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Phosphorylated hamartin–Hsp70 complex regulates apoptosis *via* mitochondrial localization

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ABSTRACT

The products of the tuberous sclerosis complex (*TSC*) genes, hamartin and tuberin, form a heterodimer. Recently we reported that hamartin directly interacted with Hsp70. However, the physiological implications of this interaction have not yet been clearly defined. Here we show that hamartin localized to the outer membrane of the mitochondria in an Hsp70-dependent manner. Moreover, phosphorylation of the T417 residue of hamartin was required for its localization to the mitochondria as well as its interaction with Hsp70. A non-phosphorylatable hamartin mutant at residue T417 was unable to localize to the mitochondria and suppress apoptosis, whereas non-phosphorylatable hamartin mutants T357A and T390A localized to the mitochondria and suppressed apoptosis. Importantly, non-phosphorylatable mutants (T357A, T390A and T417A) promoted apoptosis after treatment with Hsp 70-inhibitor KNK437. We conclude that hamartin inhibited apoptosis by localizing to the mitochondria and that its phosphorylation and binding to Hsp70 was required for facilitation of this process.

Introduction

Tuberous sclerosis complex (TSC), an autosomal dominant genetic disorder characterized by mental retardation, is assumed to be the result of somatic "second hit" mutations. Varieties of tumors, referred to as hamartomas, characteristically occur in different organs of TSC patients, including the kidney, heart, skin, and brain [1]. Two different genetic loci have been linked to TSC; the tumor suppressor gene TSC1 on chromosome 9q34 encodes a 150 kDa-protein (hamartin) while the TSC2 on chromosome 16p13.3 encodes a 200 kDa-protein (tuberin). TSC1 and TSC2 have been associated with disease occurrences and act as tumor suppressor genes [2,3].

Hamartin and tuberin form a heterodimer that negatively regulates the small GTPase, a Ras homolog enriched in the brain (Rheb), through its GTPase-activating (GAP) domain on tuberin [4,5]. Recent studies have revealed that the TSC1/2 complex activity is regulated by multi-phosphorylation sites downstream of kinases, including Akt and AMPK, both of which result in activation of Rheb/the mammalian Target of Rapamycin (mTOR)/ribosomal protein S6 kinase (S6K) pathway [6–9].

A multiple subcellular localization pattern of tuberin has previously been reported, indicating its potential to act as a multifunctional protein [10,11]. Tuberin has been implicated in the regulation of different cellular functions, such as migration, vesicular trafficking, cell cycle regulation and transcription [12–16].

On the other hand, hamartin is known to possess potential transmembrane and coiled-coil domains at the N- and C-terminal regions, respectively [1] and tuberin can bind to hamartin through both the N- and C-terminal fragments in cultured cells [17,18]. Furthermore, hamartin has been reported to prevent tuberin ubiquitination and degradation through the formation of a hetero-complex [19]. However, no enzymatic activity of hamartin has thus far been established and moreover, relatively little has been reported on any roles of hamartin that are independent to tuberin. To verify the solo (unique) function of hamartin independent to tuberin, we have reported Hsp70 as a novel hamartin-binding partner [20].

Previous experiments showed that over-expression of Hsp70 in MCF-7 cells induced a strong acceleration of cell growth and facilitated cell tumorigenicity in nude mouse [21,22]. Thus, increasing data have provided evidence that Hsp70 is one of the key players in cell survival and apoptosis. Indeed, we have reported that hamartin–Hsp70 interaction is necessary for Akt-tuberin phosphorylation during heat shock [20]. However, since these studies were conducted under heat shock induction, we probed into the physiological relevance of hamartin–Hsp70 binding in cells under normal conditions.

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Materials and methods

Cell culture. Cos-1 cells were cultured in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Equitech-Bio Inc., Kerrville, TX), 100 U/mL penicillin, and 100 μg/ml streptomycin (Gibco BRL/Invitrogen, Carlsbad, CA) at 37 °C in 5% CO_2. CACL-1-111 (renal carcinoma cell line derived from TSC1$^{+/-}$ mouse), EEF4 (a tuberin-positive embryonic fibroblast cell line derived from the Eker rat) and EEF8 (a tuberin-negative embryonic fibroblast cell line derived from the Eker rat) [23,24] were cultured in Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 Ham (Sigma) supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/ml streptomycin at 37 °C in 5% CO_2.

Reagent. KNK437 (*N*-formyl-3, 4-methylenedioxy-benzylidene-γ-butyrolaetam) from Sigma. KNK437 was dissolved in DMSO at the indicated concentration.

Antibodies. Anti-bodies for immunoblotting against tuberin (C-20), Mcl-1 and GRIM-19 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-body against mouse hamartin was purchased from ZYMED Laboratories (San Francisco, CA). Antibodies against GFP, HA and β-actin were purchased from Sigma, Hsp70 anti-body from Stressgen (Ann Arbor, MI). Cleaved caspase-3 and cleaved caspase-9 anti-bodies were from Cell Signaling Technology Inc. (Danver, MA). All primary antibodies were diluted with Tris-buffered saline with 0.05% Tween-20 (TBST) at 1:1000.

DNA constructs and transfection. Non-phosphorylatable mutant TSC1 (T357A, T390A, and T417A) were constructed by mutating wild type TSC1 cDNA using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene, San Diego, CA) with the specific primer, respectively. Wild type TSC2 was cloned into the pCMV-HA vector (BD Bioscience). Transfections of the mammalian expression vectors were performed with Lipofectamine 2000 (Invitrogen) transfection reagent following the manufacturer's instructions.

Immunoprecipitation and Immunoblotting. Cells were washed twice with PBS and lysed with 100 μL lysis buffer (20 mM Hepes, pH 7.4; 100 mM NaCl; 5 mM $MgCl_2$; 1% Triton X-100; 0.5 μg/ml leupeptin; 2 μg/ml aprotinin; and 10 μg/ml PMSF, pH 7.6). Lysates were incubated with a specific anti-body in the presence of protein A-agarose beads. The beads were washed with IP (immunoprecipitation) buffer (10-mM Tris–HCl, pH7.6; 150 mM NaCl; 1% Triton X-100; 0.5 μg/ml leupeptin; 2 μg/ml aprotinin; and 10 μg/ml PMSF, pH 7.6) and the immunoprecipitates were separated by SDS–PAGE, transferred to PVDF membrane, and incubated with specific anti-body. The immunoblot was developed using HRP-conjugated anti-rabbit/mouse IgG anti-bodies and visualized using the GE Healthcare ECL System (Piscataway, NJ).

Immunostaining. Cells were fixed with 4% formaldehyde in PBS for 10 min. Non-specific binding of antibodies was blocked using 5% sheep serum for 60 min, after which cells were incubated with primary anti-body in 5% sheep serum for 60 min [20].

Analysis of apoptosis activity. In brief [25], cells was kept in 0.2% serum for 15 h prior to 2-h serum starvation containg KNK437 or a vehicle control.

Isolation of inner or outer membrane of mitochondria. Mitochondrial fraction was isolated from Cos-1, EEF4 or EEF8 cells using a mitochondria purification kit (BioChain Institute Inc., Hayward, CA) for cultured cells, according to the supplier's instruction. The freshly isolated mitochondrial membrane fraction was suspended in homogenizing buffer (20 mM HEPES-KOH [pH 7.5], 10-mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, and 1 mM dithiothreitol [DTT]) alone or in the presence of protease K (0.1 μg/ml) or protease K plus Triton X-100 (1% final concentration) [26]. Purity of the fractions was assessed by probing for tubulin as a cytosolic marker. Mcl-1 and GRIM19 were used as mitochondrial markers.

Results

Hamartin and tuberin localized to outer membrane of the mitochondria

We first isolated the mitochondrial and non-mitochondrial fractions from Cos-1 cells, and probed for hamartin, tuberin and Hsp70 (Fig. 1A). A mitochondrial marker protein Mcl-1 and non-mitochondrial marker tubulin was also used. Hamartin, tuberin and Hsp70 were detected in the mitochondrial fraction. Moreover, immunofluorescence microscopy revealed that hamartin, tuberin and Hsp70 were co-localized with mitochondrial marker, Mcl-1 (Fig. 1B).

We then tested whether hamartin, tuberin and Hsp70 were localized on the inner or outer membrane of the mitochondria. The isolated mitochondrial fraction was incubated with proteinase K in the presence or absence of triton X-100. GRIM-19, an inner membrane protein, was resistant to proteinase K-treatment [27,28], whereas hamartin, tuberin and Hsp70 were degradated by proteinase K (Fig. 1C) indicating that both these proteins localized to the outer membrane of the mitochondria. These results indicate that hamartin, tuberin and Hsp70 were attached to the outer membrane of the mitochondria.

Hamartin localized to mitochondria in an Hsp70-dependent manner

Hamartin is known to form a hetero-complex with tuberin [4]. We therefore used EEF4 (tuberin positive) and EEF8 (tuberin negative) cell lines to test whether tuberin was required for hamartin to localize to the mitochondria. As shown in Fig 2A, hamartin and Hsp70 were localized to mitochondria even in the absence of tuberin.

In a prior report, hamartin was shown to directly interact with Hsp70 [20]. To clarify the role of Hsp70 on hamartin localization to the mitochondria, the effect of heat shock protein inhibitor KNK437 was examined in Cos-1 cells and EEF8 cells [29]. In both cells, hamartin and tuberin were not detected in the isolated mitochondrial fraction in the presence of KNK437 (Fig. 2B).

We also studied the effect of KNK437 on the intracellular localization of tuberin and hamartin. When GFP-tagged hamartin and HA-tagged tuberin were over-expressed in Cos-1 cells, both proteins co-localized with the mitochondria maker, Mcl-1 (Fig. 2C upper panel). In contrast, when the cells were treated with KNK437, the co-localization of these proteins with Mcl-1 was clearly diminished (Fig. 2C lower panel). These results suggest that the hamartin/tuberin complex localized to mitochondria in an Hsp70-dependent manner.

Phosphorylated hamartin T417 co-immunoprecipitated with Hsp70 and localized to the mitochondria

Based on a possible phosphorylation site on hamartin [17], we constructed three non-phosphorylated mutant *GFP-TSC1* constructs (*T310A*, *T357A* and *T417A*) and transfected these into Cos-1 cells. The equal expression levels of hamartin, tuberin and Hsp70 were confirmed (Fig. 3A, lower panel). We then examined the mitochondrial localization of the non-phosphorylated hamartin mutants and found that only T417A was not present in the mitochondria (Fig. 3A, upper panel). However the presence of tuberin and Hsp70 was not affected. To further confirm the interaction of non-phosphorylated mutants hamartin with Hsp70 and tuberin in the mitochondria, we carried out an immunoprecipitation assay

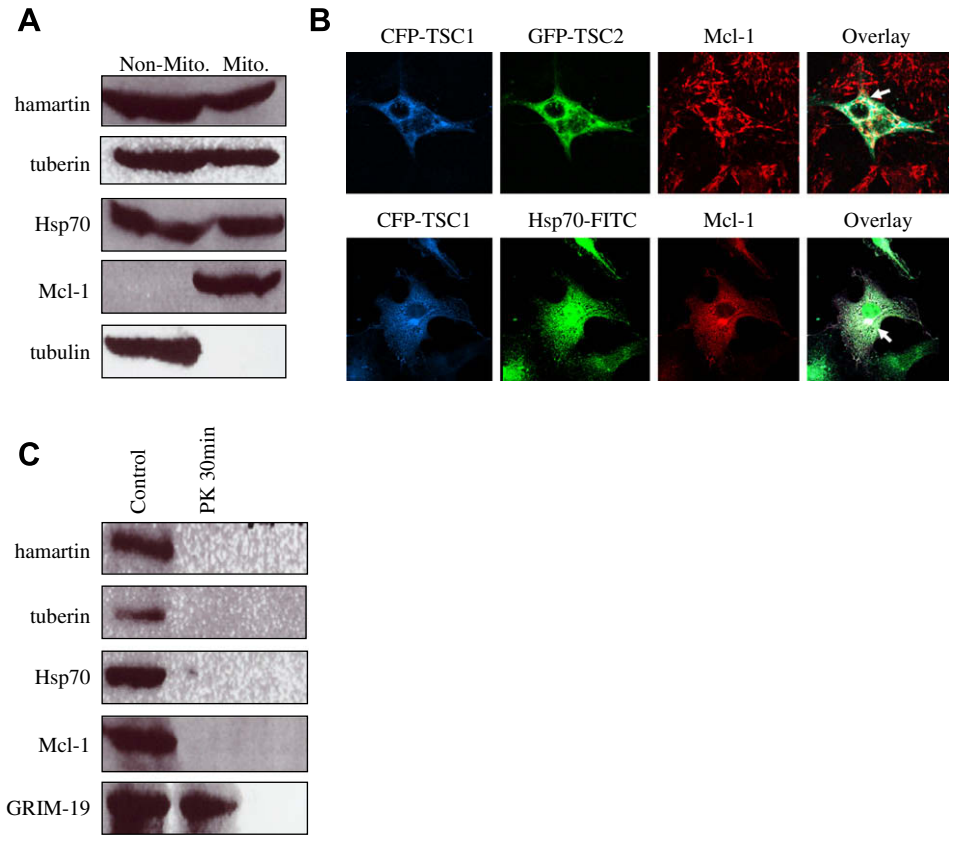

Fig. 1. Hamartin localizes to outer membrane of mitochondria. (A) Non-mitochondria (Non-Mito.) and mitochondria (Mito.) extracts were isolated. The immunoblot was hamartin, tuberin, Hsp70, tubulin (cytoplasmic marker), and Mcl-1 (mitochondrial marker). (B) CFP-TSC1 co-expressed with GFP-TSC2 or pCDNA-TSC2, respectively, in Cos-1 cells. Cells were fixed and probed with antibodies against Hsp70 or Mcl-1. (C) Isolated mitochondrial fractions were incubated with or without proteinase K (PK). To disrupt mitochondrial integrity, triton X-100 was added in the digestion buffer. GRIM-19 was used as a marker of the inner membrane of the mitochondria.

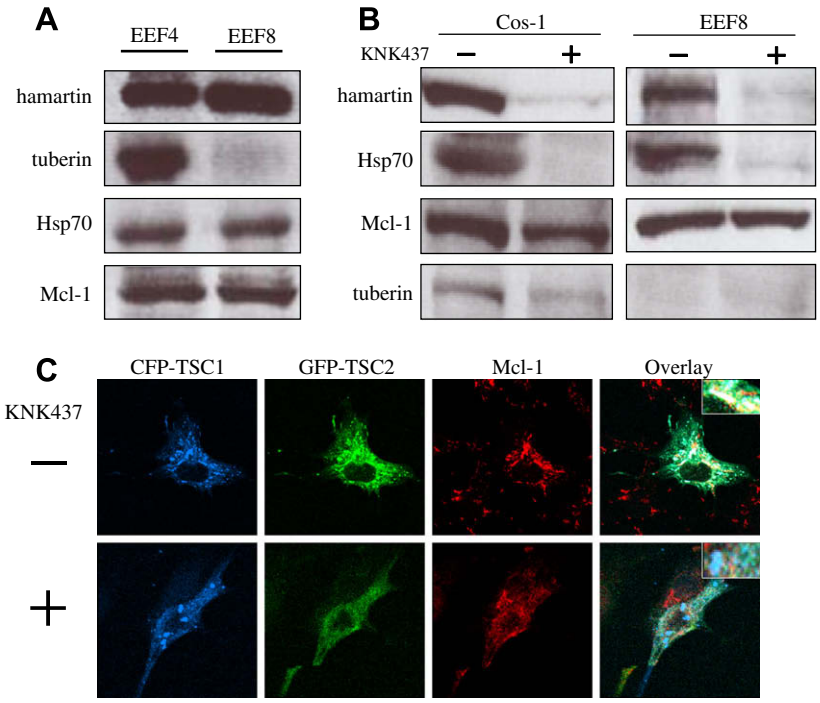

Fig. 2. Localization of TSC1 to mitochondria is dependent on Hsp70. (A) Mitochondrial fractions were isolated from EEF4 (a tuberin positive) and EEF8 (a tuberin negative) cells and then immunoblotted with indicated antibodies. (B) EEF8 and Cos-1 cells were treated with 100 μM KNK437 for 2 h. The mitochondrial fraction was isolated and immunoblotted with indicated antibodies. (C) *CFP-TSC1* (*WT*) and *GFP-TSC2* (*WT*) were transfected in Cos-1 cells. Cells were fixed and stained with antibodies against Mcl-1 with or without 100 μM KNK437 treatment for 2 h and then observed under a confocal microscope.

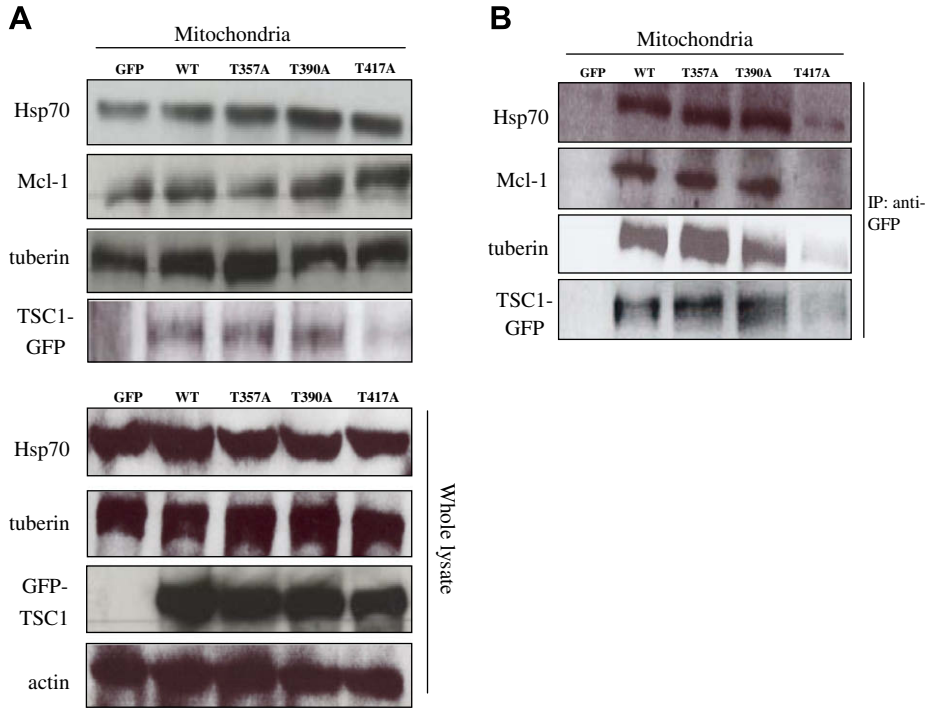

Fig. 3. Phosphorylated hamartin residue T417 is required for mitochondria localization and interacted with Hsp70. (A) Cos-1 cells were transfected with *GFP-TSC1* (*WT*) and non-phosphorylated mutants *GFP-TSC1* (*T357A*, *T390A*, and *T417A*). Immunoblotting of cell whole lysates was performed with the indicated antibodies (bottom). Isolated mitochondrial fractions from *GFP-TSC1* (*WT*) or non-phosphorylated mutants *GFP-TSC1* (*T357A*, *T390A* and *T417A*)-transfected Cos-1 cells were immunoblotted with antibodies (up). (B) After we isolated mitochondria from *GFP-TSC1* or non-phosphorylated mutants *GFP-TSC1* transfected Cos-1 cells, immunoprecipitated with anti-GFP antibody immunoblotted with indicates antibodies.

using anti-GFP anti-body. As shown Fig. 3B, three hamartin constructs (WT, T357A and T390A) were bound to tuberin and Hsp70, whereas T417A did not co-immunoprecipitate with tuberin and Hsp70 (Fig. 3B). This suggested that phosphorylation of hamartin at site T417 was required for interaction of Hsp70 and subsequent localization of the protein to the mitochondria.

Interaction of phosphorylated hamartin T417 with Hsp70 regulated apoptosis

The link between depletion of Hsp70 and apoptotic processes has been well established from previous studies [30]. On the basis of its mitochondrial localization along with Hsp70, we further investigated whether hamartin was involved in the regulation of apoptotic processes *via* Hsp70. In this experiment, we analyzed whether wild type or various non-phosphorylated mutant hamartin constructs (T357A, T390A, and T417A) inhibited stimulated apoptosis by incubating the cells under low serum conditions. Wild type hamartin or mutated hamartin constructs were over-expressed in CACL-1-111 (a *TSC1* negative cell line) with tuberin and further subjected to immuno-blotting assay using the indicated antibodies. As shown in Fig. 4A, CACL-1-111 cells underwent apoptosis based on the observation of increased caspase-9 and -3 activity. The re-entry of hamartin (WT, T357A, and T390A) in CACL-1-111 cells prevented apoptosis in the cells, indicating that hamartin exerted an anti-apoptotic activity. In contrast, we observed that only one mutant hamartin (T417A) was unable to suppress apoptosis (Fig. 4A). These results suggest that phosphorylated hamartin T417 is involved in suppression of apoptosis.

Next, to clarify that the Hsp70 was necessary for anti-apoptotic activity, we treated the cells with Hsp70 inhibitor, KNK437. Inhibition of casapase-9 and -3 activity by wild type and non-phosphorylated mutants hamartin (T357A and T390A) was no longer observed under the influence of KNK437 (Fig. 4B). Thus, we concluded that an interaction between phosphorylated hamartin T417 and Hsp70 suppressed apoptosis.

Discussion

Relatively little is known about the function of hamartin, when compared with the expanding knowledge of the function of tuberin [31,32]. In the present study we show that phosphorylated hamartin on site T417 binds to Hsp70 and is localized to the mitochondria. The results of this study also suggest that localization of hamartin to the mitochondria through its interaction with Hsp70 might inhibit the apoptotic pathway.

In this study we observed a mitochondria localization of hamartin and Hsp70 in the absence of tuberin. However, Clements et al. have reported the presence of tuberin in the mitochondria [10]. Thus, from our observation (Fig. 1A and B) we concluded that tuberin/hamartin/Hsp70 co-localize to the mitochondria under physiological conditions (Fig. 1B).

To inspect the binding of hamartin to Hsp70, we constructed several hamartin mutants based on predicted phosphorylation sites by CDC2/CyclinB, which has been previously reported [17]. We found out that a single alanine mutation at hamartin residue T417 was sufficient to disrupt hamartin-Hsp70 interaction. Therefore, we report for the first time that phosphorylation of hamartin at T417 is necessary for hamartin–Hsp70 interaction. More intriguingly, we observed that this mutant prevented the localization of hamartin to the mitochondria, indicating that the binding of Hsp70 is necessary for the hamartin localization to the mitochondria.

A well known role of Hsp70 is to serve as a molecular chaperone by binding to precursor proteins and docking directly onto the mitochondria for purposes of mitochondrial protein import [33]. We therefore investigated whether Hsp70 played a role

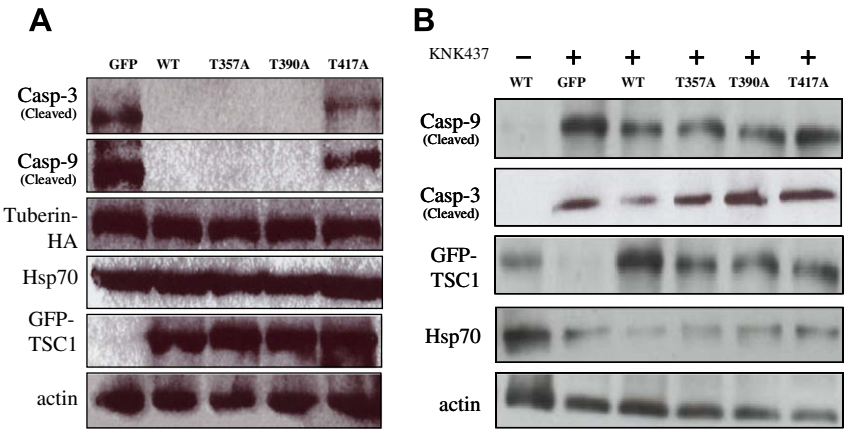

Fig. 4. Phosphorylated hamartin–Hsp70 complex suppresses the apoptotic pathway. (A) CACL-1-111 (a *TSC1* negative) cells were transfected with *GFP-TSC1* (*WT*, *T357A*, *T390A*, and *T417A*) or *GFP*-vector and incubated with 0.2% FBS overnight. (B) CACL-1-111 (a *TSC1* negative) cells were transfected with *GFP-TSC1* (*WT*), *GFP-TSC1* (*T357A*, *T390A*, and *T417A*) or GFP-vector. Transfected cells were incubated overnight with 0.2% FBS followed by 100 μM KNK437 for 2 h.

as a molecular chaperone for the internalization of hamartin into the mitochondria. In this study we show that hamartin was attached to the outer membrane of the mitochondria, indicating that Hsp70 was more likely not serving as a molecular chaperone.

The mitochondria are also known to play a key role in controlling cellular apoptosis. Outer mitochondrial membrane proteins including Bax or Bak can induce caspase activation and cell death in the cytosol [34]. Furthermore, a previous report showed that *TSC1* knock-down by siRNA in PC12 cells prompted NGF-induced cell death [35]. Therefore, we speculated that hamartin–Hsp70 interaction may be involved in the cellular apoptotic process. As shown in Fig. 4B, upon re-entry of mutant hamartin into CACL-1-111 (a *TSC1* negative cell line) cells, only the T417A mutant was unable to prevent stimulated apoptotic events due to low serum conditions. Thus, we suggest that an interaction of phosphorylated hamartin T417 with Hsp70 may be involved in the regulation of apoptosis.

Previous studies indicate that the ROS (reactive oxygen species) level was increased in *TSC1*-deficient hematopoietic stem cells and *TSC2* mutant-expressed Cos-1 cells [12,36]. In addition, mTOR activity, and more precisely mTORC1 which is negatively regulated by hamartin/tuberin complex, was tightly correlated with mitochondrial metabolism [37,38]. Therefore, the missing link between the hamartin/tuberin-mTOR pathway and mitochondrial functions, including apoptosis, mitochondrial biogenesis and regulation of genes involved in oxidative function, may possibly be a hamartin–Hsp70 interaction.

In conclusion, we have demonstrated that hamartin localized to the outer mitochondrial membrane. This is the first specific subcellular localization pattern reported for hamartin in an Hsp70-dependent manner. Furthermore, a phosphorylated form of hamartin at site T417 was shown to be required for localization to the mitochondria and interaction with Hsp70. Thus, interaction of hamartin with Hsp70 appeared to co-operatively regulate apoptosis through phosphorylation of hamartin at site T417.

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References

[1] M. Rosner, A. Freilinger, M. Hengstschläger, Proteins interacting with the tuberous sclerosis gene products, Amino Acids 27 (2004) 119–128.
[2] M.R. Gomez, J.R. Sampson, V.H. Wittemore, Tuberous Sclerosis Complex, third ed., Oxford University Press, USA, 1999.
[3] E.C.T.S. Consortium, Identification and characterization of the tuberous sclerosis gene on chromosome 16, Cell 75 (1993) 1305–1315.
[4] M. van Slegtenhorst, M. Nellist, B. Nagelkerken, J. Cheadle, R. Snell, A. van den Ouweland, A. Reuser, J. Sampson, D. Hell, P. van der Sluijs, Interaction between hamartin and tuberin, the TSC1 and TSC2 gene products, Hum. Mol. Genet. 7 (1998) 1053–1057.
[5] K. Inoki, Y. Li, T. Xu, K.L. Guan, Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling, Genes Dev. 17 (2003) 1829–1834.
[6] H.C. Dan, M. Sun, L. Yang, R.I. Feldman, X.M. Sui, C.C. Ou, M. Nellist, R.S. Yeung, D.J. Halley, S.V. Nicosia, W.J. Pledger, J.Q. Cheng, Phosphatidylinositol 3-kinase/AKT pathway regulates tuberous sclerosis tumor suppressor complex by phosphorylation of tuberin, J. Biol. Chem. 277 (2002) 35364–35370.
[7] K. Inoki, Y. Li, T. Zhu, J. Wu, K. Guan, TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signaling, Nat. Cell. Biol. 4 (2002) 648–657.
[8] E.A. Goncharova, D.A. Goncharov, A. Eszterhas, D.S. Hunter, M.K. Glassberg, R.S. Yeung, C.L. Walker, D. Noonan, D.J. Kwiatkowski, M.M. Chou, R.A. Panettieri Jr., V.P. Krymskaya, Tuberin regulates p70 S6 kinase activation and ribosomal protein S6 phosphorylation. A role for the TSC2 tumor suppressor gene in pulmonary lymphangioleiomyomatosis (LAM), J. Biol. Chem. 277 (2002) 30958–30967.
[9] K. Inoki, T. Zhu, K.L. Guan, TSC2 mediates cellular energy response to control cell growth and survival, Cell 115 (2003) 577–590.
[10] D. Clements, R.J. Mayer, S.R. Johnson, Subcellular distribution of the *TSC2* gene product tuberin in human airway smooth muscle cells is driven by multiple localization sequences and is cell-cycle dependent, Am. J. Physiol. Lung Cell Mol. Physiol. 292 (2006) 258–266.
[11] B. Gan, Z.K. Melkoumian, X. Wu, K.L. Guan, J.L. Guan, Identification of FIP200 interaction with the TSC1–TSC2 complex and its role in regulation of cell size control, J. Cell Biol. 170 (2005) 379–389.
[12] T. Suzuki, S.K. Das, H. Inoue, M. Kazami, O. Hino, T. Kobayashi, R.S. Yeung, K. Kobayashi, T. Tadokoro, Y. Yamamoto, Tuberous sclerosis complex 2 loss-of-function mutation regulates reactive oxygen species production through Rac1 activation, Biochem. Biophys. Res. Commun. 368 (2008) 132–137.
[13] H. Xiao, F. Shoarinejad, F. Jin, E.A. Golemis, R.S. Yeung, The tuberous sclerosis 2 gene product, tuberin, functions as a Rab5 GTPase activating protein (GAP) in modulating endocytosis, J. Biol. Chem. 272 (1997) 6097–6100.
[14] K.A. Jones, X. Jiang, Y. Yamamoto, R.S. Yeung, Tuberin is a component of lipid rafts and mediates caveolin-1 localization: role of TSC2 in post-Golgi transport, Exp. Cell Res. 295 (2004) 512–524.
[15] M. Rosner, A. Freilinger, M. Hanneder, N. Fujita, G. Lubec, T. Tsuruo, M. Hengstschläger, P27Kip1 localization depends on the tumor suppressor protein tuberin, Hum. Mol. Genet. 16 (2007) 1541–1556.
[16] K.W. Henry, X. Yuan, N.J. Koszewski, H. Onda, D.J. Kwiatkowski, D.J. Noonan, Tuberous sclerosis gene 2 product modulates transcription mediated by steroid hormone receptor family members, J. Biol. Chem. 273 (1998) 20535–20539.
[17] A. Astrinidis, W. Senapedis, E.P. Henske, Hamartin, the tuberous sclerosis complex 1 gene product, interacts with polo-like kinase 1 in a phosphorylation-dependent manner, Hum. Mol. Genet. 15 (2006) 287–297.
[18] A. Nakashima, K. Yoshino, T. Miyamoto, S. Eguchi, N. Oshiro, U. Kikkawa, K. Yonezawa, Identification of TBC7 having TBC domain as a novel binding

protein to TSC1–TSC2 complex, Biochem. Biophys. Res. Commun. 361 (2007) 218–223.
[19] H. Chong-Kopera, K. Inoki, Y. Li, T. Zhu, F.R. Garcia-Gonzalo, J.L. Rosa, K.L. Guan, TSC1 stabilizes TSC2 by inhibiting the interaction between TSC2 and the HERC1 ubiquitin ligase, J. Biol. Chem. 281 (2006) 8313–8316.
[20] H. Inoue, M. Ndong, T. Suzuki, M. Kazami, T. Uyama, K. Kobayashi, T. Tadokoro, Y. Yamamoto, Hamartin–Hsp70 interaction is necessary for akt-dependent tuberin phosphorylation during heat shock, Biosci. Biothechnol. Biochem. 73 (2009) 2488–2493.
[21] J.A. Barnes, D.J. Dix, B.W. Collins, C. Luft, J.W. Allen, Expression of inducible Hsp70 enhances the proliferation of MCF-7 breast cancer cells and protects against the cytotoxic effects of hyperthermia, Cell Stress Chaperones 6 (2001) 316–325.
[22] M. Jäättelä, Over-expression of Hsp70 confers tumorigenicity to mouse fibrosarcoma cells, Int. J. Cancer 60 (1995) 689–693.
[23] T. Kobayashi, O. Minowa, Y. Sugitani, S. Takai, H. Mitani, E. Kobayashi, T. Noda, O. Hino, A germ-line Tsc1 mutation causes tumor development and embryonic lethality that are similar, but not identical to, those caused by Tsc2 mutation in mice, Proc. Natl. Acad. Sci. USA 98 (2001) 8762–8767.
[24] L.D. Aicher, J.S. Campbell, R.S. Yeung, Tuberin phosphorylation regulates its interaction with hamartin. Two proteins involved in tuberous sclerosis, J. Biol. Chem. 276 (2001) 21017–21021.
[25] U. Ozcan, L. Ozcan, E. Yilmaz, K. Düvel, M. Sahin, B.D. Manning, G.S. Hotamisligil, Loss of the tuberous sclerosis complex tumor suppressors triggers the unfolded protein response to regulate insulin signaling and apoptosis, Mol. Cell. 29 (2007) 541–551.
[26] D. Chandra, G. Choy, X. Deng, B. Bhatia, P. Daniel, D. Tang, Association of active caspase 8 with the mitochondrial membrane during apoptosis: potential roles in cleaving BAP31 and caspase 3 and mediating mitochondrion-endoplasmic reticulum cross talk in etoposide-induced cell death, Mol. Cell. Biol. 24 (2004) 6592–6607.
[27] C. Lufei, J. Ma, G. Huang, T. Zhang, V.N. Diermayr, C. Thing Ong, X. Cao, GRIM-19, a death-regulatory gene product, suppresses Stat3 activity via functional interaction, EMBO J. 22 (2003) 1325–1335.
[28] J. Zhang, J. Yang, S.K. Roy, S. Tininini, J. Hu, J.F. Bromberg, V. Poli, G.R. Stark, D.V. Kalvakolanu, The cell death regulator GRIM-19 is an inhibitor of signal transducer and activator of transcription 3, Proc. Natl. Acad. Sci. USA 100 (2003) 9342–9347.
[29] S. Yokota, M. Kitahara, K. Nagata, Benzylidene lactam compound, KNK437, a novel inhibitor of acquisition of thermotolerance and heat shock protein induction in human colon carcinoma cells, Cancer Res. 60 (2000) 2942–2948.
[30] J. Nylandsted, M. Rohde, K. Brand, L. Bastholm, F. Elling, M. Jäättelä, Selective depletion of heat shock protein 70 (Hsp70) activates a tumor-specific death program that is independent of caspases and bypasses Bcl-2, Proc. Natl. Acad. Sci. USA 97 (2000) 7871–7876.
[31] Y. Yamamoto, K.A. Jones, B.C. Mak, A. Muehlenbachs, R.S. Yeung, Multicompartmental distribution of the tuberous sclerosis gene products, hamartin and tuberin, Arch. Biochem. Biophys. 404 (2002) 210–217.
[32] S.L. Cai, A.R. Tee, J.D. Short, J.M. Bergeron, J. Kim, J. Shen, R. Guo, C.L. Johnson, K. Kiguchi, C.L. Walker, Activity of TSC2 is inhibited by Akt-mediated phosphorylation and membrane partitioning, J. Cell. Biol. 172 (2006) 279–289.
[33] J.C. Young, N.J. Hoogenraad, F.U. Hartl, Molecular chaperones Hsp90 and Hsp70 deliver preproteins to the mitochondrial import receptor Tom70, Cell 112 (2003) 41–50.
[34] D.R. Green, G. Kroemer, The pathophysiology of mitochondrial cell death, Science 305 (2004) 626–629.
[35] S. Yasui, K. Tsuzaki, H. Ninomiya, F. Floricel, Y. Asano, H. Maki, A. Takamura, E. Nanba, K. Higaki, K. Ohno, The TSC1 gene product hamartin interacts with NADE, Mol. Cell. Neurosci. 35 (2007) 100–108.
[36] C. Chen, Y. Liu, R. Liu, T. Ikenoue, K.L. Guan, Y. Liu, P. Zheng, TSC-mTOR maintains quiescence and function of hematopoietic stem cells by repressing mitochondrial biogenesis and reactive oxygen species, J. Exp. Med. 205 (2008) 2397–2408.
[37] S.M. Schieke, D. Phillips, J.P. MoCoy, A.M. Aponte, R.F. Shen, R.S. Balaban, T. Finkel, The mammalian target of rapamycin (mTOR) pathway regulates mitochondrial oxygen consumption and oxidative capacity, J. Biol. Chem. 281 (2006) 27643–27652.
[38] C. Chen, Y. Liu, Y. Liu, P. Zheng, The axis of mTOR-mitochondria-ROS and stemness of the hematopoietic stem cells, Cell Cycle 8 (2009) 1158–1160.