SPANDIDOS PUBLICATIONS

Neuromuscular abundance of RB1CC1 contributes to the non-proliferating enlarged cell phenotype through both RB1 maintenance and TSC1 degradation

TOKUHIRO CHANO[1,3], MASASHI SAJI[1], HIROKAZU INOUE[2], KAHORI MINAMI[1], TOSHIYUKI KOBAYASHI[4], OKIO HINO[4] and HIDETOSHI OKABE[1]

Departments of [1]Clinical Laboratory Medicine and [2]Microbiology, Shiga University of Medical Science, Shiga 520-2192; [3]PRESTO, Japan Science and Technology Agency, Saitama 332-0012; [4]Department of Pathology, Juntendo University, Tokyo 113-8412, Japan

Received March 7, 2006; Accepted May 5, 2006

Abstract. *RB1*-inducible coiled-coil 1 (RB1CC1) is a novel tumor suppressor implicated in the regulation of RB1 expression. It is abundant in post-mitotic neuromuscular cells, which are matured and enlarged, but scarce in smaller leukocytes, indicating an association between RB1CC1 status and cell size. To clarify whether RB1CC1 is involved in cell size control, we investigated the contribution of RB1CC1 to the TSC-mTOR pathway, which plays an important role in the control through translational regulation. RNAi-mediated knockdown of *RB1CC1* reduced the activation of mTOR and S6K as well as the size of HEK293 and C2C12 cells. Such knockdown also suppressed RB1 expression and the population of G1-phase cells. Exogenous expression of *RB1CC1* maintained S6K activity and cell size, and decreased TSC1/hamartin contents under nutritionally starved conditions, which usually inhibit the mTOR-S6K pathway. Furthermore, RB1CC1 interfered with and degraded TSC1 through the ubiquitin-proteasomal pathway. A lentiviral RNAi for *RB1CC1* reduced the size of mouse leg muscles. These findings suggest that RB1CC1 is required to maintain both RB1 expression and mTOR activity. The activity of mTOR was supported by RB1CC1 through TSC1 degradation. RB1CC1 preserved cell size without cell cycle progression especially in neuromuscular tissues, and the abundance contributed to the non-proliferating enlarged cell phenotype.

Introduction

Cell proliferation, growth, differentiation and death are essential to tissue organization, and should be coordinately regulated. Although the individual components of the process have been studied, coordinate association of their components is largely unknown. We identified a novel factor, *RB1*-inducible coiled-coil 1 (RB1CC1: the symbol approved by Human Genome Organization Gene Nomenclature Committee) that is implicated in the regulation of RB1 expression (1). RB1CC1 was also designated as FIP200 (focal adhesion kinase family interacting protein of 200 kD) (2), but is referred to here as RB1CC1 conforming to HUGO. Introducing *RB1CC1* into human leukemic cells accelerated *RB1* expression and reduced cell cycle progression (1,3). Both molecules are synchronously expressed (1), and specific knockdown of *RB1CC1* represses expression of *RB1* in several cell lines (4). Thus, RB1CC1 can regulate cellular proliferative activity through the RB1 pathway. Finally, 10-20% of primary breast cancers are associated with a dysfunction in *RB1CC1*, which has the features of a classical tumor-suppressor (5,6).

Both RB1CC1 and RB1 are preferentially expressed in embryonic musculoskeletal cells, and contribute to their maturation (7,8). The expression of RB1CC1 in both cell nuclei and cytoplasms, and the intracellular localization could change according to the developing status. By altering the intracellular sub-localization, RB1CC1 contributes to the mammalian developmental process (8). We also demonstrated that RB1CC1 is a novel mediator of muscular differentiation (4). The synchronous expression of *RB1CC1* and *RB1* predicted myosin heavy chain expression during C2C12 myoblast differentiation and human embryonic development. RNAi-mediated knockdown of *RB1CC1* leads to a reduction in RB1, and C2C12 myoblasts fail to differentiate (4). These experimental data indicated that RB1CC1 plays a critical role in the repression of proliferation and induction of differentiation in rhabdomyogenic cells. However, leukocyte differentiation is independent of an increase in RB1CC1 and in fact, leukocytes do not become as enlarged as skeletal muscle cells during post-mitotic maturation. These facts suggest that RB1CC1 expression is involved in the regulation of cell size in conjunction with cell cycle arrest. To evaluate this notion, we observed the expressional status of RB1CC1 in post-mitotic embryonic spinal cord neurons and muscle cells using an immunohistochemical preparation and found a positive correlation

Correspondence to: Dr Tokuhiro Chano, Department of Clinical Laboratory Medicine, Shiga University of Medical Science, Otsu, Shiga 520-2192, Japan
E-mail: chano@belle.shiga-med.ac.jp

Key words: RB1CC1, TSC, RB1, cell size, neuromuscular cells

between cell size and the expressional status in these tissues. Based on these findings, we searched for a cascade between RB1CC1 and the TSC-mTOR pathway, which is the best understood molecular pathway with respect to cell size control (9). Mutations and depleted TSC function cause mTOR-S6K activation, upregulation of translation and enlargement of cell size in several animal species (10-16). TSC1 and TSC2 are not simply involved in cell size regulation, they are also tumor suppressors that respectively encode hamartin and tuberin, and mutations in these genes cause tuberous sclerosis (TSC), a hamartoma syndrome (9). Both genes have coiled-coil regions and form heterodimers (17), and are supposed to play a role through translational control in cell cycle progression, survival and apoptotic processes in addition to cell size regulation (9,18).

We present evidence that RB1CC1 plays an important role in cell cycle arrest and on cell enlargement through the TSC-mTOR pathway not only *in vitro*, but also in neuromuscular cells *in vivo*.

Materials and methods

Antibodies and reagents. Rabbit antiserum against RB1CC1 was generated from a GST-fusion protein containing a.a. residues 25-271 within the N-terminus of RB1CC1 as the epitope, and another anti-RB1CC1 antiserum was a gift from J.L. Guan (Cornell University, NY 14853). We purchased all antibodies from Cell Signaling except for the following: anti-TSC2 (C-20), -S6K (C-18), and -Myc (9E10, Santa Cruz Biotechnology); anti-HA (12CA5, Roche); anti-Flag (M2) and -Tubulin α (DM 1A, Sigma); anti-RB1 (G3-245, BD Pharmingen) and anti-GFP (Clontech). Cycloheximide and lactacystin were from Calbiochem.

Cell culture. HEK293, 293T and C2C12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). Horse serum (2%) induced the muscular differentiation of C2C12 cells as described (4). Cells were nutritionally starved by culture in medium containing 25 mM HEPES and 10% dialyzed FBS (Invitrogen). TSC1-/- cells were derived from murine renal carcinoma (11). Cells stably expressing TSC1 were generated from TSC1-/- cells by retroviral gene transfer with human *TSC1* cDNA (GenBank accession no. NM_000368). Cloned and expanded cells were selected in the presence of blasticidin. Against C2C12, TSC1-null and -rescued cells, additional genetic *RB1CC1* modifications were used in the lentiviral gene transfer system (Invitrogen).

Plasmid DNA and gene transfer. External and internal deletion mutants for *RB1CC1* (GenBank accession no. NM_014781) were generated by a combination of PCR-based manipulations with appropriate external primers at the positions described below and restriction enzyme digestion. The nucleotides of all constructs were confirmed by DNA sequencing. The *RB1CC1* mutants dLZ, dCC, dN and FCC contained aa 1-1363, 1-823, 1-555 and 864-1594, respectively. Transfection proceeded using Lipofectamine 2000 (Invitrogen) or FuGENE6 (Roche) according to the supplier's recommendations.

Plasmid vectors of RNAi for *RB1CC1* were prepared as described (4). The scramble RNAi sequence for *RB1CC1* was 5'-CAACTACCAAGAGCTTGCCTA-3'. Lentiviral RNAi vectors were similarly generated and the selection marker was additionally modified from EM7-Zeocin to CMV-GFP. Virus-transferring RNAi were prepared according to the manufacturer's instructions (Invitrogen).

Human *TSC1* cDNA was cloned into the pCX-bsr vector, and a combination of pCX-bsr and pCL-Ampho was applied to retroviral gene transduction (19).

Western blotting and immunoprecipitation. Cells were lysed for Western blotting as described by Sarbassov *et al* (20). Before immunoprecipitation, cells were lysed in 1X TNE buffer (20 mM Tris-HCl containing 150 mM NaCl, 5 mM EDTA, 1% NP-40 and 1 mM Na_3VO_4 and a mixture of protease inhibitors). After clearing lysed material by centrifugation at 15,000 x g for 10 min, the supernatant was rotary-incubated with anti-HA (Roche), -Myc, or -Flag (Sigma) immobilized beads for 3 h at 4˚C. The beads were washed five times with 1X TNE buffer and boiled in SDS sample buffer. Proteins resolved by SDS-PAGE were transferred to polyvinylidene difluoride (PVDF) membranes and immunoblotted with the indicated antibodies.

Cell size and cell cycle analysis. To analyze cell size and the cell cycle, the distribution of FSC and DNA contents were evaluated in individual GFP-positive cells using a Becton Dickinson FACScalibur. Flow cytometry data were analyzed with CellQuest software as described (21).

Lentiviral RB1CC1 knockdown in vivo. Lentivirus containing *RB1CC1*-RNAi was injected into the hind leg muscles of C57BL6 mice. We injected *scramble*- and *RB1CC1*-RNAi into the left and right legs, respectively and histologically evaluated the muscles 4 weeks later.

Histology and immunohistochemistry. To evaluate RB1CC1 in developing neuromuscular tissues, human embryos at 4-8 gestational weeks were processed for histological and immunohistochemical preparation as described (4,7,8). The effect of RB1CC1 knockdown in murine skeletal muscle was evaluated by comparing average cross-sectional areas (CSA) between knockdown and control muscle fibers after immunostaining for GFP. The size of CSA in muscle fibers expressing GFP was evaluated using NIH Image 1.63 software. Average nuclear numbers per cross-sectioned muscle fiber were also compared. To calculate the exact number of nuclei in each muscle fiber, the muscular basement membrane was visualized by periodic acid/Schiff's (PAS) stain after GFP immunostaining.

Results

RB1CC1 is more abundant in enlarged neuromuscular cells. Immunoreactivity to RB1CC1 was more prominent in large non-proliferative neuromuscular cells than in circulating post-mitotic small leukocytes in adult animals (data not shown) or in embryonic spinal cord neurons, and concomitantly increased with the cell enlargement induced by maturation (Fig. 1A-C). Similarly, RB1CC1 immunoreactivity became intense in skeletal muscle cells that enlarged after myotube formation (Fig. 1D). Together with the previous finding that RB1CC1

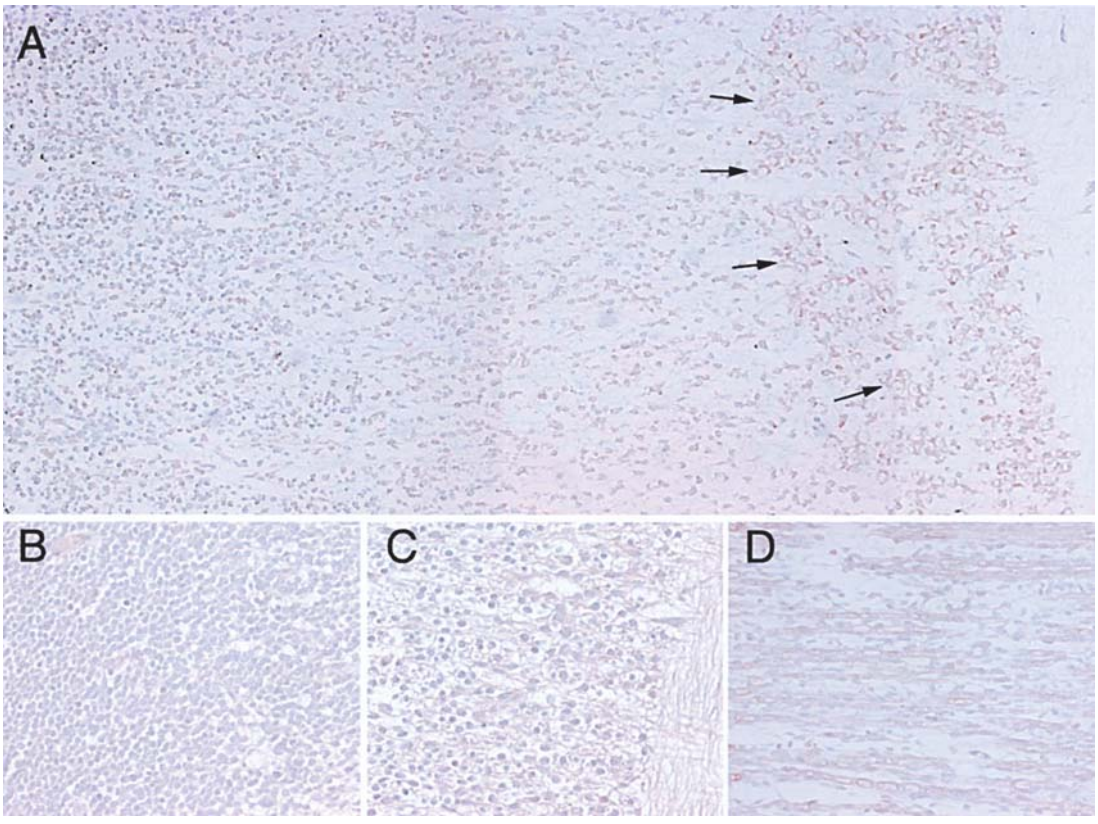

Figure 1. RB1CC1 abundance in enlarged neuromuscular cells. (A) More matured and enlarged (arrows) than proliferating or migrating neurons express abundant *RB1CC1* mRNA. *In situ* hybridization for *RB1CC1* in human embryonic spinal cord neuron. (B) Proliferating neuroblasts contain less RB1CC1. (C) Enlarged neurocytes contain increased RB1CC1. (D) RB1CC1 was detected in large and fused myocytes rather than in smaller myoblasts, during embryonic muscular development in humans. Immunohistochemical staining with anti-RB1CC1 antiserum.

is critical for musculoskeletal differentiation, these results suggested that RB1CC1 is involved in cell enlargement that is associated with differentiation in neuromuscular tissues.

RB1CC1-specific knockdown reduces mTOR-S6K activity and modulates cell size. We examined whether RB1CC1 is involved in cell size regulation through the mTOR pathway as well as cell proliferation through RB1. Fig. 2A shows that RB1CC1 knockdown in HEK293 cells mediated by 2 types of respective independent RNAi reduced the active forms of mTOR, S6K and 4EBP1, monitored as the phosphorylation of Ser2448, Thr389 and Thr37/46, respectively. Transfection with empty or scramble vectors did not affect HEK293. RB1CC1 knockdown by RNAi also decreased cell size (Fig. 2B). The effect of RNAi on the phosphorylation of proteins in the TOR pathway was dose-dependent (data not shown). RB1CC1 knockdown also repressed RB1 protein expression and the population of cells in G0-G1-phase (Fig. 2A and B). These results indicated that RB1CC1 regulates both cell size and the cell cycle through TSC-mTOR and the RB1 pathway, respectively.

The biological effect of RB1CC1 knockdown upon cell size reduction was prominent in differentiated C2C12 myocytes synchronized almost at the G1-phase, and was insignificant in exponentially proliferating cells, although the protein level was influenced similarly in both differentiated and proliferating cells. The cell size reduction of differentiated cells was associated with the decrease in phosphorylated and total forms of S6 protein, a downstream target of S6K (Fig. 3). RB1CC1 knockdown diminished the G1 fraction that is rich in differentiated cells (Fig. 3A). The reduced amount of RB1 protein caused by *RB1CC1* RNAi was also in parallel with the decreased G1 fraction in C2C12 myocytes (Fig. 3B), suggesting that significantly reduced RB1 expression fails the suppression of cell cycle especially in differentiated cells.

Exogenous RB1CC1 expression maintains S6K activities and cell size even under starvation. To confirm the contribution of RB1CC1 to the control of cell size through the mTOR pathway, we compared the effect of excess exogenous RB1CC1 expression in HEK293 cells cultured under starvation in Met- and Cys-deprived media or in media containing low (2.5 mM) or no glucose, in comparison with the complete medium supplemented with 25 mM glucose (Fig. 4A). The activity of S6K was suppressed in HEK293 cells under these starved conditions. However, exogenous RB1CC1 dose-dependently activated S6K (Fig. 4A) and recovered cell size from that reduced by low glucose (Fig. 4B). The abundance of RB1CC1 was parallel with reduced TSC1/hamartin expression and increased RB1 expression (Fig. 4A). These results further support the notion that RB1CC1 regulates cell size and the cell cycle through both the TSC-mTOR and RB1 pathways, respectively.

RB1CC1 degrades TSC1 through ubiquitin-proteasomal pathways. To analyze the molecular cascade between RB1CC1

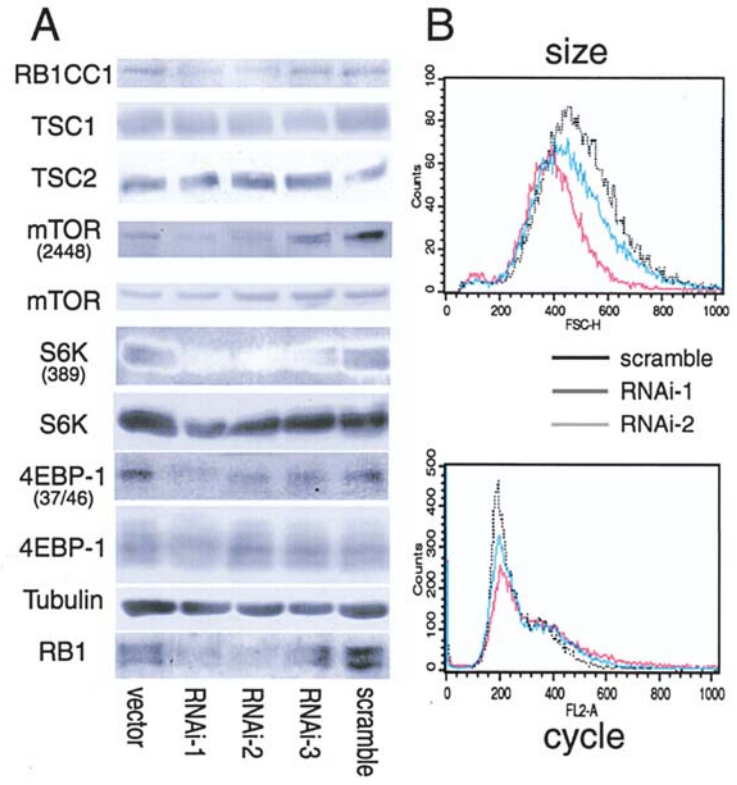

Figure 2. RB1CC1-specific knockdown reduces both mTOR activity and RB1 expression, decreases cell size and diminishes the population of G1-phase cells. (A) *RB1CC1*-RNAi reduced active forms of mTOR, S6K and 4EBP1, indicating phosphorylated Ser2448, Thr389 and Thr37/46, respectively. Knockdown also decreased RB1 expression. Two RNAi vectors (RNAi-1 and RNAi-2) similarly repressed for mTOR pathway, whereas empty or scrambled vectors had no effect. RNAi-3 had little effects for RB1 and mTOR pathways, probably depending to the insignificant knockdown for RB1CC1. Plasmid DNA (4 μg) was transferred using Lipofectamine 2000 into HEK293 cells. (B) Histograms of FSC-H indicate left-shift reflecting decreasing cell size; those of FL2-A suggest decreased number of G1-phase in RB1CC1-RNAi-treated HEK293 cells. Red and blue lines respectively indicate RNAi-1 and RNAi-2 against RB1CC1.

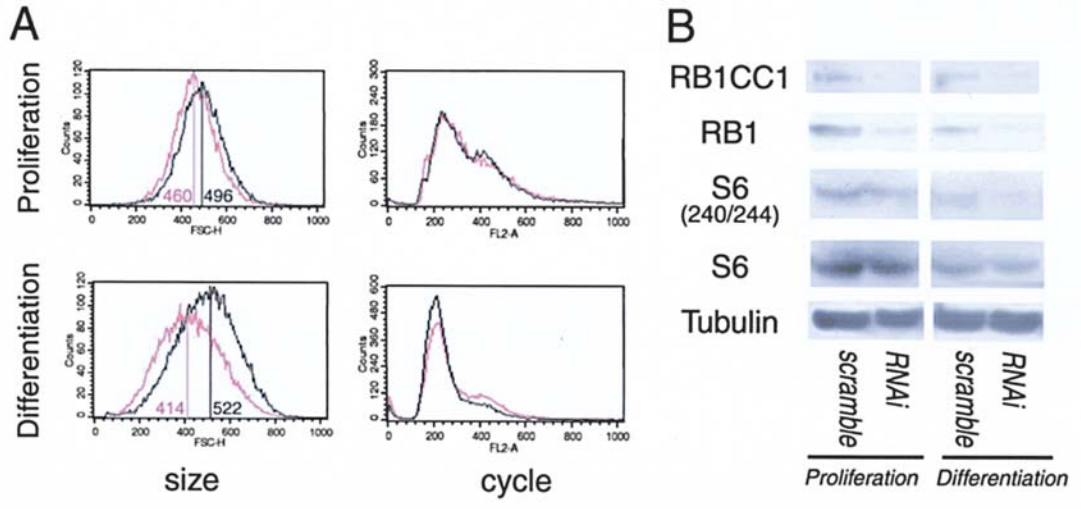

Figure 3. RB1CC1 knockdown similarly affected C2C12 myocyte size and cell cycle. (A) Decreased cell size and number of G1-phase cells in differentiated C2C12 myocytes are prominent, but not significantly different in proliferating cells. Red lines indicate lentiviral RNAi-treated cells. (B) S6 activity (Ser240/244) is reduced in RNAi-treated, proliferating and differentiated cells. Decreased numbers of G1-phase in differentiated cells might depend on decreased RB1 protein content. Proliferation or differentiation was induced in medium containing 10% FBS or 2% horse serum, respectively. C2C12 cells under each condition were cultured in parallel and analyzed by both flow cytometry and Western blotting.

and the TSC-mTOR pathway, we evaluated interactions between RB1CC1 and TSC. We stepwise introduced TSC1-2 into HEK293 cells in the presence of an excess of RB1CC1. RB1CC1 activated S6K, but an excess of TSC1-2 interfered with this activation (Fig. 5A, lanes 1-4). On the contrary, stepwise RB1CC1 overexpression did not activate S6K up to

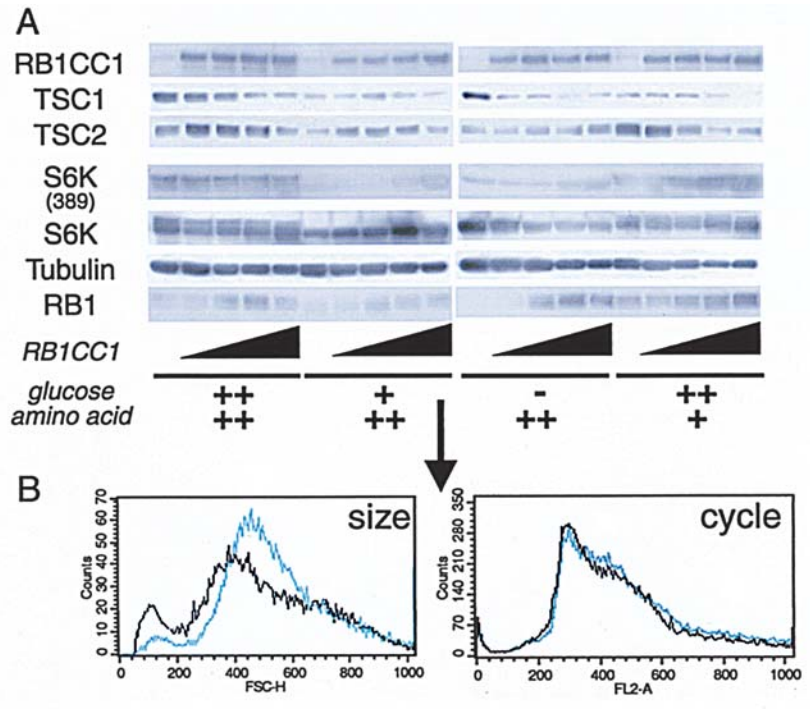

Figure 4. Exogenous *RB1CC1* maintained S6K activity, repressed TSC1 content, and protected cell size from reducing under starvation. (A) Exogenous RB1CC1 was added to the media containing 25 mM (++), 2.5 mM (+), or no (-) glucose with complete amino acids (++), or Met- and Cys-deprived medium (+). Plasmid DNA (0, 0.5, 1, 3 or 5 μg) encoding *RB1CC1* was transfected with complementary empty vectors using Lipofectamine 2000 to HEK293 cells under the above nutritionally starved conditions. With 2.5 mM or no glucose, or under partial amino-starvation, phosphorylated S6K (Thr389) was restored in response to abundant RB1CC1. Abundance of RB1CC1 correlated reciprocally with TSC1/hamartin level, and positively with RB1 expression in HEK293 cells. (B) Under 2.5 mM glucose, cell size was restored by RB1CC1, whereas cell cycle was not significantly affected. Black and blue lines represent *control*- and *RB1CC1*-transduced cells with 2.5 mM glucose, respectively.

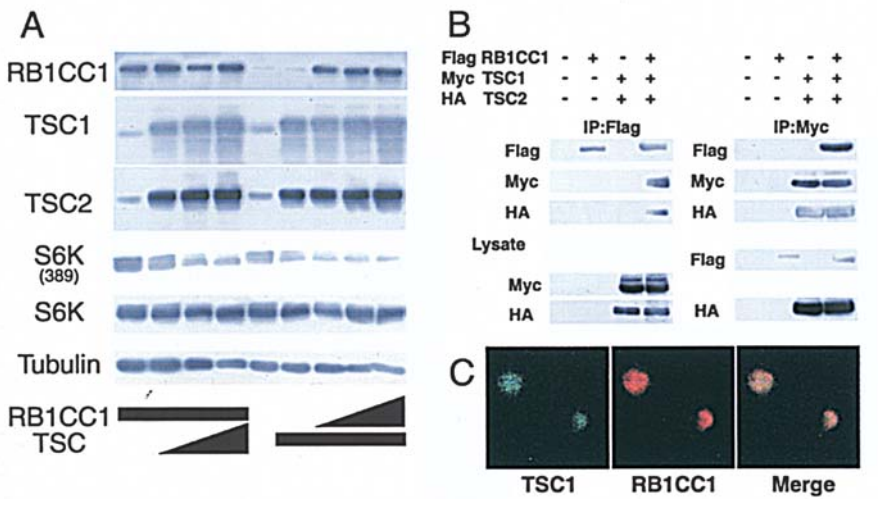

Figure 5. RB1CC1 functions directly upstream of TSC. (A) In the elevated RB1CC1 status of cells transfected with 2 μg of encoding plasmid vector, activated Thr389-S6K (lane 1) was reduced by TSC introduced with 0, 1, 3 and 5 μg of TSC1-2 vectors (lanes 1-4). In contrast, RB1CC1 induction did not activate S6K in the presence of abundant TSC caused by transfection with 2 μg TSC1-2 plasmid DNA. Exogenous amount of RB1CC1 was increased stepwise using 0, 1, 3 and 5 μg of vectors (lanes 6-9). Central lane (lane 5), control HEK293 status. (B) Interaction between RB1CC1 and TSC. HEK293T cells were cotransfected with vectors encoding Flag-RB1CC1 (+) or control (-) and plasmids encoding Myc-TSC1 and HA-TSC2. Aliquots of lysate or anti-Flag immunoprecipitated lysates (IP: Flag) were analyzed by Western blotting. Anti-Myc immunoprecipitates (IP: Myc) were also similarly analyzed. Cells were transfected with each 2 μg of DNA vector using FuGene. (C) Endogenous TSC1 and RB1CC1 were immunocytochemically colocalized in the cytoplasm, especially in ER of HEK293MSR cells, whereas RB1CC1 was also expressed in the nuclei. Indicated antibodies were probed using Alexa-Fluor 488 (green) or 555 (red), reacted with cells, and analyzed by confocal laser-scanning microscopy.

quite a high level under an excess of TSC (Fig. 5A, lanes 6-9). These results indicated that RB1CC1 functions upstream of TSC.

Immunoprecipitation and immunoblotting showed that RB1CC1 interacted with TSC1 and 2 (Fig. 5B). Yeast two-hybrid assays also confirmed binding between RB1CC1 and

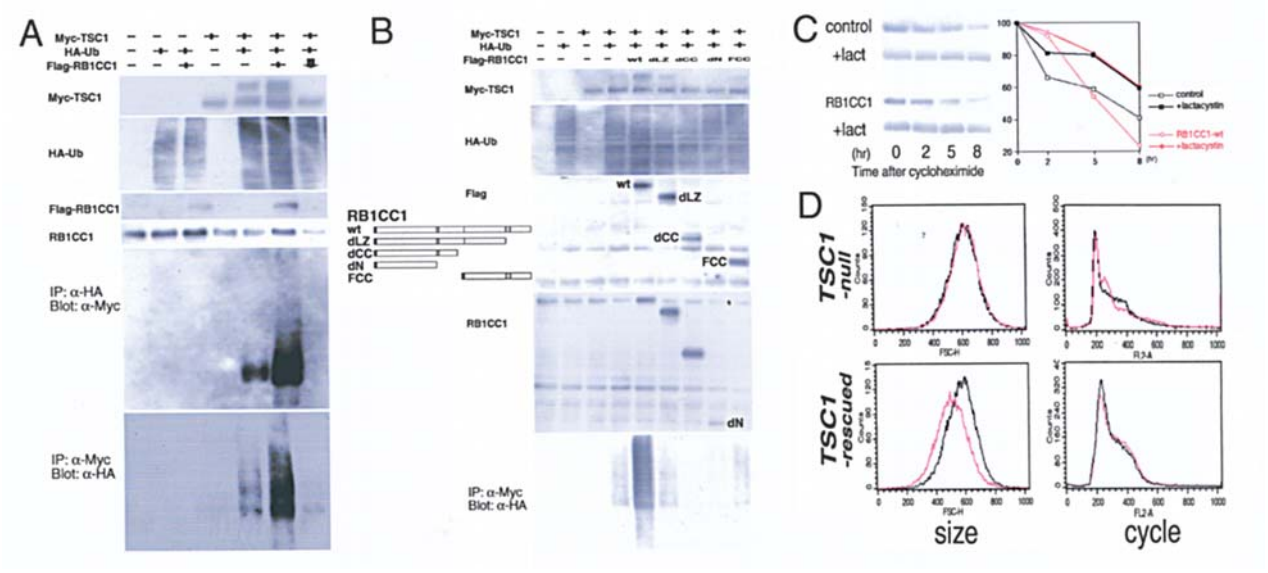

Figure 6. RB1CC1 induced TSC1 degradation through ubiquitin-proteasomal pathway. (A) HEK293 cells transfected with indicated plasmids, Myc-TSC1, HA-ubiquitin and Flag-RB1CC1. Lysates from cells were immunoprecipitated with anti-HA (ubiquitin), immunoblotted against anti-Myc (TSC1), showing in the second lowest panel, and *vice versa*, representing in the lowest panel. RB1CC1 overexpression increased and RNAi decreased poly-ubiquitination species of TSC1. (B) Wild-type RB1CC1 enhanced TSC1 ubiquitination among various RB1CC1 mutants, dLZ, dCC, dN, and FCC, whose N-terminus were tagged with Flag. Schematic diagram of RB1CC1 wild-type and mutants were also indicated on the left side. (C) Excess RB1CC1 induced more rapidly TSC1 turnover than control condition. Endogenous TSC1 contents evaluated after exposure to 10 μg/ml cycloheximide after transfection with RB1CC1 with or without lactacystin (10 μM), a proteasomal inhibitor. Quantified values of signals are plotted relative to 0 h values. Lactacystin blocked TSC1 degradation under both control and excess RB1CC1 conditions. Each plasmid vector was transfected with 6 μg DNA using Lipofectamine 2000. (D) RB1CC1 essentially required TSC1 for cell size control. RB1CC1 was knocked down using lentiviral RNAi delivery in TSC1-null and -rescued cells. Control of cell size by RB1CC1 depended upon TSC1, but knockdown induced the decrease of G1-phase cells or abnormal entry into S-phase in both types of cells. Red lines indicate lentiviral RNAi-treated cells.

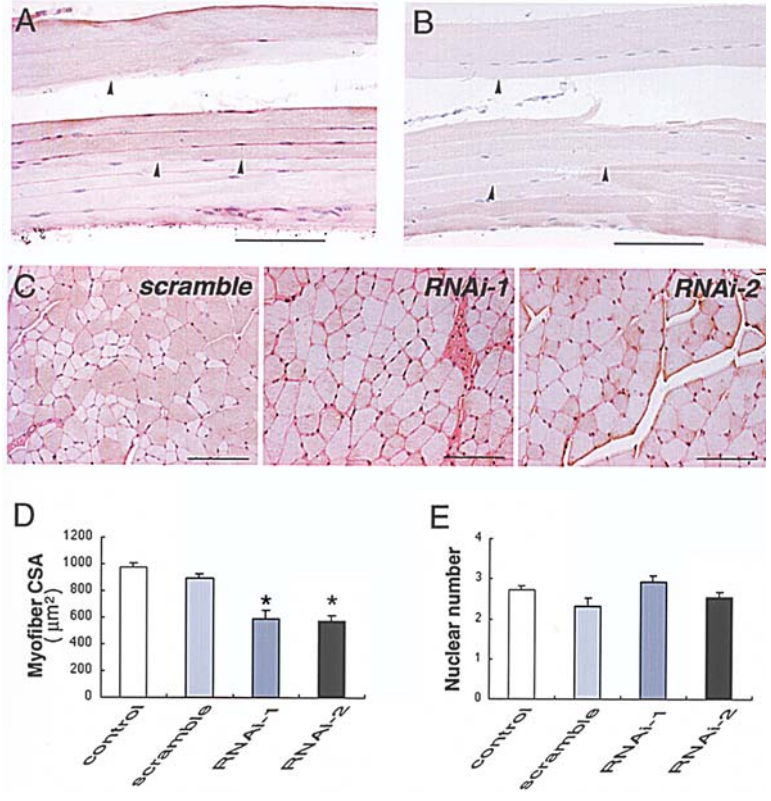

Figure 7. Muscle atrophy treated by *RB1CC1*-RNAi. (A) GFP (arrowheads) is expressed in murine muscle fibers treated by lentiviral *RB1CC1*-RNAi. (B) RB1CC1 was less abundant in GFP-positive fibers (arrowheads). Serial sections were immunostained with antibodies against GFP and RB1CC1, respectively. (C) *Scramble*-, *RNAi-1*- and *RNAi-2*-treated muscle fibers in representative sections of gastrocnemius muscles immunostained with anti-GFP antibodies and followed to PAS staining. (D) Myofiber CSA was reduced due to *RB1CC1* knockdown. CSA size was evaluated by NIH Image software. (E) Number of myonuclei per fiber section was not significantly affected by RNAi. Data are shown as means ± SE of over 100 fibers from three mice per exposure to RNAi. *Difference was statistically significant ($p<0.01$; Student's t-test). Scale bar, 100 μm.

data not shown) and partial fractions of endogenous RB1CC1 were immunocytochemically colocalized (Fig. 5C). Together with the findings that excess RB1CC1 status was reciprocally correlated with TSC1 protein content (Fig. 4A), we postulated that RB1CC1 degrades TSC1 through the ubiquitin-proteasomal pathway. To test this hypothesis, Myc-TSC1, HA-ubiquitin, and Flag-RB1CC1 were exogenously introduced into HEK293 cells, immunoprecipitated with anti-HA antibody, and blotted against anti-Myc antibody. Fig. 6A shows that RB1CC1 overexpression accelerated TSC1 ubiquitination and that the RNAi reduced it. Anti-Myc immunoprecipitation combined with anti-HA blotting produced similar results (Fig. 6A, the lowest panel). Wild-type RB1CC1 enhanced, whereas the RB1CC1 mutants, dLZ, dCC, dN, and FCC did not enhance TSC1 ubiquitination (Fig. 6B).

To examine whether RB1CC1 affects TSC1 stability, the protein contents of TSC1 were evaluated after exposure to cycloheximide with or without lactacystin (control) and an excess of RB1CC1. Fig. 6C shows that TSC1 degraded more rapidly when RB1CC1 was abundant. Lactacystin, a proteasomal inhibitor, blocked TSC1 degradation with or without an excess of RB1CC1. RNAi protected against TSC1 decay, but scramble RNAi and the RB1CC1 mutant degraded TSC1 in a manner similar to that of the control. These results indicated that RB1CC1 degrades TSC1 through the ubiquitin-proteasomal pathway.

To evaluate whether RB1CC1 essentially requires TSC1 for cell size control, RB1CC1 was knocked down in TSC1-null murine renal carcinoma and TSC1-rescued cells. The cell size control mediated by RB1CC1 was dependent on TSC1, but RB1CC1 knockdown caused the decrease of G1-phase population or irregular entry into S-phase in both cell lines (Fig. 6D).

RB1CC1 regulates cell cycle progression through the RB1, but not the TSC-mTOR pathway. Exogenous RB1CC1 positively regulated RB1 expression (Figs. 2-4). RB1CC1 knockdown also decreased G1-phase cells and caused cell cycle progression in HEK293 and C2C12 cells (Figs. 2 and 3). To determine whether the TSC-mTOR pathway is involved in cell cycle control mediated by RB1CC1, we transduced the RNAi for *RB1CC1* into TSC1-null and -rescued cells. The cell cycle alteration caused by RB1CC1 knockdown, such as reduced G1 fraction or abnormal entry into S-phase, was independent of TSC1 status, whereas cell size regulation was dependent (Fig. 6D). These findings indicated that RB1CC1 independently affects the cell cycle and cell size, and that TSC1 is essentially required for RB1CC1-mediated cell size control.

RB1CC1 knockdown decreased the size of mouse hind leg muscles. To analyze whether RB1CC1 maintains the cell or tissue size *in vivo*, we injected lentiviral RNAi for *RB1CC1* into mouse hind leg muscles, and evaluated the size of RNAi-transduced myofibers. The RNAi reduced the amount of RB1CC1 (Fig. 7A and B) and decreased the cross-sectional areas (CSA) of gastrocnemius muscles expressing GFP (Fig. 7C and D), so muscular fiber atrophy was finally caused by *RB1CC1* knockdown. The myonuclear number per transverse section of a GFP-positive fiber did not significantly differ between *scramble*- and *RB1CC1*-RNAi (Fig. 7E). These results indicate that RB1CC1 can regulate cell and tissue size in muscles *in vivo*.

Discussion

The present study offers two important findings. The study uncovered that RB1CC1 suppresses G1-S progression of the cell cycle through RB1 expression. We also discovered that RB1CC1 is abundantly expressed in post-mitotic mature neuromuscular cells throughout life and positively affects the mTOR pathway to contribute to the enlarged cell size. These findings indicate that RB1CC1 plays an important role in the maintenance of cell size without promoting cell cycle progression and contributes to the orchestrated mechanism of tissue organization, especially in neuromuscular organs.

Many tumor suppressors and oncogenes are involved in the TSC-mTOR pathway (9,14,17). The tumor suppressors, PTEN, LKB1 and TSC, negatively regulate mTOR and its targets, S6K and 4EBP1, and then reduce the translation, resulting in smaller cells (9,22,23). On the contrary, oncogenes such as Akt positively affect the mTOR pathway, and encourage translation as well as cell growth (15,16,24). These molecules play parallel roles in both cell size control and proliferation. RB1CC1 increased cell size through mTOR-S6K activation, but suppressed cell cycle progression by up-regulating RB1. The biphasic effects of RB1CC1 upon cell size and cell cycle were particularly prominent in terms of differentiation status rather than proliferation in C2C12 myogenic cells. In addition, an RB1CC1 deficiency resulted in the atrophy of muscular fibers *in vivo*. RB1CC1 is as abundant in post-mitotic large cells as in mature neuromuscular cells, but is less so in post-mitotic leukocytes, which do not grow. Although it remains unknown why neuromuscular cells can be enlarged, our data imply that a neuromuscular abundance of RB1CC1 contributes to this phenotype through both the TSC-mTOR and RB1 pathways.

The cell size control mediated by RB1CC1 depended on TSC1 status. RB1CC1 affected the cell cycle regardless of TSC1 status, but did not affect the size of TSC1-null cells, suggesting that RB1CC1 works independently on cell cycle and size, and that TSC1 is essential for the control of cell size mediated by RB1CC1. We also found that RB1CC1 positively regulated the mTOR pathway via TSC1 degradation through the ubiquitin-proteasomal pathway. The molecular mechanism through which RB1CC1 controls cell size seemed to be the accelerated degradation of TSC1. Akt might play a role in TSC degradation through TSC2 phosphorylation of the target site (24). Pam might also degrade TSC1-2 through ternary binding and by genetic linkage as a negative regulator of TSC in *Drosophila* (25). Our data indicate that a novel molecular mechanism regulates mTOR through the ubiquitination and degradation of TSC1 induced by RB1CC1.

A group who recently named RB1CC1 as FIP200, independently reported that RB1CC1 associates with TSC1 and plays a positive role in cell size control (26). They speculated that interaction between RB1CC1 and TSC1 prompts a competitively dissociative role for the complex between TSC1 and TSC2, which results in a positive mTOR function. However, they also noted that the TSC1-binding sites for RB1CC1 differ from those for TSC2 and that RB1CC1

might play another negative role for TSC1 rather than that of competitive binding against TSC2. They did not find that abundant RB1CC1 accelerated TSC1 ubiquitination. We found that the RB1CC1 mutants (dLZ, dCC) containing aa 638-859 could bind to TSC1, as previously reported (26), but did not significantly prompt TSC1 ubiquitination. This indicated that wild-type RB1CC1 does not competitively dissociate TSC through binding with TSC1, but that it actively accelerates TSC1 ubiquitination. RB1CC1 presumably recruits TSC1 to any E3 ubiquitin ligases, because it contains neither an F-box nor a RING-H2 finger domain. Furthermore, RB1CC1 can degrade various molecules in a multi-directional manner (27). RB1CC1 stabilizes p53 by protecting against self-ubiquitination, and on the contrary, also decreases cyclin D1 protein by promoting proteasome-dependent degradation. All of these findings together with that of the multi-directionality of RB1CC1 for molecular ubiquitination support the notion that RB1CC1 is a recruiter for protein ubiquitination rather than a direct ubiquitin ligase.

In summary, RB1CC1 was required for crosstalk between the mTOR and RB1 pathways, and it preserved the enlarged cell phenotype without cell cycle progression especially in neuromuscular tissues. A neuromuscular abundance of RB1CC1 contributed to the maintenance of enlarged cells through TSC1 degradation, causing activation of the mTOR pathway. In addition, RB1CC1 suppresses cell cycle progression through RB1 up-regulation. Thus, RB1CC1 plays important roles in cell proliferation, growth, differentiation and death and provides novel insight into the coordinate molecular mechanisms of tissue organization. A dysfunction of RB1CC1 causes dysregulation of these mechanisms, resulting in various pathological conditions such as cancer and neuromuscular diseases.

Acknowledgments

This study was supported in part by grants-in-aid for PRESTO, JST; Scientific Research on Priority Areas (no. 17013038) and Young Scientists (A) (no.15689008), MEXT. We thank S. Hirayama, N. Takashima, Y. Morita, N. Yagi and the staff of Central Research Laboratory, S.U.M.S, for experimental assistance. The data was presented in part at the Gordon Research Conference on Cancer Models and Mechanisms on July 25, 2005.

References

1. Chano T, Ikegawa S, Kontani K, Okabe H, Baldini N and Saeki Y: Identification of *RB1CC1*, a novel human gene that can induce *RB1* in various human cells. Oncogene 21: 1295-1298, 2002.
2. Abbi S, Ueda H, Zheng C, Cooper LA, Zhao J, Christopher R and Guan JL: Regulation of focal adhesion kinase by a novel protein inhibitor FIP200. Mol Biol Cell 13: 3178-3191, 2002.
3. Kontani K, Chano T, Ozaki Y, Tezuka N, Sawai S, Fujino S, Saeki Y and Okabe H: RB1CC1 suppresses cell cycle progression through RB1 expression in human neoplastic cells. Int J Mol Med 12: 767-769, 2003.
4. Watanabe R, Chano T, Inoue H, Isono T, Koiwai O and Okabe H: Rb1cc1 is critical for myoblast differentiation through Rb1 regulation. Virchows Arch 447: 643-648, 2005.
5. Chano T, Kontani K, Teramoto H, Okabe H and Ikegawa S: Truncating mutations of RB1CC1 in human breast cancer. Nat Genet 31: 285-288, 2003.
6. Teramoto K, Chano T, Ozaki Y, Sawai S, Tezuka N, Kontani K, Fujino S and Okabe H: Expression of RB1CC1, a novel tumor suppressor gene, is inversely correlated with the Ki-67 proliferation index in primary breast cancers. Cancer Ther 1: 103-107, 2003.
7. Chano T, Saeki Y, Serra M, Matsumoto K and Okabe H: Preferential expression of RB1-inducible coiled-coil 1 in terminal differentiated musculoskeletal cells. Am J Pathol 161: 359-364, 2002.
8. Bamba N, Chano T, Taga T, Ohta S, Takeuchi Y and Okabe H: Expression and regulation of RB1CC1 in developing murine and human tissues. Int J Mol Med 14: 583-587, 2004.
9. Inoki K, Corradetti MN and Guan KL: Dysregulation of the TSC-mTOR pathway in human disease. Nat Genet 37: 19-24, 2005.
10. Kobayashi T, Minowa O, Kuno J, Mitani H, Hino O and Noda T: Renal carcinogenesis, hepatic hemangiomatosis, and embryonic lethality caused by a germ-line Tsc2 mutation in mice. Cancer Res 59: 1206-1211, 1999.
11. Kobayashi T, Minowa O, Sugitani Y, Takai S, Mitani H, Kobayashi E, Noda T and Hino O: 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: 8762-8767, 2001.
12. Potter CJ, Huang H and Xu T: *Drosophila* Tsc1 functions with Tsc2 to antagonize insulin signaling in regulating cell growth, cell proliferation, and organ size. Cell 105: 357-368, 2001.
13. Tapon N, Ito N, Dickson BJ, Treisman JE and Hariharan IK: The *Drosophila* tuberous sclerosis complex gene homologs restrict cell growth and cell proliferation. Cell 105: 345-355, 2001.
14. Gao X, Zhang Y, Arrazola P, Hino O, Kobayashi T, Yeung RS, Ru B and Pan D: Tsc tumour suppressor proteins antagonize amino-acid-TOR signalling. Nat Cell Biol 4: 699-704, 2002.
15. Inoki K, Li Y, Zhu T, Wu J and Guan KL: TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nat Cell Biol 4: 648-657, 2002.
16. Manning BD, Tee AR, Logsdon MN, Blenis J and Cantley LC: Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/akt pathway. Mol Cell 10: 151-162, 2002.
17. Kwiatkowski DJ: Tuberous sclerosis: from tubers to mTOR. Ann Hum Genet 67: 87-96, 2003.
18. Tee AR, Manning BD, Roux PP, Cantley LC and Blenis J: Tuberous sclerosis complex gene products, Tuberin and Hamartin, control mTOR signaling by acting as a GTPase-activating protein complex toward Rheb. Curr Biol 13: 1259-1268, 2003.
19. Akagi T, Shishido T, Murata K and Hanafusa H: v-Crk activates the phosphoinositide 3-kinase/AKT pathway in transformation. Proc Natl Acad Sci USA 97: 7290-7295, 2000.
20. Sarbassov DD, Guertin DA, Ali SM and Sabatini DM: Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science 307: 1098-1101, 2005.
21. Rosner M, Hofer K, Kubista M and Hengstschlager M: Cell size regulation by the human TSC tumor suppressor proteins depends on PI3K and FKBP38. Oncogene 22: 4786-4798, 2003.
22. Groszer M, Erickson R, Scripture-Adams DD, Lesche R, Trumpp A, Zack JA, Kornblum HI, Liu X and Wu H: Negative regulation of neural stem/progenitor cell proliferation by the Pten tumor suppressor gene *in vivo*. Science 294: 2186-2189, 2001.
23. Corradetti MN, Inoki K, Bardeesy N, DePinho RA and Guan KL: Regulation of the TSC pathway by LKB1: evidence of a molecular link between tuberous sclerosis complex and Peutz-Jeghers syndrome. Genes Dev 18: 1533-1538, 2004.
24. Plas DR and Thompson CB: Akt activation promotes degradation of tuberin and FOXO3a via the proteasome. J Biol Chem 278: 12361-12366, 2003.
25. Murthy V, Han S, Beauchamp RL, Smith N, Haddad LA, Ito N and Ramesh V: Pam and its ortholog highwire interact with and may negatively regulate the TSC1-TSC2 complex. J Biol Chem 279: 1351-1358, 2004.
26. Gan B, Melkoumian ZK, Wu X, Guan KL and Guan JL: Identification of FIP200 interaction with the TSC1-TSC2 complex and its role in regulation of cell size control. J Cell Biol 170: 379-389, 2005.
27. Melkoumian ZK, Peng X, Gan B, Wu X and Guan JL: Mechanism of cell cycle regulation by FIP200 in human breast cancer cells. Cancer Res 65: 6676-6684, 2005.