# Interaction of serine/threonine protein phosphatase 5 with the protein products of tumour suppressor gene *Tsc2*

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*Tuberous sclerosis (TSC) is a tumour disease caused by mutations in Tsc1 or Tsc2 genes. Both protein products of Tsc1 and Tsc2 form an intracellular complex possessing GTPase-activating (GAP) activity towards a small GTP binding protein Rheb. The activity of TSC1/2 complex is regulated by multiple phosphorylations of TSC2 mediated by several kinases, such as PKB/Akt, AMP-activated kinase (AMPK), ERK, MK2 and RSK1. So far, very little is known about the molecular mechanisms of TSC2 dephosphorylation. In the yeast two-hybrid screening, we have identified a number of potential TSC2 binding partners including protein phosphatase 5 (PP5). In this study, we provide the evidence that the interaction between TSC2 and PP5 also occurs in mammalian cells. Using TSC2$^{+/+}$, p53$^{-/-}$ mouse embryo fibroblasts (MEFs) transiently overexpressing myc-PP5, we showed in the immunoprecipitation assay that TSC2 specifically associates with myc-PP5 in exponentially growing cells. The physiological relevance of identified interaction, especially the involvement of PP5 in the dephosphorylation of major regulatory sites is currently under investigation.*

*Keywords: TSC2, tuberous sclerosis, PP5, protein-protein interactions.*

**Introduction**. *Tsc1* and *Tsc2* are tumour suppressor genes encoding hamartin and tuberin, respectively. Both proteins form a regulatory complex which has been implicated in pathogenesis of tuberous sclerosis, a neurological disorder connected with the development of hamartomas in a wide range of tissues. Both TSC1 and TSC2 have coiled-coil regions which mediate heterodimer formation [1–3]. In contrast to TSC1 which has no known enzymatic activity, TSC2 possesses a C-terminal GAP domain for the small G protein, Ras homologue enriched in brain (Rheb) [4–9]. Recent studies have indicated that the TSC1-TSC2 complex regulates cellular functions mainly by their inhibition of mTOR and its targets S6 kinase (S6K) and 4E-BP1 [8, 10–15]. Increased S6K activity is observed in TSC mutations in *Drosophila melanogaster*, cells derived from TSC1 or TSC2 knockout mice, or cells treated with TSC1 or TSC2 small interfering RNA. Consistent with its function as a negative regulator of mTOR and its targets, the TSC complex has been found to regulate various cellular functions such as cell cycle

progression, cell size control, cell survival, and apoptosis [16–18].

TSC2 is phosphorylated on multiple sites by various kinases, including PKB/Akt, RSK1, AMPK, ERK, and MK2. However, the identity of the phosphatase(s) catalyzing dephosphorylation of these sites is unknown.

All phosphoprotein phosphatases (PPPs) are divided into three families designated PPP, PPM, and PTP, based on homology of amino acid sequences and the similarity of three-dimensional structures [19, 20]. The PPP and PPM families are comprised of phosphoserine- and phosphothreonine-specific enzymes, whereas the PTP family is comprised of phosphotyrosine-specific and/or dual specificity phosphatases. Protein phosphatase 2A (PP2A) together with PP1, PP2B (calcineurin), PP4, PP5, PP6, and PP7 are classified in the PPP family [21].

Serine/threonine protein phosphatase 5 (PP5) has a unique feature in that it consists of a single polypeptide chain containing a phosphatase catalytic domain near its C-terminus and three tetratricopeptide repeat (TPR) domains at the N terminus [22]. TPR domains mediate protein-protein interactions [23], and there is evidence that the TPR domain of PP5 targets the phosphatase to other proteins, including heat shock protein 90 – glucocorticoid receptor complex [24], apoptosis signal-regulating kinase 1 [25], the atrial natriuretic peptide receptor, Cdc 16p and Cdc 27p [26], the anaphase promoting complex [27], and PP2A [28].

To investigate the precise function of TSC1-TSC2 complex in the signal transduction and the molecular mechanism through which its tumour suppressor functions are controlled, we used yeast two-hybrid screening in order to identify cellular proteins that interact with TSC2 [29]. In this study, we report for the first time that PP5 phosphatase interacts specifically with TSC2 not only in the yeast two-hybrid system, but also in mammalian cells. It is not clear so far which sites of regulatory phosphorylations in TSC2 are targeted by PP5 phosphatase. This and other aspects of TSC2/PP5 interaction will be the subject of our future investigations.

**Materials and Methods**. *Antibodies*. Generation of D6 monoclonal antibodies towards TSC2 has been previously described (Hybridoma, in press). In brief, the C-terminal region of TSC2 was expressed as a His-tag fusion protein in bacteria, affinity purified and used as an immunogen. Hybrid myelomas were produced from the spleenocytes of immunized mice and SP2/0 myeloma cells.

Testing the specificity of cell culture supernatants from generated hybridomas towards recombinant His-TSC2C in ELISA assay allowed us to isolate a panel of positive clones. Further analysis of selected clones by Western blotting and immunoprecipitation revealed one clone, designated D6, which specifically recognized recombinant and endogenous TSC2. The specificity of generated antibody was also confirmed in $TSC2^{-/-}$, $p53^{-/-}$ and $TSC2^{+/+}$, $p53^{-/-}$ mouse embryo fibroblasts (MEFs).

*Transfection of mammalian cells*. $TSC2^{+/+}$, $p53^{-/-}$ MEFs were seeded at $4.0 10^{5}$/60-mm dish 16 h prior to transfection, followed by transfection procedure with 2 mg of plasmid DNA and 5–10 ml of ExGen 500 *in vitro* transfection reagent («Fermentas», Lithuania) according to the manufacturer's recommendations. For each transfection, plasmid DNA was diluted in 100 ml of sterile 150 mM NaCl and vortex for 10 s. The ExGen 500 reagent was added to the DNA mixture before vortex for another 10 s. The DNA/ExGen 500 mixture was incubated at room temperature for 10 min before adding into the dish with cells. Cells were grown for 48 h for the expression of the recombinant proteins.

*Cell lysate preparation and immunoblotting*. $TSC2^{+/+}$, $p53^{-/-}$ MEFs cells were lysed in buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 % Triton X-100, 50 mM NaF, 5 mM EDTA and a mixture of protease inhibitors («Roche Molecular Diagnostics», France). Protein concentration was measured by BSA assay («Pierce», USA) and equal amounts of proteins (50 μg) were resolved by SDS-PAGE. Electrophoretically separated proteins were transferred to polyvinylidene difluoride (PVDF) membrane («Millipore», USA) using wet transfer blotting apparatus. The membrane was blocked with 5 % non-fatty milk in 1 PBS containing 0.05 % Tween-20 (PBST) for 1 h at RT. D6 hybridoma or anti-myc hybridoma culture media were incubated with membranes at 4 °C overnight. After washing with PBST, HRP-conjugated anti-mouse IgG («Promega», USA) were added to the membrane for 1 h at RT.

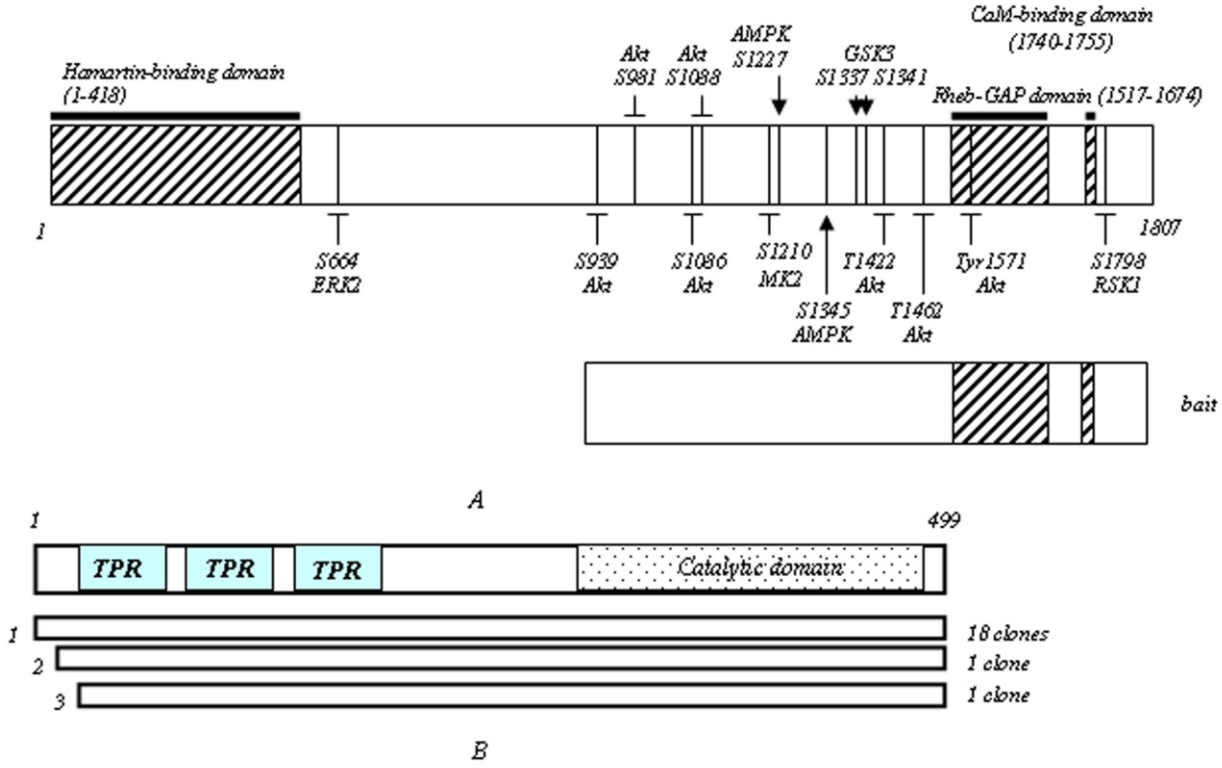


Fig. 1. *A* – Domain organization and the location of regulatory phosphorylation sites in TSC2/tuberin and the position of the C-terminal region which was used as a bait in the yeast two-hybrid screening is underlined; *B* – schematic presentation of PP5. The length of PP5 clones, isolated in the yeast two-hybrid screening with the C-terminal TSC2 is shown

Western blotting were developed using the ECL system («Amersham», Sweden) and then exposed to X-ray film.

*Immunoprecipitation*. 1 ml of D6 hybridoma media was incubated with 25 μl of 50 % suspension of Protein A-Sepharose CL-4B («Amersham», Sweden) for 2 h at 4 °C on the wheel. Then, beads were washed three times in lysis buffer described above and the supernatants (1 mg of protein) from exponentially growing $TSC2^{+/+}$, $p53^{-/-}$ MEFs transiently transfected with *pcDNA3.1* or *pcDNA3.1*/myc-PP5 were added. After incubation overnight on the wheel at 4 °C, beads were washed four times with 1 ml of lysis buffer. Immune complexes were removed from beads by boiling in Laemmli sample buffer and separated by SDS-PAGE. Resolved proteins were transferred onto PVDF membrane for immunoblotting.

**Results and Discussion**. We have previously used the yeast two-hybrid system to identify novel binding proteins for TSC2 [29]. The C-terminal region of TSC2, which possesses GAP domain and regulatory sites of phosphorylation, was used as a bait (Fig. 1, *A*). In that study, we have isolated 82 cDNA positive clones which encoded for 24 proteins. Among the isolated clones, we found a number of known TSC2 binding partners (including different isoforms of 14.3.3) as well as several potentially novel interactors with various cellular functions. Notably, 20 clones contained sequences corresponding to Ser/Thr PP5 (TSC2-BP1). The specificity of interaction between the C-terminal TSC2 bait construct and isolated PP5 clones was further confirmed by mating assay. Sequence analysis of these clones indicated that 18 clones contained full length coding sequence of PP5, while the other two clones started 2 and 3 amino acid residues downstream of the start codon (Fig. 1, *B*). These results clearly indicate that the N-terminal region of PP5, which possesses TPR regulatory domains, is possibly responsible for mediating the interaction with TSC2. Since the C-terminal region of TCS2 was used as a bait

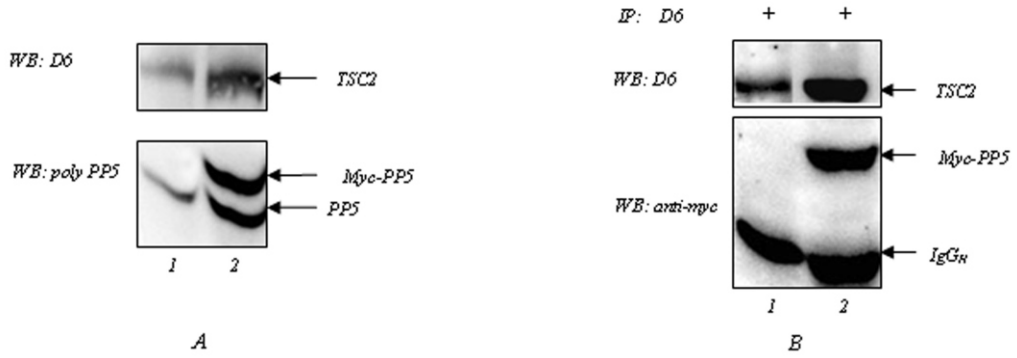


Fig. 2. Western-blot analysis of cell lysates from $TSC2^{+/+}$ $p53^{-/-}$ MEFs transfected with *pcDNA3.1* or *pcDNA3.1*/myc-PP5. 20 g of cell extracts were separated by SDS-PAGE and immunoblotted with antibodies specific for TSC2 (D6 mAb) or PP5 (affinity purified polyclonal antibody) (*A*) and co-immunoprecipitation of transiently overexpressed myc-PP5 with endogenous TSC2 (*B*). Line *1* – supernatant of exponentially growing $TSC2^{+/+}$, $p53^{-/-}$ MEFs, transiently transfected by *pcDNA3.1*; line *2* – supernatant of exponentially growing $TSC2^{+/+}$, $p53^{-/-}$ MEFs, transiently transfected by *pcDNA3.1*/PP5-myc

in the yeast two-hybrid screening, we can conclude that the TSC2/PP5 interaction is mediated through the C-terminal region of TCS2 and the N-terminal regulatory domain of PP5. Taking into account that the function of TSC1/2 tumor suppressor complex is known to be regulated by multiple phosphorylation events mediated by various signaling pathways, we hypothesized that the interaction with PP5 may control the phosphorylation status of TSC2. To verify the identified interaction in mammalian cells, we used $TSC2^{+/+}$, $p53^{-/-}$ MEFs, transiently transfected with *pcDNA3.1*/myc-PP5 plasmid. Initially, we tested in Western blotting the expression of endogenous TSC2 and transiently overexpressed myc-PP5 in MEFs.

The results shown in Fig. 2, *A*, indicate clearly that MEFs express high level of TSC2 and myc-PP5. In the immunoprecipitation analysis of TSC2/PP5complex, we used D6 monoclonal antibodies which were developed in our laboratory and found to recognize specifically endogenous TSC2 in various immunological assays (Hybridoma, in press). As shown in Fig. 2, *B*, D6 mAb specifically immunoprecipitated TSC2 from exponentially growing MEFs (line *1*) and MEFs transiently transfected with *pcDNA3.1*/myc-PP5 (line *2*). Furthermore, the immunoblotting of the TSC2 immune complexes with myc-tag antibody clearly indicated that myc-PP5 is efficiently co-immunoprecipitated with TSC2 in exponentially growing cells.

Recent studies from various laboratories indicate clearly that the function of TSC1/2 complex is regulated by phosphorylation/dephosphorylation at various sites. As a result of insulin receptor binding, a signal is conferred through PI3K kinase, which phosphorylates PKB/Akt. The latter kinase phosphorylates tuberin which negatively regulates TSC1/TSC2 complex activity [30]. A similar negative effect is also conferred by p90 ribosomal S6 kinase 1 (RSK1), which is activated in the Raf/MAPK pathway [31]. Both kinases are involved in the activation of protein synthesis and cell proliferation in response to growth factors. Tuberin is also directly phosphorylated by the p38 mitogen-activated protein kinase-activated protein kinase 2 (MAPKAPK2 or MK2). Such phosphorylation enhances the interaction between tuberin and 14-3-3 and initiates the degradation of tuberin [32]. On the other hand, there is also the mechanism of positive regulation of TSC2. LKB1, a tumor suppressor gene mutated in Peutz-Jeghers syndrome, activates TSC2 through the sensor of cellular energy status, AMP-dependent protein kinase (AMPK) [33]. However, the identity of protein phosphatases which are involved in the dephosphorylation of TSC2 is currently unknown. The data presented in this study demonstrate that PP5 might be one of them. The further study will be directed to confirmation of complex formation on the level of endogenous proteins and to identification the specific

for PP5 sites of dephosphorylation using phosphospecific antibodies.

Taking into account that PP5 interacts with TSC2 in exponentially growing cells we can predict that PP5 regulates TSC2 activity negatively.

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Взаємодія серин/треонінової протеїнфосфатази 5 з білковим продуктом гена – супресора пухлин *Tsc2*

Резюме

*Туберозний склероз (TSC) – захворювання, яке характеризується розвитком пухлиноподібних утворень і спричинене мутаціями в генах Tsc1 чи Tsc2. Обидва білкових продукти генів Tsc1 та Tsc2 утворюють внутрішньоклітинний комплекс, якому притаманна ГТФ-азна активність стосовно малого ГТФ-зв'язувального білка Rheb. Активність комплексу TSC1/TSC2 регулюється множинним фосфорилюванням TSC2 декількома кіназами, такими як PKB/Akt, AMP-активована кіназа, ERK, MK2 та RSK1. Однак майже нічого не відомо про молекулярні механізми дефосфорилювання TSC2. У попередній роботі із застосуванням дріжджової двогібридної системи нами ідентифіковано потенційні зв'язувальні партнери для TSC2, серед яких протеїнфосфатаза 5 (PP5). У даній роботі наведено докази того, що TSC2 взаємодіє з PP5 і в клітинах ссавців. Методом імунопреципітації з використанням клітин TSC2$^{+/+}$, p53$^{-/-}$ мишачих ембріональних фібробластів з тимчасово надекспресованим мус-PP5 нами встановлено, що TSC2 специфічно асоційований з PP5 у клітинах, які перебувають у фазі експоненційного росту. На даному етапі досліджень вивчається фізіологічне значення ідентифікованої взаємодії, особливо залучення PP5 до процесів дефосфорилювання багатьох регуляторних сайтів.*

*Ключові слова: TSC2, туберозний склероз, PP5, білково-білкові взаємодії.*

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Взаимодействие серин/треониновой протеинфосфатазы 5 с белковым продуктом гена – супрессора опухолей *Tsc2*

Резюме

*Туберозный склероз (TSC) – заболевание, характеризующееся развитием опухолевидных образований и вызываемое мутациями в генах Tsc1 или Tsc2. Оба белковых продукта генов Tsc1 и Tsc2 образуют внутриклеточный комплекс, обладающий ГТФ-азной активностью по отношению к малому ГТФ-связывающему белку Rheb. Активность комплекса TSC1/TSC2 регулируется множественным фосфорилированием TSC2 несколькими киназами, такими как PKB/Akt, AMP-активированная киназа, ERK, MK2 и RSK1. В настоящий момент почти ничего не известно о молекулярных механизмах дефосфорилирования TSC2. В предыдущей работе с применением дрожжевой двугибридной системы нами идентифицированы потенциальные связывающие партнеры для TSC2, среди которых протеинфосфатаза 5 (PP5). В представленной работе приведены доказательства того, что TSC2 взаимодействует с PP5 и в клетках млекопитающих. Методом иммунопреципитации с использованием клеток TSC2$^{+/+}$, p53$^{-/-}$ мышиных эмбриональных фибробластов с временно надэкспрессированным мус-PP5 нами установлено, что TSC2 специфически ассоциирован с PP5 в клетках, находящихся в экспоненциальной фазе роста. На данном этапе исследований изучается физиологическое значение идентифицированного взаимодействия, особенно вовлечение PP5 в процессы дефосфорилирования многих регуляторных сайтов.*

*Ключевые слова: TSC2, туберозный склероз, PP5, белково-белковые взаимодействия.*

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