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Title page:[Human Mutation submission]

Functional assessment of variants in the *TSC1* and *TSC2* genes identified in individuals with Tuberous Sclerosis Complex

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Running title: Functional assessment of TSC1 and TSC2 variants

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Abstract

The effects of missense changes and small in-frame deletions and insertions on protein function are not easy to predict and the identification of such variants in individuals at risk of a genetic disease can complicate genetic counselling. One option is to perform functional tests to assess whether the variants affect protein function. We have used this strategy to characterise variants identified in the *TSC1* and *TSC2* genes in individuals with, or suspected of having, Tuberous Sclerosis Complex (TSC). Here we present an overview of our functional studies on 45 TSC1 and 107 TSC2 variants. Using a standardised protocol we classified 16 TSC1 variants and 70 TSC2 variants as pathogenic. In addition we identified 8 putative splice site mutations (5 *TSC1* and 3 *TSC2*). The remaining 24 TSC1 and 34 TSC2 variants were classified as probably neutral.

Keywords: tuberous sclerosis complex, TSC1, TSC2, unclassified variants

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Introduction

Tuberous Sclerosis Complex (TSC) is an autosomal dominant disorder characterised by the development of hamartomas in a variety of organs and tissues, most notably the brain, skin and kidneys [Gomez et al., 1999]. Most individuals with TSC have epilepsy and many suffer from cognitive impairments and/or autism-spectrum disorders. Mutations in either the *TSC1* gene on chromosome 9q34 (OMIM #605284) [van Slegtenhorst et al., 1997], or the *TSC2* gene on chromosome 16p13.3 (OMIM #191092) [European Chromosome 16 Tuberous Sclerosis Consortium, 1993] cause TSC and comprehensive mutation screens in TSC patients have identified a wide variety of pathogenic mutations [Jones et al., 1999; Niida et al., 1999; Dabora et al., 2001; Sancak et al., 2005; Au et al., 2007]. Most *TSC1* and *TSC2* mutations result in premature termination of the respective open-reading frame and complete inactivation of the mutated allele. However, approximately 25% of the mutations identified in the *TSC2* gene and 1% of the mutations identified in *TSC1*, are non-terminating missense changes or small in-frame deletions or insertions [Sancak et al., 2005]. The consequences of such non-terminating changes are not easy to predict with certainty, making it difficult to distinguish disease-causing mutations from neutral (non-pathogenic) variants. In a cohort of 490 putative TSC cases, we identified 29 variants (6% of the total) that could not be classified as either pathogenic or neutral from the available clinical and genetic data [Sancak et al., 2005]. In such cases, *in vitro* functional comparisons between the wild-type and variant proteins can help determine whether an unclassified variant (UV) is pathogenic or not [Nellist et al., 2008].

The *TSC1* and *TSC2* gene products, TSC1 and TSC2, interact to form a protein complex that integrates multiple growth factor- and energy-dependent signals to help

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control cell growth [Inoki and Guan, 2009]. The N-terminal region of TSC2 is required for binding TSC1 [Li et al., 2004], while the TSC2 C-terminal region contains the active site of the complex, an "asparagine-thumb" GTPase activating protein (GAP) domain [Daumke et al., 2004]. The TSC1-TSC2 complex stimulates the GTPase activity of RHEB to promote the conversion of active, GTP-bound RHEB to inactive RHEB-GDP and thereby prevent the RHEB-GTP-dependent stimulation of the mammalian target of rapamycin (mTOR) complex 1 (TORC1) [Li et al., 2003]. In cells lacking either *TSC1* or *TSC2*, the downstream targets of TORC1, including elongation factor 4E binding protein 1 (4E-BP1), p70 S6 kinase (S6K) and ribosomal protein S6, are constitutively phosphorylated [Zhang et al., 2003; Kwiatkowski et al., 2001].

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The effects of specific amino acid changes on TSC1-TSC2 complex formation, on the activation of RHEB GTPase activity by the complex, and on the phosphorylation status of 4E-BP1, S6K and S6, have been investigated [Nellist et al., 2001; Inoki et al. 2002, Tee et al, 2002]. Previously, we used immunoblotting, double-label immunofluorescent microscopy, in-cell Western analysis and GAP assays to study the effects of 47 *TSC2* missense and in-frame insertions/deletions and 26 *TSC1* missense and in-frame insertions/deletions on TSC1-TSC2 activity [Nellist et al., 2005; Jansen et al., 2006, Jansen et al., 2008, Nellist et al., 2008, Nellist et al., 2009; Coevoets et al., 2009; Mozaffari et al., 2009]. Pathogenic missense changes in the N-terminal region of TSC1 (amino acids 50 - 224) reduced TSC1 stability [Nellist et al., 2009; Mozaffari et al., 2009], while pathogenic TSC2 missense changes had distinct effects on the TSC1-TSC2 complex, depending on the region of TSC2 that was affected. Some TSC2 amino acid substitutions prevented TSC1-TSC2 complex formation while others did not affect TSC1-TSC2 binding, but still inactivated the complex [Nellist et al., 2005]. Here we compare 73 previously tested variants to 79 new variants (19 *TSC1* and 60 *TSC2*) using

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a standardised protocol. To assess the effects of the variants on both the formation and activity of the TSC1-TSC2 complex we decided to use immunoblotting followed by infra-red scanner-based detection. In our experience, double-label microscopy and GAP assays were too labour-intensive and/or unreliable for routine use and, although the in-cell Western was a simple and reliable assay [Coevoets et al., 2009], it required large amounts of (expensive) antibodies and did not provide information on the TSC1-TSC2 interaction.

Our aim was to investigate the reliability of the assay, the degree of concordance between the *in vitro* results and computer-based predictive methods and, ultimately, to use the results of the functional assessment to determine whether variants identified in individuals with, or suspected of having, TSC were pathogenic. To assess the reliability of our functional studies we compared our results to the classifications of the variants listed in the *TSC1* and *TSC2* Leiden Open Variation Databases (LOVD) (http://www.lovd.nl/TSC1; http://www.lovd.nl/TSC2). Furthermore, we hoped to gain insight into the structural properties of TSC1 and TSC2 by comparing the properties of the different variants.

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Materials and Methods

TSC1 and TSC2 variants

The relative positions of the variants selected for functional assessment are shown in Figure 1. All variants are listed in Supporting Tables S1, S2 and S3. The variants were compared to wild-type TSC1 (Genbank AF013168.1; GI: 2331280) and TSC2 (Genbank X75621; GI:450351), as originally described [van Slegtenhorst et al., 1997; European Chromosome 16 Tuberous Sclerosis Consortium, 1993] and it is

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therefore important to note that the TSC2 wild-type lacks the amino acids encoded by the alternatively spliced exon 31. The amino acid numbering according to the *TSC2* LOVD (http://www.lovd.nl/TSC2), where this differs from the original numbering due to the inclusion of exon 31, is given in Supporting Table S3.

Nucleotide numbering reflects the cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1.

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Constructs and antisera

Expression constructs encoding the TSC1 and TSC2 variants were derived from the wild-type expression constructs [Nellist et al., 2005] using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, U.S.A.). In each case the complete open reading frame of the mutated construct was verified by sequence analysis. DNA was prepared using the Plasmid Plus Midi Purification kit (Qiagen, Venlo, The Netherlands). In most cases, multiple clones were isolated, verified and used for the assays described below. Other constructs used in this study have been described previously [Nellist et al., 2005]. Antibodies were purchased from Cell Signaling Technology (Danvers, U.S.A.) (1A5, anti T389 phospho-S6K mouse monoclonal; 9B11, anti-myc tag mouse monoclonal; anti-myc tag rabbit polyclonal), Li-Cor Biosciences (Lincoln, U.S.A.) (goat anti-rabbit 680 nm and goat anti-mouse 800 nm conjugates) or DAKO (Glostrup, Denmark) (cyanine (Cy2)-coupled secondary antibodies against mouse immunoglobulins), or have been described previously [van Slegtenhorst et al., 1998].

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Transfection-based immunoblot assay for functional assessment of TSC1 and TSC2 variants

TSC1 and TSC2 variants were assayed using essentially the same protocol. HEK 293T cells were seeded into 24-well plates and grown overnight in Dulbecco's modified Eagle medium (DMEM) (Lonza, Verviers, Belgium) supplemented with 10% foetal bovine serum, 50 U/ml penicillin and 50 µg/ml streptomycin in a 10% carbon dioxide humidified incubator. Cells at 80 - 90% confluency were transfected with 0.2 µg TSC2 expression construct, 0.4 µg TSC1 expression construct and 0.1 µg S6K expression construct using 2.1 µg polyethyleneimine (Polysciences Inc., Warrington, U.S.A.) in DMEM, as described previously [Coevoets et al., 2009]. For control transfections, pcDNA3 vector DNA was added to make a total of 0.7 µg DNA per transfection. Cells expressing the TSC2 variants were compared to cells expressing wild-type TSC1-TSC2, a known pathogenic variant (TSC2-R611Q [Nellist et al., 2005]), TSC1 and S6K only (no TSC2), and cells transfected with vector DNA only (control). Cells expressing the TSC1 variants were compared to wild-type TSC1-TSC2, a known pathogenic variant (TSC1-L117P [Nellist et al., 2009]), TSC2 and S6K only (no TSC1), TSC1 and S6K only (no TSC2) and cells transfected with vector DNA only (control).

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After 4 hours the transfection mixtures were replaced with DMEM supplemented with 10% foetal bovine serum, 50 U/ml penicillin and 50 µg/ml streptomycin. Twenty-four hours after transfection the cells were transferred to ice, washed with phosphate-buffered saline (PBS) (4°C) and harvested in 75 µl lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 50 mM NaF and 1% Triton X100, containing a protease inhibitor cocktail (Complete, Roche Molecular Biochemicals, Woerden, The Netherlands)). After centrifugation (10 000 g for 10 minutes at 4°C), the supernatant fractions were recovered, diluted in loading buffer and incubated at 96°C for 5 minutes

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prior to electrophoresis on Criterion™ 4-12% SDS-PAGE gradient gels (Bio-Rad, Hercules, U.S.A.). Proteins were transferred to nitrocellulose membranes according to the manufacturer's recommendations.

Blots were blocked for 1 hour at room temperature with 5% low-fat milk powder (Campina Melkunie, Eindhoven, The Netherlands) in PBS and incubated overnight at 4°C with the following primary antibodies: 1/15 000 dilution of 1895 (rabbit polyclonal against TSC2 [van Slegtenhorst et al., 1998]), 1/5 000 dilution of 2197 (rabbit polyclonal against TSC1 [van Slegtenhorst et al., 1998]), 1/5 000 dilution of a rabbit polyclonal against the myc epitope tag and 1/2 000 dilution of 1A5 (mouse monoclonal against p70 S6 kinase (S6K) phosphorylated at amino acid T389). Antibodies were diluted in PBS containing 0.1 % Tween 20 (PBST) (Sigma-Aldrich Fine Chemicals, Poole, U.K). After washing 3 times for 5 minutes in PBST, the blots were incubated for 1 hour at room temperature in the dark in PBST containing 1/10 000 dilutions of goat anti-rabbit 680 nm and goat anti-mouse 800 nm secondary antibodies. After washing 3 times for 5 minutes in PBST and once in PBS, the blots were scanned using the Odyssey™ Infrared Imager (Li-Cor Biosciences) at default intensity, medium quality, 169 µm resolution with 0 mm focus offset. To estimate the expression levels of the different proteins and the ratio of T389-phosphorylated S6K to total S6K, in the presence of the different TSC1 and TSC2 variants, the scans were analysed using the Odyssey quantification software. The integrated intensities of the protein bands were determined using default settings with the 3 pixel width border mean average background correction method. To correct for the detection of endogenous proteins, we subtracted the signals detected in control cells (transfected with vector DNA only) from the corresponding TSC2, TSC1, S6K and T389-phosphorylated S6K signals for all variants.

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Immunofluorescent detection of TSC1 variants

Immunofluorescent detection of TSC1 variants was performed as described previously [Mozaffari et al., 2009], using a Leica DM RXA microscope and Image Pro-Plus version 6 image analysis software.

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Prediction analysis

To investigate whether the *TSC1* and *TSC2* nucleotide changes might affect RNA splicing, 3 different splice-site prediction programs were used: www.cbs.dtu.dk/services/NetGene2, www.genet.sickkids.on.ca/~ali/splicesitefinder.html, and www.fruitfly.org/seq_tools/splice.html.

To investigate whether the TSC1 and TSC2 amino acid substitutions were likely to affect TSC1-TSC2 structure and function, the Sorting Intolerant From Tolerant (SIFT) algorithm was used [Ng and Henikoff, 2006]. SIFT scores were calculated using multiple sequence alignments of TSC1 and TSC2 from 16 different species (human, chimpanzee, maccaca, cow, dog, horse, mouse, rat (TSC1 only), chicken, zebrafish (TSC2 only), pufferfish, honey bee (TSC1 only), fruitfly, mosquito, methylotrophic yeast (TSC2 only) and fission yeast).

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Results

Immunoblot analysis

We compared 152 variants (45 TSC1 and 107 TSC2), including 79 previously untested variants (19 TSC1 and 60 TSC2). Each variant was tested in at least 4 independent transfection experiments. In each experiment, the integrated intensities of

the bands on the immunoblot corresponding to TSC2, TSC1, total S6K and T389-phosphorylated S6K were determined for each variant, relative to the integrated intensities of the bands for wild-type TSC1-TSC2. Examples of immunoblots of 13 TSC1 variants and 15 TSC2 variants are shown in Supporting Figures S1 and S2, respectively. The mean values for the TSC2, TSC1 and S6K signals and the mean T389/S6K ratios (see below) for all the variants tested are shown in Figures 2, 3 and 4.

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First, to determine whether the transfection efficiencies for the different variant constructs were approximately equal, the total S6K signal for each variant was compared to the total S6K signal for wild-type TSC1-TSC2. As shown in Figures 2D, 3D and 4D, the S6K signals were relatively constant for all the variants and controls, indicating that the transfection efficiencies were comparable between the different variants. For most variants we tested multiple DNA preparations. We did not observe large differences between the replicate DNA preparations, indicating that the quality of the different transfected DNAs was uniform (Supporting Figure S3). Furthermore, altering the amount of DNA used for transfection resulted in relatively small changes in the signals detected on the blots, indicating that the assay was robust with respect to the amount of transfected DNA (Supporting Figure S4).

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Next, the ratio of the T389-phosphorylated S6K signal to the total S6K signal was determined for each variant, relative to wild-type TSC1-TSC2. We referred to this as the T389/S6K ratio. The higher the T389/S6K ratio, the higher the estimated activity of TORC1, and therefore the lower the activity of the exogenously expressed TSC1 and TSC2. The mean T389/S6K ratios for the TSC1 and TSC2 variants are shown in Figures 2C, 3C and 4C. To determine whether the T389/S6K ratios for the variants were significantly increased relative to wild-type TSC1-TSC2, we performed Student's t-tests. This was the primary criterium for deciding whether a variant was disease-

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causing. We classified the variants with a significantly higher T389/S6K ratio than wild-type TSC1-TSC2 as pathogenic (p value < 0.05). Sixteen TSC1 variants (36%; see Supporting Table S1) and 70 TSC2 variants (65%; see Supporting Tables S2 and S3) were classified as pathogenic. For variants where the T389/S6K ratio was not significantly higher than wild-type TSC1-TSC2 (p > 0.05), we compared the T389/S6K ratio to the pathogenic variant that was included in each transfection experiment (TSC2 R611Q for TSC2 variants; TSC1 L117P for TSC1 variants). All the variants that did not have a significantly higher T389/S6K ratio than wild-type TSC1-TSC2, had a significantly lower T389/S6K ratio than the corresponding pathogenic variant, and were therefore considered to be active in our assay. We classified these variants as probably neutral.

In addition to the T389/S6K ratio, we compared the signals of the TSC1 and TSC2 variants to the corresponding signals for the wild-type proteins (Figures 2B, 3A and 4A). Variant signals significantly less than that of the wild-type indicated that the steady state expression of the variant was reduced, either because the variant mRNA was less stable or translated less efficiently than the wild-type mRNA, or that the variant protein was unstable. Therefore, although we could not distinguish between effects on mRNA or protein stability, we classified variants with a significantly reduced signal (p<0.05, paired t-test) as unstable (Supporting Tables S1, S2 and S3). We identified 16 unstable TSC1 variants and 38 unstable TSC2 variants. All of the unstable TSC1 variants had been classified as pathogenic, according to the comparisons between the variant and wild-type T389/S6K ratios. Furthermore, consistent with a previous study [Mozaffari et al., 2009], all the unstable TSC1 variants gave a diffuse cytoplasmic signal as detected by immunofluorescent microscopy, in contrast to wild-type TSC1 and the stable TSC1 variants that all gave a characteristic punctate localisation pattern (data

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Deleted: If the signal for the wild-type binding partner was significantly reduced compared to the corresponding signal for the wild-type complex then it was evidence that the variant was unable to stabilise the TSC1-TSC2 complex. We compared the signal for each variant with the corresponding signal for the wild-type protein (Figures 4B, 5A and 6A). We classified variants with a significantly lower mean signal than the corresponding wild-type protein (p < 0.05, paired t-test) as unstable (

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not shown). Of the unstable TSC2 variants, 35/38 had been defined as pathogenic according to the comparisons of the T389/S6K ratios. In the 3 remaining cases (TSC2 R1159L, TSC2 R1159Q and TSC2 R1772C), the variant inhibited S6K T389-phosphorylation as effectively as wild-type TSC2, despite being detected at lower levels (Figure 4A and C; see Discussion).

Finally, we compared the signals for wild-type TSC1 in the presence of the different TSC2 variants (Figures 3B and 4B), and the signals for wild-type TSC2 in the presence of the different TSC1 variants (Figure 2A). Coexpression of TSC2 stabilises TSC1 [Nellist et al., 1999]. Therefore we classified TSC2 variants that were associated with a significantly reduced TSC1 signal ($p < 0.05$, paired t-test) and TSC1 variants that were associated with a significantly reduced TSC2 signal ($p < 0.05$, paired t-test) as destabilising. Three TSC1 variants, L93R, N198F199delinsI and M224R, were associated with a significant reduction in the TSC2 signal (Figure 2A). In each case we had classified the variant as pathogenic due to a significantly increased T389/S6K ratio compared to wild-type TSC1 (Supporting Table S1). Thirty-eight TSC2 variants were associated with a significant reduction in the TSC1 signal (Figures 3B and 4B). We divided these variants into 2 groups. One group of 31 variants clustered within the N-terminal half of TSC2 (amino acids 98 - 897), while a smaller group of 7 variants clustered close to the C-terminus (amino acids 1525 - 1773). In all 38 cases we had classified the variant as pathogenic due to a significantly increased T389/S6K ratio compared to wild-type TSC1-TSC2 (Supporting Tables S2 and S3).

Prediction Analysis

We investigated the possibility that the nucleotide changes corresponding to variants that did not affect TSC1-TSC2 function in our assay, disrupted *TSC1* or *TSC2*

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splicing. We analysed the *TSC1* and *TSC2* nucleotide changes using 3 different splice-site prediction programs and identified 8 variants (5 *TSC1* and 3 *TSC2*) that were predicted to cause splicing abnormalities (Supporting Tables S1 and S2). In one case (*TSC2* c.1235A>T), this was confirmed by functional assessment of the predicted TSC2 splice variant, TSC2 412del8 (Figure 3, Supporting Figure S2 and Supporting Table S2; [Jansen et al., 2008]).

Next, we investigated whether the functional assessments of the TSC1 and TSC2 variants were in agreement with the Sorting Intolerant From Tolerant (SIFT) amino acid substitution prediction method [Ng and Henikoff, 2006]. The SIFT algorithm combines information from the chemical structure of the individual amino acids and the evolutionary conservation of a protein to predict whether specific substitutions can be tolerated by the protein.

SIFT analysis of TSC1 indicated that the N-terminal (amino acids 1 - 300) and C-terminal (amino acids 700 - 1164) regions were relatively intolerant to amino acid changes [Mozaffari et al., 2009]. All the TSC1 variants classified as pathogenic according to our functional assessment clustered within the substitution intolerant N-terminal region (Supporting Table S1). SIFT predicted that 18/41 (44%) of the TSC1 missense changes would not be tolerated. In 36 cases (88%), the SIFT prediction was consistent with the functional assessment. In 5 cases, R190C, R246K, R246T, H732Y and R1097H, SIFT predicted that the substitution would not be tolerated, while the functional assessment indicated that the amino acid changes were probably neutral. In the case of the R246K and R246T substitutions, splice site prediction analysis indicated that the corresponding nucleotide changes (c.737G>A and c.737G>C) were likely to cause splicing errors. Similarly, although the K121R, G305R and G305W substitutions were tolerated by the SIFT analysis and were probably neutral according to the

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functional assessment, splice site analysis predicted that the corresponding nucleotide changes were splice site mutations. The TSC1 H732Y variant has been identified in individuals without TSC (http://www.lovd.nl/TSC1) and is unlikely to cause TSC, consistent with the results of our functional assessment (Figure 2, Supporting Figure S1, and Supporting Table S1). However, this variant is associated with focal cortical dysplasia type II [Lugnier et al., 2009]. Therefore, the SIFT result may still be accurate, even though the H732Y substitution is not critical for TSC1 function in our assay. Due to reduced amino acid sequence conservation at the C-terminus of TSC1, the SIFT prediction for the TSC1 R1097H substitution was made with a low degree of confidence, and is therefore not reliable (data not shown).

SIFT analysis of TSC2 indicated that amino acids 1 - 900 and 1500 - 1807, corresponding to the TSC1-binding domain and the GAP domain respectively, were intolerant of amino acid substitutions (Supporting Figure S5). In total, the SIFT prediction and functional assessment were consistent for 77 TSC2 variants (80%)(Supporting Tables S2 and S3). SIFT tolerated 42/96 (44%) of the TSC2 amino acid substitutions subjected to functional testing. Fourteen (31%) of these tolerated changes were classified as pathogenic according to the functional assessment. In addition, splice site analysis predicted that the *TSC2* c.1118A>C (p.Q373P) variant that was tolerated by SIFT and classified as neutral according to the functional assessment, was likely to be a splice site mutation. Of the 54 substitutions that were not tolerated by SIFT, 5 were classified as neutral according to the functional assessment. For 2 of these, *TSC2* c.1235A>T (p.E412V) and *TSC2* c.1255C>T (p.P419S), splice site analysis predicted that the nucleotide changes would affect splicing.

In summary, using a transfection-based immunoblot assay we classified 16/45 TSC1 variants (36%) and 70/107 TSC2 variants (65%) as pathogenic; and 29/45

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TSC1variants (64%) and 37/107 TSC2 variants (32%) as probably neutral. In 8 cases, 5 TSC1 and 3 TSC2, the variant was neutral according to the functional assessment, but splice site analysis predicted that the corresponding nucleotide change was likely to be a pathogenic splice site mutation. Of the 79 previously untested variants, we classified 43 (54%) as pathogenic, including 5/19 TSC1 (26%) and 38/60 TSC2 variants (63%).

Discussion

Mutation analysis of individuals with, or suspected of having, a genetic disease facilitates the diagnosis, treatment and genetic counselling of those individuals and their families. However, in some cases it is not possible to determine from the genetic data whether an identified nucleotide change is disease-causing. Functional analysis of predicted protein variants provides an additional method for determining whether specific changes are pathogenic.

We have characterised the effects of 152 TSC1 and TSC2 amino acid substitutions and small in-frame insertions/deletions on the TSC1-TSC2 complex. Based on our functional assessment we classified 86 (56%) of the changes as pathogenic. In 8 (5%) cases where we did not detect an effect on protein function, the corresponding nucleotide change was predicted to cause splicing defects. In one case (*TSC2* 1235A>T), we confirmed that the splicing defect resulted in the production of an inactive TSC2 variant [Janssen et al., 2008]. In the remaining 7 cases, additional RNA studies are required to confirm the predicted effects on splicing.

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We tested 45 TSC1 variants, of which 42 were amino acid substitutions and 3 were in-frame insertion/deletion changes. In 4 cases we analysed multiple changes at the same codon. Sixteen variants were detected at significantly reduced levels compared

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to wild-type TSC1. In each case, TORC1 activity, as estimated from the ratio of T389 phosphorylated S6K to total S6K, was increased compared to the wild-type control and we classified these variants as pathogenic. Consistent with previous studies [Nellist et al., 2009, Mozaffari et al., 2009], all the variants that were detected at low levels by immunoblotting mapped to the TSC1 N-terminal region (amino acids 50 - 224) and had a distinct cytoplasmic localisation pattern compared to wild-type TSC1, confirming the importance of this region for TSC1 function, localisation and stability [Hoogeveen-Westerveld et al., 2010].

We tested 104 TSC2 variants, of which 95 were amino acid substitutions, 3 were small in-frame insertions (1 - 3 amino acids) and 6 were in-frame deletions (1 - 26 amino acids). In 12 cases we analysed multiple changes at the same codon. Fifty-five changes affected the N-terminal half of the protein (amino acids 1 - 900), and 52 affected the C-terminal region (amino acids 901 - 1784). Twenty (36%) of the changes in the N-terminal region were unstable, and 31 (56%) were associated with significantly reduced levels of TSC1, consistent with an important role for the TSC2 N-terminal region in binding and stabilising TSC1 [Li et al., 2004]. Eighteen (35%) of the changes in the C-terminal region were unstable and 7 (13%) were associated with significantly reduced levels of TSC1. The 7 C-terminal variants associated with reduced levels of TSC1 mapped between amino acids 1525 and 1729, suggesting that this region may also play a role in maintaining stable TSC1-TSC2 complexes.

For 3 TSC2 variants, R1159L, R1159Q and R1772C, the T389/S6K ratio was not significantly different from wild-type TSC1-TSC2, despite the fact that the TSC2 signals for these variants were reduced compared to wild-type TSC2. In a previous study we concluded that the R1772C variant was unlikely to be pathogenic because an individual with this variant and TSC had another *TSC2* mutation [Nellist et al., 2008].

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Furthermore, individuals with this variant and no signs of TSC have been identified (http://www.lovd.nl/TSC2). Therefore, we classified the TSC2 R1772C variant as probably neutral (Supporting Table S3). The R1159L and R1159Q variants were also classified as probably neutral because, despite the reduced signals, they were both able to inhibit S6K T389 phosphorylation as effectively as wild-type TSC1-TSC2. In the *TSC2* LOVD, the R1159L variant is classified as unknown, while the R1159Q variant is classified as having no known pathogenicity because it was identified in a TSC patient with another mutation (http://www.lovd.nl/TSC2). Nevertheless, *in vivo* it is possible that the reduced stability of the R1159L, R1159Q and R1772C variants renders them less effective at inhibiting TORC1 activity. It would be useful to have additional assays to characterise these variants in more detail.

We compared our functional assessment with the predictions of the SIFT algorithm [Ng and Henikoff, 2006] and with the classifications listed in the *TSC1* and *TSC2* LOVD (http://www.lovd.nl/TSC1 and http://www.lovd.nl/TSC2) (Supporting Tables S1, S2 and S3). Overall, there was broad agreement (>80%) between the SIFT predictions and our functional assessments for both the TSC1 and TSC2 variants. Nevertheless, there were sufficient exceptions to indicate that the results of SIFT and similar prediction algorithms should be treated with caution. In general there was also good agreement between the LOVD classifications and our functional assessment. According to the LOVD classifications, 29 variants had no known pathogenicity or were probably not pathogenic. We classified 27 (93%) of these as probably neutral. There was strong disagreement for only one TSC1 variant (F216S) and one TSC2 variant (F615S). The TSC1 F216S variant was classified as probably not pathogenic in the *TSC1* LOVD because it was identified in a TSC patient and their apparently unaffected parent, but is a pathogenic change according to our functional assessment.

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Clinical re-evaluation prompted by our findings, showed that the parent has signs of TSC, and therefore the *TSC1* c.647T>C (p.F216S) variant co-segregates with disease in this family. The status of the variant in the *TSC1* LOVD has now been updated to pathogenicity unknown, in line with current LOVD definitions. The TSC2 F615S substitution was classified as having no known pathogenicity in the *TSC2* LOVD, but is a pathogenic change according to our functional assessment, consistent with our previous studies [Nellist et al., 2001; Nellist et al., 2005]. The *TSC2* c.1844T>C (p.F615S) variant was identified in an individual with TSC and once in a control group of >100 apparently unaffected individuals [Gilbert et al., 1998]. However, as far as we are aware, the variant has not been detected in any other individuals. The status of this variant in the *TSC2* LOVD has now been updated to pathogenicity unknown.

Forty-nine of the variants tested were classified as pathogenic or probably pathogenic in the *TSC1* and *TSC2* LOVD. Forty-one (84%) of these were pathogenic according to our functional assessment. In 5 cases where the functional assessment indicated that the TSC1 or TSC2 variant was probably a neutral change, splice site analysis indicated that the corresponding nucleotide change was likely to be a splice site mutation. Therefore there was strong disagreement between the functional assessment and the LOVD classification for only 3 TSC2 variants, K599M, R951S and L1773I. The TSC2 K599M substitution is classified as pathogenic in the *TSC2* LOVD because it was reported to be a *de novo* change [Niida et al., 1999] that reduced the TSC2-dependent inhibition of 4E-BP1 phosphorylation *in vitro* [Tee et al., 2002]. However, consistent with our previous studies [Nellist et al., 2001; Nellist et al., 2005], in our assay, the K599M substitution did not affect TSC1 or TSC2 stability and inhibited S6K T389 phosphorylation as effectively as wild-type TSC2. The status of this variant in the *TSC2* LOVD has now been changed to pathogenicity unknown as it is not clear why

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there is a discrepancy between the different reports. The TSC2 R951S substitution was classified as probably pathogenic in the *TSC2* LOVD, but is probably neutral according to our functional assessment. No additional family data was available for this variant and therefore according to the current LOVD classification criteria, the variant should be classified as pathogenicity unknown. Codon 951 is encoded by the alternatively spliced exon 25 of the *TSC2* gene [Xu et al., 1995]. Very few pathogenic changes have been identified in this exon (http://www.lovd.nl/TSC2), and complete removal of this exon did not affect TSC2 function in our assay (M. Wentink, M. Nellist, unpublished observations). Finally, the TSC2 L1773I variant was classified as probably pathogenic in the *TSC2* LOVD, but is probably neutral according to our functional assessment. Although the *TSC2* c.5386C>A (p.L1773I) is listed as a *de novo* change, there is no record that paternity testing was performed, and it is possible that the *TSC2* c.5386C>A (p.L1773I) variant is a rare neutral variant.

Although in most cases it was clear whether a variant inhibited S6K T389 phosphorylation or not, for a few variants we noticed that there was more inter-experiment variation. Some variants appeared more effective at inhibiting S6K T389 phosphorylation in one experiment compared to the next. An example of this variation is shown in Supporting Figure S6. Furthermore, in some cases, the signal of the variant itself, or its binding partner, was quite variable, even though the transfection efficiency, as assessed by the S6K signal, was constant. For example, the mean signals for TSC2 in the presence of the TSC1 I76N and L93R variants are approximately equal (Figure 2A; Supporting Table S1). However, because the TSC2 signal showed more inter-experiment variation in the presence of the I76N variant, it was not significantly different from the wild-type signal, while the TSC2 signal in the presence of the L93R variant was significantly reduced compared to the wild-type. For this reason, it is

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possible that our assay missed some variants that are slightly less active or stable than wild-type TSC1 or TSC2. Therefore, although we could clearly distinguish variants that were less active than wild-type TSC1 and TSC2, it was more difficult to be certain that variants that were indistinguishable from wild-type in our assay were neutral variants, and not pathogenic. Nevertheless, we classified 29 TSC1 and 37 TSC2 variants as probably neutral because they were able to inhibit S6K T389 phosphorylation as effectively as wild-type TSC1-TSC2 in our assay. In the majority of cases these variants are most likely to be rare, neutral variants that do not cause TSC. However, we could not exclude the possibility that some variants have sufficient activity in our over-expression system to inhibit TORC1, while *in vivo* they may be inactive or unstable. Furthermore, we did not investigate any other TORC1-dependent or -independent effects of TSC1 or TSC2. Some variants might affect other TSC1-TSC2 functions than the TSC1-TSC2 interaction or TORC1 signalling to S6K. Additional analysis of the variants classified here as probably neutral is necessary to determine whether these variants affect other TSC1-TSC2 functions.

Despite these notes of caution, we conclude that our functional assessment has provided important insight into whether specific TSC1 and TSC2 variants are pathogenic. In summary, we classified 152 different TSC1 and TSC2 variants using a transfection-based immunoblot assay that distinguished pathogenic TSC1 and TSC2 variants from probable neutral variants according to whether there was increased S6K-T389 phosphorylation. Based on this functional assessment, 86 variants were classified as pathogenic and 67 as probably neutral, providing useful information for genetic counselling in the individuals carrying these variants.

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Figure 1: Overview of the TSC1 and TSC2 variants analysed as part of this study. Amino acid variants are numbered as originally described [van Slegtenhorst et al., 1997; European Chromosome 16 Tuberous Sclerosis Consortium, 1993] (Genbank AF013168.1; GI: 2331280 (TSC1) and X75621; GI:450351 (TSC2)) and according to the amino acid sequences encoded by the expression constructs used in this study. The initiation codon is codon 1.

(A) TSC1 variants. The positions of the TSC1 variants analysed as part of this study are indicated relative to the coding exons (3 - 23) of the *TSC1* gene.

(B) Variants mapping to the N-terminal half of TSC2 (amino acids 1 - 900). The positions of the variants are indicated relative to exons 1 - 23 of the *TSC2* gene.

(C) Variants mapping to the C-terminal half of TSC2 (amino acids 901 - 1784). The positions of the variants are indicated relative to exons 23 - 41 of the *TSC2* gene. The alternatively spliced exons 25 and 31 are shown in grey and the position of the GAP domain, corresponding to exon 37, is shown in black. The amino acids encoded by exon 31 were not present in the TSC2 expression constructs used in this study.

Figure 2: Functional assessment of the TSC1 variants. The signals for TSC2, TSC1, total S6K (S6K) and T389-phosphorylated S6K (T389) were determined per variant, relative to the wild-type control (TSC1) in at least 4 independent transfection experiments. The mean signals are shown for each variant. Error bars represent the standard error of the mean; variants that were significantly different from the wild-type control (TSC1) are indicated with an asterisk. The signals from cells expressing exogenous TSC2 and S6K only, and no exogenous TSC1, are also shown (TSC2).

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Figure 2: Immunoblot analysis of TSC1 variants. Cells were transfected with expression constructs encoding TSC2, S6K and wild-type TSC1 (TSC1/TSC2/S6K), TSC2, S6K and the pathogenic L117P variant (L117P), TSC2, S6K and the TSC1 variants, TSC2 and S6K only (TSC2/S6K), TSC1 and S6K only (TSC1/S6K) or with an empty vector (control) and analysed by immunoblotting. ¶
(A) Immunoblot showing signals for wild-type TSC2 (TSC2), wild-type TSC1 and the TSC1 variants (TSC1), total S6K (S6K) and T389-phosphorylated S6K (T389).¶
(B) Quantification of the TSC2 signals shown in (A), relative to the wild-type control (TSC1/TSC2/S6K).¶
(C) Quantification of the TSC1 signals shown in (A), relative to the wild-type control (TSC1/TSC2/S6K).¶
(D) Quantification of the total S6K signals shown in (A), relative to the wild-type control (TSC1/TSC2/S6K).¶
(E) Ratio of T389-phosphorylated S6K to total S6K in the presence of the TSC1 variants. The ratio of the signal for T389 phosphorylated S6K (T389) to the total S6K protein (S6K) was determined relative to the wild-type control (TSC1/TSC2/S6K; T389/S6K ratio = 1) for each variant shown in (A).¶
¶
Figure 3: Immunoblot analysis of TSC2 variants. Cells were transfected with expression constructs encoding TSC1, S6K and wild-type TSC2 (TSC2), TSC1, S6K and the pathogenic R611Q variant (R611Q), TSC1, S6K and the TSC2 variants, or with an empty vector (control), and analysed by immunoblotting. ¶
(A) Immunoblot showing signals for wild-type TSC2 and the TSC2 variants (TSC2), wild-type TSC1 (TSC1), total S6K (S6K) and T389-phosphorylated S6K (T389).¶
(B) Quantification of the TSC2 signals shown in (A), relative to the wild-type control (TSC2).¶
(C) Quantification of the TSC1 signals shown in (A), relative to the wild-type control (TSC2).¶
(D) Quantification of the total S6K signals shown in (A), relative to the wild-type control (TSC2).¶
(E) Ratio of T389-phosphorylated S6K to total S6K in the presence of the TSC2 variants. The ratio of the signal for T389 phosphorylated S6K (T389) to the total S6K protein (S6K) was determined relative to the wild-type control (TSC2; T389/S6K ratio = 1) for each variant shown in (A).¶

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(A) Mean TSC2 signals in the presence of the TSC1 variants, relative to wild-type TSC1-TSC2 (TSC1; TSC2 signal = 1).

(B) Mean signals for the TSC1 variants, relative to wild-type TSC1 (TSC1; TSC1 signal =1).

(C) Mean T389/S6K ratios for the TSC1 variants, relative to wild-type TSC1-TSC2 (TSC1; T389/S6K ratio = 1).

(D) Mean total S6K signals in the presence of the TSC1 variants, relative to wild-type TSC1-TSC2 (TSC1; S6K signal = 1).

Figure 3: Functional assessment of the TSC2 variants mapping to the N-terminal half of TSC2 (amino acids 1 - 900). The signals for TSC2, TSC1, total S6K (S6K) and T389-phosphorylated S6K (T389) were determined per variant, relative to the wild-type control (TSC2) in at least 4 independent transfection experiments. The mean signals are shown for each variant. Error bars represent the standard error of the mean; variants that were significantly different from the wild-type control (TSC2) are indicated with an asterisk.

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(A) Mean signals for the TSC2 variants, relative to wild-type TSC1-TSC2 (TSC2; TSC2 signal =1).

(B) Mean TSC1 signals in the presence of the TSC2 variants, relative to wild-type TSC1-TSC2 (TSC2; TSC1 signal = 1).

(C) Mean T389/S6K ratios for the TSC2 variants, relative to wild-type TSC1-TSC2 (TSC2; T389/S6K ratio = 1).

(D) Mean total S6K signals in the presence of the TSC2 variants, relative to wild-type TSC1-TSC2 (TSC2; S6K signal = 1).

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Figure 4: Functional assessment of the TSC2 variants mapping to the C-terminal half of TSC2 (amino acids 901 - 1784). The signals for TSC2, TSC1, total S6K (S6K) and T389-phosphorylated S6K (T389) were determined per variant, relative to the wild-type control (TSC2) in at least 4 independent transfection experiments. The mean signals are shown for each variant. Error bars represent the standard error of the mean; variants that were significantly different from the wild-type control (TSC2) are indicated with an asterisk.

(A) Mean signals for the TSC2 variants, relative to wild-type TSC2 (TSC2; TSC2 signal =1).

(B) Mean TSC1 signals in the presence of the TSC2 variants, relative to wild-type TSC1-TSC2 (TSC2; TSC1 signal = 1).

(C) Mean T389/S6K ratios for the TSC2 variants, relative to wild-type TSC1-TSC2 (TSC2; T389/S6K ratio = 1).

(D) Mean total S6K signals in the presence of the TSC2 variants, relative to wild-type TSC1-TSC2 (TSC2; S6K signal = 1).

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Figure 1

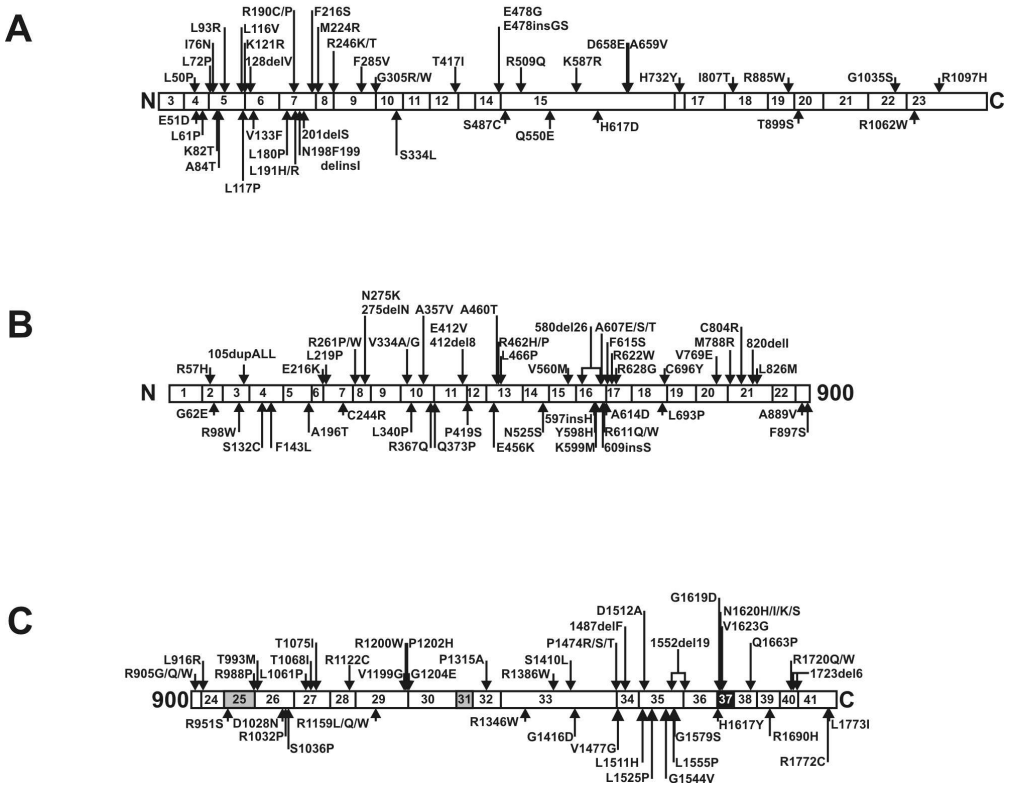

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Figure 2

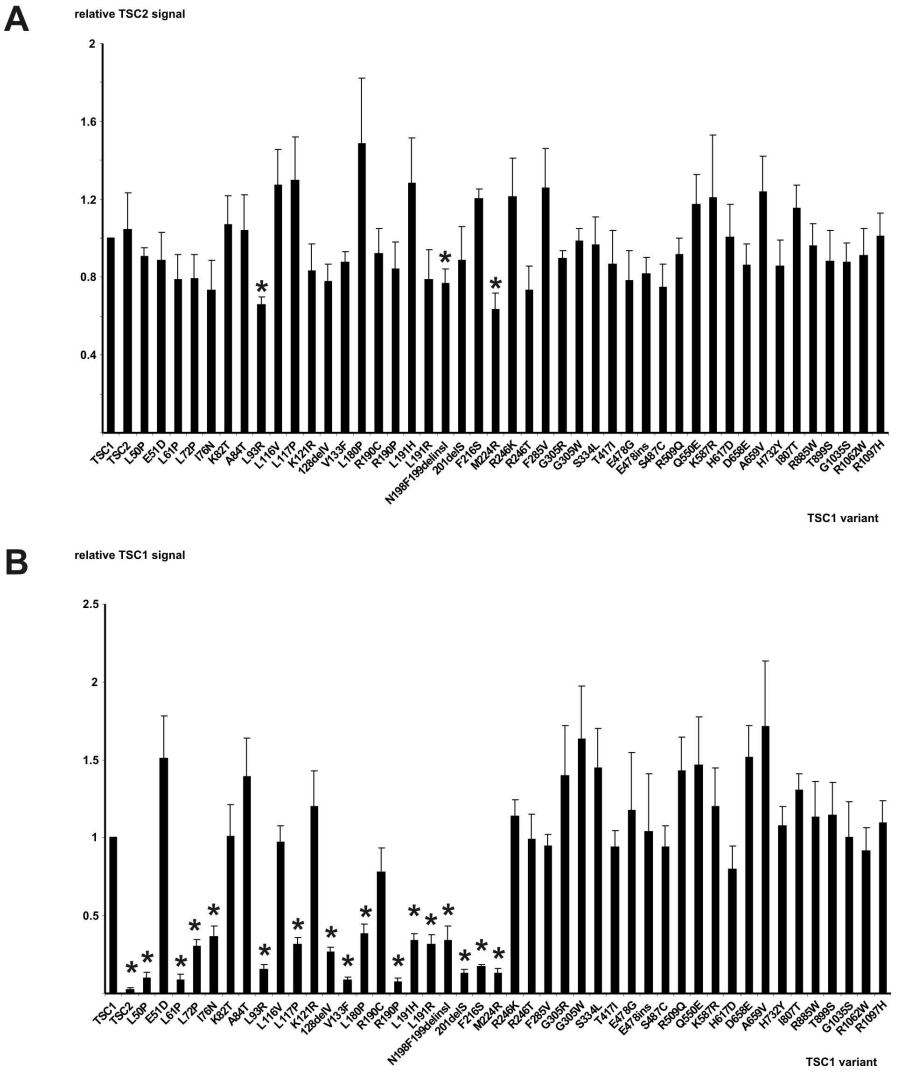

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Figure 2

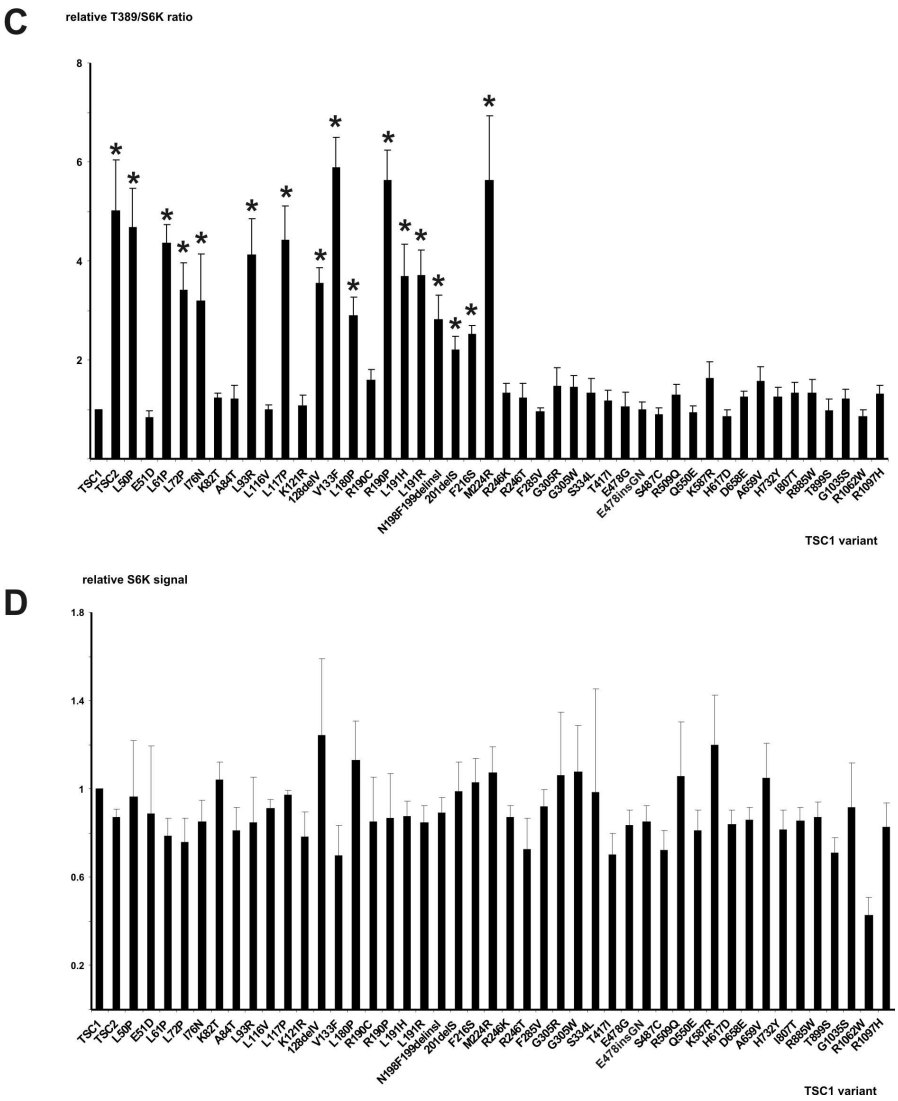

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Figure 3

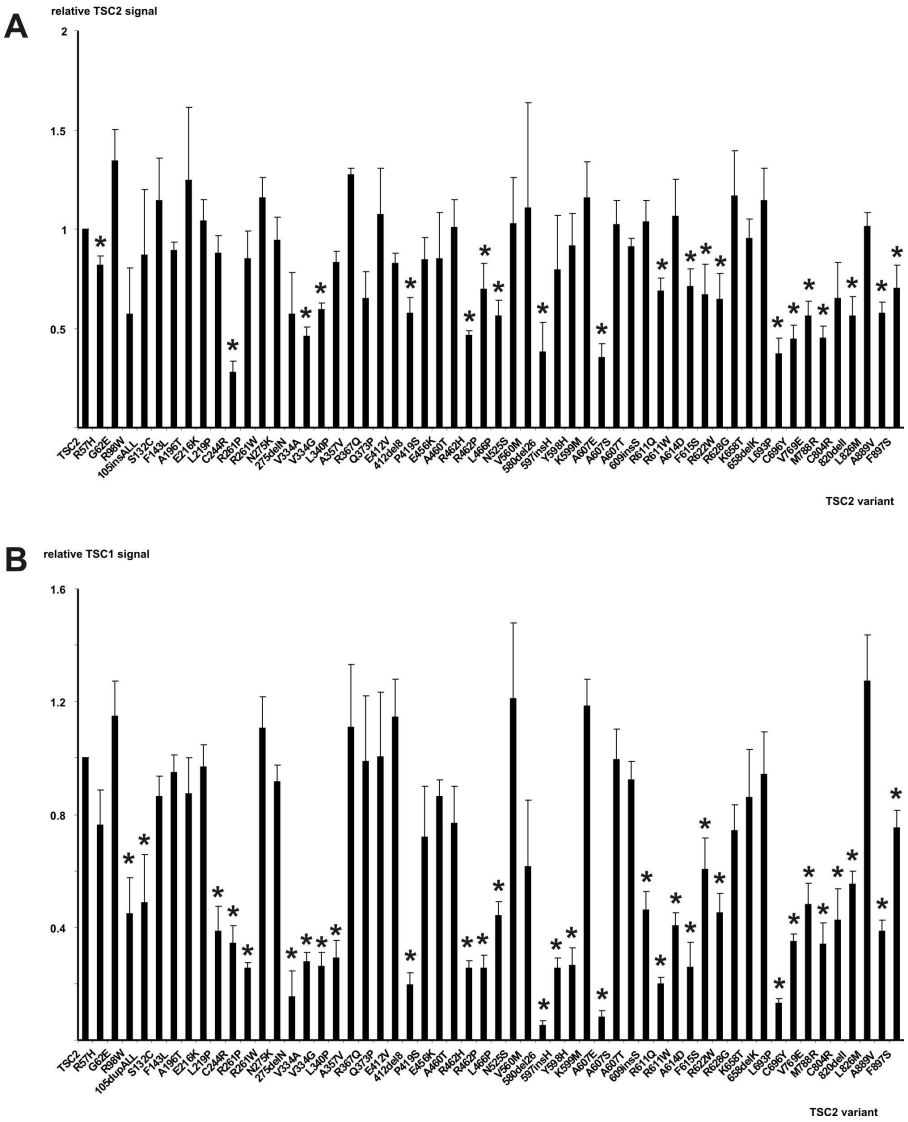

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Figure 3

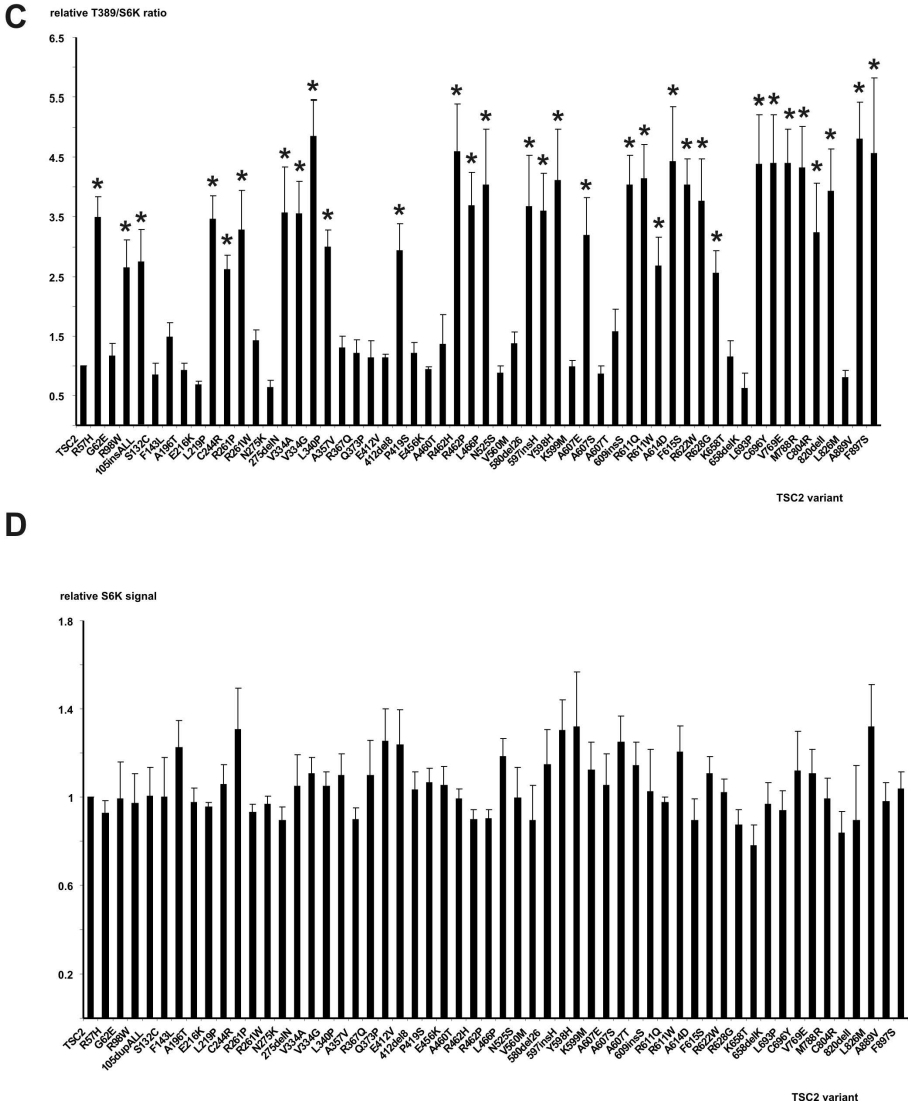

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Figure 4

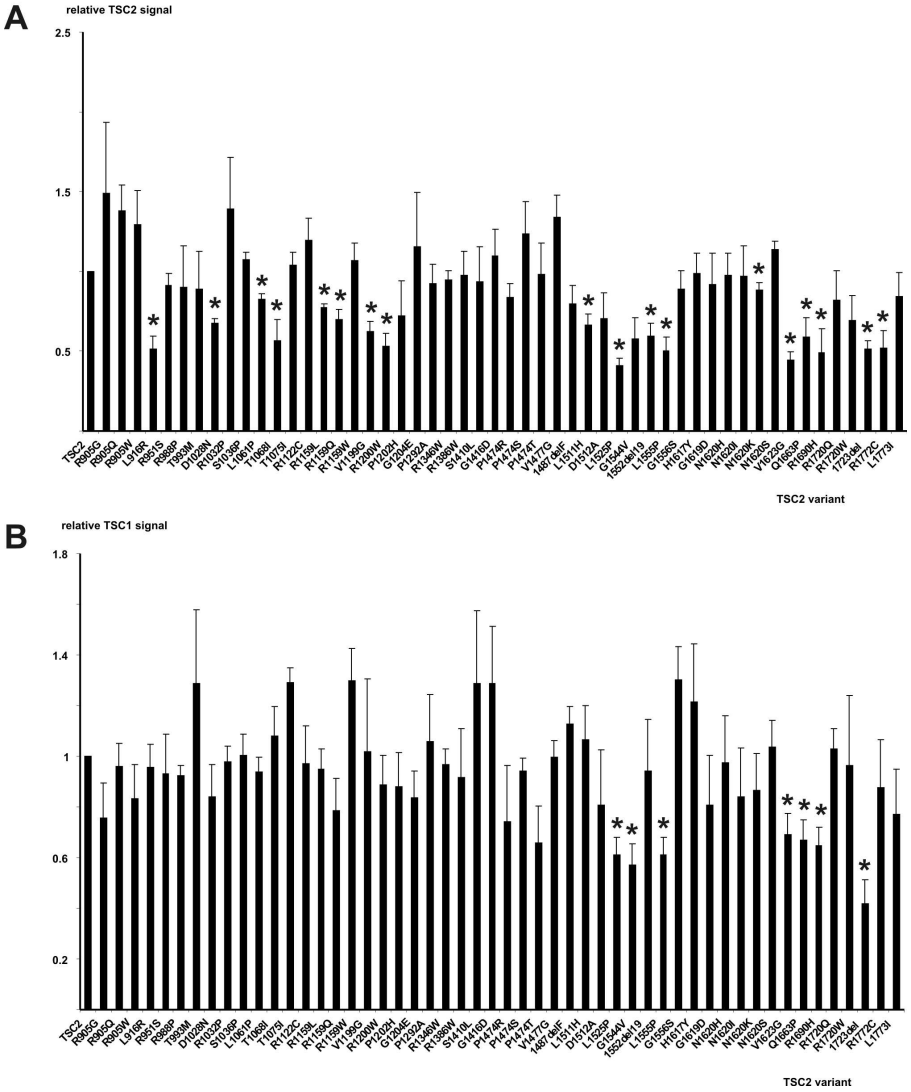

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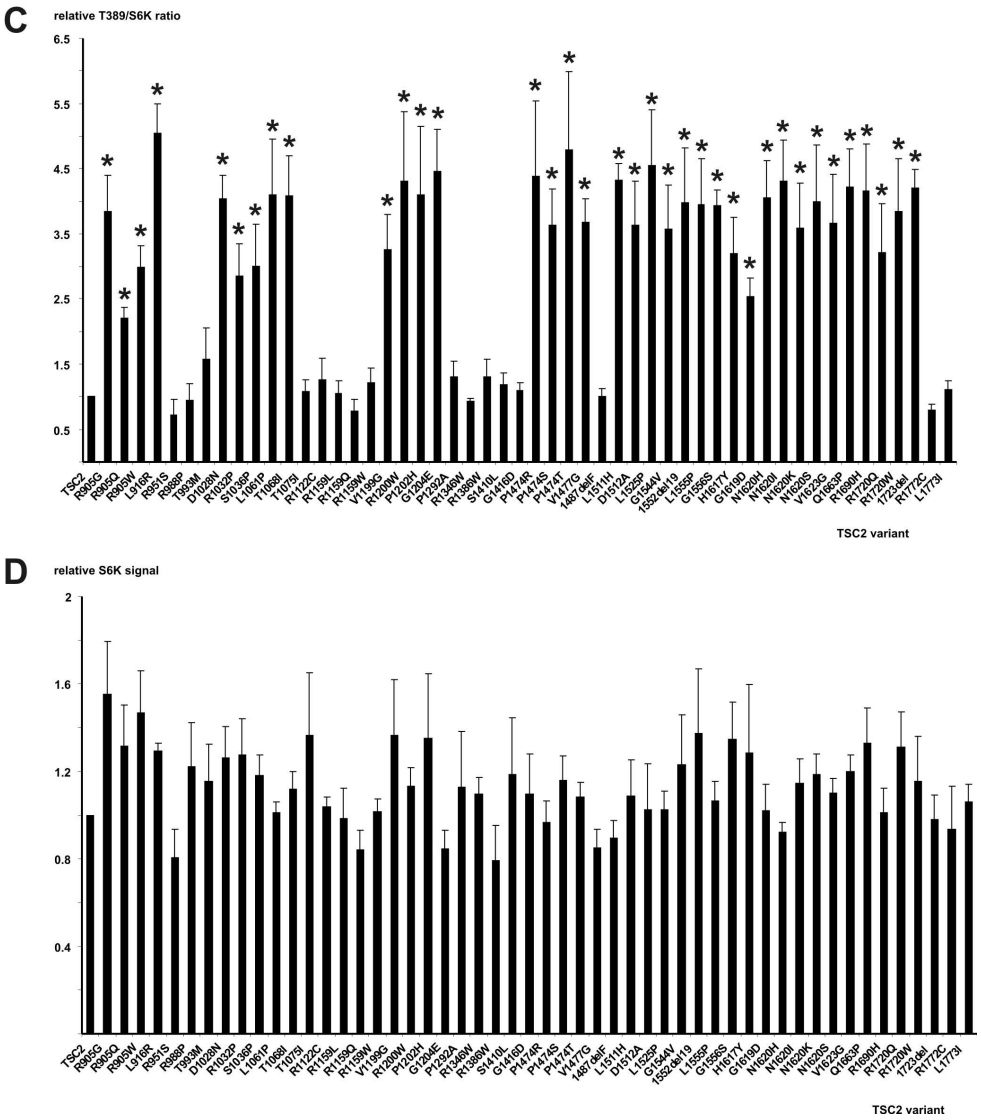
Figure 4



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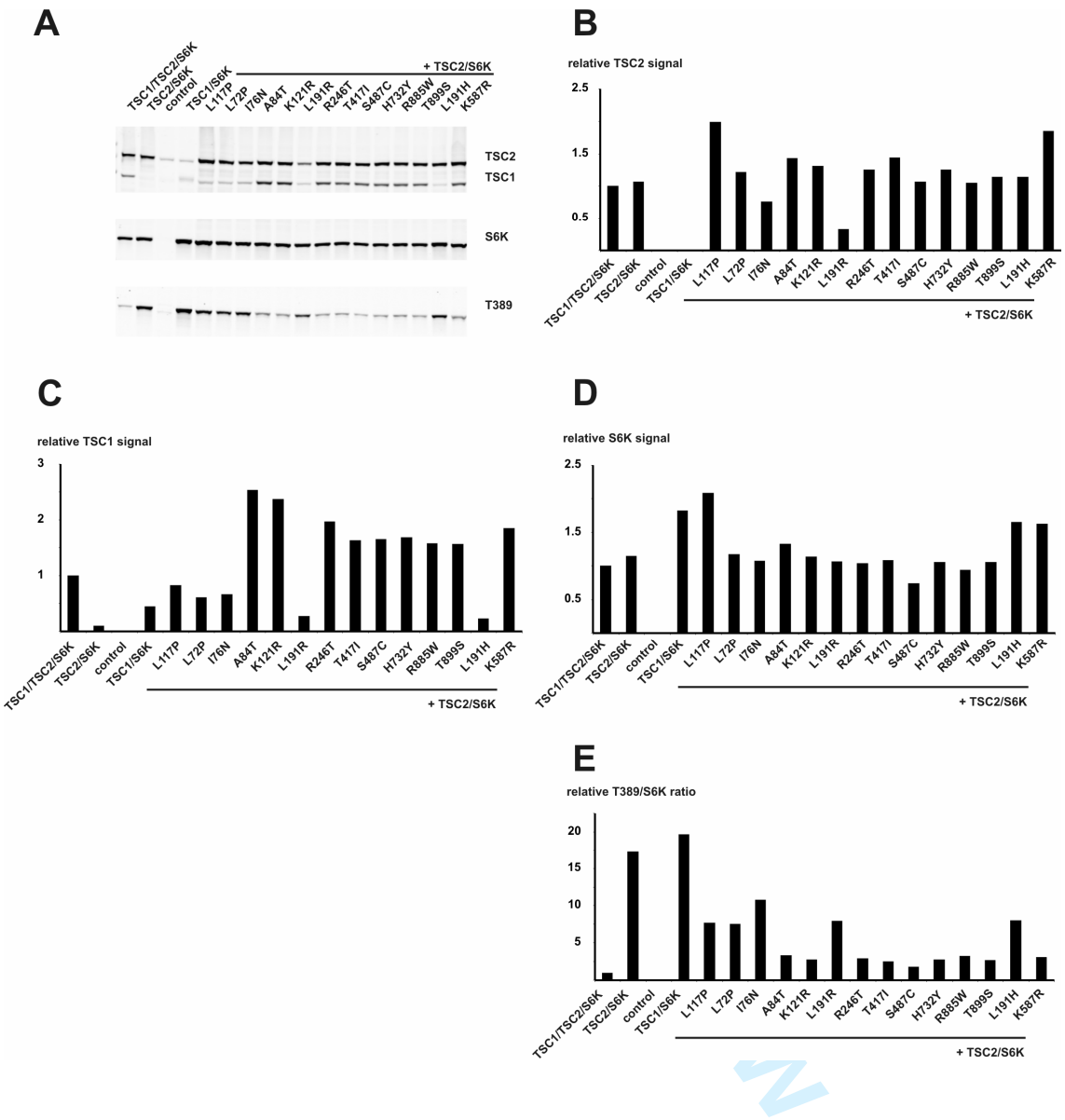
Supporting Figure S1:



Immunoblot analysis of TSC1 variants. Cells were transfected with expression constructs encoding TSC2, S6K and wild-type TSC1 (TSC1/TSC2/S6K), TSC2, S6K and the pathogenic L117P variant (L117P), TSC2, S6K and the TSC1 variants, TSC2 and S6K only (TSC2/S6K), TSC1 and S6K only (TSC1/S6K) or with an empty vector (control) and analysed by immunoblotting.

(A) Immunoblot showing signals for wild-type TSC2 (TSC2), wild-type TSC1 and the TSC1 variants (TSC1), total S6K (S6K) and T389-phosphorylated S6K (T389).

(B) Quantification of the TSC2 signals shown in (A), relative to the wild-type control (TSC1/TSC2/S6K).

(C) Quantification of the TSC1 signals shown in (A), relative to the wild-type control (TSC1/TSC2/S6K).

(D) Quantification of the total S6K signals shown in (A), relative to the wild-type control (TSC1/TSC2/S6K).

(E) Ratio of T389-phosphorylated S6K to total S6K in the presence of the TSC1 variants. The ratio of the signal for T389 phosphorylated S6K (T389) to the total S6K protein (S6K) was determined relative to the wild-type control (TSC1/TSC2/S6K; T389/S6K ratio = 1) for each variant shown in (A).

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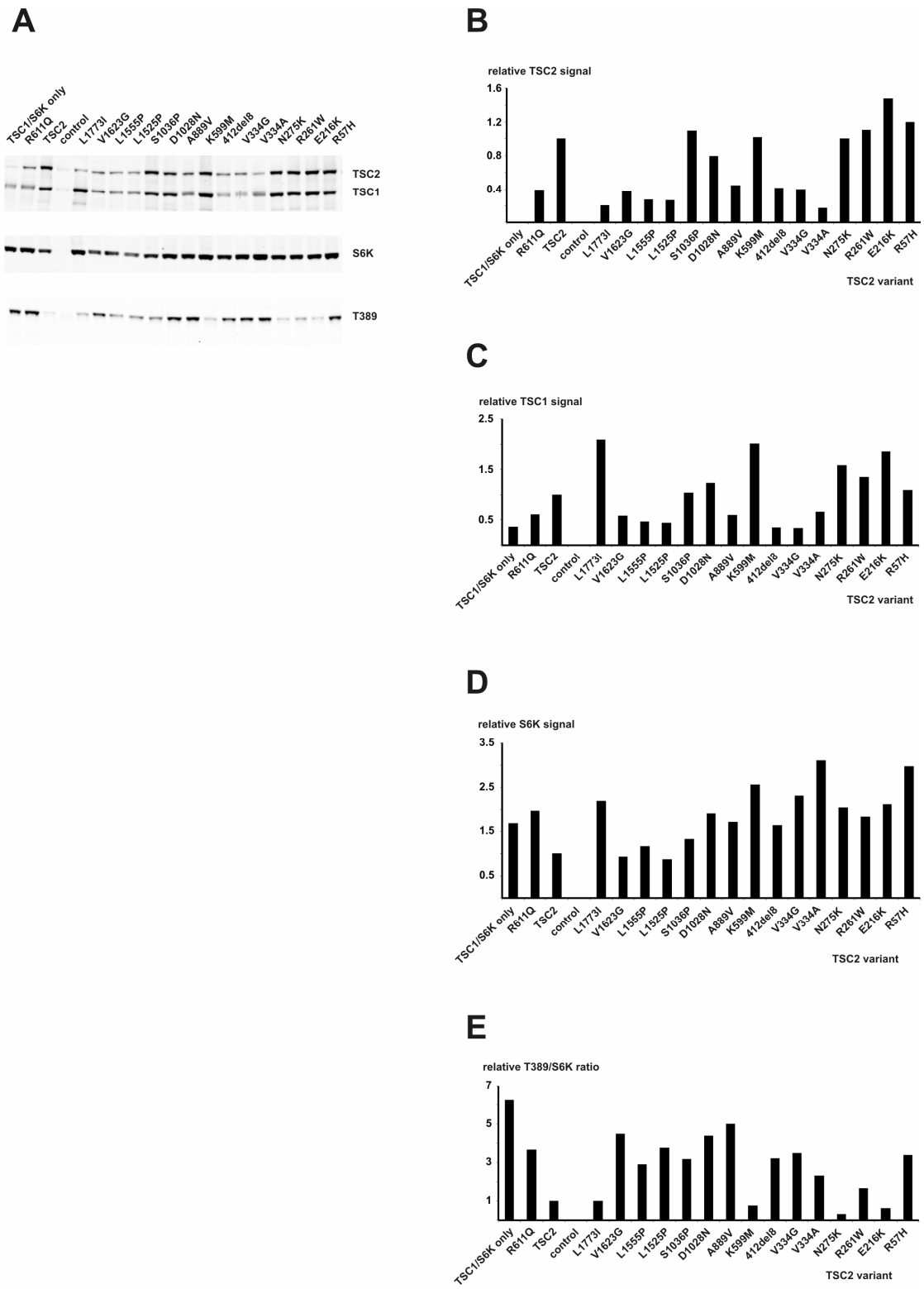
Supporting Figure S2:



Immunoblot analysis of TSC2 variants. Cells were transfected with expression constructs encoding TSC1, S6K and wild-type TSC2 (TSC2), TSC1, S6K and the pathogenic R611Q variant (R611Q), TSC1, S6K and the TSC2 variants, or with an empty vector (control), and analysed by immunoblotting.

(A) Immunoblot showing signals for wild-type TSC2 and the TSC2 variants (TSC2), wild-type TSC1 (TSC1), total S6K (S6K) and T389-phosphorylated S6K (T389).

(B) Quantification of the TSC2 signals shown in (A), relative to the wild-type control (TSC2).

(C) Quantification of the TSC1 signals shown in (A), relative to the wild-type control (TSC2).

(D) Quantification of the total S6K signals shown in (A), relative to the wild-type control (TSC2).

(E) Ratio of T389-phosphorylated S6K to total S6K in the presence of the TSC2 variants. The ratio of the signal for T389 phosphorylated S6K (T389) to the total S6K protein (S6K) was determined relative to the wild-type control (TSC2; T389/S6K ratio = 1) for each variant shown in (A).

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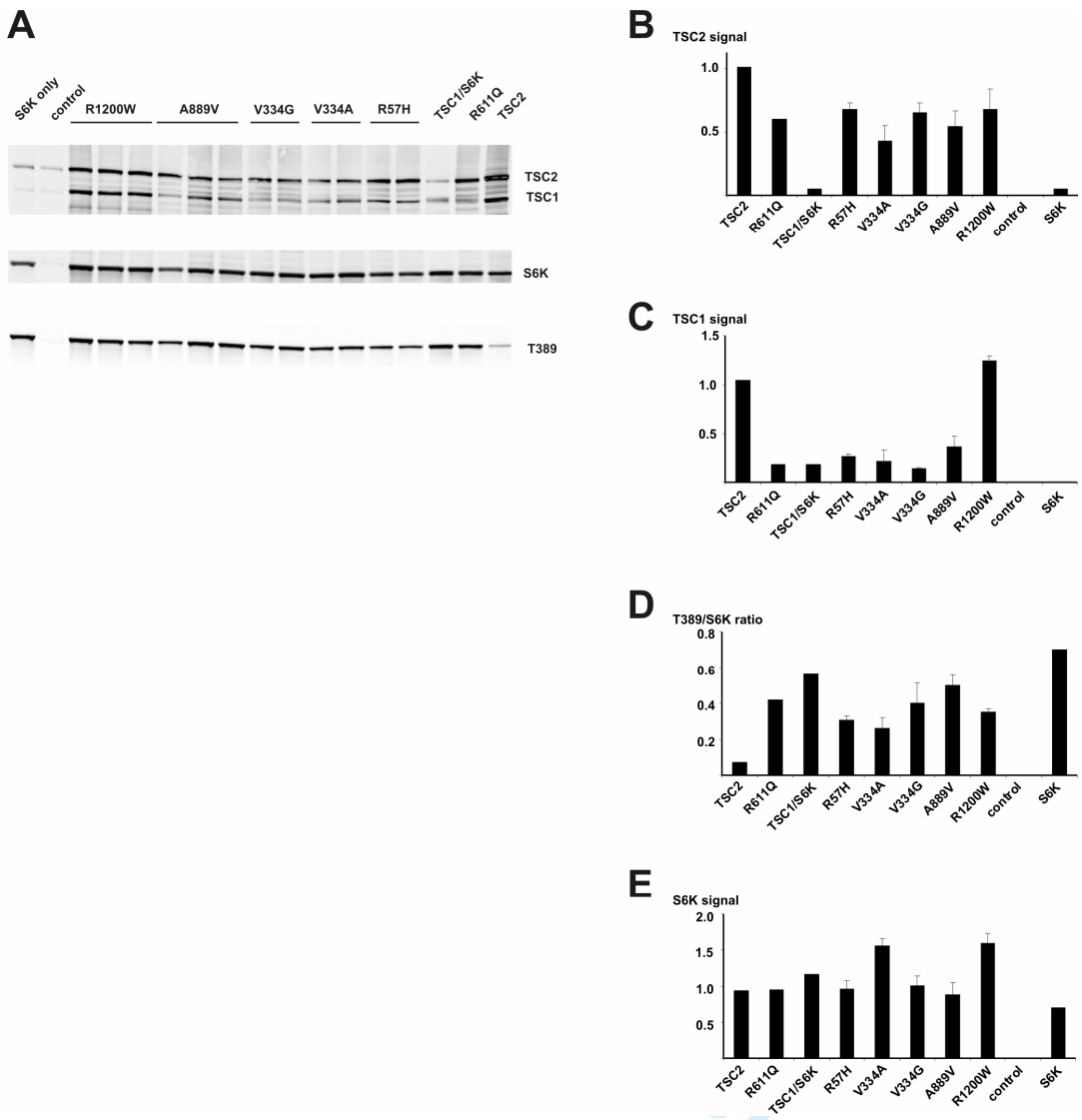
Supporting Figure S3:



Immunoblot analysis of different DNA preparations of the TSC2 R1200W, A889V, V334G, V334A and R57H variants. Cells were transfected with expression constructs encoding TSC1, S6K and wild-type TSC2 (TSC2), TSC1, S6K and the pathogenic R611Q variant (R611Q), TSC1, S6K and the TSC2 variants, or with an empty vector (control), and analysed by immunoblotting. Three different preparations of the TSC2 R1200W and A889V variant expression constructs and 2 different preparations of the V334G, V334A and R57H variant constructs were tested.

(A) Immunoblot showing signals for wild-type TSC2 and the TSC2 variants (TSC2), wild-type TSC1 (TSC1), total S6K (S6K) and T389-phosphorylated S6K (T389).

(B) Quantification of the TSC2 signals shown in (A). Mean signals for the different variants are shown relative to the wild-type control (TSC2). Error bars indicate the standard error of the mean.

(C) Quantification of the TSC1 signals shown in (A), relative to the wild-type control (TSC2).

(D) Quantification of the total S6K signals shown in (A), relative to the wild-type control (TSC2).

(E) Ratio of T389-phosphorylated S6K to total S6K in the presence of the TSC2 variants. The ratio of the signal for T389 phosphorylated S6K (T389) to the total S6K protein (S6K) was determined relative to the wild-type control (TSC2; T389/S6K ratio = 1) for each variant shown in (A).

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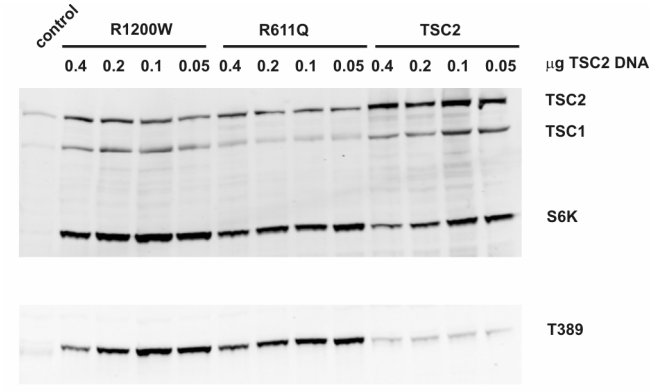
Supporting Figure S4:



Immunoblot analysis of wild-type TSC2 and the TSC2 R1200W and R611Q variants. Cells were transfected with expression constructs encoding TSC1 (0.4 µg), S6K (0.1 µg) and the indicated amounts of the TSC2 expression constructs, and analysed by immunoblotting. Control cells (control) were transfected with 0.7 µg empty vector. The effects of the TSC2 R1200W and R611Q variants on S6K T389 phosphorylation are clearly distinguishable from wild-type TSC2 across the whole range of transfected TSC2 expression construct DNA (0.05 - 0.4 µg).

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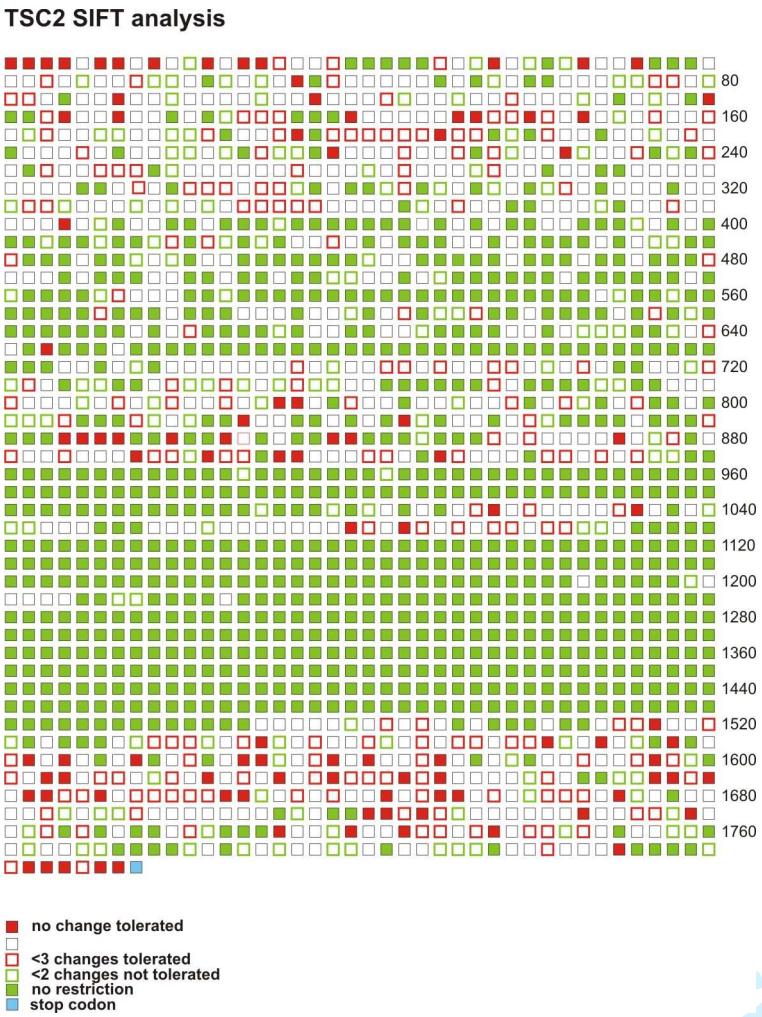
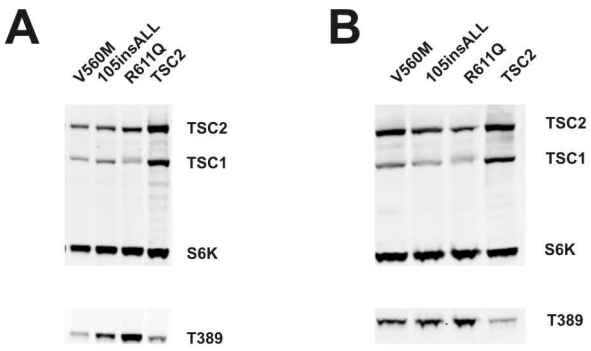
Supporting Figure S5:



SIFT analysis of TSC2. The TSC2 amino acid sequence from the *TSC2* LOVD was analysed using the SIFT algorithm. The number of tolerated and not tolerated substitutions was determined for each amino acid residue. In the figure each amino acid position is represented by a box. Solid green boxes represent positions that are completely tolerant (all substitutions possible, according to SIFT); open green boxes represent positions where 1 or 2 substitutions are not tolerated. Solid red boxes represent intolerant positions (no substitutions tolerated); open red boxes represent positions where 3 or fewer substitutions are tolerated. Empty boxes represent positions where between 3 and 17 substitutions are tolerated.

Supporting Figure S6:



Immunoblot analysis of the TSC2 105insALL and V560M variants. Cells were transfected with expression constructs encoding TSC1, S6K and wild-type TSC2 (TSC2) or the TSC2 105insALL, V560M or R611Q variants, and analysed by immunoblotting in 2 separate experiments, as shown in A and B. Note the apparent increase in the T389-phosphorylated S6K signal for the V560M variant in B compared to A.

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Supporting Table S1:

***TSC1* variants.** *TSC1* nucleotide changes and exon numbers are given according to the *TSC1* LOVD (http://www.lovd.nl/TSC1). Nucleotide numbering reflects the *TSC1* cDNA numbering (Genbank AF013168.1; GI: 2331280) with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines (www.hgvs.org/mutnomen). Amino acid changes are numbered accordingly; the initiation codon is codon 1. Nucleotide changes predicted to cause splicing defects are marked with an asterisk.

Amino acid substitutions predicted by SIFT to be tolerated (T) or not tolerated (NT) are indicated. Previously untested variants are indicated in bold. Links to the original references describing the different variants can be accessed via the *TSC1* LOVD. Variants not previously listed in the *TSC1* LOVD are indicated as 'not listed'.

Exon	**Variant**	**SIFT prediction**	**LOVD classification**	**Functional assessment**
4	c.149T>C (p.L50P)	NT	unknown	unstable, pathogenic
4	c.153A>C (p.E51D)	T	probably no pathogenicity	probably neutral
4	c.182T>C (p.L61P)	NT	probably pathogenic	unstable, pathogenic
5	**c.215T>C (p.L72P)**	**NT**	**probably pathogenic**	**unstable, pathogenic**

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5	**c.227T>A (p.I76N)**	**NT**	**unknown**	**unstable, pathogenic**
5	**c.245A>C (p.K82T)**	**T**	**unknown**	**probably neutral**
5	**c.250G>A (p.A84T)**	**T**	**probably no pathogenicity**	**probably neutral**
5	c.278T>G (p.L93R)	NT	probably pathogenic	unstable, destabilising, pathogenic
5	**c.346T>G (p.L116V)**	**T**	**not listed**	**probably neutral**
5	c.350T>C (p.L117P)	NT	probably pathogenic	unstable, pathogenic
5	**c.362A>G* (p.K121R)**	**T**	**unknown**	**probably neutral (predicted splice mutation)**
6	c.381_383delTGT* (p.128delV)	-	unknown	unstable, pathogenic
6	c.397G>T (p.V133F)	NT	unknown	unstable, pathogenic
7	c.539T>C (p.L180P)	NT	probably pathogenic	unstable, pathogenic
7	c.568C>T (p.R190C)	NT	no known pathogenicity	probably neutral
7	c.569G>C	NT	unknown	unstable, pathogenic

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	(p.R190P)			
7	c.572T>A (p.L191H)	NT	probably pathogenic	unstable, pathogenic
7	**c.572T>G (p.L191R)**	**NT**	**unknown**	**unstable, pathogenic**
7	c.593_595delACT (p.N198F199delinsI)	-	probably pathogenic	unstable, destabilising, pathogenic
7	**c.602_604delCCT (p.S201del)**	**-**	**unknown**	**unstable, pathogenic**
7	**c.647T>C (p.F216S)**	**NT**	**probably no pathogenicity; updated to unknown**	**unstable, pathogenic**
8	c.671T>G (p.M224R)	NT	probably pathogenic	unstable, destabilising, pathogenic
8	c.737G>A* (p.R246K)	NT	probably pathogenic	probably neutral (predicted splice mutation)
8	**c.737G>C* (p.R246T)**	**NT**	**probably pathogenic**	**probably neutral (predicted splice mutation)**
9	**c.853T>G (p.F285V)**	**T**	**not listed**	**probably neutral**

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9	c.913G>A* (p.G305R)	T	probably pathogenic	probably neutral (predicted splice mutation)
9	c.913G>T* p.G305W	T	probably pathogenic	probably neutral (predicted splice mutation)
10	c.1001C>T p.S334L	T	probably no pathogenicity	probably neutral
12	**c.1250C>T (p.T417I)**	**T**	**no known pathogenicity**	**probably neutral**
14	c.1433A>G* (p.E478G)	T	unknown	probably neutral
14	c.1433A>G* (p.E478GinsGN)	-	unknown	probably neutral
15	**c.1460C>G (p.S487C)**	**T**	**not listed**	**probably neutral**
15	c.1526G>A (p.R509Q)	T	probably no pathogenicity	probably neutral
15	c.1648C>G (p.Q550E)	T	unknown	probably neutral
15	**c.1760A>G (p.K587R)**	**T**	**no known pathogenicity**	**probably neutral**
15	**c.1849C>G (p.H617D)**	**T**	**not listed**	**probably neutral**
15	c.1974C>G	T	probably no	probably neutral

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	(p.D658E)		pathogenicity	
15	c.1976C>T (p.A659V)	T	probably no pathogenicity	probably neutral
17	**c.2194C>T (p.H732Y)**	**NT**	**no known pathogenicity**	**probably neutral**
19	c.2420T>C (p.I807T)	T	unknown	probably neutral
21	**c.2653C>T (p.R885W)**	**T**	**unknown**	**probably neutral**
21	**c.2696C>G (p.T899S)**	**T**	**unknown**	**probably neutral**
23	c.3103G>A (p.G1035S)	T	no known pathogenicity	probably neutral
23	**c.3184C>T (p.R1062W)**	**T**	**unknown**	**probably neutral**
23	c.3290G>A (p.R1097H)	NT	probably no pathogenicity	probably neutral

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Supporting Table S2:

***TSC2* variants (amino acids 1 - 900).** *TSC2* nucleotide changes and exon numbers are given according to the *TSC2* LOVD (http://www.lovd.nl/TSC2). Nucleotide numbering reflects the *TSC2* cDNA numbering (Genbank X75621; GI:450351) with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines (www.hgvs.org/mutnomen). Amino acid changes were numbered accordingly; the initiation codon is codon 1. Nucleotide changes predicted to cause splicing defects are marked with an asterisk.

Amino acid substitutions predicted by SIFT to be tolerated (T) or not tolerated (NT) are indicated. Links to the original references describing the different variants can be accessed via the *TSC2* LOVD. Variants not previously listed in the *TSC2* LOVD are indicated as 'not listed'. Previously untested variants are indicated in bold.

Exon	Variant	SIFT prediction	LOVD classification	Functional assessment
2	**c.170G>A (p.R57H)**	**NT**	**not listed**	**unstable, pathogenic**
2	c.185G>A (p.G62E)	T	unknown	probably neutral
3	c.292C>T (p.R98W)	NT	unknown	destabilising, pathogenic
3	**c.307_315dup (p.105insALL)**	**-**	**unknown**	**destabilising, pathogenic**
4	c.395C>G (p.S132C)	T	probably no pathogenicity	probably neutral

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4	c.447C>G (p.F143L)	T	probably no pathogenicity	probably neutral
5	c.586G>A (p.A196T)	T	probably no pathogenicity	probably neutral
6	**c.646G>A (p.E216K)**	**T**	**not listed**	**probably neutral**
7	**c.656T>C (p.L219P)**	**NT**	**not listed**	**destabilising, pathogenic**
7	c.730T>C (p.C244R)	NT	probably pathogenic	unstable, destabilising, pathogenic
8	**c.782G>C (p.R261P)**	**T**	**probably pathogenic**	**destabilising, pathogenic**
8	**c.781C>T (p.R261W)**	**NT**	**probably no pathogenicity**	**probably neutral**
8	**c.825C>G (p.N275K)**	**T**	**not listed**	**probably neutral**
8	c.824_826del (p.275delN)	-	unknown	destabilising, pathogenic
10	**c.1001T>C (p.V334A)**	**NT**	**not listed**	**unstable, destabilising, pathogenic**
10	**c.1001T>G (p.V334G)**	**NT**	**probably pathogenic**	**unstable, destabilising, pathogenic**

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10	**c.1019T>C (p.L340P)**	**NT**	**not listed**	**pathogenic**
10	**c.1070C>T (p.A357V)**	**T**	**not listed**	**probably neutral**
10	c.1100G>A (p.R367Q)	T	no known pathogenicity	probably neutral
10	c.1118A>C* (p.Q373P)	T	unknown	probably neutral (predicted splice mutation)
11	c.1235A>T* (p.E412V)	NT	unknown	probably neutral (splice mutation)
11	c.1235A>T* (p.412del8)	-	unknown	unstable, destabilising, pathogenic
11	**c.1255C>T* (p.P419S)**	**NT**	**probably pathogenic**	**probably neutral (predicted splice mutation)**
13	**c.1366A>G (p.E456K)**	**T**	**not listed**	**probably neutral**
13	**c.1378G>A (p.A460T)**	**T**	**unknown**	**probably neutral**
13	**c.1385G>A (p.R462H)**	**NT**	**not listed**	**unstable, destabilising, pathogenic**
13	**c.1385_1386del**	**NT**	**not listed**	**unstable,**

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	insCT (p.R462P)			**destabilising, pathogenic**
13	**c.1397T>C (p.L466P)**	**T**	**unknown**	**unstable, destabilising, pathogenic**
14	c.1574A>G (p.N525S)	T	no known pathogenicity	probably neutral
15	**c.1678G>A (p.V560M)**	**T**	**not listed**	**pathogenic**
16	c.1736del78 (p.580del26)	-	unknown	unstable, destabilising, pathogenic
16	c.1790insCAC (p.597insH)	-	unknown	destabilising, pathogenic
16	c.1792T>C (p.Y598H)	T	probably pathogenic	destabilising, pathogenic
16	c.1796A>T (p.K599M)	T	pathogenic; updated to unknown	probably neutral
16	c.1820C>A (p.A607E)	T	unknown	unstable, destabilising, pathogenic
16	**c.1819G>T (p.A607S)**	**T**	**not listed**	**probably neutral**
16	**c.1819G>A**	**T**	**probably no**	**probably neutral**

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	(p.A607T)		**pathogenicity**	
16	c.1826_1828dup (p.609insS)	-	pathogenic	destabilising, pathogenic
16	c.1832G>A (p.R611Q)	NT	pathogenic	unstable, destabilising, pathogenic
16	c.1831C>T (p.R611W)	NT	pathogenic	destabilising, pathogenic
17	c.1841C>A* (p.A614D)	T	probably pathogenic	unstable, destabilising, pathogenic
17	c.1844T>C (p.F615S)	T	no known pathogenicity; updated to unknown	destabilising, pathogenic
17	**c.1864C>T (p.R622W)**	**NT**	**unknown**	**unstable, destabilising, pathogenic**
17	**c.1882C>G* (p.R628G)**	**T**	**unknown**	**pathogenic**
18	**c.1973A>C (p.K658T)**	**T**	**not listed**	**probably neutral**
18	**c.1972_1974del (p.658delK)**	**-**	**no known pathogenicity**	**probably neutral**

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18	**c.2078T>C (p.L693P)**	**NT**	**probably pathogenic**	**unstable, destabilising, pathogenic**
18	c.2087G>A (p.C696Y)	NT	unknown	unstable, destabilising, pathogenic
20	c.2306T>A (p.V769E)	T	probably pathogenic	unstable, destabilising, pathogenic
21	**c.2363T>G (p.M788R)**	**NT**	**probably pathogenic**	**unstable, destabilising, pathogenic**
21	**c.2410T>C (p.C804R)**	**NT**	**probably pathogenic**	**destabilising, pathogenic**
21	c.2458_2460del (p.820delI)	-	pathogenic	unstable, destabilising, pathogenic
21	c.2476C>A (p.L826M)	NT	no known pathogenicity	probably neutral
23	**c.2666C>T (p.A889V)**	**NT**	**probably pathogenic**	**unstable, destabilising, pathogenic**
23	**c.2690T>C (p.F897S)**	**NT**	**probably pathogenic**	**unstable, destabilising, pathogenic**

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Supporting Table S3:

***TSC2* variants (amino acids 900 - 1807).** *TSC2* nucleotide changes and exon numbers are given according to the *TSC2* LOVD (http://www.lovd.nl/TSC2). Nucleotide numbering reflects the *TSC2* cDNA numbering (Genbank X75621; GI:450351) with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines (www.hgvs.org/mutnomen). Amino acid changes are numbered according to the sequence of the wild-type TSC2 expression construct used [European Chromosome 16 Tuberous Sclerosis Consortium, 1993] (Genbank X75621; GI:450351). Amino acid changes according to the *TSC2* LOVD are also given, where this differs from the original sequence due to the inclusion of 23 amino acids encoded by the alternatively spliced exon 31 (Genbank X75621.1; GI:4071057). Nucleotide changes predicted to cause splicing defects are marked with an asterisk.

Amino acid substitutions predicted by SIFT to be tolerated (T) or not tolerated (NT) are indicated. Previously untested variants are indicated in bold. Links to the original references describing the different variants can be accessed via the *TSC2* LOVD. Variants not previously listed in the *TSC2* LOVD are indicated as 'not listed'.

Exon	**Variant**	**SIFT prediction**	**LOVD classification**	**Functional assessment**
23	c.2713C>G (p.R905G)	NT	probably pathogenic	pathogenic
23	c.2714G>A (p.R905Q)	NT	pathogenic	pathogenic
23	c.2713C>T	NT	pathogenic	pathogenic

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	(p.R905W)			
24	c.2765T>G (p.L916R)	NT	probably pathogenic	unstable, pathogenic
25	**c.2853A>T (p.R951S)**	**T**	**probably pathogenic; updated to unkown**	**probably neutral**
25	**c.2963G>C (p.R988P)**	**T**	**not listed**	**probably neutral**
26	c.2978C>T (p.T993M)	T	probably no pathogenicity	probably neutral
26	**c.3082G>A (p.D1028N)**	**NT**	**not listed**	**unstable, pathogenic**
26	**c.3095G>C (p.R1032P)**	**NT**	**probably pathogenic**	**pathogenic**
26	**c.3106T>C (p.S1036P)**	**NT**	**probably pathogenic**	**pathogenic**
27	**c.3182T>C (p.L1061P)**	**NT**	**not listed**	**unstable, pathogenic**
27	c.3203C>A (p.T1068I)	NT	unknown	pathogenic
27	c.3224C>T (p.T1075I)	NT	unknown	probably neutral
28	**c.3382C>T (p.R1122C)**	**T**	**not listed**	**probably neutral**

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29	**c.3476G>T (p.R1159L)**	**T**	**unknown**	**unstable, probably neutral**
29	**c.3476G>A (p.R1159Q)**	**T**	**no known pathogenicity**	**unstable, probably neutral**
29	**c.3475C>T (p.R1159W)**	**T**	**unknown**	**probably neutral**
29	c.3596T>G (p.V1199G)	T	unknown	unstable, pathogenic
29	**c.3598C>T (p.R1200W)**	**NT**	**pathogenic**	**unstable, pathogenic**
29	**c.3605C>A (p.P1202H)**	**T**	**not listed**	**pathogenic**
30	**c.3611G>A* (p.G1204E)**	**NT**	**probably pathogenic**	**pathogenic**
32	c.3943C>G (p.P1315A/ P1292A)	T	unknown	probably neutral
33	**c.4105C>T (p.R1369W/ R1346W)**	**T**	**probably no pathogenicity**	**probably neutral**
33	**c.4225C>T (p.R1409W/ R1386W)**	**T**	**no known pathogenicity**	**probably neutral**
33	c.4298C>T (p.S1433L/ S1410L)	T	unknown	probably neutral

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33	c.4316G>A (p.G1439D/G1416D)	T	unknown	probably neutral
33	**c.4490C>G (p.P1497R/P1474R)**	**NT**	**pathogenic**	**pathogenic**
33	**c.4489C>T (p.P1497S/P1474S)**	**T**	**not listed**	**pathogenic**
33	**c.4489C>A (p.P1497T/P1474T)**	**NT**	**probably pathogenic**	**pathogenic**
34	**c.4499T>G (p.V1500G/V1477G)**	**T**	**probably pathogenic**	**pathogenic**
34	**c.4525_4527del (p.1510delF/ 1487delF)**	**-**	**no known pathogenicity**	**probably neutral**
35	c.4601T>A p.L1534H/L1511H)	NT	probably pathogenic	unstable, pathogenic
35	c.4604A>C (p.D1535A/D1512A)	NT	unknown	pathogenic
35	**c.4643T>C (p.L1548P/L1525P)**	**NT**	**unknown**	**unstable, destabilising, pathogenic**
36	c.4700G>T (p.G1567V /G1544V)	NT	unknown	destabilising, pathogenic
36	c.4726_4783del (p.1575del19/	-	unknown	unstable, pathogenic

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	1552del19)			
36	**c.4733T>C (p.L1578P/L1555P)**	**NT**	**probably pathogenic**	**unstable, destabilising, pathogenic**
36	c.4735G>A (p.G1579S/G1556S)	NT	probably pathogenic	pathogenic
37	c.4918C>T (p.H1640Y/H1617Y)	NT	unknown	pathogenic
37	**c.4925G>A (p.G1642D/G1619D)**	**NT**	**unknown**	**pathogenic**
37	**c.4927A>C (p.N1643H/N1620H)**	**NT**	**unknown**	**pathogenic**
37	**c.4928A>T (p.N1643I/N1620I)**	**NT**	**probably pathogenic**	**pathogenic**
37	**c.4929C>G (p.N1643K/N1620K)**	**NT**	**probably pathogenic**	**unstable, pathogenic**
37	**c.4928A>G (p.N1643S/N1620S)**	**NT**	**not listed**	**pathogenic**
37	c.4937T>G (p.V1646G/V1623G)	NT	unknown	unstable, destabilising, pathogenic
38	**c.5057A>C (p.Q1686P/Q1663P)**	**T**	**not listed**	**unstable, destabilising, pathogenic**
39	**c.5138G>A**	**NT**	**unknown**	**unstable,**

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	(p.R1713H/R1690H)			**destabilising, pathogenic**
40	c.5228G>A (p.R1743Q/R1720Q)	NT	pathogenic	pathogenic
40	c.5227C>T (p.R1743W/ R1720W)	NT	pathogenic	pathogenic
40	**c.5238_5255del (p.1746del6/ 1723del6)**	**-**	**pathogenic**	**unstable, destabilising, pathogenic**
41	c.5383C>T (p.R1795C/R1772C)	NT	no known pathogenicity	probably neutral
41	**c.5386C>A (p.L1796I/L1773I)**	**T**	**probably pathogenic; updated to unknown**	**probably neutral**

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