Original research

mTOR-S6K1 pathway mediates cytoophidium assembly

Zhe Sun a, Ji-Long Liu a, b, *

a School of Life Science and Technology, ShanghaiTech University, Shanghai, 201210, China
b MRC Functional Genomics Unit, Department of Physiology, Anatomy and Genetics, University of Oxford, Oxford, OX1 3PT, United Kingdom

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A B S T R A C T

CTP synthase (CTPS), the rate-limiting enzyme in de novo CTP biosynthesis, has been demonstrated to assemble into evolutionarily conserved filamentous structures, termed cytoophidia, in Drosophila, bacteria, yeast and mammalian cells. However, the regulation and function of the cytoophidiun remain elusive. Here, we provide evidence that the mechanistic target of rapamycin (mTOR) pathway controls cytoophidium assembly in mammalian and Drosophila cells. In mammalian cells, we find that inhibition of mTOR pathway attenuates cytoophidium formation. Moreover, CTPS cytoophidium assembly appears to be dependent on the mTOR complex 1 (mTORC1) mainly. In addition, knockdown of the mTORC1 downstream target S6K1 can inhibit cytoophidium formation, while overexpression of the constitutively active S6K1 reverses mTOR knockdown-induced cytoophidium disassembly. Finally, reducing mTOR protein expression results in a decrease of the length of cytoophidium in Drosophila follicle cells. Therefore, our study connects CTPS cytoophidium formation with the mTOR signaling pathway.

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1. Introduction

CTP not only serves as the building blocks for nucleic acid synthesis, but also contributes to the synthesis of membrane phospholipids and protein sialylation (Huang and Graves, 2003; Higgins et al., 2007). Low intracellular concentration makes CTP one of the rate-limiting molecules for nucleic acid biosynthesis and other CTP-dependent events (Traut, 1994). Therefore, understanding the precise control of CTP production is crucial for cell metabolism and many growth-related processes.

CTP can be generated through either the de novo synthesis pathway or the salvage pathway in mammalian cells. CTP synthase (CTPS) is the rate-limiting enzyme that catalyzes the conversion of UTP to CTP using glutamate or ammonia as the nitrogen source (Levitizki and Koshland, 1971). It has been demonstrated in a number of studies that CTPS can be assembled into filamentous structures, termed cytoophidia, in different organisms, including fruit fly, bacteria, yeast and mammalian cells (Ingeron-Mahar et al., 2010; Liu, 2010; Noree et al., 2010; Carcamo et al., 2011; Chen et al., 2011).

Recent studies have established a link between cytoophidium and CTPS enzymatic activity (Aughey et al., 2014; Barry et al., 2014; Noree et al., 2014; Strochlic et al., 2014; Lynch et al., 2017). In Drosophila, inhibition of the proto-oncogene Cbl disrupts cytoophidium formation, and the protein level of the oncogene c-Myc is correlated with cytoophidium abundance and size (Wang et al., 2015; Aughey et al., 2016). Moreover, CTPS activity was found to be elevated in various cancers such as hepatoma and lymphoma (Williams et al., 1978; Ellum et al., 1983). Recently, we also observed the presence of CTPS cytoophidia in a variety of human cancer tissues (Chang et al., 2017). These findings suggest that the formation of cytoophidia is an evolutionarily conserved property of CTPS.

In mammals, the mechanistic target of rapamycin (mTOR) is the key serine/threonine protein kinase, which can interact with several proteins to form two distinct molecular complexes, called mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (Saxton and Sabatini, 2017). mTORC1 controls cell growth and metabolism by regulating protein synthesis, lipid and glucose metabolism, and protein turnover (Saxton and Sabatini, 2017). In
contrast, mTORC2 regulates cell proliferation and survival primarily through phosphorylating Akt and several members of the AGC (PKA/PKG/PKC) family of proteins (Sarbassov et al., 2005; Saxton and Sabatini, 2017). Deregulation of the mTOR signaling pathway is associated with a number of human diseases, including cancer, type 2 diabetes, obesity, and neurodegeneration (Saxton and Sabatini, 2017).

Recent studies have established a direct link between mTOR pathway and nucleotide metabolism (Ben-Sahra et al., 2013, 2016; Robitaille et al., 2013). In this study, to get a better understanding of the regulation of cytophodium, we used a human cancer cell line and Drosophila as model systems to investigate the regulation of cytophodium assembly by mTOR. We show that inhibiting mTOR pathway results in cytophodium disassembly without affecting CTPS protein expression. In addition, the mTOR pathway controls CTPS cytophodium assembly mainly via the mTORC1/S6K1 signal axis. Thus, this study links mTOR-S6K1 pathway to the polymerization of the pyrimidine metabolic enzyme CTPS.

2. Results

2.1. mTOR regulates CTPS cytophodium assembly

To investigate whether the mTOR pathway regulates CTPS cytophodium formation, we screened various cell lines. We observed that CTPS cytophodia were present in ~40% SW480 (a human colorectal cancer cell line) cells under normal culture conditions (Fig. 1A). However, it is hard to detect cytophodia in other colorectal cancer cell lines, including LoVo, RKO, DLD1, HCT116 and a normal human colon mucosal epithelial cell line NCM460 (Fig. S1). Therefore, we used the SW480 cell line as a model for investigating the correlation between the CTPS cytophodium and mTOR pathway activity.

We first treated SW480 cells with the mTOR inhibitors rapamycin or everolimus, and then labeled CTPS with anti-CTPS antibody. Immunofluorescence analysis showed that CTPS cytophodia were present in 34.6% of control cells, while the percentage of cells with CTPS cytophodia was reduced to 17% and 15.8% upon rapamycin or everolimus treatment, respectively (Fig. 1B and C). Inhibition of mTOR pathway was confirmed by the decreased level of phosphorylation at T389 of S6K1, a marker of active mTOR signaling (Fig. 1D). Further analysis showed that rapamycin and everolimus inhibit CTPS cytophodium formation in a time- and dose-dependent manner (Fig. 1E–H). Previous studies have shown that Myc and Cbl regulate cytophodium formation in Drosophila. Here we investigate if mTOR mediates cytophodium assembly through the reduction of c-Myc or Cbl. Our data showed that the mRNA levels of c-Myc and Cbl were not changed when cells were treated with rapamycin (Fig. S2A and B). Moreover, the protein levels of c-Myc were not changed upon rapamycin treatment either (Fig. S2C), suggesting that mTOR does not regulate cytophodium formation via c-Myc or Cbl.

To confirm the correlation between mTOR pathway and cytophodium assembly, we constructed a stable cell line expressing shRNA targeting mTOR and investigated the impact of mTOR knockdown on cytophodium formation. Immunofluorescence results showed that the percentage of cells with CTPS cytophodia dramatically decreased in cells expressing mTOR shRNA in comparison with the cells expressing control shRNA (Fig. 2A and B, 39.2% versus 11.5%; P < 0.0001). mTOR knockdown efficiency was confirmed by the decreased protein level of mTOR (Fig. 2C). A similar result was observed in an mTOR siRNA experiment. Compared with control siRNA, transfection of mTOR siRNA decreased the expression of mTOR protein (Fig. 2F), which was accompanied by a reduced proportion of cells presenting the CTPS cytophodia (Fig. 2D and E, 35.4% versus 23.2%; P < 0.0001). The expression level of CTPS has been recognized as a critical factor for cytophodium assembly (Ingerson-Mahar et al., 2010; Chen et al., 2011; Azzam and Liu, 2013; Liu, 2016). We next determined whether mTOR pathway inhibition reduces cytophodium assembly through decreasing CTPS protein expression. Our data showed that neither rapamycin nor everolimus treatment affected CTPS protein expression (Fig. 3A and B). Inhibition of mTOR pathway was confirmed by the decreased level of phosphorylation at T389 of S6K1. In addition, knockdown of mTOR either by siRNA or by shRNA did not decrease CTPS protein expression (Fig. 3C and D).

2.2. mTORC1 controls CTPS cytophodium assembly

mTOR can be incorporated into both mTORC1 and mTORC2, and is essential for them to exert their biological functions (Saxton and Sabatini, 2017). Rapamycin binds to FK506-binding protein 12 (FKBP12) and inhibits mTORC1 activity directly. Although the rapamycin-FKBP12 complex does not directly bind to and inhibit mTORC2, long-time rapamycin treatment attenuates mTORC2 signaling, likely because the rapamycin-bound mTOR cannot be incorporated into a new mTORC2 complex (Sarbassov et al., 2006). Therefore, we next determined which complex plays a dominant role in controlling CTPS cytophodium assembly. For this purpose, we constructed two other stable cell lines expressing shRNA targeting a specific component of mTORC1 (Raptor) or a specific component of mTORC2 (Rictor) (Saxton and Sabatini, 2017). Immunofluorescence data showed that knockdown of Rictor did not change the proportion of cells with cytophodia (Fig. 4A and B). In contrast, the percentage of cells presenting cytophodia was reduced from 34.1% to 12.7% in Raptor knockdown cells as compared with control cells, and the degree of reduction is comparable to cells expressing mTOR shRNA (Fig. 4A and B). The knockdown efficiency was confirmed by Western blotting assay (Fig. 4C). For further confirmation of this phenomenon, we conducted a siRNA experiment. We found no difference in the percentage of cells with cytophodia when cells were transfected with Rictor siRNA as compared with control siRNA. However, the transfection of Raptor siRNA significantly decreased the proportion of CTPS cytophodia-positive cells from 32% to 20% (Fig. 4D and E), which is similar to the transfection of mTOR siRNA. The knockdown efficiency of the indicated genes was verified by Western blotting (Fig. 4F). Taken together, these results show that mTORC1 plays a dominant role in controlling CTPS cytophodium assembly.

2.3. mTORC1 controls CTPS cytophodium formation through S6K1

Recent studies showed that mTORC1 could promote purine and pyrimidine synthesis through the ATF4/MTHFD2 and S6K1 pathway, respectively (Ben-Sahra et al., 2013, 2016). To further understand the mechanisms by which mTORC1 regulates CTPS cytophodium formation, we analyzed the effects of ATF4, MTHFD2 or S6K1 knockdown on CTPS cytophodium formation. In comparison with cells transfected with control siRNA, no significant difference in the percentage of cells with cytophodia was observed in cells transfected with ATF4 or MTHFD2 siRNA. Yet, transfection of S6K1 siRNA significantly decreased the proportion of CTPS cytophodia-positive cells from 36.1% to 19.8% (Fig. 5A and B). Western blotting was used to verify the knockdown efficiency of the indicated genes (Fig. 5C). The role of S6K1 in cytophodium assembly was further confirmed by lentiviral shRNA targeting S6K1. Immunofluorescence results showed that the percentage of cells expressing S6K1 shRNA-1 or shRNA-2 which contained CTPS...
cytoophidia dropped significantly from 41.1% to 6% and 15%, respectively, in comparison with cells expressing control shRNA (Fig. 5D and E). Cells stably expressing S6K1 shRNA-1 or shRNA-2 showed significantly reduced S6K1 protein expression (Fig. 5F).

2.4. mTOR is required for cytoophidium assembly in Drosophila

We further investigated the correlation between mTOR pathway and CTPS cytoophidium assembly in vivo. Two independent UAS driven shRNA were used to knock down the expression of mTOR in follicle cell epithelium of the Drosophila egg chambers. Compared with the neighboring cells, the cells expressing mTOR shRNA showed reduced nuclear size (Fig. 6A and B). The nuclear size in mTOR knockdown cells is less than 50% of that in neighboring cells (Fig. 6H), which is in agreement with the well-known function of mTOR in cell size control (Zhang et al., 2000; Fingar et al., 2002). Meanwhile, the expression of mTOR shRNAs resulted in a decrease of the cytoophidium length in GFP-positive clones as compared to the normal cytoophidium formation observed in their neighboring cells (Fig. 6C‒G and I). Statistical analysis showed that the length of cytoophidia is less than 50% of the length of cytoophidia in their neighboring cells, suggesting that mTOR is required for cytoophidium assembly in vivo.
the mTOR pathway and CTPS cytoophidium assembly. mTOR has emerged as an important regulator of nucleotide metabolism (Bensahra et al., 2013, 2016; Robitaille et al., 2013) and is implicated in multiple human cancer types (Saxton and Sabatini, 2017). Mutations in mTOR itself are observed in various cancer subtypes (Sato et al., 2010; Grabiner et al., 2014). mTOR also serves as a downstream effector for many frequently mutated prooncogenic pathways, such as Ras/Raf/MAPK pathway, resulting in the hyper-activation of mTOR pathway in numerous human cancers. However, single-agent therapies using mTORC1 inhibitors, including rapamycin and everolimus, only showed limited anti-cancer activity, mainly due to the inhibition of mTORC1 generally has
cytostatic but not cytotoxic effects in cancer cells. Elevated CTP levels and increased CTPS enzyme activity have been reported in many types of cancer such as hepatomas, leukemia and colorectal cancer (Williams et al., 1978; Kizaki et al., 1980; Weber et al., 1980; Ellims et al., 1983; van den Berg et al., 1993). Knockdown of CTPS reduced tumorigenesis in a Drosophila tumor model (Willoughby et al., 2013), indicating that CTPS plays a functional role in tumor metabolism. In fact, CTPS has been an attractive anti-cancer target for decades. However, treatment with CTPS inhibitors such as acivicin and 6-Diazo-5-oxo-L-norleucine (DON) often provokes some unacceptable side effects, such as neurotoxicity, nausea and vomiting, which has hindered their further applications (Lynch et al.,

Fig. 4. Reduced cytoophidium formation is dependent on mTORC1. A: SW480 cells expressing shRNA targeting mTOR, Raptor, Rictor, or RFP (as control) were analyzed by immunofluorescence staining for the presence of CTPS cytoophidium. B: Quantitative data of percentage of cells with cytoophidia shown in (A). C: Western blotting analysis of mTOR, Raptor and Rictor protein expression in the cells expressing shRNA of the indicated genes. D: SW480 cells transfected with siRNA targeting mTOR, Raptor, Rictor, or scrambled control siRNA were subjected to immunofluorescence staining with anti-CTPS antibody. E: Quantitative data for SW480 cells with CTPS cytoophidia shown in (D). F: Western blotting analysis of mTOR, Raptor and Rictor protein expression in cells transfected with scrambled control siRNA or siRNA targeting mTOR, Raptor or Rictor with appropriate antibodies. β-Actin was used as a loading control in (C) and (F). Mean ± S.E.M., n.s., not significant; *P < 0.05, **P < 0.01; ****P < 0.0001 versus control. Scale bars = 20 µm. One of four to six similar experiments is shown.
1982; Rubin et al., 1983; Earhart et al., 1990; Falkson et al., 1990; Maroun et al., 1990). A recent study also reported that inactivation of CTPS caused imbalance of dNTP pools and increased mutagenesis in Saccharomyces cerevisiae (Schmidt et al., 2017). The assembly of CTPS into cytophoidium has been suggested as a way for modulating its enzymatic activity. Polymerization of CTPS inhibits its catalytic activity in S. cerevisiae and Escherichia coli (Barry et al., 2014; Noree et al., 2014). However, an in vitro study showed that filamentation of CTPS increases its enzymatic activity (Lynch et al., 2017).

We recently reported the increased abundance of CTPS cytophoidium in various human cancers including colon, prostate and live cancers (Chang et al., 2017). A larger nucleotide pool is required to support fast cancer cell growth. The potential advantage of the cytophoidium formation is to increase enzyme activity rapidly, provided that polymerization is faster than transcription. Inhibition of the rate-limiting enzyme in guanylate nucleotide synthesis, inosine monophosphate dehydrogenase (IMPDH), selectively kills mTORC1–activated cancer cells, implying that targeting nucleotide metabolism is promising for treating tumors with elevated mTOR signaling (Valvezan et al., 2017). Therefore, it will be interesting to determine whether inhibition of CTPS filamentation could suppress mTOR hyperactive cancer cell growth in future studies. If this is true, further identification of small molecules to disrupt CTPS polymerization may be a promising strategy for combatting mTOR-driven cancers.

The mechanisms by which mTOR pathway controls CTPS filamentation is likely through direct and indirect manners. In this study, we understand that the regulation of CTPS filamentation seems not through reducing CTPS protein expression, which has been recognized as a critical factor for cytophoidium assembly (Ben-Sahra et al., 2013). We showed evidence supporting that the regulation of cytophoidium formation by mTOR pathway is being carried out mainly by S6K1 kinase. When S6K1 was knocked down by siRNA, there is an approximate 50% reduction in the number of cells containing cytophidia (Fig. 5A and B), and a further reduction in the percentage of cells stably expressing S6K1 shRNA (Fig. 5D and E). Importantly, exogenous expression of a constitutively active S6K1 mutant (CA-S6K1) rescued mTOR knockdown-induced cytophoidium disassembly (Fig. 5G and H). Both transcriptional and post-transcriptional mechanisms especially phosphorylation can regulate CTPS enzymatic activity (Park et al., 1999, 2003; Choi et al., 2003; Han et al., 2005; Chang et al., 2007; Higgins et al., 2007). Indeed, filamentous CTPS can be recognized by a phosphospecific antibody against CTPS phosphorylated on serine 36 (Liu, 2010), whose mutation causes a decrease in CTPS catalytic activity (Park et al., 2003). These findings raise the possibility that phosphorylation could regulate CTPS activity by influencing cytophoidium assembly. Interestingly, a previous phosphoproteomics study identified several phosphorylation sites at the C-terminal of CTPS in the mTOR pathway-activated mouse embryonic fibroblasts (MEFs) (Robitaille et al., 2013). We recently reported that deletion of the conserved N-terminal of Drosophila CTPS, targets of multiple post-translational modifications including phosphorylation, is sufficient to interfere with cytophoidium assembly (Huang et al., 2017). Therefore, it will be interesting to determine if phosphorylation has a direct effect on CTPS filamentation in the future.

mTOR plays a central role in regulating cell size, cell cycle progression and cell proliferation in Drosophila and many other species (Zhang et al., 2000; Fingar et al., 2002; Lloyd, 2013). A previous study reported that reduction of CTPS could result in a decrease in nuclear size in Drosophila follicle cells (Wang et al., 2015). Our recent investigation in Drosophila also showed that CTPS is required for Myc-dependent cell size control (Aughney et al., 2016). It is worth to note that several nucleotide metabolizing enzymes are phosphorylated or upregulated in response to mTOR activation in mammalian cells leading to increased intracellular pools of pyrimidines and purines for DNA and RNA synthesis (Ben-Sahra et al., 2013, 2016; Robitaille et al., 2013; Valvezan et al., 2017), the relevance of this relationship in normal animal development has not been well defined. In this study, we observed a connection between mTOR expression and the length of CTPS cytophoidium in Drosophila oogenesis.

Together, using the colorectal cancer cell line SW480 and Drosophila as model systems, we show that the mTOR pathway regulates CTPS cytophoidium assembly. We have found that pharmacological inhibition of the mTOR pathway or knockdown of mTOR protein expression significantly reduces cytophoidium formation without affecting CTPS protein expression. In addition, the mTOR pathway controls CTPS cytophoidium assembly mainly via the mTORC1/S6K1 signal axis. Collectively, our results show a connection between the mTOR pathway and CTPS cytophoidium assembly.

4. Materials and methods

4.1. Regents and antibodies

Antibodies for CTPS (15914-1-AP), Raptor (20984-1-AP), Rictor (27248-1-AP), S6K1 (14485-1-AP), AT4 (10835-1-AP) and MTHFD2 (12270-1-AP) were purchased from ProteinTech (China). Antibodies for mTOR (#29283) and Phospho-S6K1 (#9205) were purchased from Cell Signaling Technology (USA). Antibodies for c-Myc (ab32072) and β-Actin (ab6276) were purchased from Abcam (USA). Antibody for HA (sc-7392) and normal mouse IgG (sc-2025) were from Santa Cruz Biotechnology (USA). Antibody for α-tubulin (T5168) was purchased from Sigma-Aldrich (USA). Antibodies for Drosophila CTPS (134457 and 33304) were purchased from Santa Cruz. Antibody for Hu-li tai shao (Hts) (7H9 1B1) was purchased from Developmental Studies Hybridoma Bank (USA). Ramaycin (S1039) and everolimus (S1120) were from Selleck Chemicals (China).

4.2. Cell culture

293T, SW480 and NCM460 cells were cultured in Dulbecco's...
modified Eagle’s medium (DMEM, SH30022.01, Hyclone, China), whereas LoVo, RKO, DLD-1 and HCT116 cells were cultured in Roswell Park Memorial Institute Medium 1640 (RPMI 1640, SH30809.01, Hyclone) supplemented with 10% fetal bovine serum (04-001, Biological Industries, Israel) and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin, and SV30010, Hyclone), in a humidified atmosphere containing 5% CO₂ at 37 °C (normal culture conditions). Cell transfections were carried out by using Lipofectamine 2000 (11668019, Invitrogen, USA) or R0531 (Thermo Fisher Scientific, USA) according to the manufacturer’s instructions.

4.3. Drosophila husbandry

All stocks were maintained on standard Drosophila medium at 25 °C. w¹¹¹⁸ (Bloomington stock centre) was used as a wild-type control in all our experiments. All RNAi stocks were from the TRiP collection (Bloomington Stock Center, USA).

4.4. Lentiviral shRNA cloning, production, and infection

Desalted oligonucleotides were cloned into pPLK/GFP + Puro purchased from the Public Protein/Plasmid Library (Nanjing, China) with the BamHI/EcoRI sites at the 3’ end of the human H1 promoter. The target sequences for mTOR, Raptor and Rictor are 5’-CCGCATTGTCTCTATCAAGTT-3’, 5’-CGAGTCCTCTTTCACTACAAT-3’ and 5’-CCGCAGTTACTGGTACATGAA-3’, respectively. The target sequences for S6K1 are 5’-ACACACAGAAAATCCTCAGACA-3’ and 5’-CCCATGATCTCCAAACGGCCA-3’. Plasmids were propagated in and purified from top 10 bacterial cells and co-transfected together with psPAX2 and pMD2.G into HEK 293T cells. Virus-containing
supernatants were collected at 48 h after transfection, and then filtered with 0.45 μm PES filters (Millipore, USA). Cells were infected with appropriate lentiviruses in the presence of 8 μg/mL polybrene (Millipore) for 48 h. The GFP-positive cells were purified by flow cytometry and then cultured in normal medium containing 0.5 μg/mL puromycin for 1 week. The resulting puromycin-resistant cells were used for further analysis.

4.5. siRNA and transfection

Small interfering RNA (siRNA) duplexes against mTOR (stQ0004935-1), Raptor (stQ0012651-1), Rictor (stQ0016785-1), S6K1 (stQ0004595-1), ATF4 (stQ0005631-1) and MTHFD2 (stQ0002930-1) were purchased from Ribobio (Guangzhou, China). Three siRNA duplexes were used for one target gene to achieve greater knockdown efficiency and lower off-target effects. A 2 μL or 5 μL aliquot of 20 μM siRNA per well was transfected into cells seeded in 24-well or 6-well plates, respectively, with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol.

4.6. Immunofluorescence

For mammalian, cells were cultured on glass slides and fixed with 4% paraformaldehyde in PBS for 10 min, and then permeabilized with 0.1% Triton X-100 for 10 min at room temperature. After washed with PBS, samples were blocked with 5 mg/mL bovine serum albumin in PBS for 1 h, followed by incubation with anti-CTP antibodies overnight at 4 °C. After the primary antibody reaction, samples were washed and incubated with FITC-labeled secondary antibodies for 1 h. Finally, samples were washed and mounted with medium containing 4′,6-diamidino-2-phenylindole (DAPI), which was used to visualize nuclei. The images were taken under a confocal laser scanning microscope (Carl Zeiss, German).

For Drosophila, tissues were dissected into Grace’s Insect Medium, and then fixed in 4% paraformaldehyde for 10 min. After that, tissues were washed with PBT (1× PBS + 0.5% horse serum + 0.3% Triton X-100), followed by overnight incubation with primary antibodies at room temperature. After primary antibody reaction, tissues were washed with PBT, and then incubated at room temperature overnight in secondary antibodies. Nuclei were labeled by Hoechst 33342. All samples were imaged using a Leica SP5II confocal microscope.

4.7. Western blotting

Cell lysates were prepared with NP-40 lysis buffer (150 mmol/L NaCl, 1% NP-40, 50 mmol/L Tris [pH 8.0]), and equal amounts of lysates were electrophoresed on a 10% SDS-PAGE gel. PVDF membranes (Roche) were used for protein transfer. The membranes were then blocked with 5% nonfat milk in TBST (150 mmol/L NaCl, 50 mmol/L Tris-HCl [pH 7.4], and 0.1% Tween 20) for 1 h, followed by incubation with appropriate primary antibodies at 4 °C overnight. After primary antibody reaction, the membranes were washed with TBST three times and then incubated with HRP-labeled secondary antibody at room temperature for 1 h. After washed again with TBST for three times, the signals of secondary antibodies were detected by an enhanced chemiluminescence system.

4.8. RNA extraction and quantitative real-time PCR

Total RNAs were extracted by Trizol (Invitrogen). The first-strand cDNA synthesis was conducted with RevertAid First-Strand cDNA synthesis kits (Fermentas, USA). qRT-PCR reactions were performed using SYBR Green dye and the Applied Biosystems 7500 Fast Real-Time PCR System. Primers used for c-Myc (forward primer, 5′-GGCTCCTGGCAAAAGGTA-3′; reverse primer, 5′-CTGCG TAGTTGCTGTATG-3′), Chl (forward primer, 5′-TGTTGGGTT GTGTCAGAAC-3′; reverse primer, 5′-GTTAGGTTGCTGTAG CAGGC-3′) and β-actin (forward primer, 5′-CATGTACCTGCTATC CAGGC-3′; reverse primer, 5′-CTCTTAAATGCAAGCACT-3′). The resulting values were normalized to β-actin expression.

4.9. Co-immunoprecipitation assay

For Co-IP assay, SW480 cells stably expressing HA-CA-S6K1 were cultured in 10 cm dishes for 48 h, and then cell lysates were prepared with Co-IP lysis buffer (Hepes–NaOH 50 mmol/L [pH7.5], NaCl 100 mM, EDTA 2.5 mM, NP-40 0.5%, DTT 1 mM/L and proteasome inhibitors). Cell lysates were incubated with the appropriate antibody for 1 h, and subsequently incubated with protein A-Sepharose beads overnight at 4 °C. The protein–antibody complexes recovered on beads were subjected to Western blotting using appropriate antibodies after separation by SDS-PAGE.

4.10. Statistical analysis

Two-tailed unpaired Student’s t-test was used for comparisons between two groups and ordinary one-way ANOVA with Tukey’s multiple comparison post-test was used to compare variables among three or more groups. The quantification of the percentage of cells containing cytoophidia was from at least three independent experiments, and more than 200 cells were counted for each quantification. P < 0.05 was considered statistically significant. All analyses in human cells were performed using GraphPad Prism version 6.00 (GraphPad Software, San Diego, CA, USA, www.graphpad.com). For Drosophila data, image processing and analysis was conducted using Leica Application Suite Advanced Fluorescence Lite and ImageJ. Each group over 60 Drosophila follicle cells were quantified. Nuclear sizes or the length of cytoophidia are expressed as a ratio of the average nuclear size or cytoophidium length in GFP marked clones to neighbouring cells (GFP negative).

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Supplementary data

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Z. Sun, J.-L. Liu / Journal of Genetics and Genomics 46 (2019) 65–74