LETTER TO JMG

Expression of wild type and mutant *TSC2*, but not *TSC1*, causes an increase in the G1 fraction of the cell cycle in HEK293 cells

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Tuberous sclerosis complex (TSC) is a tumour suppressor gene syndrome whose manifestations include seizures, mental retardation, autism, and tumours of the brain, retina, kidney, heart, and skin.[1] Mutations in two tumour suppressor genes, *TSC1* on chromosome 9q34 and *TSC2* on chromosome 16p13, cause TSC. *TSC2* encodes tuberin, a 190 kDa protein with homology to the catalytic domain of a GTPase activating protein (GAP) for Rap1.[2] *TSC1* encodes hamartin, a 130 kDa protein.[3] Tuberin and hamartin have been shown to directly interact, both in mammalian cells[4 5] and in *Drosophila*.[6 7] This is consistent with the nearly identical spectrum of disease seen in humans with *TSC1* and *TSC2* germline mutations, and with the identical phenotypes of *Drosophila TSC1*[6–8] and *TSC2*[9] homologue mutants. The mouse models of *TSC1* and *TSC2* also have similar phenotypes; renal carcinoma and renal cysts develop in heterozygous animals of the Eker rat model of *TSC2*[10 11] and in the knock out mouse models of both *TSC1*[12] and TSC2,[13 14] all of which are embryonically lethal in the homozygous form.

The cellular pathways through which germline *TSC1* or *TSC2* mutations result in tumorigenesis are not completely understood. Mammalian tuberin and hamartin have been shown to suppress cell growth, accompanied by an increase in cells in the G1 phase of the cell cycle.[15–17] The importance of cell cycle regulation to human TSC is not known. In this study, we used a sensitive fluorescence activated cell sorting approach to investigate the cell cycle effects of wild type hamartin and tuberin, two patient derived mutant forms of tuberin, and a carboxy-terminus construct of tuberin containing the region of GTPase activating protein homology. Similar overexpression approaches using cell sorting have been used to elucidate the cell cycle effects of numerous proteins including the retinoblastoma protein and its family members, PTEN, cyclin dependent kinases, cyclin dependent kinase inhibitors, and substrates of the cyclin dependent kinases.[18–26]

Our goal was to determine the impact of transient expression of wild type tuberin and hamartin, and mutant and truncated forms of tuberin, on the cell cycle. Previously, transient expression of wild type full length tuberin was shown to increase the G1 fraction of cells[16 17] and stable expression of hamartin suppressed cell growth accompanied by an increase in G1.[15]

MATERIAL AND METHODS

We cotransfected cells with *TSC1* and/or *TSC2* in excess of a construct encoding the cell surface protein CD19, and sorted transfected cells by FACS using an FITC conjugated anti-CD19 antibody. Transfections were performed using calcium-phosphate precipitation. The *TSC1* and/or *TSC2* constructs were transfected in five-fold molar excess of the expression vector encoding the cell surface protein CD19, as previously described.[27] After transfection, the cells were washed with phosphate buffered saline and cultured in fresh media until trypsinisation at 48 hours post-transfection. A construct encoding the cyclin dependent kinase inhibitor p21(WAF1/CIP1) was used as a positive control for an increase in the G1 phase of the cell cycle.[27] A summary of the constructs used in these experiments is shown in fig 1A. The C-terminus construct has been previously shown to reduce the in vitro proliferation and in vivo tumorigenicity of Eker rat renal carcinoma cells.[28] Site directed PCR based mutagenesis was performed using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) to generate *TSC2* mutants which were then confirmed by DNA sequencing. To generate the exon 16 (G1832A) *TSC2* mutant construct, the primers were 5′-GAG CAG CAT CCA GCT GCA GGC C-3′ and 5′-GGC CTG CAG CTG GAT GCT GCT C-3′. The G1832A missense change in exon 16 changes the amino acid at position 611 from arginine to glutamine (R611Q). This is a frequent naturally occurring germline mutation in TSC patients[29] and has recently been found as a somatic mutation in tumour cells from women with the sporadic form of pulmonary lymphangiomyomatosis.[30] To generate the exon 38 *TSC2* mutant (C5024T), the primers were 5′-CAC GTG ATC GTC ACC CTG CTG GAC TAC GAG TGC-3′ and 5′-GCA CTC GTA GTC CAG CAG GGT GAC GAT CAC GTG -3′. Constructs were cloned into pcDNA 3.1+ (Invitrogen, Carlsbad, CA). The C5024T missense change in exon 38 changes the amino acid at position 1675, within the GAP related domain, from proline to leucine (P1675L) (fig 1B). This mutation occurs at a highly conserved residue and has been reported in at least 10 unrelated TSC patients (see the TSC Variation Website (http://expmed.bwh.harvard.edu/ts/) for specific references and further details for both mutations).

Overexpression of the appropriate protein(s) was confirmed by western immunoblot analysis (fig 2A). A polyclonal tuberin C20 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), a polyclonal affinity, purified hamartin antibody developed in our laboratory,[5] a p27(KIP1) antibody (Oncogene Research Products, San Diego, CA), and an actin antibody (Sigma, St Louis, MO) were used to detect proteins by enhanced chemiluminescence (Amersham, Piscataway, NJ).

Key points

- To understand the relevance of p27 and cell cycle regulation to the clinical manifestations of TSC, we analysed HEK293 cells overexpressing wild type *TSC1*, wild type *TSC2*, or disease causing mutant forms of *TSC2*.
- Expression of wild type *TSC2*, but not *TSC1*, caused an increase in the G1 fraction of the cell cycle. Expression of the *TSC2* mutants also resulted in a G1 fraction increase.

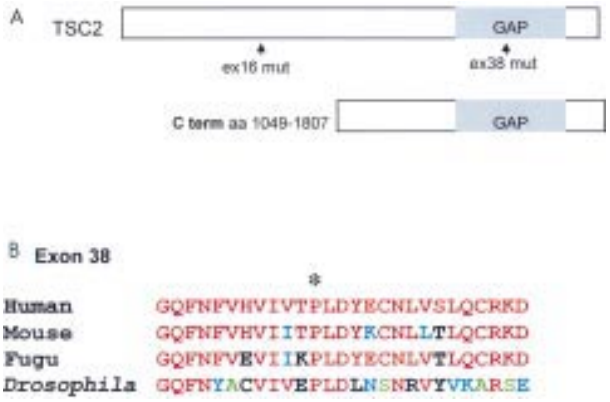


Figure 1 (A) *TSC2* constructs. Tuberin contains a GTPase activating protein (GAP) related domain near its carboxy terminus. The relative positions of the exon 16 and exon 38 missense mutations and the carboxy-terminus construct (aa 1049-1807) are indicated. This C-terminus construct has been previously shown to reduce the in vitro proliferation and in vivo tumorigenicity of Eker rat renal carcinoma cells.[28] (B) Evolutionary conservation of *TSC2* exon 38. The exon 38 mutant results in a Pro1675Leu change (*) within the GAP related domain of tuberin. This mutation has been found in at least 10 unrelated TSC patients. It occurs at a residue that is identical in human, mouse, Fugu, and *Drosophila*. Residues that are identical in the four species are in red, residues that represent conservation of strong groups are in blue, residues representing conservation of weak groups are in green, and residues that are different are in black.

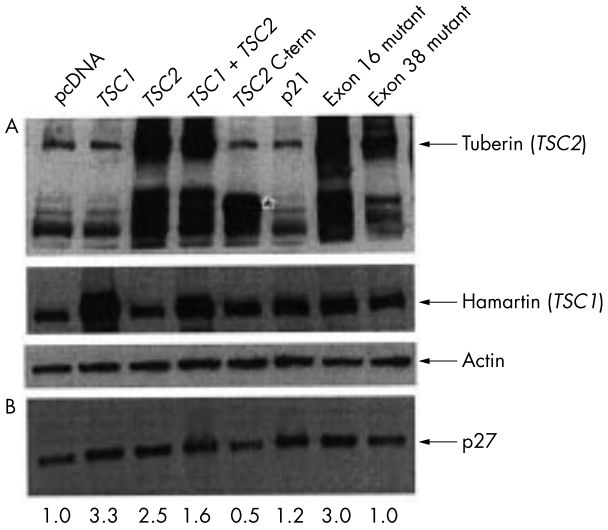


Figure 2 Western immunoblot analysis confirming expression of the *TSC1* and *TSC2* constructs. (A) Overexpression of full length hamartin (migrating at approximately 130 kDa) is seen in the cells transfected with *TSC1* or *TSC1* + *TSC2*, overexpression of full length tuberin (migrating at approximately 190 kDa) is seen in the cells transfected with *TSC2* or *TSC1* + *TSC2*. Overexpression of the carboxy-terminus of *TSC2* is seen as a band migrating at the expected size of approximately 80 kDa (indicated with an asterisk), with no change in the expression of full length tuberin in this lane. Overexpression of the exon 16 or exon 38 mutant forms of tuberin results in a band at the same mobility as wild type tuberin, as expected. In each lane with overexpression of wild type or mutant full length tuberin, two additional bands are seen migrating just above and below the carboxy-terminus band. The functional significance of these smaller products is not known. Beta-actin is shown as a loading control. (B) An increase in p27(KIP1) expression was present when *TSC1*, *TSC2*, *TSC1*, and *TSC2*, or the *TSC2* exon 16 mutant were overexpressed, but not when the carboxy-terminus of *TSC2* or the *TSC2* exon 38 mutant were overexpressed. The number below each lane indicates the relative level of p27 (KIP1) expression (normalised to actin expression) as determined by densitometry. The level of expression in the pcDNA control transfection was arbitrarily set to 1.0.

Two colour fluorescence activated cell sorting (FACS) was used to determine the DNA content of cells transiently transfected with plasmids encoding CD19 and *TSC1* or *TSC2*.[27] After harvest, the cell pellet was resuspended in 100 µl of phosphate buffered saline containing 5 µl of FITC conjugated anti-CD19 antibody (Caltag Laboratories, Burlingame, CA), incubated on ice for one hour, and washed twice with PBS containing 0.1% BSA. The cells were then fixed with 70% ethanol at 4°C. Before flow cytometry analysis, the cells were pelleted, washed in phosphate buffered saline, and stained with 20 µg/ml of propidium iodide (Sigma) containing 9.5 mg/ml RNase (Sigma). Flow cytometry analysis was performed on a Becton-Dickinson FACScan machine. The intensity of propidium iodide staining was analysed on FITC positive cell populations. The percentages of cells in the G1, S, and G2/M phases of the cell cycle were determined using ModFit cell cycle analysis software version 3.1 (Verity Inc, Topsham, ME). Statistical analysis was performed using a one sided Student's *t* test. The intensity of PI staining was analysed in the CD19 positive cells and used to determine the percentages of transfected cells in the G1, S, and G2/M phases of the cell cycle.

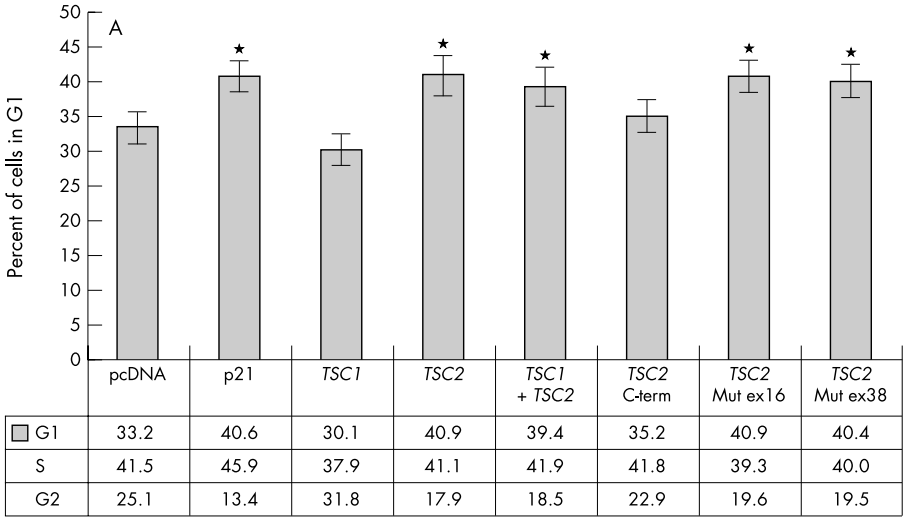
RESULTS AND DISCUSSION

We initially analysed U2OS cells, which were derived from a human osteosarcoma. In U2OS cells, the expected increase in G1 was seen with p21(WAF1/CIP1) transfection, which was used as a positive control, but no change in G1 was found with transfection of *TSC1*, *TSC2*, or both (data not shown).

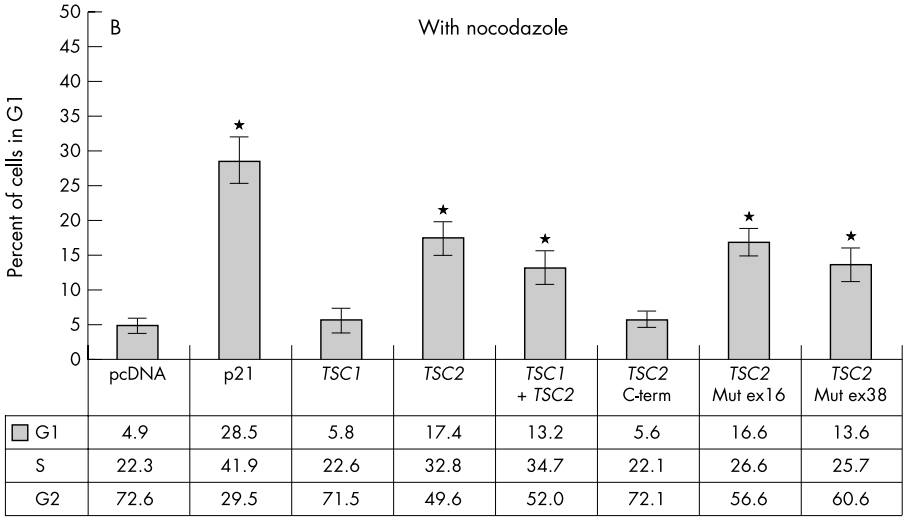
We then studied HEK293 cells, which were derived from human embryonic kidney cells. A small but significant increase in the G1 phase of the cell cycle was observed when wild type *TSC2* was overexpressed in HEK293 cells (fig 3). The percentage of cells in G1 was 33.2% for cells transfected with CD19 and the empty pcDNA vector, compared with 40.6% for p21(WAF1/CIP1) transfection, 40.9% for *TSC2*, and 39.4% for *TSC1* plus *TSC2*. In each case, the increase in the G1 fraction was accompanied by a decrease in the G2 fraction. No significant change in the G1 fraction was found when *TSC1* (30.1% in G1) or the carboxy-terminus of *TSC2* (35.2% in G1) were overexpressed. This is, to our knowledge, the first time that the cell cycle effects of hamartin have been studied using transient, rather than stable, expression, and the first time that the effects of *TSC1* and *TSC2* have been directly compared. Our data suggest that tuberin, not hamartin, is responsible for G1 regulation by the hamartin-tuberin complex.

We chose to study HEK293 cells in part because the kidney is a major site of disease in TSC patients. Renal disease in TSC can include angiomyolipomas, cysts, and renal cell carcinomas,[31] with angiomyolipomas by far the most common renal manifestation.[29] Renal disease is an important cause of mortality in TSC patients.[32] The fact that we observed an effect of *TSC2* in HEK293 cells but not U2OS cells could reflect intrinsic differences between these cell types, and these differences could be related to the high incidence of renal manifestations among patients with TSC. It is more likely, though, that U2OS cells contain mutations in other genes that block the effect of *TSC2*. Similar differences between cell lines have been observed for many tumour suppressor genes, including RB. In some cases, the downstream genes that distinguish "sensitive" cells from insensitive cells have been elucidated.[22 23]

The effect of *TSC2* on the G1 phase of the cell cycle that we observed in HEK293 cells was substantially enhanced by treating the cells with the anti-microtubule agent nocodazole, which induces a G2/M block. Cells were treated with 70 ng/ml of nocodazole (Sigma, St Louis, MO) for another 18.5 hours to induce a G2/M phase block. After nocodazole treatment the vector transfected control cells accumulated in G2/M (as



	pcDNA	p21	*TSC1*	*TSC2*	*TSC1* + *TSC2*	*TSC2* C-term	*TSC2* Mut ex16	*TSC2* Mut ex38
G1	33.2	40.6	30.1	40.9	39.4	35.2	40.9	40.4
S	41.5	45.9	37.9	41.1	41.9	41.8	39.3	40.0
G2	25.1	13.4	31.8	17.9	18.5	22.9	19.6	19.5



	pcDNA	p21	*TSC1*	*TSC2*	*TSC1* + *TSC2*	*TSC2* C-term	*TSC2* Mut ex16	*TSC2* Mut ex38
G1	4.9	28.5	5.8	17.4	13.2	5.6	16.6	13.6
S	22.3	41.9	22.6	32.8	34.7	22.1	26.6	25.7
G2	72.6	29.5	71.5	49.6	52.0	72.1	56.6	60.6

Figure 3 *TSC2* expression causes an increase in cells in the G1 phase of the cell cycle. (A) The fraction of cells in the G1, S, and G2 phases of the cell cycle for each transfection is indicated. The percentage of cells in G1 is indicated by the bar height. The standard error of the mean (SEM) is shown in white. An increase in the G1 fraction was seen in cells transfected with p21(WAF1/CIP1), *TSC2*, *TSC1* plus *TSC2*, the *TSC2* exon 16 mutant (*TSC2* Mut Ex16), and the *TSC2* exon 38 mutant (*TSC2* Mut Ex38). There was no change in the G1 fraction for cells transfected with *TSC1* or the *TSC2* carboxy-terminus (*TSC2* C-term). *p<0.05 relative to CD19/pcDNA control. (B) With nocodazole treatment, the majority of the pcDNA/CD19 control transfected cells were in G2/M, as expected. An increase in the G1 fraction was seen in cells transfected with p21(WAF1/CIP1), *TSC2*, *TSC1* plus *TSC2*, the *TSC2* exon 16 mutant (*TSC2* Mut Ex16), and the *TSC2* exon 38 mutant (*TSC2* Mut Ex38). There was no change in the G1 fraction for cells transfected with *TSC1* or the *TSC2* carboxy-terminus (*TSC2* C-term). *p <0.05 relative to the CD19/pcDNA control .

expected) accompanied by a decrease in the G1 population, while the *TSC2* transfected cells had a significant accumulation in G1 (figs 3 and 4). The percentage of cells in G1 was 4.9% for cells transfected with CD19 and the empty pcDNA vector, compared with 28.5% for p21 (WAF1/CIP1) transfection, 17.4% for wild type *TSC2*, and 13.2% for *TSC1* plus *TSC2* ($p<0.05$ for each transfection compared with the vector control). In each case the increase in the G1 fraction was accompanied by a decrease in the G2 fraction. No change in any phase of the cell cycle was found when *TSC1* (5.8% in G1) or the carboxy-terminus of *TSC2* (5.6% in G1) were overexpressed.

The mechanism through which nocodazole enhances the cell cycle effect of tuberin expression may simply involve cell synchronisation. It is also possible that a protein such as p53 whose activity or level is increased by nocodazole is a key downstream effector of tuberin. We did not observe an increase in p53 levels in nocodazole treated HEK293 cells, but this does not exclude an increase in the transcriptional activity of p53.[33]

We hypothesised that naturally occurring *TSC2* missense mutations would not result in the G1 increase seen with wild type *TSC2* if perturbation of this pathway is a primary event in disease pathogenesis. Approximately 20% of germline *TSC2* mutations are missense changes.[29 34] For reasons that are not yet understood, all identified *TSC1* mutations result in premature protein truncation, with no known missense mutations. The functional characterisation of mutant forms of *TSC2* is at a very early stage. In our study, expression of two of the most common naturally occurring *TSC2* missense mutants, in exons 16 (R611Q) and 38 (P1675L), resulted in an increase in the G1 fraction of similar magnitude to that mediated by wild type *TSC2*. As with wild type *TSC2*, this effect was enhanced by exposure of the cells to nocodazole (figs 3 and 4). The percentage of cells in G1 with nocodazole treatment was 4.9% for the pcDNA/CD19 control, 17.4% for wild type *TSC2*, 16.6% for the

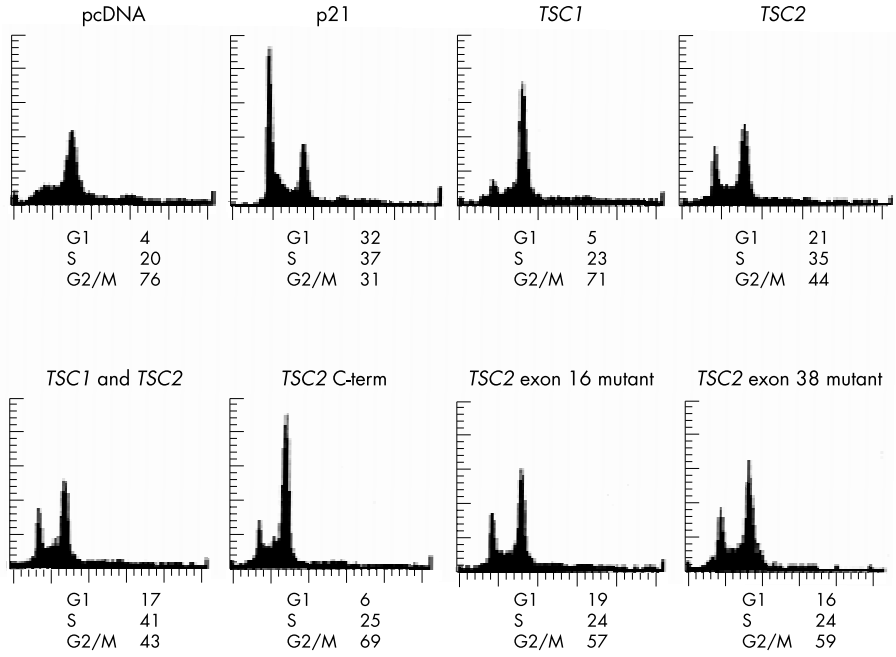


Figure 4 Representative fluorescence activated cell sorting analyses of cells cotransfected with *CD19* and *TSC1* or *TSC2* and treated with nocodazole. Examples of cell cycle profiles are shown for each of the transfections in the presence of nocodazole. Each panel represents cells cotransfected with the indicated construct and CD19, and sorted for CD19 positivity. The percentage of cells in each phase of the cell cycle, as determined by ModFit analysis, is shown below the appropriate panel.

TSC2 exon 16 mutation, and 13.6% for the *TSC2* exon 38 mutation ($p<0.05$ for each construct compared with the vector control). These results support recent work in which three mutations within the GAP domain (N1643K, N1651S, and N1681K) were found to have effects on the cell cycle that were similar to those of wild type *TSC2*,[35] consistent with our results for the P1675L GAP domain mutant. Because these mutations occurred in patients with TSC, these data suggest that cell cycle regulation is not the primary mechanism through which tuberin functions as a tumour suppressor gene.

In addition to cell cycle regulation, mammalian tuberin and/or hamartin appear to be involved in other cellular pathways that may contribute to tumorigenesis: Rap1 activation,[36] vesicular trafficking,[37] transcriptional activation by steroid hormones,[38] and focal adhesion formation.[39] The in vivo significance of these pathways and their contribution to the abnormal cell proliferation and abnormal cell cycle kinetics in TSC are not yet known. Clearly, it will be important to test the function of naturally occurring *TSC2* missense mutations in these pathways.

While the exon 16 and exon 38 *TSC2* mutants resulted in an increase in cells in the G1 phase of the cell cycle, the carboxy-terminus construct containing the wild type GAP domain did not. An identical carboxy-terminus construct has been shown previously to reduce the in vitro proliferation of two Eker rat derived cell lines at a level similar to that of full length *TSC2*, and suppressed the in vivo tumorigenicity of one of the cell lines (as did full length *TSC2*).[28] Our data may indicate that the carboxy-terminus of *TSC2* is not required for the cell cycle effects of *TSC2* overexpression, but is involved in growth suppression through other cellular pathways.

Other groups have observed increases in p27(KIP1) protein levels when *TSC1* or *TSC2* is overexpressed.[15 17] We also found increases in p27(KIP1) levels when *TSC1* or *TSC2* was expressed (fig 2B), although only when the cells were exposed to nocodazole. The relative increase in p27(KIP1) compared with the control transfected cells, as determined by densitometry, was 3.3 for *TSC1* transfection, 2.5 for *TSC2* transfection, and 3.9 for the *TSC2* exon 16 mutant transfection. An increase in p27(KIP1) was not seen when the *TSC2* carboxy-terminus or the *TSC2* exon 38 mutant construct was transfected. Similar results were seen in multiple independent transfections. Interestingly, in our study the p27(KIP1) increase was seen in *TSC1* transfected cells, despite the fact that *TSC1* did not affect the cell cycle profile. The exon 38 mutant (P1675L), which resulted in an increase in the G1 fraction, did not affect the p27(KIP1) level. In contrast, the exon 16 mutant (R611Q) increased both G1 and p27(KIP1). These results functionally separate cell cycle regulation (predominantly mediated by tuberin and intact in the GAP domain mutant construct) from p27(KIP1) up regulation (mediated by tuberin and hamartin and lost in the GAP domain mutant construct).

In summary, we examined the cell cycle impact of transient expression of *TSC1* and/or *TSC2* using a two colour FACS assay. We found that expression of wild type *TSC2*, but not *TSC1*, increased the percentage of cells in the G1 phase of the cell cycle. No additive or synergistic effect occurred when *TSC1* and *TSC2* were coexpressed. This is the first time that the cell cycle effects of *TSC1* and *TSC2* have been studied in parallel, and suggests that the cell cycle effects of the tuberin-hamartin complex derive primarily from tuberin, not hamartin. We also tested two naturally occurring mutant forms of *TSC2*, one within the region of GAP homology (P1675L) and one in exon 16 (R611Q). We found that expression of either mutant also induced an increase in the G1 fraction, similar in magnitude to that of wild type *TSC2*. A similar result was recently reported for three other mutations in the GAP homology domain.[35] There are several competing hypotheses that could explain the unexpected finding that expression of mutant forms of *TSC2* increases the G1 fraction. First, it is possible that the domains affected by these mutations (including the GAP domain) are not involved in tuberin's cell cycle regulation. Second, overexpression of the mutant proteins could compensate for decreased activity in vivo at endogenous expression levels. Finally, it is possible that the G1 increase seen with expression of mutant *TSC2* (as well as wild type) indicates that disruption of the cell cycle is not an important pathway of tumorigenesis in TSC, despite a number of studies indicating that wild type *TSC2* and *TSC1* regulate the G1 phase of the cell cycle.

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