

Interaction between hamartin and tuberin, the *TSC1* and *TSC2* gene products

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Received February 13, 1998; Revised and Accepted March 27, 1998

Tuberous sclerosis (TSC) is an autosomal dominant disorder caused by a mutation in either the *TSC1* or *TSC2* tumour suppressor gene. The disease is characterized by a broad phenotypic spectrum that can include seizures, mental retardation, renal dysfunction and dermatological abnormalities. *TSC2* encodes tuberin, a putative GTPase activating protein for rap1 and rab5. The *TSC1* gene was recently identified and codes for hamartin, a novel protein with no significant homology to tuberin or any other known vertebrate protein. Here, we show that hamartin and tuberin associate physically *in vivo* and that the interaction is mediated by predicted coiled-coil domains. Our data suggest that hamartin and tuberin function in the same complex rather than in separate pathways.

INTRODUCTION

Tuberous sclerosis (TSC) is characterized by the widespread development of hamartomatous growths in many tissues and organs. The brain, eyes, kidneys, heart and skin are frequently affected, but the lungs, skeleton and endocrine glands may also be involved (1). The lack of clues as to cellular functional abnormalities has meant that efforts to identify the primary underlying defect in TSC patients have focused on positional cloning.

TSC is genetically heterogeneous, with loci on chromosomes 9q34 (*TSC1*) and 16p13.3 (*TSC2*) (2). The *TSC2* gene was isolated in 1993 (3) and codes for tuberin, a 200 kDa (1807 amino acid) protein. Tuberin contains a relatively hydrophobic N-terminal domain (4) and a conserved 163 amino acid region close to the C-terminus, which is homologous to the GTPase activating proteins (GAP) rap1GAP and mSpa1 (5).

The *TSC1* gene was recently identified (6) and codes for hamartin, a 130 kDa (1164 amino acid) hydrophilic protein with no significant homology to tuberin or other known vertebrate proteins.

We tested whether hamartin and tuberin could interact using the yeast two-hybrid system (7) and transfection assays. Further-

more, in human cells in culture, we investigated the association between endogenous hamartin and tuberin by coimmunoprecipitation. Our data demonstrate that hamartin and tuberin associate physically *in vivo* suggesting that both proteins play a closely related role in an as yet undetermined physiological process.

RESULTS

The predicted coiled-coil domain in hamartin interacts with a putative coiled-coil domain in tuberin

The predicted amino acid sequences of hamartin and tuberin were analysed for potential interaction domains. COILS version 2.1 (8) identified a more extensive coiled-coil structure in hamartin than reported previously (6) (amino acids 719–998, window size 28) (Fig. 1a), while a less stringent analysis (window size 14) of the original tuberin sequence (3) predicted two coiled-coil domains at amino acid positions 346–371 and 1008–1021 (Fig. 1a). As coiled-coil domains have the capacity to form homophilic and heterophilic protein complexes (8), these domains were made the focus of subsequent yeast two-hybrid experiments.

A construct coding for tuberin (amino acids 1–1784) (3) fused to the GAL4 DNA-binding domain was tested against constructs coding for the GAL4 transactivating domain fused to the N-terminal (XB1, amino acids 23–357) and C-terminal (EE1a, amino acids 334–1153) domains of hamartin (Fig. 1b). A strong, specific interaction was detected between tuberin and EE1a, containing the C-terminal, putative coiled-coil domain of hamartin. No interaction was detected between tuberin and the N-terminal domain of hamartin. Self-activation of GAL4 activity was not observed for any of the constructs used in this study.

To define the binding domain within hamartin more precisely, a series of truncated constructs was analysed (Fig. 1b). Only construct ESA (amino acids 334–673), lacking the entire coiled-coil domain, did not interact with tuberin. Construct EE2 (amino acids 334–788) tested positive, suggesting that the first seven heptad structures in the coiled-coil domain were sufficient for hamartin to interact with tuberin.

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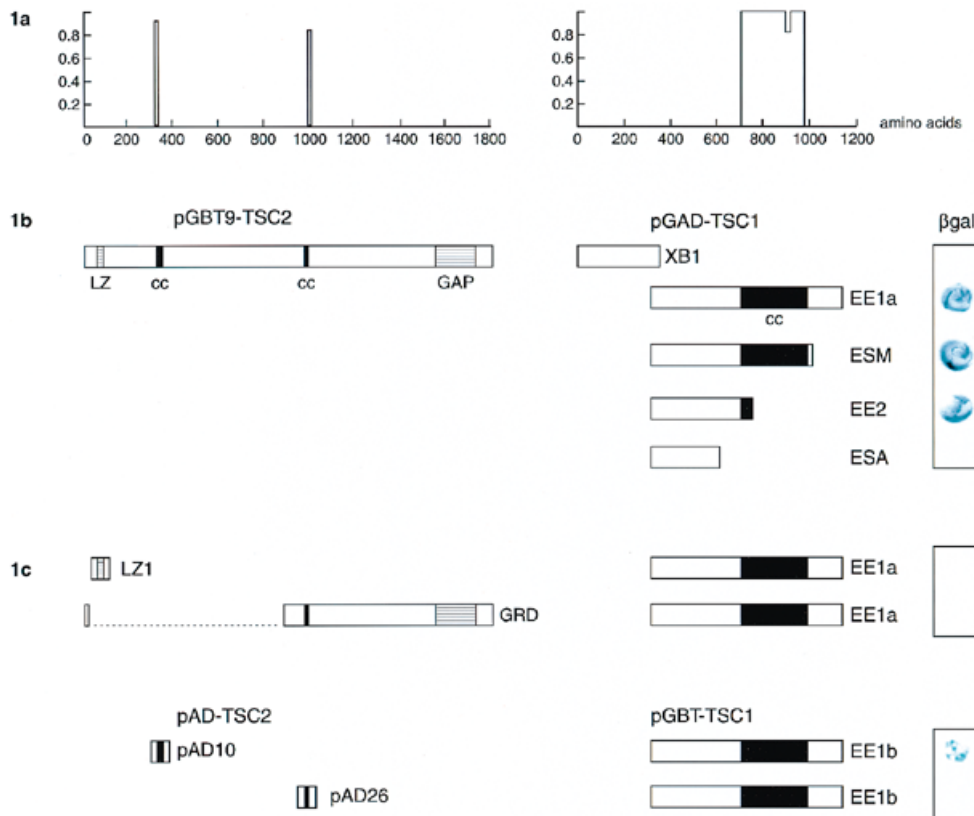


Figure 1. Coiled-coil predictions and mapping of the interacting domains of hamartin and tuberin in the yeast two-hybrid system. (a) Plot showing the position and the probability of the coiled-coil motifs in tuberin and hamartin; COILS version 2.1 window size 28 for hamartin and window size 14 for tuberin. (b) One N-terminal and four C-terminal *TSC1* constructs, fused to the DNA transactivation domain of GAL4 (XB1, EE1a, ESM, EE2 and ESA, respectively), were assayed for interaction with a full-length *TSC2* construct, fused to the DNA-binding domain of GAL4. The deletion constructs of EE1a were created with the internal restriction sites *SmaI* (ESM), *EcoRI* (EE2) and *SaII* (ESA). The putative coiled-coil domains (cc; shaded boxes) in hamartin and tuberin, and the N-terminal leucine zipper and C-terminal GAP-related domain in tuberin (hatched boxes), are indicated. The bait and prey constructs were cotransformed in yeast strain YGH1 bearing a *lacZ* reporter. Interaction was detected with the β -galactosidase assay, positives resulting in a blue colour (last column). (c) The N-terminal leucine zipper (LZ1) and C-terminal construct (GRD), fused to the GAL4 DNA-binding domain, and the two putative coiled-coil structures fused to the GAL4 transactivation domain (pAD10 and pAD26), were tested against the EE1 hamartin construct fused to either the GAL4 DNA-binding (EE1b) or transactivation domain (EE1a).

The interaction domain in tuberin was also defined using partial constructs (Fig. 1c). A potential leucine zipper (amino acids 81–121), the two putative coiled-coil domains (amino acids 346–371 and 1008–1021) and a deletion construct (GRD; amino acids 1–41 and 861–1784) containing the GAP-related domain were tested against the EE1 (amino acids 334–1153) hamartin construct. Only the most N-terminal coiled-coil construct (amino acids 346–371) tested positive. The specificity of the coiled-coil interaction was investigated in the two-hybrid assay using another coiled-coil containing protein, giantin (9), against hamartin and tuberin. No GAL4 activation occurred (data not shown), indicating that the interaction detected between the coiled-coil domains in hamartin and tuberin was specific.

Hamartin and tuberin colocalize in transfected mammalian cells

In order to confirm the two-hybrid results, the localization of hamartin and tuberin in transfected cells was studied using immunofluorescent microscopy. A full-length *TSC1* cDNA in the pcDNA3.1 expression vector was transfected into COS cells. A distinct labelling pattern was observed, consisting of discrete

structures in the cytoplasm (Fig. 2A). In contrast, expression of an epitope tagged full-length *TSC2* construct produced a general cytoplasmic labelling pattern (Fig. 2B). When COS cells were cotransfected with the *TSC1* and *TSC2* construct, both hamartin and tuberin localized to the same structures as well as to the cell cytoplasm (Fig. 2C–E). Untransfected COS cells did not stain with the hamartin- and tuberin-specific antisera. Similar results were obtained in transfected HeLa and CHO cells (data not shown). To investigate whether the colocalization was due to overexpression of hamartin and tuberin, several control proteins including the fragile X mental retardation protein, acid α -glucosidase and the C-terminal domain of polycystin, containing a predicted coiled-coil structure (10), were co-expressed with hamartin in COS cells. None of the controls colocalized to the hamartin positive structures, confirming that the colocalization of hamartin and tuberin was specific.

Hamartin and tuberin coimmunoprecipitate *in vivo*

In order to investigate whether the observed association between hamartin and tuberin also occurred *in vivo*, the endogenous proteins were immunoprecipitated from HeLa cells and cultured

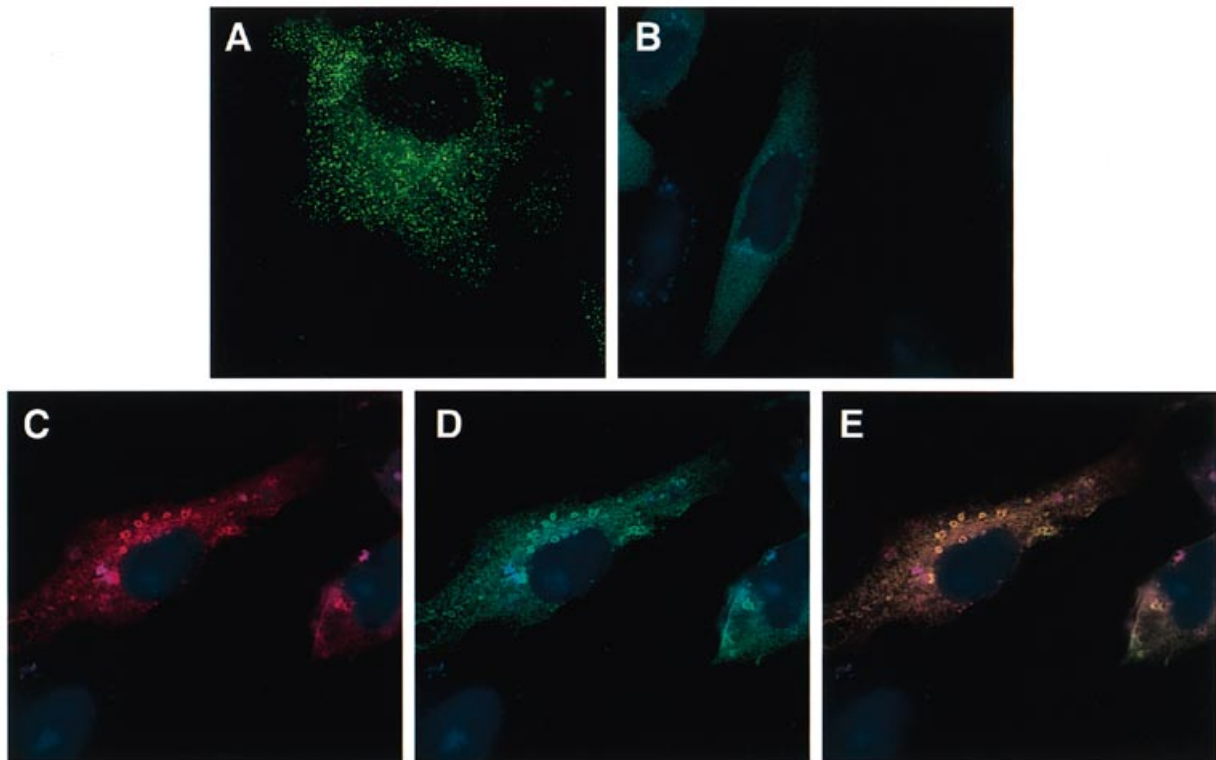


Figure 2. Colocalization of hamartin and tuberlin in COS cells. (A) COS cell, transfected with the full-length *TSC1* construct. (B) COS cell, transfected with the full-length *TSC2* construct. Both proteins were detected with specific rabbit polyclonal primary antisera, followed by anti-rabbit IgG secondary antisera conjugated to fluorescein (FITC). (C–E) Cotransfection of full-length *TSC1* and *TSC2* constructs. Cells were double labelled as follows. (C) Hamartin was detected with a specific rabbit polyclonal antiserum [as in (A)], followed by an anti-rabbit IgG–Texas Red-coupled secondary antibody, and (D) tuberlin was visualized with a mouse monoclonal antibody against an N-terminal epitope tag sequence, followed by an anti-mouse IgG–FITC conjugate (Xpress; Invitrogen). (E) Colocalization of hamartin and tuberlin in the cytoplasm of COS cells (yellow).

human fibroblasts. Hamartin could be recovered from the immunoprecipitates of antisera specific for tuberlin, while tuberlin coimmunoprecipitated with hamartin when an antiserum specific for hamartin was used (Fig. 3). Identical results were obtained with different anti-hamartin and anti-tuberlin antisera (data not shown). Preimmune sera and a control antiserum (against human acid α -glucosidase) were negative, demonstrating that the observed coimmunoprecipitation of tuberlin and hamartin was specific, and confirming that the association detected by the two-hybrid assay occurs in mammalian cells.

DISCUSSION

To investigate the molecular mechanism underlying TSC, we tested for an interaction between tuberlin and hamartin using three independent methods. In each case, we showed that the proteins are partners. In view of the phenotypic overlap observed between TSC patients with either a *TSC1* or *TSC2* mutation, this suggests that inactivation of hamartin or tuberlin may prevent the formation of a functional protein complex. A comparable scenario has been reported recently for the PKD1 and PKD2 proteins (10,11). However, unlike polycystin 1 and 2, no regions of homology between tuberlin and hamartin have been detected (6).

The interaction between tuberlin and hamartin is mediated by potential coiled-coil domains. A predicted N-terminal coiled-coil domain in tuberlin interacts with only a small part of an extensive

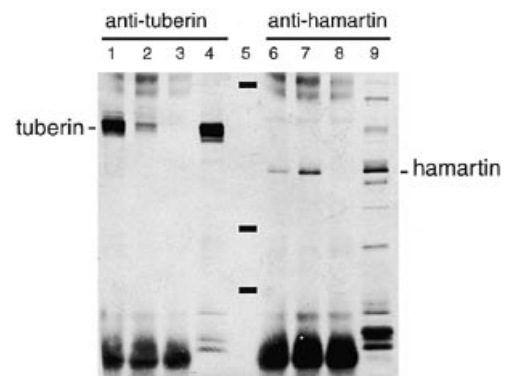


Figure 3. *In vivo* coimmunoprecipitation of tuberlin and hamartin. Tuberlin and hamartin were immunoprecipitated from fibroblast lysates with tuberlin-specific antiserum (lanes 1 and 6), hamartin-specific antiserum (lanes 2 and 7) and an antiserum against human acid α -glucosidase (lanes 3 and 8). Lanes 4 and 9 contain the lysate prior to immunoprecipitation. The molecular weight marker is in lane 5 (from top to bottom: 230, 100, 80 kDa). Lanes 1–4 were incubated with anti-tuberlin antibody, and lanes 6–9 with anti-hamartin antibody. IgG heavy chains (50 kDa) are visible in lanes 1–3 and 6–8. The additional lower molecular weight bands in lane 9 are probably degradation products of hamartin, not consistently observed (data not shown).

coiled-coil region in hamartin. Preliminary results indicate that the coiled-coil domain in hamartin can form a homophilic complex (data not shown). We are currently investigating whether the additional coiled-coils in hamartin mediate interactions with additional proteins important in the pathogenesis of TSC.

Overexpression of hamartin in COS cells showed a distinct labelling pattern in the cytoplasm, while tuberin produced a general cytoplasmic labelling. When hamartin and tuberin were cotransfected in mammalian cells, tuberin was recruited in a specific manner to the hamartin positive structures and the proteins colocalized more generally in the cytoplasm. These data, together with the *in vivo* association of hamartin and tuberin detected by coimmunoprecipitations, support the results of the two-hybrid system and provide strong evidence that hamartin and tuberin exist as a complex.

Recently, it has been demonstrated by the two-hybrid system that the C-terminal part of tuberin interacts with rabaptin-5 (12). Rabaptin-5 is a 115 kDa cytosolic protein that is an effector for the endosomal small GTPase rab5 and therefore involved in endocytic fusion events (13). Consistent with the tuberin-rabaptin-5 interaction, tuberin has been shown to act as a GTPase activating protein for rab5, and to reduce the rate of fluid-phase endocytosis (12). It will be important to establish whether binding between hamartin and tuberin regulates the rab5 GAP activity of tuberin and to investigate the effect of hamartin expression on fluid-phase endocytosis and early endosome fusion. We are currently investigating the nature of the hamartin-containing structures detected in the transfection experiments. It is possible that the identification of additional endosomal proteins that interact with either hamartin, tuberin or both may help to clarify whether dysregulation of endocytosis is important in the aetiology of TSC.

MATERIALS AND METHODS

TSC1 and *TSC2* constructs

A full-length *TSC2* cDNA (nucleotides 1–5474) was derived from previously identified partial cDNAs (3) and cloned into the pGBT9 (Clontech) and pcDNA3.1HisA (Invitrogen) vectors. The *TSC2* C-terminal GAP domain construct (GRD) was made by digestion with *SacII*, leading to an in-frame deletion of amino acids 42–860. The full-length *TSC1* cDNA was amplified by RT-PCR with oligonucleotides 5'-TGAGGTAAACAGCTGAGGGG-3' and 5'-AAGGTCAAGAGGCATTTC-3' and cloned into pGEM-T Easy (Promega) and subsequently into pcDNA3.1. The remaining *TSC1* and *TSC2* constructs were derived by PCR, using primers with linkers for direct restriction site cloning. pAD26, pAD10 and LeuZip were amplified from a *TSC2* cDNA clone using the primer pairs 5'-CTCGAATTC-CACGCAGTGGGAAGCACTCTG-3' and 5'-CTCGGATCCG-GAAGGGTAATCCTTGATGACC-3' for LeuZip, 5'-GGAAT-TCCAGACGTCCCTCACCAGTGC-3' and 5'-GCTCTAGAA-GCCGTGAAGTTGGAGAAGA-3' for pAD26, 5'-GGAATTC-GAGATCGTCTGTCCATCAC-3' and 5'-GCTCTAGACG-CACATCTCTCCACCAGTT-3' for pAD10. The *TSC1* deletion constructs were amplified by RT-PCR with the primer pairs 5'-CCCGGGGACGACGTGACAGCTGTCTTT-3' and 5'-CC-CGGGGAGTGGTCATACCACAAACCAT-3' for XB1, 5'-GGA-TCCCATGATGAGTCTCATTGTAGTC-3' and 5'-GGATCC-GACACGGCTGATAACTGAACCA-3' for EE1a, 5'-GGATCC-

CTCATGATGAGTCTCATTGTAGT-3' and 5'-GGATCCCGA-CACGGCTGATAACTGAACCA-3' for EE1b. pAD26 and pAD10 were cloned into pAD-2.1 (Stratagene), LeuZip and EE1b into pGBT9 (Clontech) and XB1 and EE1a into pGADGH (Clontech). Three deletion constructs (ESM, EE2 and ESA) were generated using internal restriction sites (*SmaI*, *EcoRI* and *Sall*, respectively). All constructs were checked by sequencing and, where appropriate, by *in vitro* coupled transcription-translation assay (TnT system; Promega).

Yeast two-hybrid assay

Yeast host strain (YGH1) was cotransformed with 2.5 µg of each plasmid according to the SBEG method (14). Transformants were plated on minimal media lacking Trp and Leu. After 3 days, three colonies per interaction were plated on media lacking His, Trp and Leu, and growing colonies were tested for β-galactosidase activity with the filter assay.

Generation of tuberin- and hamartin-specific antisera

Two fusion proteins containing N-terminal histidine tag sequences and amino acids 1535–1784 from tuberin and 543–1087 from hamartin were overexpressed in bacteria and affinity purified under denaturing conditions according to the manufacturer's protocol (Qiagen GmbH). The final eluates were concentrated through an Amicon PM-10 filter and dialysed against phosphate-buffered saline. New Zealand white rabbits were immunized with 150 µg of purified fusion protein suspended in Freund's complete adjuvant, and boosted at 4-week intervals with 150 µg fusion protein in Freund's incomplete adjuvant. Serum was collected 10 days after injection of the immunogen. The resulting polyclonal sera were checked for specificity by western blot and transfection experiments.

Immunofluorescence

Expression constructs were transfected into COS cells with lipofectamine, as recommended by the manufacturer (Gibco BRL). For immunocytochemistry, cells were fixed in 3% paraformaldehyde (10 min, room temperature), quenched with 50 mM NH₄Cl (10 min) and permeabilized in 0.1% Triton X-100 (5 min). Cells were incubated with primary antibodies, followed by fluorescein (FITC)- or Texas Red (TRITC)-coupled secondary antibodies. Images were captured using the Power Gene FISH system on a Leica DM RXA microscope. Images were processed using a filter wheel (Chroma Technology) and the Adobe Photoshop software package. In addition to the polyclonal sera against hamartin and tuberin, generated as part of this study, antibodies against FMRP, polycystin and α-glucosidase were used for control experiments.

Coimmunoprecipitations

Washed cells (one 10 cm plate) were lysed in 700 µl TNE buffer (40 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40 containing 0.2 mM PMSF), according to standard procedures (15) and cleared by centrifugation (10 000 g, 10 min, 4°C). The supernatant was recovered and incubated with 2 µl antisera for 60 min on ice before the addition of 30 µl 50% protein A-Sepharose suspension. After gentle rotation for 60 min at 4°C, the immunoprecipitates were washed extensively with TNE buffer. Immunoblotting was performed according to standard procedures

(15) and coimmunoprecipitating proteins were detected using the appropriate antibodies and enhanced chemiluminescence (Amersham).

ACKNOWLEDGEMENTS

We are grateful to Bert Eussen and Tom de Vries-Lensch for help with the figures, to Arjenne Hesselting-Janssen and Shelley Idziaszyk for technical assistance, and Professors H. Galjaard and D. Lindhout for support. We thank Filippo Tamanini and Miriam Goedbloed for providing us with the *FMRP* and *PKD1* constructs and antibodies. This work was funded by the Dutch Organization for Scientific Research (NWO), Dutch Kidney Foundation (Nierstichting), Dutch Cancer Society (NKB), Tuberous Sclerosis Association of Great Britain, National Tuberous Sclerosis Association (USA), Medical Research Council and Cancer Research Wales. P.v.d.S. is an investigator of the Royal Netherlands Academy of Arts and Sciences

REFERENCES

- Gomez, M.R. (1980) *Tuberous Sclerosis*, 2nd edn. Raven Press, New York.
- Janssen, L.A.J., Sampson, J.R., van der Est, M., Deelen, W., Verhoef, S., Daniels, I., Hesselting, A., Brook-Carter, P., Nellist, M., Lindhout, D., Sandkuijl, L.A. and Halley, D.J.J. (1994) Refined localization of *TSC1* by combined analysis of 9q34 and 16p13 data in 14 tuberous sclerosis families. *Hum. Genet.*, **94**, 437–440.
- The European Chromosome 16 Tuberous Sclerosis Consortium (1993) Identification and characterization of the tuberous sclerosis gene on chromosome 16. *Cell*, **75**, 1305–1315.
- Maheshwar, M., Sandford, R., Nellist, M., Cheadle, J.P., Sgotto, B., Vaudin, M. and Sampson, J.R. (1996) Comparative analysis and genomic structure of the tuberous sclerosis 2 (*TSC2*) gene in human and pufferfish. *Hum. Mol. Genet.*, **5**, 131–137.
- Maheshwar, M., Cheadle, J.P., Jones, A.C., Myring, J., Fryer, A.E., Harris, P.C. and Sampson, J.R. (1997) The GAP-related domain of tuberin, the product of the *TSC2* gene, is a target for missense mutations in tuberous sclerosis. *Hum. Mol. Genet.*, **6**, 1991–1996.
- van Slegtenhorst, M., de Hoogt, R., Hermans, C., Nellist, M., Janssen, B., Verhoef, S., Lindhout, D., van den Ouweland, A., Halley, D., Young, J., Burley, M., Jeremiah, S., Woodward, K., Nahmias, J., Fox, M., Ekong, R., Osborne, J., Wolfe, J., Povey, S., Snell, R.G., Cheadle, J.P., Jones, A.C., Tachataki, M., Ravine, D., Sampson, J.R., Reeve, M.P., Richardson, P., Wilmer, F., Munro, C., Hawkins, T.L., Sepp, T., Ali, J.B.M., Ward, S., Green, A.J., Yates, J.R.W., Kwiatkowska, J., Henske, E.P., Short, M.P., Haines, J.H., Jozwiak, S. and Kwiatkowski, D.J. (1997) Identification of the tuberous sclerosis gene *TSC1* on chromosome 9q34. *Science*, **277**, 805–807.
- Fields, S. and Song, O. (1989) A novel genetic system to detect protein–protein interactions. *Nature*, **340**, 245–246.
- Lupas, A., Van Dyke, M. and Stock, M. (1991) Predicted coiled-coils from protein sequences. *Science*, **252**, 1162–1164.
- Lindsted, A.D., Foguet, M., Renz, M., Seelig, H.P., Glick, B.S. and Hauri, H.P. (1995) A C-terminally-anchored Golgi protein is inserted into the endoplasmic reticulum and then transported to the Golgi apparatus. *Proc. Natl Acad. Sci. USA*, **92**, 5102–5105.
- Qian, F., Germino, F.J., Cai, Y., Zhang, X., Somlo, S. and Germino, G.G. (1997) *PKD1* interacts with *PKD2* through a probable coiled-coil domain. *Nature Genet.*, **16**, 179–183.
- Tsiokas, L., Kim, E., Arnould, T., Sukhatme, V.P. and Walz, G. (1997) Homo- and heterodimeric interactions between the gene products of *PKD1* and *PKD2*. *Proc. Natl Acad. Sci. USA*, **94**, 6965–6970.
- Xiao, G.-H., Shoarinejad, F., Jin, F., Golemis, E.S. and Yeung, R.S. (1997) The *TSC2* gene product, tuberin, functions as a Rab5 GTPase-activating protein (GAP) in modulating endocytosis. *J. Biol. Chem.*, **272**, 6097–6100.
- Stenmark, H., Vitale, G., Ullrich, O. and Zerial, M. (1995) Rabaptin-5 is a direct effector of the small GTPase Rab5 in endocytic membrane fusion. *Cell*, **83**, 423–432.
- Klebe, R.J., Harriss, J.V., Sharp, Z.D. and Douglas, M.G. (1983) A general method for polyethylene-glycol-induced genetic transformation of bacteria and yeast. *Gene*, **25**, 333–341.
- Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY.