Sec6 modulates PP2A phosphatase activity through alteration of the binding affinity of PP2A in A and C subunits

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Abstract

Dephosphorylation of the activated the regulator of a-fetoprotein (Raf)-MAP/ERK kinase (MEK)-the extracellular signal-regulated protein kinase (ERK)-p90 ribosomal protein 6 kinase (RSK) cascade plays an essential role in regulating the magnitude and duration of kinase activation and the nature of the physiological response. The protein phosphatase 2A (PP2A), which is one of the major serine/threonine phosphatases, plays a pivotal role in various signaling pathways including cell cycle, metabolism, migration, and cell death. The impairment of PP2A activity is associated with various diseases including neurodegenerative disorders, autoimmune diseases, type II diabetes, and tumors. However, little is known about how cells control the assembly of PP2A subunits and stabilize PP2A phosphatase activity. In the present study, we demonstrate that Sec6 regulates PP2A phosphatase activity by modulating the binding affinity of the A and C subunits of PP2A, thereby modulating the phosphorylation of p90RSK at Thr359 and Ser380, glycogen synthase kinase 3b (GSK3b) at Ser9, and the expression of zinc finger E-box binding homeobox 1 (ZEB1), vimentin, and zonula occludens 3 (ZO-3).

Background

Protein phosphorylation, which is a major regulatory mechanism of enzyme activity and stability, is the most widespread class of post-translational modification and involves the addition of a phosphate group onto serine, threonine, or tyrosine [1]. The extracellular signal-regulated protein kinase 1 and 2 (ERK1/2) are members of the mitogen-activated protein kinase (MAPK) intracellular signaling pathway and are ubiquitously expressed as hydrophilic serine/threonine kinases that participate in the regulation of a-fetoprotein (Raf)-MAP/ERK kinase (MEK)-ERK-p90 ribosomal protein 6 kinase (RSK) signal transduction cascade [2]. Although the Raf-MEK-ERK-p90RSK cascade is associated with the regulation of various intracellular events including adhesion, cell cycle, migration, metabolism, proliferation, and transcription, the aberration of Raf-MEK-ERK-p90RSK signaling occurs in approximately one-third of all human tumors [2]. The dephosphorylation of activated Raf-MEK-ERK-p90RSK plays an essential role in regulating the magnitude and duration of kinase activation and the nature of the physiological response [3]. Because many proteins in the Raf-MEK-ERK-p90RSK cascade contain phosphoserine and phosphothreonine, protein phosphatase 2A (PP2A), which is a major serine/threonine phosphatase, has the potential to regulate several steps in the overall pathway.

PP2A is responsible for up to 90% of the serine/threonine phosphatase activity together with protein phosphatase 1 (PP1) in various cell types [4–7]. Because PP2A plays a pivotal role in many signaling pathways, such as cell cycle [8], metabolism [9], migration [10], and cell death [11], the impairment of PP2A is associated with various diseases including neurodegenerative disorders [12], autoimmune diseases [13], type II diabetes [14], and tumors [15]. PP2A consists of a structural subunit A, a regulatory/variable subunit B, and a catalytic subunit C. The C subunit of PP2A binds directly to the A subunit, which acts as scaffolding to form the so-called PP2A dimeric core [16]. The PP2A dimeric core serves as a platform for the association of the regulatory B subunit to generate a trimeric complex, which is important for substrate recruitment and subcellular targeting. Although the specificity of PP2A

phosphatase activity requires a trimeric complex, the manner in which cells control the assembly of the PP2A subunits and the stability of PP2A phosphatase activity remains largely unknown.

Sec6 is one of the subunits of the exocyst, an evolutionarily conserved eight-protein complex consisting of Sec3 (EXOC1), Sec5 (EXOC2), Sec6 (EXOC3), Sec8 (EXOC4), Sec10 (EXOC5), Sec15 (EXOC6), Exo70 (EXOC7), and Exo84 (EXOC8) subunits [17]. Recently, several studies have demonstrated that Sec6 is associated with various cellular mechanisms including neurotransmission [18, 19], neurite growth [20], cell adhesion [21], Ca^{2+} signaling [22], cell polarity [23], insulin secretion [24], glucose transporter type 4 (Glut4) trafficking [25], cell migration [26], cytokinesis [27], ciliogenesis [28], amylase release [29], cell cycle [30], nuclear factor- κ B (NF- κ B) signaling [31], and apoptosis [32]. These functions may be dependent or independent on Sec6 during exocytosis.

However, the relationship between Sec6 and PP2A in the Raf-MEK-ERK-p90RSK signaling cascade is unclear. In this study, we demonstrate that Sec6 regulates PP2A phosphatase activity by modulating the binding affinity of the A and C subunits, thereby modulating the phosphorylation of p90RSK at Thr359 and Ser380, glycogen synthase kinase 3 β (GSK3b) at Ser9, and attenuating the expression of zinc finger E-box binding homeobox 1 (ZEB1), vimentin, and zonula occludens 3 (ZO-3).

Materials And Methods

Cell culture

The A549 human lung adenocarcinoma epithelial cell line and the Saos2 human osteosarcoma cell line cell were maintained in Dulbecco's modified Eagle's medium (Wako, Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum and 100 U/ml penicillin and streptomycin. Cells were routinely cultured at 37°C in a humidified atmosphere of 5% CO₂.

RNA interference

Two sets of small interfering RNA (siRNA) duplexes for human Sec6 were prepared as previously described [31]. AllStars Negative Control siRNA (Qiagen, Hilden, Germany) was used as the control. Cells were transfected with RNAi duplexes using Lipofectamine RNAiMAX (Invitrogen). Experiments were performed 48 h after transfection.

Plasmid construction

The full-length human Sec6 cDNA from HSC3 cells was cloned into the *HindIII* and *KpnI* sites of the pEGFP-C3 vector (Clontech, Mountain View, CA). GFP-Sec6 was mutated using the Quick Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) to be resistant to siSec6-2 (GFP-Sec6 pm #2) or siSec6-3 (GFP-Sec6 pm #3). The following primers were used: 5'-CACCATCTTGGAGAGGACTGTCACGACCAGAATTGAGGGCAC-3' and 5'-GTGCCCTCAATTCTGGTCGTGACAGTCTCTCCAAGATGGTG-3' for point mutation of GFP-Sec6#2 or 5'-

CGTACATGTCCACGCTCACTTCTAACATCATCGCGTGGCTGCGGAAAGCG-3' and 5'-CGCTTTCCGCAGCCACGCGATGATGTTAGAAGTGAGCGTGGACATGTACG-3' for point mutation of GFP-Sec6#3. Constructs were confirmed by performing restriction enzyme analysis and sequencing.

Immunoblot analysis

Transfected cells were lysed in a lysis buffer (20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM Na₃VO₄, 50 mM NaF, 1% Triton X-100, and protease inhibitor cocktail). Protein concentration was determined using the BCA Protein Assay Reagent (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. The samples were boiled for 10 min in an SDS sample buffer (New England Biolabs, Inc., Beverly, MA, USA). Equal amounts of protein lysate were separated using SDS-PAGE and electrophoretically transferred onto PVDF membranes (Millipore, Billerica, MA, USA). The PVDF membranes were then blocked with 5% skim milk in PBS containing Tween 20. The PVDF membranes were immunoblotted using primary antibodies against Sec6 (1:1,000; ab156568; abcam), ERK1/2 (1:1,000; 4695; Cell Signaling Technology), phospho-ERK1/2 (Thr202/Tyr204) (1:1,000; 4370; Cell Signaling Technology), protein kinase d (PKCd) (1:1,000; 9616; Cell Signaling Technology), phospho-PKCd (Thr505) (1:1,000; 9255; Cell Signaling Technology), phospho-PKCd (Thr634) (1:1,000; 9376; Cell Signaling Technology), b-actin (1:1,000; 3700; Cell Signaling Technology), phospho-MEK1/2 (Ser217/221) (1:1,000; 9154; Cell Signaling Technology), MEK1 (1:1,000; 9146; Cell Signaling Technology), MEK2 (1:1,000; 9147; Cell Signaling Technology), PP2A in the A subunit (1:1,000; 2041; Cell Signaling Technology), PP2A in the B subunit (1:1,000; 2290; Cell Signaling Technology), PP2A in the C subunit (1:1,000; 2259; Cell Signaling Technology), dual specificity phosphatase 6 (DUSP6) (1:1,000; 3058; Cell Signaling Technology), ubiquitin (Ub) (1:1,000; 3936; Cell Signaling Technology), cullin3 (Cul3) (1:1,000; 2759; Cell Signaling Technology), ubiquitin protein ligase E3 component N-recognin 5 (UBR5) (1:1,000; 65344; Cell Signaling Technology), target of rapamycin signaling pathway regulator-like 1 (TIPRL1) (1:1,000; ab70795; abcam), GFP (1:1,000; 2956; Cell Signaling Technology), p90RSK (1:1,000; 9355; Cell Signaling Technology), phospho-p90RSK (Thr359) (1:1,000; 8735; Cell Signaling Technology), phospho-p90RSK (Ser380) (1:1,000; 11989; Cell Signaling Technology), phospho-p90RSK (Thr573) (1:1,000; 9346; Cell Signaling Technology), GSK3b (1:1,000; 9315; Cell Signaling Technology), phospho-GSK3b (Ser9) (1:1,000; 9336; Cell Signaling Technology), liver kinase B1 (LKB1) (1:1,000; 3050; Cell Signaling Technology), phospho-LKB1 (Ser428) (1:1,000; 3482; Cell Signaling Technology), vimentin (1:1,000; 5741; Cell Signaling Technology), ZEB1 (1:1,000; 3396; Cell Signaling Technology), Snail (1:1,000; 3879; Cell Signaling Technology), ZO-1 (1:1,000; 13663; Cell Signaling Technology), ZO-2 (1:1,000; 2847; Cell Signaling Technology), ZO-3 (1:1,000; 3704; Cell Signaling Technology). Immunoreactive complexes were visualized using the chemiluminescent HRP Substrate ImmobilonTM Western (Millipore).

Immunofluorescence microscopy

Cells were cultured on a micro-cover glass and rinsed with PBS. They were then fixed with 4% paraformaldehyde, washed with PBS three times, and permeabilized using 0.1% Triton/PBS. The cells were incubated in 10% normal donkey serum for blocking and incubated with the anti-PP2A in the C

subunit (1:100; 2038; Cell Signaling Technology) or anti-PP2A in the A subunit (1:100; 2041; Cell Signaling Technology) overnight at room temperature in a humidified chamber. For immunofluorescence analysis, the cells were washed with PBS three times and incubated with secondary antibodies for 1 h. 4',6-diamidino-2-phenylindole (DAPI) was used as a nuclear stain. Images were obtained using a confocal laser-scanning microscope (LSM-700, Carl Zeiss, Jena, Germany).

Immunoprecipitation analysis

Cells were lysed in a lysis buffer. Cell lysates (20,000 mg) were pre-cleaned with protein A sepharose beads (GE health care) for 2 h at 4 °C and incubated with 4 mg PP2A in the C subunit (1:1,000; 2259; Cell Signaling Technology) or alpha4 (1:1,000; 5699; Cell Signaling Technology) antibody overnight at 4 °C and reacted to protein A sepharose beads for 5 h. After being washed four times using the lysis buffer, the immunoprecipitated complex was boiled for 10 min in the SDS sample buffer (New England Biolabs). It was then separated using SDS-PAGE and transferred onto a PVDF membrane (Millipore) and subjected to an immunoblot analysis.

Cell migration assay

A cell migration assay was performed as previously described [33]. After 48 h of transfection with siRNAs, cell migration was assessed using Thincert™ cell culture chambers (Greiner Bio-one, Frickenhausen, Germany). The underside of a polycarbonate membrane insert (8 µm pores) was coated with 20 µg/ml of fibronectin at 4°C overnight. A total of 20,000 cells in serum-free media were added to the insert of each well, and the cells were incubated for 12 h. The cells were fixed with methanol for 10 min and stained with Giemsa (Muto Pure Chemicals, Tokyo, Japan). The number of cells that had migrated to the lower surface of the filters was determined microscopically. The experiments were performed in triplicate and repeated at least three times.

Quantitative real-time reverse transcription-PCR

Quantitative real-time reverse transcription PCR was performed as previously described [32]. The primers used for RT-PCR were: 5'-GCCAACCGCGAGAAGATGA-3' and 5'-CATCACGATGCCAGTGGTA-3' for b-actin [34], 5'-TCGTTGTGGTAACCAAGCTG-3' and 5'-AACATGTGGCTCGCCTCTAC-3' for the PP2A in the C subunit [35].

PP2A immunoprecipitation phosphatase assay

The PP2A phosphatase assay was performed using a PP2A immunoprecipitation phosphatase assay kit (Millipore) according to the manufacturer's instructions.

Statistical analysis

Data are expressed as means ± standard deviations from three or more independent experiments. Statistical analyzes were performed using Student's *t* test at a significance level of $P < 0.001$ (***), $P <$

0.01 (**), $P < 0.05$ (*), or n.s. (not statistically significant).

Results

We previously demonstrated that Sec8 knockdown significantly decreased phosphorylation of ERK [33] and that knockdown of Sec8 decreased the levels of Sec6 protein [21]. Thus, we first investigated whether the phosphorylation status of ERK1/2 changed in response to Sec6 knockdown. Depletion of Sec6 by siRNA resulted in reduced phosphorylation of ERK1/2 compared with A549 or Saos2 cells transfected with a negative control siRNA [upper band: ERK1 (p44), Figs. 1a, b, d and e; lower band: ERK2 (p42), Figs. 1a, c, d, and f)]. However, total expression of ERK did not change in response to Sec6 knockdown in A549 (Fig. 1a and Suppl Figs. 1a, b) or Saos2 (Fig. 1d, Suppl Figs. 2a, b) cells. Because activation of PKC δ promotes the activation of ERK [36], we determined whether the phosphorylation status or total expression of PKC δ was altered in response to Sec6 knockdown. Immunoblot analysis revealed that the phosphorylation status of PKC δ and the total expression of PKC δ did not change in response to Sec6 knockdown in A549 (Fig. 1a, Suppl Figs. 1c-e) or Saos2 (Fig. 1d, Suppl Figs. 2c-e) cells. These results indicated that Sec6 is associated with ERK phosphorylation.

Because Sec6 knockdown suppressed the phosphorylation of ERK1/2 (Fig. 1), and the phosphorylation of MEK1/2 promotes the phosphorylation of tyrosine and threonine in ERK1 (p44) and ERK2 (p42) [37], we next examined whether Sec6 knockdown may influence the phosphorylation status of MEK1/2. Figure 2 illustrates that the phosphorylation of MEK1/2 decreased in A549 and Saos2 cells transfected with Sec6 siRNAs compared with cells transfected with the negative control siRNA (Figs. 2a-f). However, total expression of MEK1/2 did not change in response to Sec6 knockdown (Suppl Figs. 3a, b, and Suppl Figs. 4a, b).

PP2A, which is the major serine/threonine phosphatase, consists of the structural subunit (A), regulatory subunit (B), and catalytic subunit (C) [4] and is involved in ERK inactivation [38, 39]. Therefore, we investigated whether the expression of PP2A in each subunit changed in response to Sec6 knockdown. Immunoblot analysis revealed that Sec6 knockdown did not alter the expression of the A and B subunits (Figs. 3a, c, Suppl Figs. 3c, d, and Suppl Figs. 4c, d). However, the expression of the C subunit increased in A549 or Saos2 cells transfected with Sec6 siRNAs compared with cells transfected with the negative control siRNA (Figs. 3a-d). Moreover, we performed an immunofluorescence analysis to confirm the increase in PP2A C subunit expression in cells transfected with Sec6 siRNAs. We found that the PP2A C subunit was markedly increased in cells transfected with Sec6 siRNAs compared to that in cells transfected with the negative control siRNAs (Fig. 3e). By contrast, the expression of the PP2A C subunit did not change in A549 or Saos2 cells transfected with Sec6 siRNAs at the mRNA level (Suppl Fig. 5). Furthermore, the immunofluorescence analysis revealed that Sec6 knockdown did not change the expression of the PP2A A subunit (Suppl Fig. 6). Previous studies demonstrated that DUSP6 is a cytoplasmic MAP kinase phosphatase (MKP) that can inactivate ERKs [40]. We next determined whether the expression of DUSP6 changed in response to Sec6 knockdown. The immunoblot analysis indicated that Sec6 knockdown did not alter the expression of DUSP6 compared with A549 or Saos2 cells

transfected with the negative control siRNA (Figs. 3a, c, Suppl Fig. 3e and Suppl. Figure 4e). To determine whether the quantity of Sec6 affects the PP2A C subunit, we examined the expression of the PP2A C subunit, which changed concomitantly with the quantity of Sec6. As illustrated in Fig. 3f, although the PP2A C subunit was unchanged in cells transfected with a GFP vector, the C subunit of PP2A gradually decreased and was dependent on the quantity of GFP-Sec6 (Fig. 3f). These results indicated that Sec6 is associated with the phosphorylation of ERK and MEK1/2 by controlling PP2A C subunit expression.

The PP2A C subunit needs to be stabilized as a free C subunit, but it may be unstable and quickly degraded [41, 42]. Therefore, we examined whether the association of PP2A subunits C and A changes in response to Sec6 knockdown. Interestingly, immunoprecipitation analysis revealed that Sec6 knockdown increased the binding affinity of the C and A subunits (Fig. 4a). Furthermore, although there was an accumulation of a poly-ubiquitinated C subunit in cells transfected with the negative control siRNA, the poly-ubiquitinated C subunit was suppressed in response to Sec6 knockdown (Fig. 4a). Previous studies revealed that Cul3 [43] and UBR5 [44] were identified as E3 ligases that interact with the C subunit. As illustrated in Fig. 4a, the binding affinity of the C subunit and Cul3 decreased in cells transfected with Sec6 siRNAs. However, the binding affinity the C subunit and UBR5 did not change in cells transfected with Sec6 siRNAs compared to that in cells transfected with negative control siRNAs (Fig. 4a). Moreover, overexpression of Sec6 decreased the binding affinity of the PP2A A and C subunits. Furthermore, the binding affinity of the PP2A C subunit and Cul3 was strongly dependent on the quantity of Sec6 (Fig. 4b). Previous studies revealed that alpha4 functions as a potential modulator of the PP2A C subunit stability, folding, and translation [45, 46] and that TIPRL1 is part of the trimolecular alpha4-PP2A C subunit complex and controls the assembly of active alpha4-PP2A complexes [47]. Therefore, we determined whether the association of alpha4, PP2A C subunit, and TIPRL1 changed in response to Sec6 knockdown. The immunoprecipitation analysis revealed that Sec6 knockdown did not affect the binding affinity of the three proteins (Suppl Fig. 7). These data indicate that Sec6 promotes poly-ubiquitination and degradation of the PP2A C subunit via Cul3 by inhibiting the binding of the C and A subunits. Next, we investigated whether the phosphatase activity of PP2A changed in response to Sec6 knockdown. As illustrated in Figs. 4c, d, Sec6 knockdown increased the phosphatase activity of PP2A. Furthermore, we investigated when restoration was made using the GFP-Sec6 point mutant (pm) #2 and #3, which mutant was resistant to siSec6-2 or siSec6-3, altered the phosphatase activity of PP2A. Figure 4e demonstrated that the phosphatase activity of PP2A decreased by adding back the GFP-Sec6 point mutants (Fig. 4e).

Sec6 knockdown decreased the phosphorylation of ERK (Fig. 1), which phosphorylates p90RSK [48]. Next, we determined whether the phosphorylation status of p90RSK changed in response to Sec6 knockdown. As illustrated in Fig. 5, depletion of Sec6 by siRNA resulted in reduced phosphorylation of p90RSK at Thr359 (Figs. 5a, b, d, and e) and Ser380 (Fig. 5a, c, d, and f), compared to that in A549 or Saos2 cells transfected with the negative control siRNA. However, total expression of p90RSK and phosphorylation of p90RSK at Thr573 did not change in response to Sec6 knockdown in A549 cells (Fig. 5a, Suppl Figs. 8a, b) or Saos2 cells (Fig. 5d, Suppl Figs. 8c, d).

Previous studies revealed that p90RSK regulates protein synthesis by phosphorylating GSK3b [49] and LKB1 [50]. Thus, we investigated whether the phosphorylation status of GSK3b and LKB1 changed in response to Sec6 knockdown. The immunoblot analysis revealed that although Sec6 knockdown did not change the expression of total GSK3b and LKB1 (Figs. 6a, c, Suppl Figs. 9a–d), Sec6 knockdown decreased the phosphorylation of GSK3b at Ser9 compared with that in cells transfected with the negative control siRNA (Figs. 6a–d). By contrast, the phosphorylation status of LKB1 at Ser428 did not change in response to Sec6 knockdown (Figs. 6a, c, Suppl Figs. 9e, f).

Because our previous studies demonstrated that Sec6 knockdown suppressed cell migration [21, 51] and the suppression of phosphorylated GSK3b at Ser9 inhibited epithelial mesenchymal transition (EMT) but promoted mesenchymal epithelial transition (MET) [52, 53], we performed an immunoblot analysis to determine whether EMT (ZEB1, vimentin, and Snail) or MET (ZO-1, ZO-2, and ZO-3) markers are altered following transfection with Sec6 siRNA compared with the negative control siRNA. As illustrated in Fig. 7, Sec6 knockdown suppressed the expression of ZEB1 and vimentin compared with that in cells transfected with the negative control siRNA (Figs. 7a–f). However, we confirmed that Sec6 knockdown did not affect the expression of Snail protein levels compared with that in cells transfected with the negative control siRNA (Figs. 7a, d, Suppl Fig. 10). By contrast, Sec6 knockdown increased the expression of ZO-3 compared with that in cells transfected with the negative control siRNA (Figs. 8a–d); however, Sec6 knockdown did not change the expression of ZO-1 and ZO-2 compared with that in cells transfected with the negative control siRNA (Figs. 8a, c, Suppl Fig. 11). Taken together, these data indicate that Sec6 modulates the expression of ZEB1, vimentin, and ZO-3 via the GSK3b-p90RSK-MEK1/2-ERK axis by regulating PP2A phosphatase activity.

Because downregulation of ZEB1 and vimentin decrease cell migration [54], we performed cell migration assays to determine whether cell migration was decreased under Sec6 knockdown conditions. Predictably, cell migration was decreased in cells transfected with Sec6 siRNAs (Fig. 9). Next, we investigated whether adding back the GFP-Sec6 point mutant (pm) #2 or #3, which is resistant to siSec6-2 or siSec6-3, respectively, restored cell migration. As illustrated in Fig. 9, cell migration was restored by adding back the GFP-Sec6 point mutant both in A549 cells (Figs. 9a, c) and Saos2 cells (Figs. 9b, d).

Discussion

In the present study, we observed that Sec6 regulated the phosphorylation of ERK1/2 and MEK1/2 and the phosphatase activity of PP2A through degradation of the PP2A C subunit via Cul3 by inhibiting the binding of the PP2A C and A subunits. Moreover, Sec6 knockdown suppressed the expression of ZEB1 and vimentin but up-regulated the expression of ZO-3 through the phosphorylation of p90RSK at Thr359 and Ser380 and GSK3b at Ser9. The schema for the present study is presented in Fig. 10.

PP2A has an essential role in maintaining homeostasis in cells by acting as a phosphatase that regulates most protein kinase-related intracellular signaling. Because functional inactivation and alteration of the assembly of the PP2A holoenzyme are required for cancer development, PP2A is

considered to be a tumor suppressor [55]. The PP2A A subunit, which exhibits a scaffolding function with the PP2A B and C subunits, is mutated in high-grade endometrial tumors [56], type I ovarian tumors [57], type I uterine carcinomas [57], primary gliomas [57], lung cancer, breast cancer, and melanoma [4]. Mutation of the PP2A A subunit results in decreased binding to the PP2A B and C subunits [58, 59]. We found that Sec6 promoted the poly-ubiquitination and degradation of the C subunit via Cul3 by inhibiting the binding of the C and A subunits, thereby modulating the phosphatase activity of PP2A (Fig. 4). These results indicate that Sec6 may be involved in the development of PP2A-related tumors.

Previous studies demonstrated that alpha4 serves as a binding partner with the PP2A C subunit that not only renders it catalytically inactive but also contributes to the ability of the C subunit to be assembled into functional PP2A phosphatase complexes [60]. However, our results indicated that Sec6 knockdown did not affect the binding affinity of alpha4 and the PP2A C subunit (Suppl Fig. 7). How does Sec6 affect the binding affinity of the PP2A C and A subunits? One possibility is that Sec6 directly inhibits the interaction between the A and C subunits. Our previous studies revealed that Sec6 binds to Jab1, Siah1, Hsp27, and p38MAPK [30, 51]. It is possible that Sec6 competitively and directly binds to the A or C subunit and disrupts the interaction between these subunits. Another possibility is that Sec6 is one of the components in the multimolecular signaling complexes of the PP2A-ERK-MEK-p90RSK axis. The multimolecular signaling complexes, which are also called “signalosomes,” are an assembly of multiple intracellular signaling proteins [61]. Because the signalosomes have features in which the domains responsible for assembly are parts of enzymes or binding sites of enzymes, the local concentration of the bound enzyme domains increases at the spatial clustering in the signalosomes [61]. Moreover, the signalosomes have distinctive functions in signal amplification, decline of biological noise, and threshold response, thereby conferring spatial and temporal control of intracellular signaling [61]. In the signalosomes, the active intracellular signaling may be terminated by feedback activation of phosphatases and deubiquitinases, thereby promoting disassembly of multiple intracellular signaling proteins. The signalosomes appear to exist everywhere in intracellular signaling pathways. For example, p90RSK3-PP2A-muscle A-kinase anchoring protein b (mAKAPb)-serum response factor (SRF) signalosomes regulate SRF phosphorylation and control a transcriptional program responsible for modulating changes in cardiac myocyte morphology that occur secondary to pathological stressors [62]. In p90RSK3-PP2A-mAKAPb-SRF signalosomes, the phosphorylation of SRF at Ser103 is differentially controlled by binding both kinases and phosphatases. Moreover, the anchoring disruptors of RSK3 and PP2A may inhibit each binding partner sharing the mAKAPb-binding interface, potentially modulating the activity of the enzyme, kinase, and phosphatase [62].

Because our results indicated that Sec6 regulates the phosphorylation of ERK1/2 and MEK1/2 and the phosphatase activity of PP2A through the degradation of the PP2A C subunit via Cul3 and by inhibiting the binding of the C and A subunits, Sec6 functions as an anchoring disruptor or modulator in PP2A-ERK-MEK-p90RSK signalosomes. Moreover, depletion of Sec6 suppresses the phosphorylation of ERK1/2, MEK1/2, p90RSK at Thr359 and Ser380 and GSK3b but not PKCd, p90RSK at Thr573 or LKB1 (Figs. 1, 2, 5, and 6). These precise selectivities for the phosphorylation of proteins may be related to the function of the anchoring disruptor or modulator in Sec6-PP2A-ERK-MEK-p90RSK signalosomes, which result from

the regulation of EMT or MET protein expression. Therefore, understanding the anchoring disruptor or modulator of Sec6 in Sec6-PP2A-ERK-MEK-p90RSK signalosomes may provide a new therapeutic strategy for treating PP2A-related tumors. However, further studies are needed to determine the precise role of Sec6 in Sec6-PP2A-ERK-MEK-p90RSK signalosomes.

Conclusions

The results of the present study suggest that Sec6 regulated the phosphorylation of ERK1/2 and MEK1/2 and the phosphatase activities of PP2A through the degradation of the PP2A C subunit via Cul3 and by inhibiting the binding of the C and A subunits, which resulted in the modulation of ZEB1, vimentin, and ZO-3 expression through the phosphorylation of p90RSK at Thr359 and Ser380 and GSK3b at Ser9.

Declarations

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Conflict of interests

All authors declare that they have no financial interests.

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Availability of data and material

The datasets are available from the corresponding author upon reasonable request.

Author contributions

T.T. performed all experiments. T.T. and M.I. designed the experiments and wrote the manuscript. All authors checked the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Abbreviations

ERK1/2

extracellular signal-regulated protein kinase1/2; Raf:regulator of a-fetoprotein; MEK:MAP/ERK kinase; RSK:ribosomal protein 6 kinase; PP2A:Protein phosphatase 2A; PP1:protein phosphatase 1; Glut4:glucose transporter type 4; NF- κ B:Nuclear factor- κ B; GSK3 β :glycogen synthase kinase 3 β ; ZEB1:zinc finger E-box binding homeobox 1; ZO:zonula occludens; siRNA:small interfering RNA; PKC δ :protein kinase d; DUSP6:dual Specificity Phosphatase 6; Ub:ubiquitin; Cul3:cullin3; UBR5:ubiquitin protein ligase E3 component N-recogin 5; TIPRL1:target of rapamycin signaling pathway regulator-like 1; LKB1:liver kinase B1; DAPI:4',6-diamidino-2-phenylindole; MKP:MAP kinase phosphatase; EMT:epithelial mesenchymal transition; MET:mesenchymal epithelial transition; mAKAPb:muscle A-kinase anchoring protein b; SRF:serum response factor

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Figures

Figure 1

Sec6 knockdown results in reduced ERK phosphorylation but not PKC δ phosphorylation. A549 (a–c) or Saos2 cells (d–f) were transfected with the negative control or Sec6 siRNAs. After 48 h, whole cell lysates were analyzed by immunoblotting using the indicated antibodies. β -actin was used as a loading control. Histograms showing the relative expression of phospho-p44/total p44 in A549 (b) or Saos2 cells (e) and phospho-p42/total p42 in A549 (c) or Saos2 cells (f). Results represent the means $\pm$ standard deviations

of three separate experiments. Asterisks indicate significant differences compared with cells transfected with the negative control siRNA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Student's *t* test).

Figure 2

Depletion of Sec6 suppresses the phosphorylation of MEK1/2. A549 (a–c) or Saos2 (d–f) cells were transfected with negative control or Sec6 siRNAs. After 48 h, whole cell lysates were analyzed by immunoblotting using the indicated antibodies. β-actin was used as a loading control. Histograms

showing the relative expression of phospho-MEK1/2/total MEK1 in A549 (b) or Saos2 (e) cells and phospho-MEK1/2/total MEK2 in A549 (c) or Saos2 (f) cells. Results represent the means $\pm$ standard deviations of three separate experiments. Asterisks indicate significant differences compared with cells transfected with negative control siRNA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Student's t test).

Figure 3

Sec6 knockdown results in reduced PP2A C subunit but not DUSP6. A549 (a, b) or Saos2 (c, d) cells were transfected with negative control or Sec6 siRNAs. After 48 h, whole cell lysates were analyzed by immunoblotting using the indicated antibodies. β -actin was used as a loading control. (b, d) Histograms of the relative expression of PP2A C/ β -actin in A549 (b) or Saos2 (d) cells. Results represent the means $\pm$ standard deviations of three separate experiments. Asterisks indicate significant differences compared with cells transfected with negative control siRNA. n.s., not significant, * $P < 0.05$, ** $P < 0.01$ (Student's t test). (e) Saos2 cells were transfected with negative control or Sec6 siRNAs. After 48 h, cells were fixed with 4% paraformaldehyde and stained with the anti-PP2A C subunit antibody (red). DAPI was used for nuclear staining (blue). Scale bar = 20 μ m. (f) A549 cells were transfected with GFP vector or GFP-Sec6 gradually. After 48 h, whole cell lysates were analyzed by immunoblotting using the indicated antibodies. β -actin was used as a loading control.

Figure 4

Sec6 affects PP2A phosphatase activity by modulating the binding affinity between the PP2A A subunit, PP2A C subunit, and Cul3. (a) A549 cells were transfected with negative control or Sec6 siRNAs. After 48 h, whole cell lysates were immunoprecipitated with an anti-PP2A C subunit antibody. Immunoprecipitants were subjected to immunoblotting using the indicated antibodies. IP, immunoprecipitation; IB, immunoblotting. (b) A549 cells were transfected with GFP or GFP-Sec6 vectors. After 24 h, total A549 cell lysates were immunoprecipitated with anti-PP2A C subunit antibody. Cell lysates and immunoprecipitants were subjected to immunoblotting using the indicated antibodies. IP, immunoprecipitation; IB, immunoblotting. (c, d) A549 (c) or Saos2 (d) cells were transfected with negative control or Sec6 siRNAs. After 48 h, whole cell lysates were immunoprecipitated with the anti-PP2A C subunit antibody. Immunoprecipitants were analyzed by a PP2A immunoprecipitation phosphatase assay. Results represent the means $\pm$ standard deviations of three separate experiments. Asterisks indicate significant differences compared with cells transfected with negative control siRNA. ** $P < 0.01$, *** $P < 0.001$ (Student's *t* test). (e) A549 cells were transfected with Sec6-2 siRNA and GFP or Sec6-3 siRNA and GFP or Sec6-2 siRNA and GFP-Sec6 pm #2 or Sec6-3 siRNA and GFP-Sec6 pm #3. After 48 h; whole cell lysates were immunoprecipitated with the anti-PP2A C subunit antibody. Immunoprecipitants were analyzed using a PP2A immunoprecipitation phosphatase assay. Results are presented as the mean $\pm$ SD of three independent experiments. Asterisks indicate the significant variations observed by comparison between cells transfected with GFP-Sec6 pm #2 or GFP-Sec6 pm #3. *** $P < 0.001$ (Student's *t* test).

Figure 5

Depletion of Sec6 suppresses the phosphorylation of p90RSK. A549 (a–c) or Saos2 (d–f) cells were transfected with negative control or Sec6 siRNAs. After 48 h, whole cell lysates were analyzed by immunoblotting using the indicated antibodies. β -actin was used as a loading control. Histograms of the relative expression of phospho-p90RSK Thr359/total p90RSK in A549 (b) or Saos2 (e) cells and phospho-p90RSK Ser380/total p90RSK in A549 (c) or Saos2 (f) cells. Results are means $\pm$ standard deviations of

three separate experiments. Asterisks indicate significant differences compared with cells transfected with the negative control siRNA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Student's t test).

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Figure 6

Sec6 knockdown suppresses the phosphorylation of GSK3 β at Ser9. A549 (a, b) or Saos2 (c, d) cells were transfected with negative control or Sec6 siRNAs. After 48 h, whole cell lysates were analyzed by immunoblotting using the indicated antibodies. β -actin was used as a loading control. Histograms

showing the relative expression of phospho-GSK3 β Ser9/total GSK3 β in A549 (b) or Saos2 (d) cells. Results are means $\pm$ standard deviations of three separate experiments. Asterisks indicate significant differences compared with cells transfected with the negative control siRNA. * $P < 0.05$, ** $P < 0.01$ (Student's t test).

Figure 7

Sec6 knockdown results in reduced ZEB1 and vimentin expression. A549 (a–c) or Saos2 (d–f) cells were transfected with negative control or Sec6 siRNA. After 48 h, whole cell lysates were analyzed by immunoblotting using the EMT markers. β -actin was used as a loading control. Histograms showing the relative expression of vimentin/ β -actin in A549 (b) or Saos2 (e) cells and ZEB1/ β -actin in A549 (c) or Saos2 (f) cells. Results are means $\pm$ standard deviations of three separate experiments. Asterisks indicate significant differences compared with cells transfected with the negative control siRNA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Student's t test).

Fig. 8A-D Tanaka et al.

Figure 8

Sec6 knockdown results in increased ZO-3 expression. A549 (a, b) or Saos2 (c, d) cells were transfected with negative control or Sec6 siRNA. After 48 h, whole cell lysates were analyzed by immunoblotting using the MET markers. β -actin was used as a loading control. Histograms showing the relative expression of ZO-3/ β -actin in A549 (b) or Saos2 (d) cells. Results are means $\pm$ standard deviations of three separate experiments. Asterisks indicate significant differences compared with cells transfected with the negative control siRNA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Student's t test).

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Figure 9

Overexpression of Sec6 point mutant reverted cell migration. (a–d) A549 (a) or Saos2 (b) cells were transfected with Sec6-2 siRNA and GFP or Sec6-3 siRNA and GFP or Sec6-2 siRNA and GFP-Sec6 pm#2 or Sec6-3 siRNA and GFP-Sec6 pm#3. After 48 h, the number of cells that migrated to the lower filter surfaces were determined. A549 (c) or Saos2 (d) cells that migrated to the bottom of the dish were stained with Giemsa. Results are presented as the mean $\pm$ SD of three independent experiments. Asterisks indicate the significant variations observed by comparison between cells transfected with GFP-Sec6 pm#2 or GFP-Sec6 pm#3. ** $P < 0.01$, *** $P < 0.001$ (Student's *t* test).

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Figure 10

A schematic model showing the mechanism of PP2A phosphatase activity through the regulation of binding affinity between the PP2A A subunit, C subunit, and Cul3 by Sec6.

Supplementary Files

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