



HAL
open science

Analysis of TSC1 truncations defines regions involved in TSC1 stability, aggregation and interaction

Marianne Hoogeveen-Westerveld, Carla Exalto, Anneke Maat-Kievit, Ans Van den Ouweland, Dicky Halley, Mark Nellist

► **To cite this version:**

Marianne Hoogeveen-Westerveld, Carla Exalto, Anneke Maat-Kievit, Ans Van den Ouweland, Dicky Halley, et al.. Analysis of TSC1 truncations defines regions involved in TSC1 stability, aggregation and interaction. *Biochimica et Biophysica Acta - Molecular Basis of Disease*, 2010, 1802 (9), pp.774. 10.1016/j.bbadis.2010.06.004 . hal-00608983

HAL Id: hal-00608983

<https://hal.science/hal-00608983>

Submitted on 17 Jul 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Accepted Manuscript

Analysis of TSC1 truncations defines regions involved in TSC1 stability, aggregation and interaction

Marianne Hoogeveen-Westerveld, Carla Exalto, Anneke Maat-Kievit, Ans van den Ouweland, Dicky Halley, Mark Nellist

PII: S0925-4439(10)00114-6
DOI: doi: [10.1016/j.bbadis.2010.06.004](https://doi.org/10.1016/j.bbadis.2010.06.004)
Reference: BBADIS 63112

To appear in: *BBA - Molecular Basis of Disease*

Received date: 4 May 2010
Revised date: 7 June 2010
Accepted date: 8 June 2010



Please cite this article as: Marianne Hoogeveen-Westerveld, Carla Exalto, Anneke Maat-Kievit, Ans van den Ouweland, Dicky Halley, Mark Nellist, Analysis of TSC1 truncations defines regions involved in TSC1 stability, aggregation and interaction, *BBA - Molecular Basis of Disease* (2010), doi: [10.1016/j.bbadis.2010.06.004](https://doi.org/10.1016/j.bbadis.2010.06.004)

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Title page:

Analysis of TSC1 truncations defines regions involved in TSC1 stability, aggregation and interaction.

Marianne Hoogeveen-Westerveld¹, Carla Exalto¹, Anneke Maat-Kievit¹, Ans van den Ouweland¹, Dicky Halley¹ and Mark Nellist^{1,2}

¹ Department of Clinical Genetics, Erasmus Medical Centre, Rotterdam, The Netherlands.

² To whom correspondence should be addressed: Dr. Mark Nellist, Department of Clinical Genetics, Erasmus Medical Centre, Dr. Molewaterplein 50, 3015 GE Rotterdam, The Netherlands, Tel: +31 10 7044628; Fax: +31 10 7044736; email: m.nellist@erasmusmc.nl

Abbreviations: tuberous sclerosis complex (TSC), ras homolog expressed in brain (RHEB), target of rapamycin complex 1 (TORC1), p70 S6 kinase (S6K), GTPase activating protein (GAP)

Financial support was provided by the U.S. Department of Defense Congressionally-Directed Medical Research Program (grant #TS060052). The authors report no conflicts of interest. The funding source had no role in the project.

Abstract

Tuberous sclerosis complex (TSC) is an autosomal dominant disorder characterised by the development of hamartomas in a variety of organs and tissues. The disease is caused by mutations in either the *TSC1* gene on chromosome 9q34, or the *TSC2* gene on chromosome 16p13.3. The *TSC1* and *TSC2* gene products, TSC1 and TSC2, interact to form a protein complex that inhibits signal transduction to the downstream effectors of the target of rapamycin complex 1 (TORC1). Here we investigate TSC1 structure and function by analysing a series of truncated TSC1 proteins. We identify specific regions of the protein that are important for TSC1 stability, localisation, interactions and function.

Keywords: tuberous sclerosis complex, TSC1, TSC2, TORC1

1. Introduction

Tuberous sclerosis complex (TSC) is an autosomal dominant disorder characterised by the development of hamartomas in a variety of organs and tissues, including the brain, skin and kidneys [1]. Mutations in either the *TSC1* gene on chromosome 9q34 [2], or the *TSC2* gene on chromosome 16p13.3 [3] cause TSC.

The *TSC1* and *TSC2* gene products, TSC1 and TSC2, interact to form a protein complex [4]. TSC2 contains a GTPase activating protein (GAP) domain, and the TSC1-TSC2 complex has been shown to have GAP activity for a GTPase called ras homolog expressed in brain (RHEB). The TSC1-TSC2 complex stimulates the hydrolysis of RHEB-bound GTP to GDP, thereby inhibiting the RHEB-GTP-dependent stimulation of the target of rapamycin complex 1 (TORC1) [5]. TORC1 regulates a wide array of cellular processes, including transcription, translation and autophagy [6]. Inactivation of the TSC1-TSC2 complex results in the phosphorylation of TORC1 targets, including p70 S6 kinase (S6K) and elongation factor 4E binding protein 1 and, consequently, increased protein synthesis and cell growth [6]. TSC1 has limited homology with other proteins [2] and the exact role of TSC1 in the TSC1-TSC2 complex is not completely clear. Some studies have shown that TSC1 is required for TSC2 GAP activity [7 - 9], while others suggest that TSC1 is not essential for GAP activity but is necessary to maintain the stability, activity and correct intracellular localisation of the TSC1-TSC2 complex [10, 11]. TSC1 has been shown to be involved in recruitment of the TSC1-TSC2 complex to cell membranes [12] and may integrate multiple inputs to help regulate TORC1 activity, or perform other, independent functions [13].

In the absence of TSC2, over-expressed TSC1 forms Triton X100-insoluble aggregates or inclusions due to interactions between the coiled coil regions (amino

acids 719 - 998) of different TSC1 molecules [14]. Coexpression of TSC2 prevents the formation of these inclusions, resulting in a shift of TSC1 to Triton X100-soluble cell lysate fractions [15]. Stable TSC1-TSC2 complexes can be immunoprecipitated from these cell fractions, demonstrating that TSC2 acts as a molecular chaperone, maintaining TSC1 in a stable cytosolic form [14]. Recently, we identified TSC1 amino acid substitutions in TSC patients that prevented TSC1 inclusion formation *in vitro* [16, 17]. Surprisingly, none of these changes mapped to the coiled coil region, indicating that other regions of TSC1 are also important for the expression and localisation of the protein.

To gain additional insight into the structure and function of TSC1, we compared full-length TSC1 to a series of TSC1 N- and C-terminal truncation proteins. Our *in vitro* analysis demonstrates that the N-terminal region is important for TSC1 stability and that both the N-terminal and coiled coil regions are required for TSC1 aggregation. Furthermore, we show that multiple regions of TSC1 are required for binding TSC2 and that some TSC1 truncations can co-operate with TSC2 to inhibit TORC1 activity. This information will be useful for the functional characterisation of unclassified *TSC1* variants identified in individuals with TSC.

2. Materials and Methods

2.1 Generation of constructs and antisera

To derive expression constructs encoding myc-tagged TSC1 C-terminal truncations a KpnI site was introduced into a full-length myc-tagged TSC1 expression construct by site-directed mutagenesis (QuikChange, Stratagene, La Jolla, CA, U.S.A.). After digestion with KpnI to excise the 3' portion of the *TSC1* open reading frame (ORF), the vector and remaining ORF was re-circularised so that the epitope tag

remained in-frame with the last codon of the truncated *TSC1* ORF. N-terminal truncations were derived in a similar way. A *NheI* site was introduced to allow excision of the 5' portion of the *TSC1* ORF. In each case the complete open reading frame of the new construct was verified by sequence analysis. Expression constructs were derived for 4 C-terminal truncation proteins: Q900 (*TSC1* amino acids 1 – 900, molecular mass 100 kDa), R692 (amino acids 1 – 692, 80 kDa), R509 (amino acids 1 – 509, 60 kDa) and T339 (amino acids 1 – 339, 35 kDa); one N-terminal truncation protein, M351 (amino acids 351 – 1164, 100 kDa), and one truncation protein, M351-Q900, lacking both termini (amino acids 351 – 900, 65 kDa). The pathogenic L117P and M224R missense changes were introduced by site directed mutagenesis, as described previously [15]. An overview of the expressed *TSC1* truncation proteins is shown in Figure 1. Other constructs used in this study have been described elsewhere [4, 15, 18].

Antibodies were purchased from Cell Signaling Technology (Danvers, MA, U.S.A.), except for a mouse monoclonal antibody against *TSC2* which was purchased from Zymed Laboratories (San Francisco, CA, U.S.A.), or were described previously [4]. Secondary antibodies for infra-red detection proteins on immunoblots were obtained from Li-Cor Biosciences (Lincoln, NE, U.S.A.).

2.2 Immunoblot analysis of cells over-expressing TSC1 truncations.

HEK 293T cells in 3.5 cm dishes were transfected with expression constructs using polyethylenimine (Polysciences Inc., Warrington, PA, U.S.A.) [19]. Twenty-four hours after transfection the cells were washed with cold phosphate buffered saline (PBS) and lysed in 0.15 ml lysis buffer (50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 50 mM NaF, 1 % Triton X100 and a protease inhibitor cocktail (Complete, Roche Molecular Biochemicals)) for 10 minutes on ice. The cell lysates were cleared by

centrifugation (10 000 g for 10 minutes at 4°C) and the supernatant (Triton X100-soluble) and pellet (Triton X100-insoluble) fractions were recovered. The pellet fractions were resuspended in 0.15 ml lysis buffer and sonicated at 12 µm for 15 seconds prior to immunoblot analysis using the Criterion SDS-PAGE system (BioRad, Hercules, CA, U.S.A.). Protein expression levels were estimated by near infra-red detection and quantification of the blotted proteins on an Odyssey™ scanner (Li-Cor Biosciences).

To estimate the effect of the TSC1 truncation proteins on S6K T389 phosphorylation HEK 293T cells were transfected with a 4:2:1 mixture of the TSC1, TSC2 and S6K expression constructs and harvested as above. The Triton X100-soluble fractions were analysed by immunoblotting, as described previously [16, 17].

2.3 Immunofluorescent detection of TSC1 truncations.

HEK 293T cells were seeded onto glass coverslides coated with poly-L-lysine (Sigma-Aldrich, Carlsbad, CA, U.S.A.), transfected and processed for immunofluorescent microscopy as described previously [16]. Fixed, permeabilised cells were incubated with a primary mouse monoclonal antibody specific for the myc-epitope tag, followed by a cyanine (Cy2)-coupled secondary antibody (DAKO, Carpinteria, CA, U.S.A.). Cells were mounted in Mowiol (Calbiochem, La Jolla, CA, U.S.A.) and studied using a Leica DM RXA microscope and Image Pro-Plus version 6 image analysis software.

2.4 Coimmunoprecipitation of TSC2 with the TSC1 truncations.

Immunoprecipitations were performed using the Triton X100-soluble cell lysate fractions. EZ Red anti-myc affinity beads (Sigma-Aldrich) were pre-equilibrated in

lysis buffer prior to incubation with the cell lysates for 3 hours at 4°C with gentle rotation. The beads were washed at least 3 times with >20-fold excess of lysis buffer per wash step. Beads were recovered by gentle centrifugation (1000 g for 15 seconds at 4°C) between each wash step, and resuspended in sample buffer prior to immunoblot analysis.

3. Results

3.1 *TSC1* truncation proteins

We derived expression constructs for 4 C-terminal *TSC1* truncations that we called: Q900, R692, R509 and T339. The Q900 truncation consists of *TSC1* amino acids 1 - 900 and corresponds to a *TSC1* c.2919C>T (p.Q900X) mutation, the most distal *TSC1* mutation identified in our patient cohort [20]. We identified this mutation in 2 unrelated cases of TSC. Clinical data was available for one of these individuals (Table 1). The R692 and R509 truncations consist of amino acids 1 - 692 and 1 - 509 respectively, and correspond to the *TSC1* c.2295C>T and c.1746C>T mutations. Together these 2 mutations account for >5% of the *TSC1* variants listed in the Leiden Open Tuberous Sclerosis Complex Variation Database [21]. We identified the *TSC1* c.2295C>T mutation in 6 families in our cohort, and in 5 cases had clinical data on one or more individuals (Table 1). We identified 6 cases with the *TSC1* c.1746C>T mutation in our cohort. Clinical data was available for one individual (Table 1.). The T339 truncation corresponds to a *TSC1* c.1015dupA mutation identified in an individual with very mild peripheral features of TSC, epilepsy and cognitive disturbances (Table 1.) (P. de Vries, personal communication).

In addition to the C-terminal truncations, we expressed 2 proteins lacking 350 amino acids from the N-terminus of *TSC1*. Truncation protein M351 consisted of *TSC1*

amino acids 351 - 1164, and truncation protein M351-Q900 consisted of amino acids 351 - 900. A schematic overview of the truncation proteins is shown in Figure 1.

Full-length TSC1 and the TSC1 truncation proteins were expressed as fusion proteins containing a C-terminal myc epitope tag. This tag did not interfere with either the TSC1-TSC2 interaction, or with the function of the TSC1-TSC2 complex [15 - 19]. We compared the relative expression levels of the truncation proteins in the Triton X100-soluble and -insoluble fractions by immunoblotting. Full-length TSC1 and the Q900 truncation were detected predominantly in the Triton X100-insoluble fraction, while the remaining truncation proteins were detected predominantly in the soluble fraction (Figure 2A and C).

3.2 TSC1 truncation prevents aggregate formation

The Q900 truncation protein localised to cytoplasmic punctate inclusions or aggregates in HEK 293T cells (Figure 3A) and was detected predominantly in Triton X100-insoluble cell lysate fractions, similar to full-length TSC1 (Figure 2A and C). In contrast, truncation proteins R692, R509 and T339 that lack the entire coiled coil region, and the M351 truncation protein, that contains the coiled coil region but lacks the TSC1 N-terminal, were detected predominantly in Triton X100-soluble cell lysate fractions (Figure 2A and C) and did not form cytoplasmic inclusions (Figure 3C and D, and data not shown) We concluded that the coiled coil region was necessary but not sufficient for aggregate formation.

3.3 The N-terminal region is required for TSC1 stability

Pathogenic amino acid substitutions in the N-terminal region of TSC1 prevent TSC1 aggregation and reduce expression levels *in vitro* [16, 17]. To determine whether

this destabilisation is due to effects on local protein folding, or due to long-range interactions between the N- and C-terminal regions of TSC1, a pathogenic N-terminal amino acid substitution (M224R) [17] was introduced into the R692 truncation protein. that lacks the C-terminal region (amino acids 693 - 1164). As shown in Figure 4, the M224R substitution reduced the amount of both full-length TSC1 and the R692 truncation detected in the Triton X100 soluble fraction, demonstrating that the M224R substitution had the same effect irrespective of the presence or absence of amino acids 693 – 1164. We concluded that the low expression levels of TSC1 missense mutants [16, 17, 22] are more likely due to autonomous effects on the N-terminal region than disruption of long-range interactions with the TSC1 C-terminal, containing the predicted coiled coil regions.

3.4 TSC1 truncation reduces the stability of the TSC1-TSC2 complex

The formation of stable, Triton X100-soluble TSC1-TSC2 complexes prevents TSC1 aggregation [14]. We coexpressed TSC2 with the TSC1 truncations in HEK 293T cells and estimated the proportions of the expressed proteins in the Triton X100-soluble fractions (Figure 5A and C). As expected, the proportion of full-length TSC1 detected in the Triton X100-soluble fraction was increased by coexpression of TSC2, as estimated by immunoblotting (compare Figures 2C and 5C). TSC2 coexpression had a similar effect on the Q900 truncation, increasing the proportion detected in the soluble fraction (compare Figures 2C and 5C).

To investigate whether the TSC1 truncation proteins interacted directly with TSC2, we coexpressed TSC2 with the different TSC1 truncations and immunoprecipitated the truncation proteins from the Triton X100-soluble cell lysate fractions. We determined whether TSC2 was coimmunoprecipitated by

immunoblotting. As shown in Figure 6, all the TSC1 truncation proteins were immunoprecipitated efficiently, even those that were expressed at low levels. TSC2 coimmunoprecipitated with TSC1 and with the M351 and Q900 truncations, and to a lesser extent with the R692 and R509 truncations. TSC2 was not coimmunoprecipitated with the M351-Q900 or T339 truncations or with a control protein (myc-tagged β -lactamase).

The interaction detected between the Q900 truncation and TSC2 was consistent with the observed shift of the Q900 truncation from the insoluble to the soluble fraction (compare Figures 2 and 5). However, the TSC2-Q900 interaction appeared weaker than that between TSC2 and full-length TSC1 (Figure 6D). Similarly, TSC2 was coimmunoprecipitated with the M351 truncation, but less efficiently than with full-length TSC1 (Figure 6D). Therefore, we concluded that multiple regions of TSC1 are necessary for the interaction with TSC2 and to form a stable TSC1-TSC2 complex.

3.5 The central region of TSC1 is involved in TSC1-TSC1 interactions

Interactions between the TSC1 coiled coil regions cause TSC1 aggregation [14]. We sought additional evidence for TSC1-TSC1 interactions by coimmunoprecipitation. As shown in Figure 7, the myc-tagged TSC1 truncations were immunoprecipitated from Triton X100-soluble fractions. Coimmunoprecipitated full-length (untagged) TSC1 was detected by immunoblotting. TSC1 was coimmunoprecipitated with the M351 and M351-Q900 truncations that contain part or all of the coiled coil region, and, to a lesser extent, by the R509 and R692 truncations, that do not (Figure 7A and C). TSC1 was not detected in the T339 and Q900 immunoprecipitates, or in the control immunoprecipitate. We were unable to detect stable TSC1-Q900 complexes by coimmunoprecipitation because both full-length TSC1 and the Q900 truncation

localised predominantly to the Triton X100-insoluble cell lysate fractions. We concluded that in addition to the coiled coil region, the central region of TSC1 (amino acids 351 - 509) is involved in the TSC1-TSC1 interaction.

3.6 TSC1 truncation proteins help TSC2 suppress TORC1 activity

To determine whether the TSC1 truncations could affect the TSC2-dependent inhibition of S6K-T389 phosphorylation, TSC2, S6K and the TSC1 truncations, were expressed in HEK 293T cells and the T389 phosphorylation status of S6K was estimated by immunoblotting, as shown in Figure 8. The R509 truncation was not included in this analysis as it comigrated with S6K, making it difficult to detect and quantify separate R509 and S6K signals.

Although the expression levels of the different TSC1 truncation proteins were variable (Figure 8B), the expression levels of TSC2 (Figure 8C) and S6K (Figure 8E) were relatively constant. This indicated that coexpression of the different TSC1 truncations did not affect TSC2 expression levels. In cells coexpressing TSC2 and full-length TSC1, S6K-T389 phosphorylation was reduced approximately 7-fold compared to cells expressing neither protein, or to cells expressing TSC1 only (Figure 8D). Expression of TSC2 only (without TSC1) was less effective than coexpression of TSC1 and TSC2: S6K-T389 phosphorylation was approximately 5-fold higher in TSC2 expressing cells, than in TSC1-TSC2 expressing cells. Coexpression of the TSC1 T339 and M351-Q900 truncations did not affect the ability of TSC2 to reduce S6K-T389 phosphorylation (Figure 8D). In contrast, coexpression of TSC2 with the a pathogenic TSC1-L117P variant [17] reduced S6K-T389 phosphorylation to a level approximately 3-fold higher than TSC1-TSC2 expressing cells, indicating that this variant can partially inhibit TORC1 activity *in vitro*. Interestingly, the M351, Q900 and R692 truncations

had a similar effect. In each case, S6K-T389 phosphorylation was increased approximately 3-fold with respect to the TSC1-TSC2 expressing cells, but reduced with respect to cells expressing TSC2 only. Therefore, in this *in vitro* assay, the TSC1 R692, M351 and Q900 truncation proteins all increased the TSC2-dependent inhibition of S6K-T389 phosphorylation, albeit not as effectively as full-length TSC1.

4. Discussion

TSC1 and *TSC2* encode the TSC1-TSC2 protein complex, an essential regulator of TORC1 [23]. TSC2 contains the active site of the TSC1-TSC2 complex and therefore has a clearly defined functional role. In contrast, the role of TSC1 is less clear. Some studies suggest that TSC1 is required for TSC2 GAP activity, while others indicate that TSC1 does not affect the catalytic activity of the TSC1-TSC2 complex, but prevents the degradation of the complex [7 - 11]. To gain more insight into the structure and function of TSC1, we compared the characteristics of a series of TSC1 truncation proteins with full-length, wild-type TSC1.

We found that deletion of the complete TSC1 coiled coil region (amino acids 719 - 998) prevented TSC1 aggregation and that sequences close to the N-terminal were required for both stability and aggregate formation. Deletion of amino acids 901 - 1164, containing the C-terminal part of the coiled coil region, did not prevent aggregate formation but did reduce the strength of the TSC1-TSC2 interaction and the activity of the TSC1-TSC2 complex, consistent with the identification of truncating mutations in this region of the *TSC1* gene in TSC patients [20, 21].

Deletion of the N-terminal region (amino acids 1 - 350) had the same effect on TSC1 expression levels as pathogenic missense changes to this region [16, 17, 22]. When one of these mutations (M224R) [17] was introduced into the R692 truncation

protein, the expression level of the truncation protein was reduced, indicating that the N-terminal region has an autonomous effect on TSC1 stability. It has been suggested that a putative transmembrane domain in the N-terminal region of TSC1 (amino acids 127 - 144) may be important for the correct localisation of the TSC1-TSC2 complex [12]. One possibility is that recruitment of TSC1 to membranes might help stabilise the TSC1-TSC2 complex. Mutant TSC1-TSC2 complexes, that are unable to be recruited to the membrane, might therefore be degraded more rapidly.

To investigate the interactions between TSC2 and the TSC1 truncation proteins we performed coimmunoprecipitation experiments. Together, deletion of the N- and C-terminal regions of TSC1 (amino acids 1 -350 and 901 - 1164) prevented TSC2 binding, while truncation at either terminal only reduced the interaction, indicating that both the N- and C-terminal regions of TSC1 are necessary for the formation of stable TSC1-TSC2 complexes. The M351 and Q900 truncations that were able to bind TSC2, were also able to promote the TSC2-mediated inhibition of TORC1 activity, as assayed by the phosphorylation status of the TORC1 substrate, S6K.

Full-length TSC1 coimmunoprecipitated with the R509 and M351-Q900 truncations, confirming that TSC1 interacts with itself [14], and indicating that the central region of the protein (amino acids 351 - 509) is required for this interaction. In these experiments we only over-expressed TSC1 and the TSC1 truncations, not TSC2. Furthermore, the M351-Q900 truncation did not interact with TSC2. Therefore, it is unlikely that the detected TSC1-TSC1 interactions are a consequence of the formation of TSC1-TSC2 complexes containing multiple TSC1 and TSC2 subunits. Nevertheless, the TSC1-TSC1 interaction indicates that it is possible that TSC1-TSC2 complexes consist of multiple TSC1 and TSC2 subunits *in vivo*. It will be interesting to investigate

whether substitutions in the central region of TSC1 (amino acids 351 - 900) disrupt TSC1-TSC1 binding, and whether such changes can cause the TSC phenotype.

Recently it has been proposed that different *TSC1* and *TSC2* mutations may result in different neurocognitive manifestations of TSC, depending on the exact effects of the mutations on the regulation of signalling by the TSC1-TSC2 complex [24]. One requirement of this hypothesis is that mutant TSC1 and TSC2 isoforms are expressed. Although all our experiments were performed *in vitro* on over-expressed proteins, our data suggest that if truncated TSC1 isoforms are expressed, then they may indeed have distinct effects on TSC1-TSC2 function *in vivo*. Only limited clinical data were available for the individuals with mutations corresponding to the truncation proteins analysed. However, we did not observe any obvious differences between the individuals with a *TSC1* c.2295C>T (p.R692X), *TSC1* c.2919C>T (p.Q900X) or *TSC1* c.1015dupA (p.T339X) mutation. More work is required to establish whether the distinct functional characteristics of truncated TSC1 isoforms influence the (neurocognitive) phenotype in TSC.

5. Conclusions

The N-terminal region of TSC1 (amino acids 1 - 350) is required to stabilise TSC1 and maintain expression levels in the cell. The central (amino acids 351 - 509) and C-terminal (amino acids 693 - 1164) regions are required for TSC1-TSC1 interactions.

Multiple regions of TSC1, including the N- and C-terminal regions, are required for binding TSC2, and the binding between truncated TSC1 proteins and TSC2 is sufficient to promote the ability of TSC2 to inhibit TORC1 activity *in vitro*. This

information will be useful for the detailed functional characterisation of unclassified *TSC1* missense variants identified in individuals with TSC.

Acknowledgments

P. de Vries (University of Cambridge, U.K.) is thanked for providing information on the *TSC1* c.1015dupA (p.T339X) mutation, and for useful comments on the manuscript.

References

1. Gomez M, Sampson J, Whittemore V, eds. *The tuberous sclerosis complex*. Oxford University Press, Oxford, UK, 1999.
2. van Slegtenhorst M, de Hoogt R, Hermans C, Nellist M, Janssen LAJ, Verhoef S, Lindhout D, van den Ouweland AMW, Halley DJJ, Young J, Burley M, Jeremiah S, Woodward K, Nahmias J, Fox M, Ekong R, Wolfe J, Povey S, Osborne J, Snell RG, Cheadle JP, Jones AC, Tachataki M, Ravine D, Sampson JR, Reeve MP, Richardson P, Wilmer F, Munro C, Hawkins TL, Sepp T, Ali JBM, Ward S, Green AJ, Yates JRW, Short MP, Haines JH, Jozwiak S, Kwiatkowska J, Henske EP and Kwiatkowski DJ. Identification of the tuberous sclerosis gene *TSC1* on chromosome 9q34. *Science* 277 (1997) 805 - 808.
3. The European Chromosome 16 Tuberous Sclerosis Consortium. Identification and characterization of the tuberous sclerosis gene on chromosome 16. *Cell* 75 (1993) 1305 - 1315.
4. van Slegtenhorst M, Nellist M, Nagelkerken B, Cheadle J, Snell R, van den Ouweland A, Reuser A, Sampson JR, Halley D, van der Sluijs P Interaction between

- hamartin and tuberlin, the *TSC1* and *TSC2* gene products. *Hum. Mol. Genet.* 7 (1998) 1053 - 1057.
5. Li Y, Corradetti MN, Inoki K, Guan K-L. TSC2: filling the GAP in the mTOR signaling pathway. *Trends Biochem. Sci.* 28 (2003) 573 - 576.
 6. Guertin DA and Sabatini DM. Defining the role of mTOR in cancer. *Cancer Cell* 12 (2007) 9 - 22.
 7. Zhang Y, Gao X, Saucedo LJ, Ru B, Edgar BA, Pan D. Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins. *Nat. Cell Biol.* 5 (2003) 578 - 581.
 8. Garami A, Zwartkuis FJ, Nobukuni T, Joaquin M, Roccio M, Stocker H, Kozma SC, Hafen E, Bos JL, Thomas G. Insulin activation of Rheb, a mediator of mTOR/S6K/4E-BP signaling, is inhibited by TSC1 and 2. *Mol. Cell* 11 (2003) 1457 - 1466.
 9. Tee AR, Manning BD, Roux PP, Cantley LC, Blenis J.. Tuberous sclerosis complex gene products, tuberlin and hamartin, control mTOR signaling by acting as a GTPase-activating protein complex toward Rheb. *Curr. Biol.* 13 (2003) 1259 - 1268.
 10. Castro AF, Rebhun JF, Clark GJ, Quilliam LA Rheb binds tuberous sclerosis complex 2 (TSC2) and promotes S6 kinase activation in a rapamycin- and farnesylation-dependent manner. *J. Biol. Chem.* 278 (2003) 32493 - 32496.
 11. Inoki K, Li Y, Xu T, Guan K-L. Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. *Genes Dev.* 17 (2003) 1829 - 1834.
 12. Cai SL, Tee AR, Short JD, Bergeron JM, Kim J, Shen J, Guo R, Johnson CL, Kiguchi K, Walker CL. Activity of TSC2 is inhibited by AKT-mediated phosphorylation and membrane partitioning. *J. Cell Biol.* 173 (2006) 279 - 289.

13. Rosner M, Hanneder M, Siegel N, Valli N, Hengstschlager M. The tuberous sclerosis gene products hamartin and tuberin are multifunctional proteins with a wide spectrum of interacting partners. *Mut. Res.* 658 (2008) 234 - 246.
14. Nellist M, van Slegtenhorst MA, Goedbloed M, van den Ouweland AMW, Halley DJJ, van der Sluijs P. Characterization of the cytosolic tuberin-hamartin complex: tuberin is a cytosolic chaperone for hamartin. *J. Biol. Chem.* 274 (1999) 35647 - 35652.
15. Nellist M, Verhaaf B, Goedbloed MA, Reuser AJJ, van den Ouweland AMW and Halley DJJ. *TSC2* missense mutations inhibit tuberin phosphorylation and prevent formation of the tuberin-hamartin complex. *Hum. Mol. Genet.* 10 (2001) 2889-2898.
16. Mozaffari M, Hoogeveen-Westerveld M, Kwiatkowski D, Sampson J, Ekong R, Povey S, den Dunnen JT, van den Ouweland A, Halley D, Nellist M. Identification of a region required for *TSC1* stability by functional analysis of *TSC1* missense mutations found in individuals with tuberous sclerosis complex. *BMC Med. Genet.* 10 (2009) e88.
17. Nellist M, van den Heuvel D, Schluep D, Exalto C, Goedbloed M, Maat-Kievit A, van Essen T, van Spaendonck-Zwarts K, Jansen F, Helderma P, Bartalini G, Vierimaa O, Penttinen M, van den Ende J, van den Ouweland A and Halley D. Missense mutations to the *TSC1* gene cause tuberous sclerosis complex. *Eur. J. Hum. Genet.* 17 (2009) 319 - 328.
18. Nellist M, Sancak O, Goedbloed MA, Rohe C, van Netten D, Mayer K, Tucker-Williams A, van den Ouweland AMW, Halley DJJ. Distinct effects of single amino acid changes to tuberin on the function of the tuberin-hamartin complex. *Eur. J. Hum. Genet.* 13 (2005) 59 - 68.
19. Coevoets R, Arican S, Hoogeveen-Westerveld M, Simons E, van den Ouweland A, Halley D, Nellist M. A reliable cell-based assay for testing unclassified *TSC2* gene variants. *Eur. J. Hum. Genet.* 17 (2009) 301 - 310.

20. Sancak O, Nellist M, Goedbloed M, Elfferich P, Wouters C, Maat-Kievit A, Zonnenberg B, Verhoef S, Halley D, van den Ouweland A. Mutational analysis of the TSC1 and TSC2 genes in a diagnostic setting: genotype-phenotype correlations and comparison of diagnostic DNA techniques in tuberous sclerosis complex. *Eur. J. Hum. Genet.* 13 (2005) 731 - 741.
21. Tuberous sclerosis database - Leiden Open Variation Database [www.chromium.liacs.nl/lovd/index.php?select_db=TSC1].
22. Pymar LS, Platt FM, Askham JM, Morrison EE, Knowles MA. Bladder tumour-derived somatic TSC1 missense mutations cause loss of function via distinct mechanisms. *Hum. Mol. Genet.* 17 (2008) 2006-2017.
23. Inoki K, Guan K-L. Tuberous sclerosis complex, implication from a rare genetic disease to common cancer treatment. *Hum. Mol. Genet.* 18 (2009) 94-100.
24. de Vries P., Howe CJ. The tuberous sclerosis complex proteins - a GRIPP on cognition and neurodevelopment. *Trends Mol. Med.* 13 (2007) 319-326.

Figure 1. TSC1 truncation proteins. Schematic diagram illustrating the TSC1 truncation proteins used as part of this study. The positions of the predicted transmembrane domain (amino acids 127 - 144), coiled coil region (amino acids 719 - 998), and the L117P and M224R missense mutations identified in individuals with TSC are indicated. Numbers indicate the positions of the first and last amino acids, relative to the full-length protein (top).

Figure 2. Immunoblot analysis of TSC1 truncation proteins. Wild-type myc-tagged TSC1 (TSC1) and the myc-tagged truncations were overexpressed in HEK 293T cells. Triton X100-soluble and -insoluble cell lysate fractions were separated on 4-12% gradient SDS-PAGE gels, blotted and incubated with a monoclonal mouse antibody against the myc epitope tag.

(A) Immunoblot of Triton X100-soluble and -insoluble cell lysate fractions. Bands marked with an asterisk (*) are due to leakage of the signal from the R692 truncation.

(B) Total expression of the TSC1 truncation proteins. Truncation protein levels were quantified in at least 3 separate experiments, and the mean expression levels, relative to full-length myc-tagged TSC1, were derived. Standard deviations are indicated.

(C) Relative expression of the TSC1 truncations in Triton X100-soluble and -insoluble cell lysate fractions. The signal detected in the soluble fraction was divided by the total signal (soluble + insoluble) for each protein in at least 3 separate experiments. Standard deviations are indicated.

Figure 3. Immunofluorescence analysis of TSC1 truncation protein expression.

Transfected HEK 293T cells expressing the different truncation proteins were fixed and incubated with an antibody against the myc-epitope tag.

(A) Punctate, cytoplasmic localisation of truncation protein Q900.

(B) Punctate, cytoplasmic localisation of full-length TSC1.

(C) Diffuse, cytoplasmic localisation of truncation protein M351.

(D) Diffuse, cytoplasmic localisation of truncation protein R509.

Figure 4. Effect of the pathogenic M224R amino acid substitution on expression of the R692 truncation protein. Wild-type myc-tagged TSC1 (TSC1), the TSC1-M224R

mutant, the R692 truncation protein, and the R692-M224R mutant truncation were coexpressed with TSC2 in HEK 293T cells. Triton X100-soluble and -insoluble cell lysate fractions were separated on 4-12% gradient SDS-PAGE gels, blotted and incubated with antibodies specific for TSC2 and the myc epitope tag.

(A) Immunoblot showing expression of full-length TSC1 and the R692 truncation protein compared to the TSC1-M224R and R692-M224R mutants. The levels of the TSC1-M224R and R692-M224R mutants detected in the Triton X100 soluble fraction is reduced compared to wild-type TSC1 and the R692 truncation. Coexpressed TSC2 is shown as a control for transfection efficiency.

(B) Total expression of the TSC1-M224R and R692-M224R mutants. Protein levels in the Triton X100 soluble and insoluble fractions were quantified and summed in at least 3 separate experiments, and the mean expression levels, relative to full-length myc-tagged TSC1, were calculated. Standard deviations are indicated.

(C) Relative expression of the TSC1-M224R and R692-M224R mutants in Triton X100-soluble and -insoluble cell lysate fractions. The signal detected in the soluble fraction was divided by the total signal (soluble + insoluble) for each protein in at least 3 separate experiments. Standard deviations are indicated.

Figure 5. Immunoblot analysis of coexpression of TSC2 and the TSC1 truncation proteins. Wild-type myc-tagged TSC1 (TSC1) and the myc-tagged truncations were coexpressed with TSC2 in HEK 293T cells. Triton X100-soluble and -insoluble cell lysate fractions were separated on 4-12% gradient SDS-PAGE gels, blotted and incubated with antibodies specific for TSC2 and the myc epitope tag.

(A) Immunoblot of Triton X100-soluble and -insoluble cell lysate fractions prepared from cells coexpressing TSC2 and the TSC1 truncation proteins.

(B) Effect of TSC2 coexpression on expression levels of the TSC1 truncation proteins.

Protein levels were quantified as in Figure 2B. Standard deviations are indicated.

(C) Effect of TSC2 coexpression on the relative expression levels of the TSC1

truncation proteins in Triton X100-soluble and -insoluble cell lysate fractions.

Expression levels were quantified as in Figure 2C. Standard deviations are indicated.

Figure 6. Immunoblot analysis of TSC1-TSC2 interactions. TSC1 truncation proteins were coexpressed with TSC2 in HEK 293T cells. The truncation proteins were immunoprecipitated from Triton X100-soluble cell lysate fractions using an anti-myc epitope affinity matrix, and coimmunoprecipitated TSC2 was detected by immunoblotting. Each interaction was tested at least 3 times.

(A and B) Immunoblots showing coimmunoprecipitation of TSC2 with full-length TSC1 and the M351, Q900, R692 and R509 truncations, but not with the M351-Q900 or T339 truncations, or the myc-tagged β -lactamase control protein (lac).

(C) Mean total signal intensity of the immunoprecipitated truncations, relative to full-length TSC1. Standard deviations are indicated.

(D) Mean total signal intensity of coimmunoprecipitated TSC2, relative to the TSC2 signal detected after coimmunoprecipitation with full-length TSC1. Standard deviations are indicated.

Figure 7. Immunoblot analysis of TSC1-TSC1 interactions. The myc-tagged TSC1 truncations were coexpressed with full-length TSC1 (untagged) in HEK 293T cells. The truncations were immunoprecipitated from Triton X100-soluble cell lysate fractions using an anti-myc epitope affinity matrix. Coimmunoprecipitated, untagged TSC1 was detected by immunoblotting. Each interaction was tested at least 3 times.

(A) Immunoblot showing coimmunoprecipitation of full-length TSC1 with the M351-Q900, M351, R692 and R509 truncations, but not with the Q900 or T339 truncations, or the myc-tagged β -lactamase control protein (lac).

(B) Mean total signal intensity of the immunoprecipitated truncations, relative to the β -lactamase control protein (lac). Standard deviations are indicated.

(C) Mean total signal intensity of coimmunoprecipitated TSC1, relative to the signal detected after coimmunoprecipitation with the M351 truncation. Standard deviations are indicated.

Figure 8. Inhibition of S6K-T389 phosphorylation by the TSC1 proteins truncations.

(A) Immunoblot analysis of cells expressing S6K, TSC2 and either wild-type TSC1 (TSC1), mutant TSC1 (L117P), or the TSC1 truncation proteins (Q900, M351, R692, T339 and M351-Q900). Cells transfected with vector only were included as a control (control).

(B) Expression of the TSC1 truncation proteins. The signals for each truncation protein, relative to wild-type TSC1, were determined in 3 independent experiments. Standard deviations are indicated.

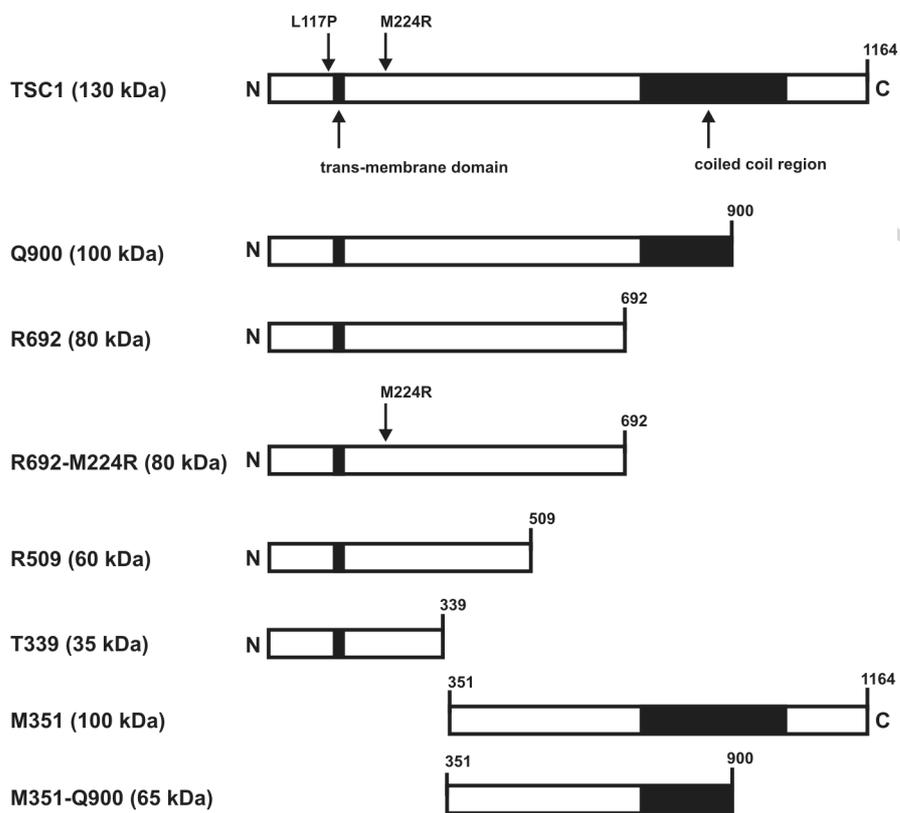
(C) Expression of TSC2 in the presence of the TSC1 truncation proteins. The TSC2 signals in the presence of the different TSC1 truncations, relative to the TSC2 signal in the presence of wild-type TSC1 were compared. No large differences were observed.

(D) Inhibition of S6K T389 phosphorylation in the presence of different TSC1 truncations. The ratio of the T389 S6K phosphorylation signal intensity to the total S6K signal intensity (T389/S6K), relative to the wild-type TSC1 (wild-type TSC1 T389/S6K ratio = 1) was determined for each truncation. Standard deviations are indicated.

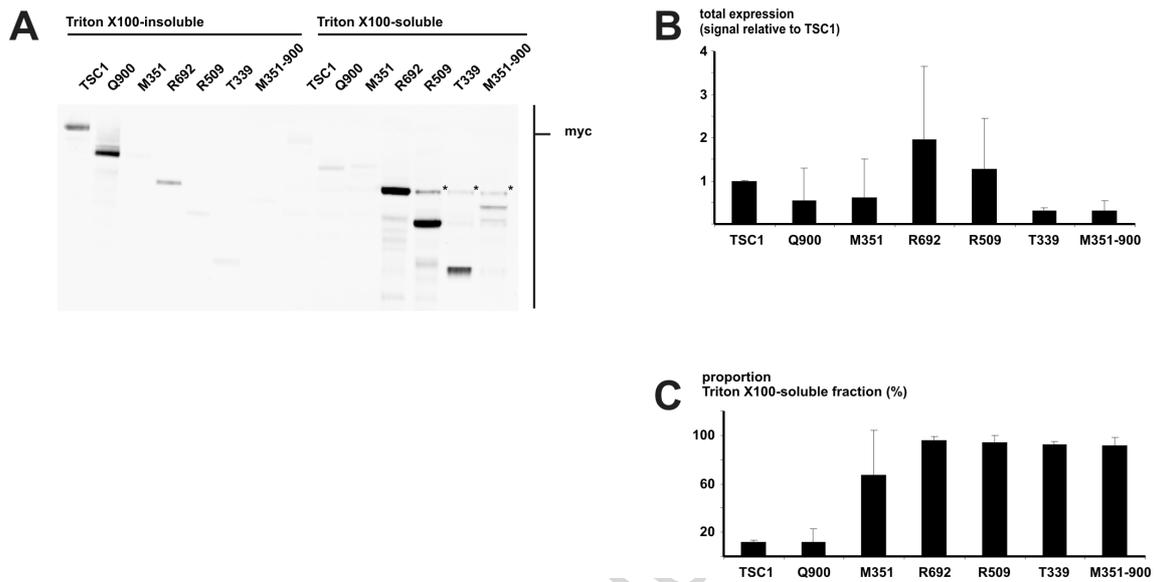
(E) Expression of S6K in the presence of the TSC1 truncations. The total S6K signal in the presence of each of the different truncations, relative to the signal in the presence of wild-type TSC1 (TSC1), was determined for each truncation. Standard deviations are indicated. The total S6K signals in the presence of the different truncations were similar, indicating that transfection efficiency and gel-loading was consistent between samples.

Table 1. Clinical findings in individuals with the *TSC1* c.2919C>T (p.Q900X), *TSC1* c.2295C>T (p.R692X) and *TSC1* c.1015dupA (p.T339X) mutations.

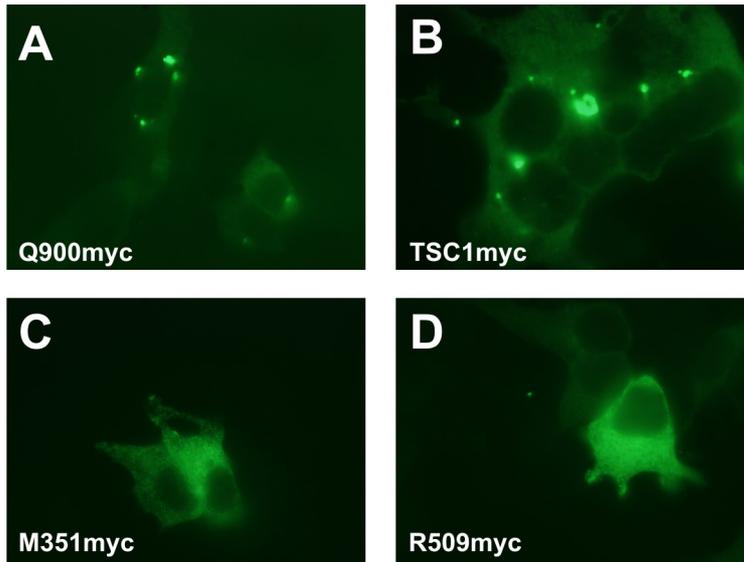
Key: -, not present, or not recorded; RC, renal cysts; CR, cardiac rhabdomyoma; DP, dental pit; RP, retinal phakoma; WPW, Wolff-Parkinson-White syndrom; HM, hypomelanotic macule; SP, shagreen patch; UF, ungual fibroma; FA, facial angiofibroma; E, epilepsy; MR, mental retardation; BD, behavioural disturbance; SEN, subependymal nodule; CT, cortical tuber; SEGA, subependymal giant cell astrocytoma.

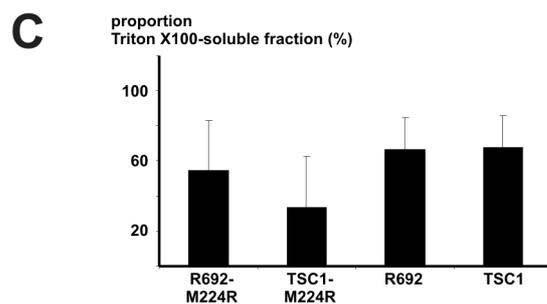
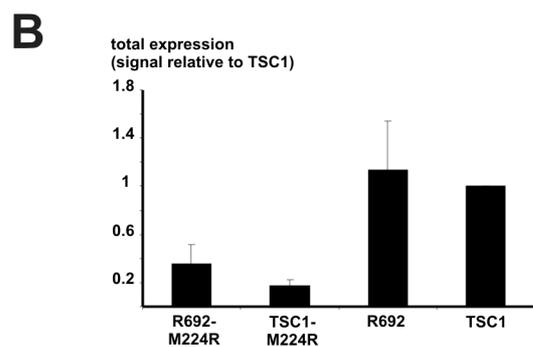
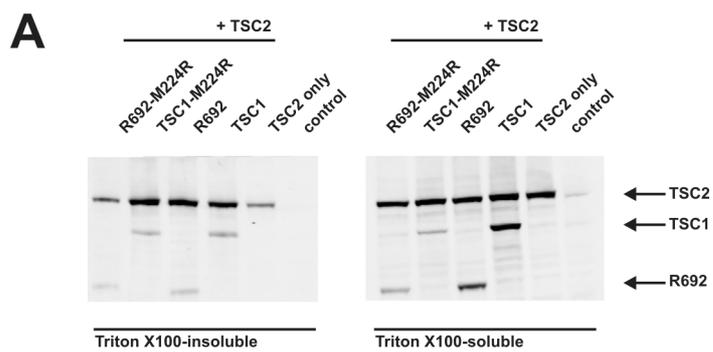


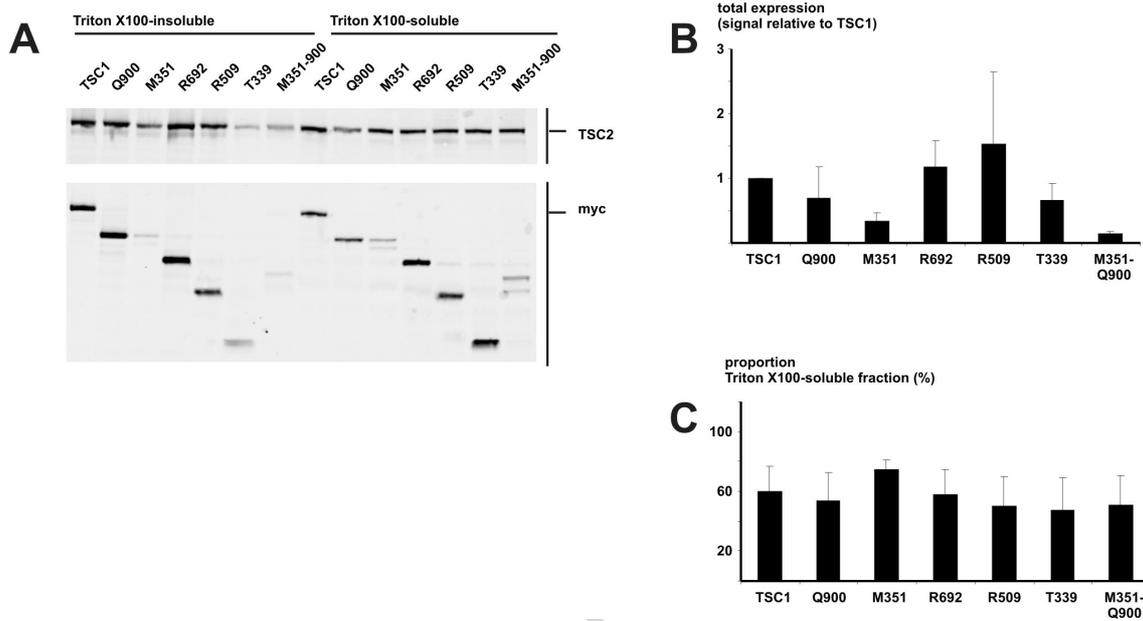
ACCEPTED



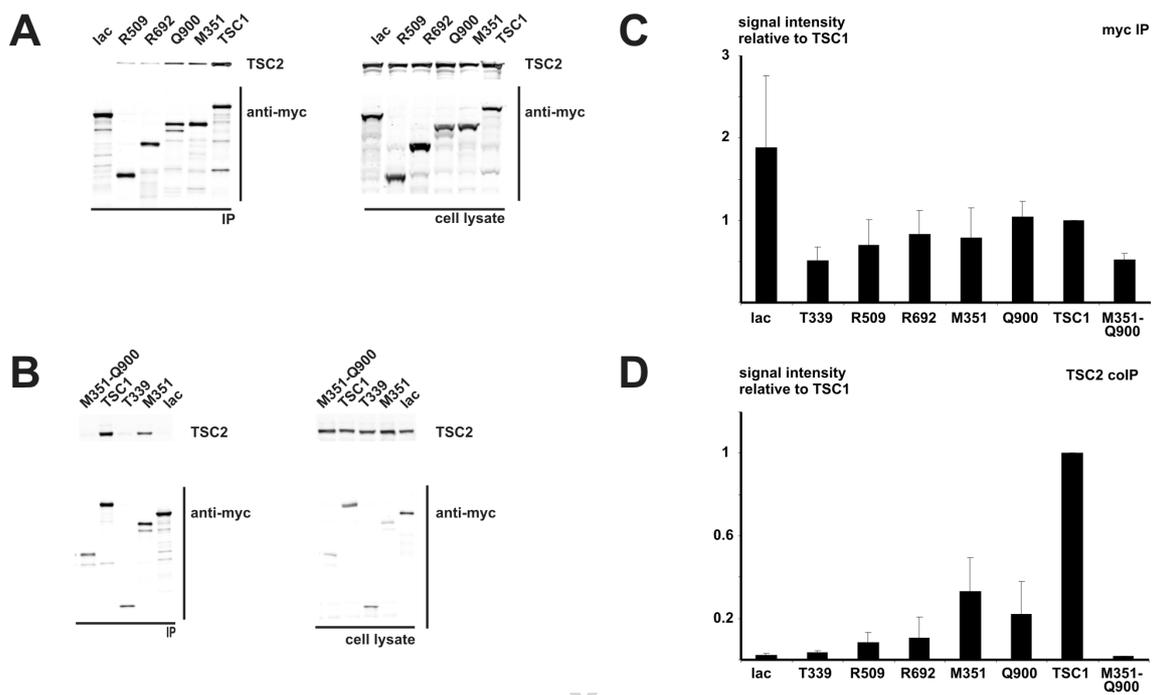
ACCEPTED MANUSCRIPT



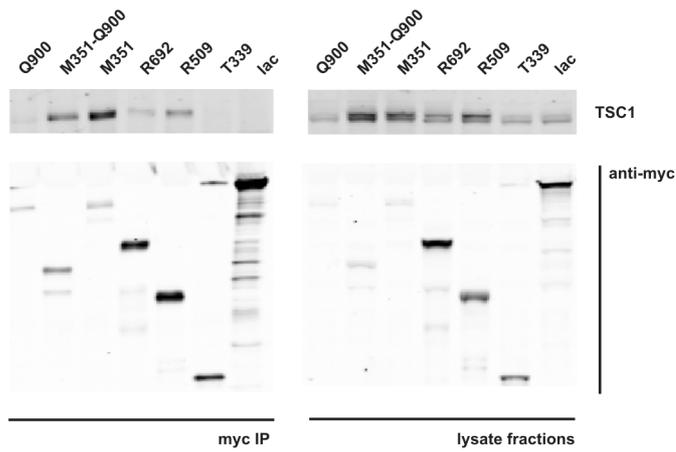
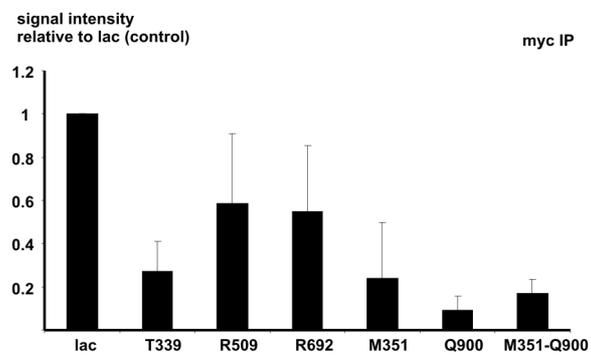
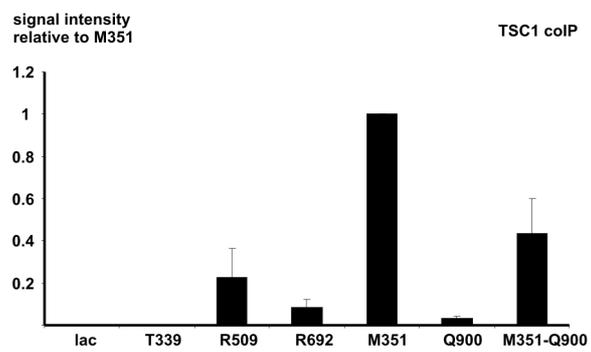


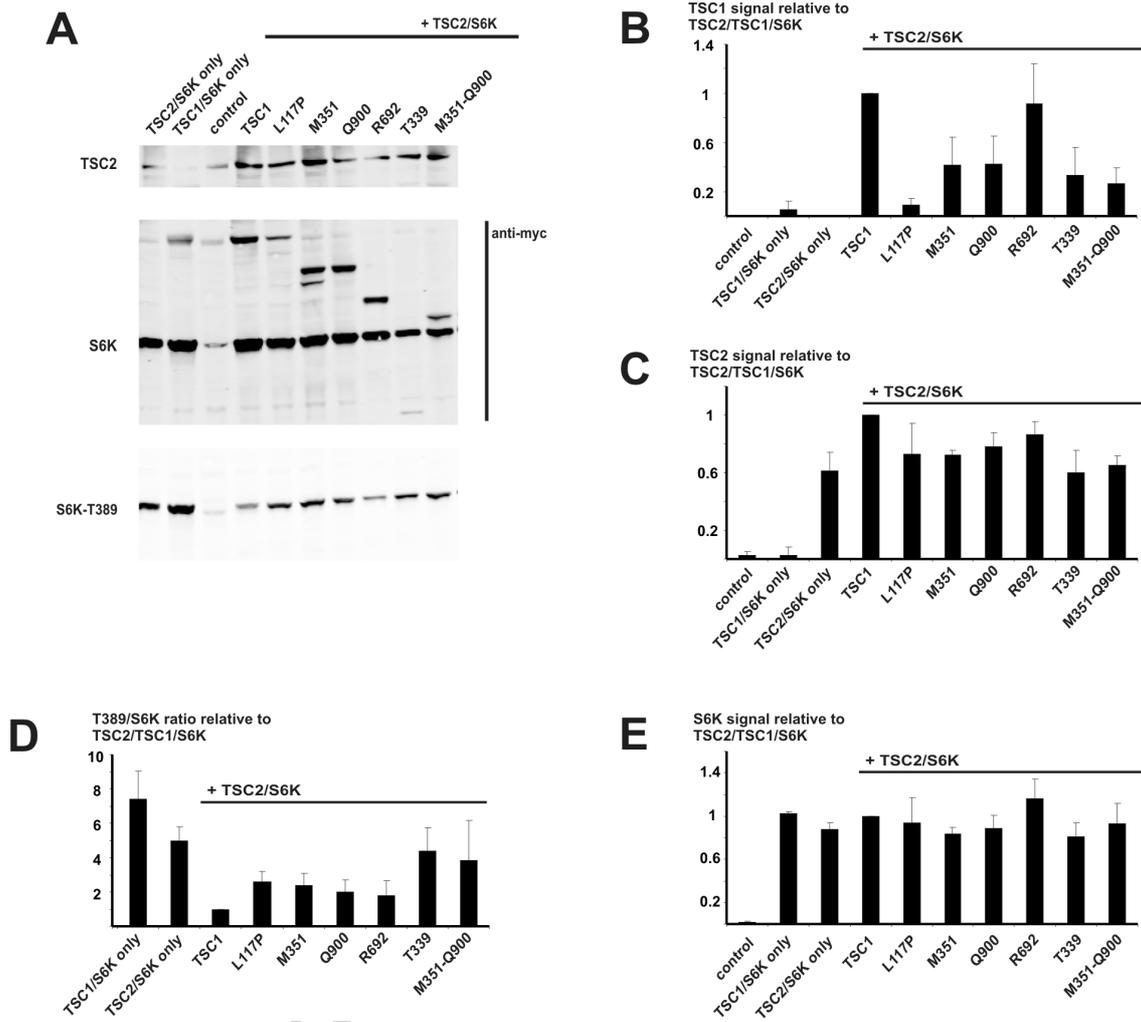


ACCEPTED MAI



ACCEPTED MANUSCRIPT

A**B****C**



Case	Mutation	Renal	Other	Skin	Neurological	Brain
1	c.2919C>T	RC	-	HM, FA, SP	E, MR, BD	SEN, CT
2	c.2295C>T	-	CR	HM	E, MR	SEN, CT
3	c.2295C>T	-	-	HM	E	SEN, CT
4.1	c.2295C>T	-	-	FA, HM	E	SEGA
4.2	c.2295C>T	-	-	UF	E	SEN
5	c.2295C>T	-	CR	HM, SP	E, BD	-
6	c.2295C>T	-	DP, RP	FA, UF	E, BD	SEN, CT
7	c.1746C>T	RC	CR	-	-	SEN, CT
8	c.1015dupA	-	WPW	HM	E, BD	-