition of mTORC2 by Rictor depletion was associated with a marked reduction in the expression of MITF and its target genes PGC-1α and TYR, as well as reduced melanin production and pigmentation formation in iHPMs and SK-MEL-30 cells (Figure 2, M–P and Supplemental Figure 2, M–P). In contrast, inhibition of mTORC1 by shRaptor did not reduce pigmentation, but marginally increased MITF expression (Figure 2, M–P, and Supplemental Figure 2, M–P). In summary, these results indicated that rapamycin, but not Torin-1, rescued the reduced pigmentation seen in melanocytes lacking the TSC complex and that loss of Rictor had effects similar to those seen with loss of the TSC complex in reducing pigmentation. In aggregate, these findings suggested that the reduced AKT activity observed with loss of both the TSC complex and Rictor might be responsible for the reduction in pigment biosynthesis.

**TSC protein complex controls melanogenesis through stabilization of β-catenin.** Because MITF expression is controlled at multiple levels, we considered 2 possible mechanisms for the reduction of MITF in response to loss of the TSC protein complex and downstream mTORC1 activation in these cells: a) accelerated degradation of MITF due to phosphorylation or other events secondary to mTORC1 activation; and b) altered transcriptional regulation. To examine the possibility of accelerated MITF degradation, we used the proteasome inhibitor MG132 (25 μM) to treat cells expressing the most efficient TSC1 and TSC2 shRNAs. In both iHPMs and SK-MEL-30 cells, MG132 treatment did not restore the expression of MITF, PGC-1α, or TYR (Figure 1N and Supplemental Figure 1N). As an internal control, TSC2 levels were increased in the MG132-treated TSC1-knockdown cells, indicating effective proteasome blockade. Thus, accelerated MITF degradation did not appear to contribute to the reduction in MITF levels. Second, to explore whether altered transcriptional cues following loss of the TSC complex alters MITF levels, we examined AKT activation and the effects on its downstream substrate GSK3β (57, 58). We observed a marked reduction in p-AKT (Thr308), p-AKT (Ser473), and p-GSK3β (Ser9) levels in iHPMs with knockdown of either TSC1 or TSC2 (Figure 3A), with similar findings in SK-MEL-30 cells (Supplemental Figure 3A). As noted above, free GSK3β is regulated by AKT and is also present in cells as an essential component of the APC destruction complex that drives β-catenin degradation (59). Since increased levels of active GSK3β would be expected to lead to phosphorylation and degradation of β-catenin, reducing nuclear β-catenin–TCF/LEF complex levels to reduce MITF transcription (42, 60, 61), we examined β-catenin levels. Knockdown of either TSC1 or TSC2 led to increased levels of p–β-catenin (Ser33/37 and Thr41) and reduced levels of β-catenin in iHPMs and SK-MEL-30 cells (Figure 3A and Supplemental Figure 3A). Furthermore, rapamycin treatment of the knockdown cells decreased p–β-catenin and increased β-catenin levels (Figure 3B and Supplemental Figure 3B), suggesting that this effect was due to mTORC1 activity. The nuclear fraction of β-catenin was markedly diminished in knockdown cells to a greater extent than was seen with total levels, consistent with a consequent lack of transcriptional activity (Figure 3C and Supplemental Figure 3C), and nuclear levels of β-catenin were also restored by rapamycin treatment (Figure 3D and Supplemental Figure 3D).

To examine the effects of TSC complex loss on MITF transcription in greater detail, we expressed an MITF-luciferase reporter construct (42). MITF promoter activity was significantly reduced in both iHPMs and SK-MEL-30 cells with either TSC1 or TSC2 knockdown and was fully restored by treatment with rapamycin (Figure 3G and Supplemental Figure 3G). In addition, mRNA levels of MITF and its target TYR were considerably diminished by TSC1 or TSC2 knockdown and were restored to higher-than-normal levels by treatment with rapamycin (Figure 3H and Supplemental Figure 3H). To confirm that loss of nuclear β-catenin had transcriptional effects that would lead to loss of MITF expression, we used the TK-TOP reporter in parallel assays (62). We observed that TSC1/TSC2-deficient cells had a 5-fold reduction of TCF/LEF transcriptional activity (Figure 3E and Supplemental Figure 3E). These experiments suggested that β-catenin–TCF/LEF transcriptional activity is markedly attenuated in melanocytes with TSC1/TSC2 loss, explaining the marked reduction of MITF mRNA levels. Rapamycin treatment fully restored β-catenin–TCF/LEF transcriptional activity (Figure 3F and Supplemental Figure 3F), indicating that this series of events was due to aberrant mTORC1 activation.

To confirm that changes in β-catenin expression and nuclear localization were the main driver of reduced MITF expression in iHPMs and SK-MEL-30 cells, we examined the effects of expression of WT and constitutively active (CA) mutant (S33Y) β-catenin. WT and CA β-catenin expression in both TSC1- and TSC2-knockdown iHPMs and SK-MEL-30 cells led to increased expression of MITF, PGC-1α, and TYR, as well as a return of pigmentation (Figure 4, A–D and Supplemental Figure 4, A–D).

**TSC protein complex regulates β-catenin by inhibition of GSK3β.** Guided by previous studies linking GSK3β with both AKT and β-catenin, we next sought to explain the mechanism by which reduced AKT activity affected β-catenin levels. To directly assess the role of GSK3β, we used 2 approaches. First, shRNA-mediated GSK3β depletion within cells with suppressed TSC1 or TSC2 markedly reduced the phosphorylation of β-catenin at Ser33/37 and Thr41 and restored total β-catenin protein to levels similar or higher than those seen in control cells (Figure 4E, and Supplemental Figure 4E). Furthermore, expression of MITF and its target genes PGC-1α and TYR was significantly increased at both RNA and protein levels, as was MITF promoter activity and pigment formation in the shRNA-GSK3β–derivative cell lines (Figure 4, E–H and Supplemental Figure 4, E–H). In addition, reduced
GSK3β expression had no effect on mTORC1 activation, measured as p-S6, in the TSC1/TSC2-knockdown cells (Figures 4E, and Supplemental Figure 4E), indicating that this effect was independent of mTORC1. These results suggested that hypophosphorylated GSK3β was phosphorylating β-catenin, leading to its destruction.

As a second approach to confirm the role of GSK3β in the regulation of β-catenin in these melanocyte cell lines, we used pharmacological inhibitors specific to GSK3β. Using the pan-GSK3 inhibitor CHIR-99021 (63–65), we observed effects similar to those of shRNA against GSK3β. CHIR-99021 treatment reduced levels of
p–β-catenin (Ser33/37 and Thr41) and increased both total and nuclear β-catenin levels in both control and TSC1/TSC2-depleted SK-MEL-30 and iHPM cell lines (Figure 5, A and D, and Supplemental Figure 5, A and B). Corresponding to this increase in β-catenin levels, CHIR-99021 treatment reversed the effects induced by TSC protein complex depletion on: mRNA and protein levels of MITF and its downstream targets PGC-1α and TYR (Figure 5A, Figure 6B, and Supplemental Figure 5, A and D); MITF promoter activity (Figure 6A and Supplemental Figure 5C); and pigment formation (Figure 5F and Supplemental Figure 5E). To confirm that these effects of the inhibitor were specific to effects on GSK3β, we tested 2 other structurally unrelated GSK3β-specific inhibitors, AR-A014418 (66, 67) and 6-bromoindirubin-3’-oxime (BIO) (68), and found that both were identical in their ability to reverse the effects of loss of the TSC protein complex (Figure 5, B, C, E, G, and H, Figure 6, C–F, and Supplemental Figure 5, F–N).

Taken together, our data demonstrate that in melanocytes and melanoma cells, the TSC protein complex suppresses mTORC1 signaling, leading to physiological activity of mTORC2 and AKT, inhibition of GSK3β, stabilization and nuclear transport of β-catenin to activate the TCF/LEF1 transcription complex to cause transcription of MITF, production of MITF’s downstream melanogenic genes, and production of pigment.

mTORC1 is hyperactive in melanocytes from TSC patients’ hypomelanotic macules due to loss of TSC complex activity. Having established a mechanistic connection between normal TSC complex activity and pigmentation in melanocytes in vitro, we examined our hypothesis that spontaneous loss of the TSC complex occurred in hypomelanotic macules from patients with TSC. Skin biopsy samples were obtained from 6 hypomelanotic macules from 7 patients with TSC, with nearby normally pigmented skin available from 5 of these patients. Primary melanocyte and fibroblast cultures were prepared. Since these were primary cell cultures without immortalization, the melanocytes grew for a variable period of time prior to the onset of senescence, which limited the amounts of material available for various studies.

Melanocytes cultured from the hypomelanotic macules had features consistent with those observed in our studies of TSC1- and TSC2-knockdown iHPM and SK-MEL-30 cell lines (Figure 7A and Supplemental Figure 5A). In comparison with normal skin melanocytes, hypomelanotic macule melanocyte cultures showed a marked reduction in TSC2, but not TSC1, protein expression (Figure 7A and Supplemental Figure 6A). mTORC1 was activated, as assessed by phosphorylation of its downstream targets S6, S6K1, and 4E-BP1 (Figure 7A and Supplemental Figure 6A). AKT activity was reduced, as assessed by reduced phosphorylation at Thr308 and Ser473. Levels of p-GSK3β were reduced, while total GSK3β levels were increased, and p–β-catenin was increased, while total β-catenin was markedly reduced (Figure 7A and Supplemental Figure 6A). Protein levels of MITF and its target genes PGC-1α...
could be identified. The occurrence of a germline mutation in 
TSC2 in 5 of 6 patients was concordant with the observation that 
TSC2 expression was reduced, while TSC1 expression was normal, 
in all of these hypomelanotic macule melanocyte cultures. 
One hypomelanotic macule melanocyte culture (P35) had 
2 mutations in TSC2: c.2098-1G>A and c.1595-1597delAGA p.(Lys533del). The fibroblast culture from the same biopsy 
showed only the TSC2 c.2098-1G>A mutation, consistent with 
its occurrence as a germline event. These observations clearly 
imply that the c.1595-1597delAGA p.(Lys533del) mutation was a 
somatic mutation, occurring only in the hypomelanotic macule 
melanocytes. However, no second mutations were seen in any of 
the other melanocyte cultures.

We also analyzed the expression of MITF and the phosphor-
lation of S6 in paraffin-embedded skin biopsy samples from 2 
TSC patients’ hypomelanotic macules and nearby normal skin. 
Normal skin had melanin (brown) in the basal layer of the epider-
mis, including melanocytes, which were identified by strong MITF 
and TYR were markedly reduced (Figure 7A and Supplemental 
Figure 6A). Pigmentation was also markedly reduced in melano-
cytes from the hypomelanotic macules, in contrast to those from 
normal skin (Figure 7B and Supplemental Figure 6B).

We also examined mRNA levels of TSC1, TSC2, MITF, and 
MITF target genes in 5 of these cultures from which there was 
sufficient material. In comparison with normal skin melanocytes, 
hypomelanotic macule melanocytes showed markedly reduced 
expression of TSC2, MITF, and several downstream MITF target 
genes (Figure 8C).

In contrast, fibroblasts cultured from 2 of the hypomelanotic 
macules showed no difference in TSC1 or TSC2 expression and 
had normal regulation of mTORC1 activity (Figure 7D), indicating 
that the defect in TSC2 expression and the downstream effects 
were specific to the melanocytes.

We analyzed all melanocyte cultures for mutations in TSC1/ 
TSC2. Five of six patients carried a mutation in TSC2, while in one 
patient with two specimens, no mutation in either TSC1 or TSC2 
could be identified. The occurrence of a germline mutation in 
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expression (Figure 7C, top left). The hypomelanotic macule biopsy showed much less melanin and somewhat reduced expression of MITF (Figure 7C, bottom left). Furthermore, melanocytes in the hypomelanotic macule had increased p-S6 and reduced MelanA (Figure 7C, bottom right) compared with melanocytes from normal skin (Figure 7C, top right).

To further characterize the TSC2-deficient melanocytes, we examined the cultured cells by transmission electron microscopy (TEM). TEM sections showed that melanocytes from the hypomelanotic macules of 3 patients accumulated fewer pigmented melanosomes than did control skin (from 2 patients, Figure 8A). Furthermore, blinded melanosome grading and counting of multiple sections of melanocytes showed that hypomelanotic macules had a reduced proportion of mature, stage-IV melanosomes and a higher proportion of immature stage-I melanosomes (Figure 8B).

In aggregate, these results are consistent with our hypothesis that TSC hypomelanotic macules occur as a result of secondary events leading to a near-complete loss of TSC1/TSC2 during a melanoblast-to-melanocyte differentiation that results in clonal skin patches containing immature melanocytes with mTORC1 activation and reduced MITF-dependent pigment production.

The lack of distinguishable somatic mutations in the majority of the cultures suggests that hitherto unidentified epigenetic mechanisms may occur as a “second hit” in a significant fraction of hypomelanotic macules and thus result in monoallelic expression of mutant TSC2.

Discussion

TSC is a tumor-suppressor gene syndrome with diverse manifestations, many of which are tumors that occur in multiple organ systems including brain, skin, kidneys, heart, and lungs (1, 69). Although TSC tumors are typically histologically benign, their progressive growth in the brain, kidneys, or lungs can lead to severe consequences including death, if not managed properly. Historically, surgery has been used as the primary treatment approach for TSC tumors, but recent clinical trials have shown that rapalog therapy has clinical benefit in controlling the growth of TSC-related tumors (70–74).

Here, we present evidence that a functional TSC complex is required for normal pigment formation by melanocytes both in vitro and in vivo (modeled in Figure 9). We demonstrate that loss of the TSC protein complex, through loss of either of its constituent proteins, TSC1 or TSC2, leads to GSK3β activation, with a consequent loss of nuclear β-catenin and a marked reduction in MITF transcription and expression, resulting in loss of pigmentation. The conclusion that loss of TSC1 or TSC2 results in reduced pigmentation is supported by a recent clinical study, in which topical rapamycin treatment restored the pigmentation of TSC hypomelanotic macules (25). Our findings support the model that
second-hit loss of TSC1 or TSC2, corresponding to the germline mutation present in an individual with TSC, occurs in melanoblast lineage cells during embryonic development and leads to clonal patches of hypopigmented skin. This model is supported by the clinical observation that TSC hypomelanotic macules can be observed at birth through the use of UV illumination, which led to their use as a reliable and early marker of TSC for decades (22, 24, 75). This 2-hit mechanism is analogous to that underlying café-au-lait macules in neurofibromatosis, in which the melanocytes sustain 2 mutations in \( NF1 \) (76). The timing of the second-hit event leading to clonal patches in TSC may determine the size and shape of the hypopigmented skin, predicted to be earlier for the occasional large, segmental lesions and later for scattered small, confetti-like lesions. These patterns resemble the results of developmental studies in mice showing that single-cell–derived clonal melanocyte clusters occupy patches of skin in the mouse (49). Our results also suggest that, in melanocytes, TSC protein complex loss is accompanied by relatively little, if any, proliferative effect, consistent with the relative absence of TSC1/TSC2 mutations observed in melanoma genome–sequencing studies (77). Hence, these observations indicate that TSC complex loss does not lead to a proliferative advantage in all cell types, but rather that its effects are cell context dependent, helping explain the limited distribution and distinctive pathology of TSC-related tumors.

We were surprised to discover that only 1 of 7 hypomelanotic macule melanocyte cultures examined showed a second somatic mutation in \( TSC1 \) or \( TSC2 \). Four of six other patients’ cultures showed a single mutation in \( TSC2 \), which was consistent with the reduced expression of \( TSC2 \) by those cells. To examine whether an altered epigenetic status could explain the nearly completely extinguished expression of \( TSC2 \) in these cultures, we performed bisulfite treatment, followed by sequencing of the \( TSC2 \) CpG-rich promoter region, but found no evidence of CpG methylation. We suspect that long-range epigenetic or chromatin-mediated

Figure 7. mTORC1 is hyperactive in melanocytes isolated from TSC patients’ hypomelanotic macules. (A) Immunoblot analysis of primary melanocytes isolated from hypomelanotic macules (HMs) and nearby normal skin from TSC patients P35 and P38. Note the activation of mTORC1 in melanocytes from the hypomelanotic macules, with reduced MITF, PGC-1α, TYR, and β-catenin and increased GSK3β. (B) Pigmentation of the melanocytes analyzed in A. (C) Immunohistochemical staining of TSC hypomelanotic macules and nearby normal skin. Note that melanin was present in the basal layer of the epidermis of normal skin (faint brown stain) and was reduced in HM skin. MITF staining in HM melanocytes was also somewhat reduced. Normal skin melanocytes had higher expression of MelanA and reduced expression of p-S6 compared with HM skin melanocytes. Original magnification, ×200 and ×400 (insets). (D) Immunoblot analysis of HM and normal skin fibroblast cultures from the same TSC patients studied in A and B. Immunoblots show similar regulation of mTORC1 in both sets of cells in response to serum deprivation and addback. The immunoblots presented in the figure include replicate samples run on parallel gels.
Previous studies have explored the interaction between TSC1/TSC2, the Wnt signaling pathway, and GSK3β. β-Catenin levels have been reported to be high in kidney cystadenomas occurring in Tsc2+/– rat and mouse models (79, 80) as well as in human kidney angiomyolipomas and pulmonary lymphangioleiomyomatosis (LAM) (80, 81). All of these tumors have been shown to have 2-hit inactivation of Tsc2/TSC2 or Tsc1/TSC1. In vitro studies have changes on the intact WT allele of TSC2 (not the one bearing the germline mutation) had occurred and explain the lack of TSC2 expression by these cultures. Random monoallelic expression has been identified in a substantial proportion of human genes in different developmental contexts (78) and may occur in human melanocytes for TSC2. However, further investigations to prove this model will be required.
mTORC1 activation due to loss of the TSC protein complex, S6K may phosphorylate GSK3β at the same Ser9 site (86).

It is important to note that all studies examining pigmentation in this report were performed under chronic growth conditions, a minimum of 4 days, allowing development of long-term effects from knockdown or enhanced protein expression and/or drug exposure (e.g., rapamycin, CHIR-99021). Thus, under these chronic conditions, it is likely that there is an exchange of GSK3β within different cell compartments, contributing to the inhibition of β-catenin signaling downstream of AKT inactivation, as we have shown. Nonetheless, apart from the obvious cell lineage differences, it remains unclear why β-catenin expression is high in TSC-related kidney angiomyolipomas and pulmonary LAM and low in melanocytes from hypomelanotic macules, in which the same genetic lesion (loss of TSC1 or TSC2) has been sustained.

Some clues to this discrepancy may relate to the biology of MITF in melanocytes and its relationship to Wnt/β-catenin. In contrast to neural tissues, in which Wnt/β-catenin signaling is associated with proliferation and reduced differentiation, in the melanocyte lineage, this pathway supports both proliferation and differentiation through MITF (42). Indeed, the lineage commitment of early melanocytes from the neural crest requires Wnt-mediated β-catenin coactivation of MITF expression (60). However, the requirement for this signaling cascade during develop-

Figure 9. Model of interactions in mTOR signaling in normal and TSC melanocytes. (A) In normal melanocytes, there is physiological activation of PI3K and AKT and inactivation of the TSC complex (TSC1/TSC2), leading to mTORC1 activity, but retained AKT activity, which then leads to phosphorylation of GSK3β, reduced β-catenin phosphorylation, nuclear entry of β-catenin, activation of TCF/LEF1 enhancer activity, expression of MITF, and expression of MITF target genes, all of which lead to pigment formation. (B) In TSC hypomelanotic macule melanocytes, there is complete loss of either TSC1 or TSC2, leading to high levels of RHEB-GTP, mTORC1 activation, S6K1/2 activation, inactivation of AKT by several mechanisms, activation of GSK3β, phosphorylation of β-catenin, and reduced levels of nuclear β-catenin, leading to reduced transcription of MITF and reduced pigmentation. For both A and B, arrows indicate activating interactions, while solid lines indicate inhibitory interactions. Dotted lines indicate interactions attenuated in hypomelanotic macule melanocytes; green arrows indicate proteins with enhanced activity, and red arrows indicate those with reduced activity. P, phosphorylation; PRAS40, AKT1 substrate 1; mLST8, mTOR-associated protein, LST8 homolog; mSIN1, mammalian stress–activated protein kinase–interacting protein.
ment appears to be transient (87). Nonetheless, MITF expression remains important for pigment biosynthesis as well as survival of melanocytes after development, although MITF levels are modulated by the differentiation state and bioenergetic needs of the cell (53, 88). Combined with our observations, we speculate that heightened signaling through mTORC1/GSK3β may support a less differentiated phenotype in melanocytes with reduced MITF-regulated pigment biosynthetic pathway activity, while mTORC2/ AKT signaling may promote increased differentiation and pigment biosynthesis. This dual regulatory mechanism, with 1 branch of mTOR signaling (mTORC1) reducing pigment synthesis and the other (mTORC2) promoting it, has not, to our knowledge, been appreciated previously. An earlier study did report that high MITF expression enhances mTORC1 activity, suggesting that there may be a reciprocal regulatory relationship, such that high MITF levels promote mTORC1 activity, while mTORC1 inhibits MITF activity (89). However, these latter effects are mainly seen in cell types other than melanocytes, since melanocyte-specific MITF does not have the same Rag-binding sites as other MITF isoforms or the transcription factors TFE2 or TFE3 (89). In aggregate, these findings indicate that the TSC/mTORC1/AKT/GSK3β/β-catenin/MITF axis plays a central role in regulating melanogenesis in humans. Thus, interventions that enhance or diminish mTORC1 activity in melanocytes have a potentially broad role in the modification of pigmentation. Furthermore mTORC1 inhibitors may have a role in the treatment of the subset of malignant melanoma in which growth is dependent on MITF expression.

Methods

Subjects. Five of six subjects were patients with TSC who were seen at the NHLBI’s NIH Clinical Center. One patient with TSC provided samples from an off-site location. A diagnosis of TSC was based on the presence of at least 2 major or 1 major and 2 or more minor diagnostic features (2).

Cell culture. The human melanoma cell line SK-MEL-30 and HEK293T cells were obtained from the Broad Institute of Harvard and MIT (Cambridge, Massachusetts, USA) and the American Type Culture Collection (ATCC), respectively, and were cultured in high-glucose DMEM supplemented with 10% FBS and incubated at 37°C in 5% CO2. iHPSs transduced with pBABE-hygro-hTERT, pLNCX2-CDK4(R24C), and pBABE-puro-p3DD were generated as described previously (51) and cultured in Medium 254 (Invitrogen, Thermo Fisher Scientific). Lentiviruses were collected after 48 hours, filtered through 0.22 μm filters, and subsequently selected with puromycin (2 μg/ml) for 3 days.

To generate cells with stable expression of WT TSC2, P429S-mutant TSC2, MITF, WT β-catenin, and the CA form of β-catenin (S33Y), 293T cells were cotransfected with the corresponding cDNA constructs in pBabe, VSV-G, and pUMVC (plasmid 8449; Addgene) plasmids using Lipofectamine 2000. Retroviruses were harvested after 48 hours and sterile filtered. SK-MEL-30 cells and iHPSs expressing shTSC1 or shTSC2 were infected with retroviruses in the presence of polybrene (8 μg/ml) and subsequently selected with puromycin (8 μg/ml) for 3 days.

Plasmids. Retroviral IRES-hygro constructs expressing human TSC2 or a patient-derived TSC2 missense were designed previously (86). Retroviral pBMN-2 β-catenin WT and a CA form of β-catenin (S33Y) constructs were gifts of Ken Inoki (University of Michigan, Ann Arbor, Michigan, USA) (85). pLKO1 shRNA constructs against TSC1, TSC2, and GSK3β were purchased from GE Healthcare with the following sequences: shTSC1-1 (RHS3979-201793839), 5′-CCGGAAAAGAGAACAGTTGTCTCTCATCAGAGCATAAC-3′; shTSC2-1 (RHS3979-201768589), 5′-CCGGGCAAGGGTTCCTGGAAGCATAAC-3′; shRaptor (RHS3979-201792689), 5′-CCGGAAAGAAAGCAGGCTCACTTCTCATCAGAGCATAAC-3′; shRictor (RHS3979-200795893), 5′-AAACCCAGGCGTGGTCTGTTTTCATCTTTT-3′; shTSC2-1 (RHS3979-201768589), 5′-AAACCCAGGCGTGGTCTGTTTTCATCTTTT-3′; shTSC2-2 (RHS3979-200795893), 5′-AAACCCAGGCGTGGTCTGTTTTCATCTTTT-3′; shGSK3β-1 (RHS3979-201768854), 5′-AAACCCAGGCGTGGTCTGTTTTCATCTTTT-3′; shGSK3β-2 (RHS3979-201768852), 5′-AAACCCAGGCGTGGTCTGTTTTCATCTTTT-3′; and shGSK3β-3 (RHS3979-201742129), 5′-AAACCCAGGCGTGGTCTGTTTTCATCTTTT-3′. shRaptor and shRictor constructs were gifts of David Sabatini (MIT, Boston, Massachusetts, USA). Plasmids 1858 and 1853 were obtained from Addgene.

Real-time quantitative RT-PCR. Total RNA was isolated using TRIzol reagent (catalog 15596026; Invitrogen, Thermo Fisher Scientific) following the manufacturer’s instructions. cDNA was synthesized using 500 ng total RNA and the cDNA Synthesis Kit with SuperScript III RT (catalog 11752050; Life Technologies, Thermo Fisher Scientific) following the manufacturer’s instructions. cDNA was synthesized using 500 ng total RNA and the cDNA Synthesis Kit with SuperScript III RT (catalog 11752050; Life Technologies, Thermo Fisher Scientific) following the manufacturer’s instructions. cDNA was diluted 1:100 before quantification by real-time quantitative PCR (qPCR). The PCR reactions contained 10 μl 2× iQ SYBR Green Supermix (catalog 170-882; Bio-Rad), 0.25 μM of each primer, and 3 μl diluted cDNA. Reactions were processed for 1 cycle at 94°C for 3 minutes, then for 45 cycles at 94°C for 10 seconds, and for 40 seconds at 68°C using the Applied Biosystems Step One Plus instrument. The primers used in this study, including MITF-M, TYR, TYRPI, DCT, SOX10, and SILV (53) and TSC1 (91) were described previously. The primers used for TSC2 expression were as follows: forward, 5′-GAAGTGCAGGAAACACAAGATGGTCTCTTGT-3′; reverse, 5′-TGTGTCTCAGATGGTGTTTCTCGAGAGA-3′. Relative expression was measured using the 2^ΔΔCt method, and data are represented as fold levels over control.

Immunoblot analysis. Preparation of protein extracts and immunoblot analysis were performed as described previously (92). The following antibodies used in Western blotting were purchased from Cell Signaling Technology: Flag (product 2368); TSC1 (product 4906); TSC2 (product 4906).
Subcellular fractionations. Cytoplasmic and nuclear fractionations were isolated as described previously (92). β-Tubulin and Topo IIβ were used as controls for cytoplasmic and nuclear fractions, respectively.

IHC. Immunohistochemical studies were performed on paraffin-embedded skin biopsies from TSC patients’ hypomelanotic macules and adjacent normal-appearing skin. Primary antibodies used included anti-MITF (I:50; M362129-2; Dako); anti-p-S6 (Ser240/244) (I:100; 2215; Cell Signaling Technology); β-catenin (1:200; ab731; Abcam). MITF and p-S6 were detected using the Dako DAB Detection Kit, and MelanA was detected with the AEC Detection Kit (Dako EnVision+ System HRP). Costaining for p-S6 (Ser240/244) and β-catenin, and anti-MelanA (1:200; ab731; Abcam) was performed. All immunohistochemical studies were performed at the histopathology core of Brigham and Women’s Hospital.

Luciferase reporter assays. For luciferase reporter assays, SK-MEL-30 cells or iHPMs with shScrambled (shScr) or shTSC1 or shTSC2 were seeded in a 12-well plate in triplicate and transfected the following day using Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific). Each transfection contained 250 ng of the firefly luciferase reporter plasmid pGL3-MiFT-M or TK-FOP/TK-TOP and 12.5 ng pRl-CMV Renilla luciferase control, and the total DNA was adjusted to 2 μg with pcDNA3. Where indicated, rapamycin (20 nM; Figure 5, A, D and F; Figure 6, A and B; Supplemental Figure 5, B, E and G; Figure 6, C and D; Supplemental Figure 5, K–N) was added for 72 hours prior to measuring luciferase levels. The next day, cells were lysed in 200 μl of 1× passive lysis buffer (Promega), and luciferase levels were measured using a Dual-Glo Luciferase Kit (catalog E2920; Promega) or a GloMax 20/20 luminometer (Promega). Luciferase values were normalized to constitutive Renilla luciferase, and for the TK-FOP/TK-TOP experiments, values were normalized to pRL-TK (Promega) as described previously (42).

DNA isolation and next-generation sequencing. Genomic DNA was isolated from the fibroblasts and melanocytes cultured from the hypomelanotic macules and paired normal skin from TSC patients using a DNeasy Blood and Tissue Kit (catalog 69504; QIAGEN). Next-generation sequencing of the DNA samples from cultured fibroblasts and melanocytes for the mutational status of TSC1 and TSC2 was performed as described previously (21) (dbGaP phs001236.v1).

DNA methylation analysis. Genomic DNA extracted from primary melanocytes from hypomelanotic macules and paired normal skin was subjected to bisulfite treatment using the EpiTect Bisulfite Kit (catalog 59104; QIAGEN) according to the manufacturer’s instructions. The primers used for amplification of regions of the CpG island upstream of the human TSC2 locus were as follows: DMTSC2F, 5′-TTTGGTATAGGGGTTATAGAAT-3′; DMTSC2R, 5′-ACACTACRAAATCCRCCTCTC-3′; 1272F, TTGTGTTTATATG-GGTAGAGAGAGA; 1484R, ACTCCCTAAAATCTCAACTA-CAAC; 391F, GGATGATAGTTTGTAGGAGG; and 683R, AAATAAAGACTAAAATCAATACCC (corresponding chromosome [chr] 16:2047897-2048119, chr16: 2048260-2048472, and chr16: 2047379-2047646). PCR products were subjected to Sanger sequencing and compared with a control, universal methylated human DNA standard (catalog D5011; Zymo Research).

Melanin content measurement. Melanin content was measured as described previously (93). Briefly, the cells were harvested, pelleted, and washed in PBS. Half of the cells were used for protein amount determination. The other half was resuspended in 400 μl of 1 N NaOH solution and incubated at room temperature for 2 hours, and 100 μl of the solution was then loaded onto a 96-well plate. The melanin content was determined by measuring the absorbance of the solution between 405 and 490 nm and compared with a melanin standard (product M8631; Sigma-Aldrich). Melanin content was expressed as micrograms per milligram of protein.

TYR activity assays. TYR activity was determined as described previously (94). Briefly, cells were sonicated in protein lysis buffer. After protein quantification, 250 μg total protein lysate in a total volume of 100 μl lysis buffer was loaded onto a 96-well plate, followed by the addition of 100 μl of 1 mM L-DOPA (product D9628; Sigma-Aldrich). The plate was incubated at 37°C for 1 hour, and the absorbance at 475 nm was recorded. TYR activity was expressed as a percentage of the control.

TEM. Nonimmortalized primary melanocytes at passage 2 isolated from hypomelanotic macule biopsies and paired normal skin from patients with TSC were fixed in 2% glutaraldehyde/2% formaldehyde in cacodylate buffer, followed by 1% osmium tetroxide. The samples were embedded in epoxy resin, cut into 60- to 80-nm sections, and viewed with an FEI Tecnai 12 TEM operated at 80 kV and ×9,300 magnification (Harvard Medical School Electron Microscopy core facility). The mean of stage I–IV melanosomes was quantified in a minimum of 10 cells from each melanocyte culture and used to calculate the percentages of stage I–IV melanosomes as described previously (43).

Statistics. All data are presented as the mean ± SD from at least 3 independent experiments. A 2-tailed Student’s t test was used to compare group differences in all cases, and a P value of less than 0.05 was considered statistically significant.

Study approval. This study was approved by the IRB of the NHLBI (protocols 96–H-0100 and 00-H-0051). All subjects providing biopsy samples were aged 18 years or older and provided written informed consent.

Author contributions. JC designed the research studies, conducted experiments, acquired data, analyzed data, and wrote the manuscript. MET conducted experiments, acquired data, analyzed data, and reviewed the manuscript. JM provided samples from the TSC patients seen at the NIH and reviewed the manuscript. TND acquired biopsy samples from the patients seen at the NIH and reviewed the data and manuscript. HRW helped design the studies, contributed many reagents, acquired, analyzed, and reviewed the data, and helped
write the manuscript. DJK designed the research studies, analyzed data, and wrote the manuscript.

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