



Title	The mTOR pathway controls cell proliferation by regulating the Fox03a via SGK1 kinase
Author(s)	森, 俊介
Citation	大阪大学, 2014, 博士論文
Version Type	VoR
URL	https://doi.org/10.18910/34064
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**The mTOR pathway controls cell proliferation by regulating
the FoxO3a via SGK1 kinase**

(mTOR シグナル経路は SGK1 を介した FoxO3a の制御により
細胞増殖を調節する)

A Doctoral Thesis
by
Shunsuke Mori

**Department of Oncogene Research,
Research Institute for Microbial Diseases,
Osaka University**

**Graduate School of Science,
Osaka University**

January 2014

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General Introduction

In 1965, Rapamycin was discovered in a soil sample from Easter Island in the South Pacific, a small island famous for its big stone statues called Moai. Rapamycin is a macrocyclic lactone produced by the microorganism *Streptomyces hygroscopicus* and possesses antifungal and antibiotic properties (Vézina et al., 1975). Since its discovery, Rapamycin has been found to have antiproliferative activity. Although the mechanism of its action remained unknown for many years, Rapamycin has been used as a potent immunosuppressive drug in solid organ transplantation for the prevention of acute rejection (Thomson et al., 1989). The antiproliferative effects of Rapamycin and its analogs (Rapalogs) have been evaluated in several types of cancers (Yu et al., 2010). Rapamycin is also widely used to coat stents to reduce post-stenting restenosis phenomenon after coronary angioplasty (Beyar et al., 2001).

Studies on the mechanisms of action of Rapamycin in *S. cerevisiae* led to the discovery of Target Of Rapamycin (TOR) (Kunz et al., 1993) and its mammalian analogs, mTOR (Brown et al., 1994; Sabatini et al., 1994). mTOR is a serine/threonine kinase, which belongs to the phosphatidylinositol-3 kinase (PI3K)-related kinases family. mTOR forms two large protein complexes; mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 consists of regulatory-associated protein of mTOR (Raptor) (Hara et al., 2002), DEP domain-containing mTOR-interacting protein (Deptor) (Peterson et al., 2009), mammalian lethal with SEC13 protein 8 (mLST8) (Kim et al., 2003), and TELO2 interacting protein 1/Telomere maintenance 2 (Tti1/Tel2) (Kaizuka et al., 2010), while mTORC2 forms by the association of Rapamycin-insensitive companion of mTOR (Rictor) (Sarbassov et al., 2004), mammalian stress-activated protein kinase-interaction protein 1 (mSIN1) (Frias et al., 2006), protein observed with Rictor-1/2 (Proter1/2) (Pearce et al., 2007), Deptor, mLST8, and Tti1/Tel2. These components of the protein complexes are known to differentially regulate the kinase activity and subcellular localization of mTORC1 and mTORC2. Rapamycin preferentially inhibits mTORC1, although longer treatment can also inhibit mTORC2 (Sarbassov et al., 2006). Rapamycin interacts

with FK506-binding protein (FKBP12), a protein-folding chaperon that belongs to the immunophilin family, generating a complex that binds to the FKBP-rapamycin-binding (FRB) domain of mTOR, resulting in inhibition of the interaction with Raptor (Chen et al., 1995; Sabatini et al., 1994). However, the underlying molecular mechanisms of Rapamycin-mediated inhibition of mTORC1/2 activity remain under debate (Wang et al., 2011).

Because Rapamycin preferentially acts on mTORC1, the function and regulation of mTORC1 is relatively well characterized compared with those of mTORC2. mTORC1 mediates diverse cellular functions by phosphorylating multiple substrates, such as p70S6K (S6K), eukaryotic initiation factor 4-binding protein 1 (4E-BP1) (Hara et al., 1998; Liu et al., 2006), fatty liver dystrophy (fld/lipin-1) (Peterson et al., 2011), sterol regulatory element-binding protein 1/2 (SREBP1/2) (Porstmann et al., 2008), and transcription factor EB (TFEB) (Peña-Llopis et al., 2011). S6K and 4E-BP1 regulate the initiation of translation of a large set of proteins including cyclin D1 (cell-cycle progression) (Averous et al., 2008), c-Myc (cell growth) (Babcock et al., 2013), B cell leukemia/lymphoma 2 (Bcl-2) (apoptosis) (Zou et al., 2013), matrix metallopeptidase 9 (MMP9) (cell migration) (Gulhati et al., 2011), and hypoxia-inducible factor 1-alpha (HIF1- α) (Energy metabolism) (Hudson et al., 2002). Lipin-1 ordinarily localized to the nucleus, inhibiting nuclear localization or activation of SREBP1/2. However, upon activation of mTORC1, phosphorylated Lipin-1 is exported from the nucleus, allowing nuclear entry of SREBP1/2. SREBP1/2 is further activated by phosphorylation via S6K and/or mTORC1. The activated SREBP1/2 promotes expression of genes related to lipid biosynthesis (Düvel et al., 2010; Porstmann et al., 2008). TFEB nuclear import is also regulated by mTORC1 phosphorylation. When mTORC1 activity is decreased, TFEB is translocated into the nucleus, where it acts as a transcription factor to regulate lysosomal biosynthesis by controlling expression of genes encoding lysosomal proteins, such as lysosomal-associated membrane protein 1 (LAMP1) and vacuolar-type H⁺-ATPase (V-ATPases) (Peña-Llopis et al., 2011; Song et al., 2013). As such, mTORC1 is involved in the regulation of a wide range of cellular functions,

including protein synthesis, lipid biosynthesis, and energy metabolism, which are required for cell growth and proliferation.

In contrast, mTORC2 regulates survival signals and the actin cytoskeleton. mTORC2 phosphorylates Akt or SGK1 (Pearce et al., 2010), which induces the activation of survival signal-related substrates including forkhead transcriptional factor (FoxO) (Tzivion et al., 2011), glycogen synthases kinase 3 beta (GSK3 β) (Pap et al., 1998), and BCL2-associated agonist of cell death (BAD) (S, 1997). mTORC2 also localizes upstream of the mTORC1 pathway. Akt phosphorylates and suppresses the activation of tuberous sclerosis 2 (TSC2) (Inoki et al., 2002) or prorine-rich Akt substrate of 40kDa (PRAS40) (Sancak et al., 2007), which are known as mTORC1 suppressors, resulting in activation of mTORC1. Moreover, mTORC2 regulates the actin cytoskeleton via phosphorylation of protein kinase C alpha (PKC- α) (Ikenoue et al., 2008). Reduction of Rictor expression causes suppression of mTORC2 activity, resulting in decreased formation of actin stress fibers.

Taken together, it is evident that mTOR serves as a central regulator not only in cell growth and proliferation but also in the maintenance of cellular homeostasis. The crucial role of mTOR has been confirmed in the analyses of mTOR knock-out mice, which show lethality in early embryonic stages (Murakami et al., 2004); however, the regulatory mechanisms underlying the mTOR pathway remain unclear. Although the activation of mTORC1 occurring downstream of growth factor receptors (such as insulin and insulin-like growth factor receptors) has been well described, the upstream pathway of mTORC2 has not yet been identified. Interestingly, it was shown that the activation of mTORC1 requires localization on the lysosomal surface, where it can be directly activated by interaction with the Ras homolog enriched in brain (Rheb) GTPase (Inoki et al., 2003). However, the mechanisms underlying the specific localization of mTORC1 to the lysosome and why mTORC1 is anchored to the lysosomal surface remain unknown.

Scaffold proteins that specifically anchor signaling components are involved in the spatio-temporally regulation of the localization and specificity of intracellular signaling pathways.

In an analysis of the role of c-Src tyrosine kinase in the regulation of EGF receptor signaling, we previously identified a new scaffold protein (termed p18) as a potential target of c-Src downstream of the EGF receptor (Nada et al., 2009). To evaluate the physiological role of p18, we generated p18 knock-out mice. The p18-deficient embryos died at an early embryonic stage (around embryonic day 7) due to severe defects in lysosomal development in the visceral endoderm cells, indicating the indispensable role of p18 in mouse development. To investigate the cellular function of p18, we then obtained p18 deficient (p18KO) cells from p18-deficient mouse embryos. Cell biological analysis revealed that p18 is specifically localized to lysosomal membranes. A pull-down assay identified the MEK partner 1 (MP1)/p14 heterodimer as a stable binding partner of p18. The MP1/p14 complex was previously identified as a scaffold for MEK1 in the MAPK signaling pathway (Teis et al., 2002). Indeed, p18KO cells exhibited a decrease in MAP kinase activity. Moreover, a recent study showed that the p18-MP1-p14 complex (termed Ragulator) acts as a scaffolding protein complex for Ras-related GTP-binding protein (Rag) GTPases (RagA/B and RagC/D), which is activated in response to amino acids (Sancak et al., 2010). Amino acids stimulate the exchange from RagA/B-GDP: RagC/D-GTP to RagA/B-GTP: RagC/D-GDP. Activated RagA/B recruits Raptor, and, subsequently, mTORC1 localizes to the lysosomal surface (Sancak et al., 2008). mTORC1 is then activated by Rheb GTPase, which is regulated by insulin signals through the Akt-TSC2 axis, the underlying mechanism to which remains unclear. These findings indicate that mTORC1 activity is regulated on the lysosomal surface by nutrients (amino acids) and growth factor signaling (insulin and IGF-1), and that the p18 complex serves as a crucial scaffold for the activation of mTORC1 on the lysosomal surface.

Upon activation, mTORC1 phosphorylates S6K to positively regulate protein synthesis. In turn, the activated S6K phosphorylates growth factor receptor bound protein 10 (Grb10) to inactivate insulin receptor substrate (IRS), resulting in suppression of growth factor signaling (Yu et al., 2011). This negative feedback mechanism mediates the activity of the mTORC1 pathway. In addition, recent reports showed that inactivation of mTORC1 induces the expression of Rictor,

a crucial component of the mTORC2 complex, indicating that the functions of mTORC1 and mTORC2 are mutually regulated (Chen et al., 2010); thus, the balance of mTOR signaling is fine-tuned by these feedback mechanisms.

The activation of the insulin receptor leads to activation of the PI3K-PDK1 axis, which induces phosphorylation of Akt and SGK1 at Thr308 and Ser422 (Dangelmaier et al., 2013; Kobayashi et al., 1999), respectively. mTORC2 also phosphorylates Akt (Ser473) and SGK1 (Ser422) (García-Martínez et al., 2008; Sarbassov et al., 2005), which both belong to the AGC family kinase and recognize the same phosphorylation consensus site (RXRXXS/T), and have common substrates, such as FoxO, GSK3 β , and p27^{Kip1}. Although the role of Akt in the mTOR pathway has been well established, the contribution of SGK1 to this pathway remains unclear.

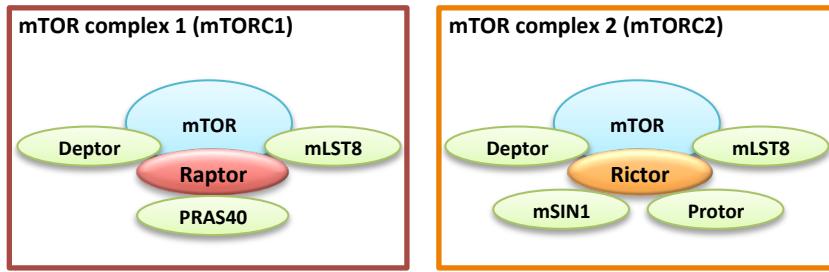
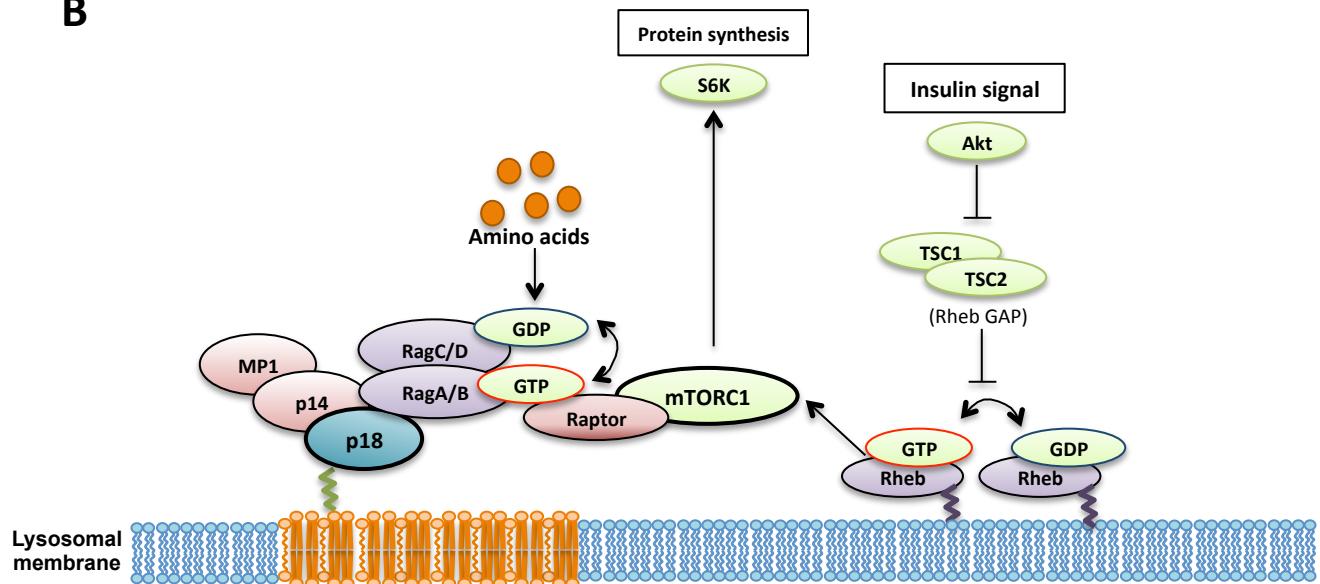
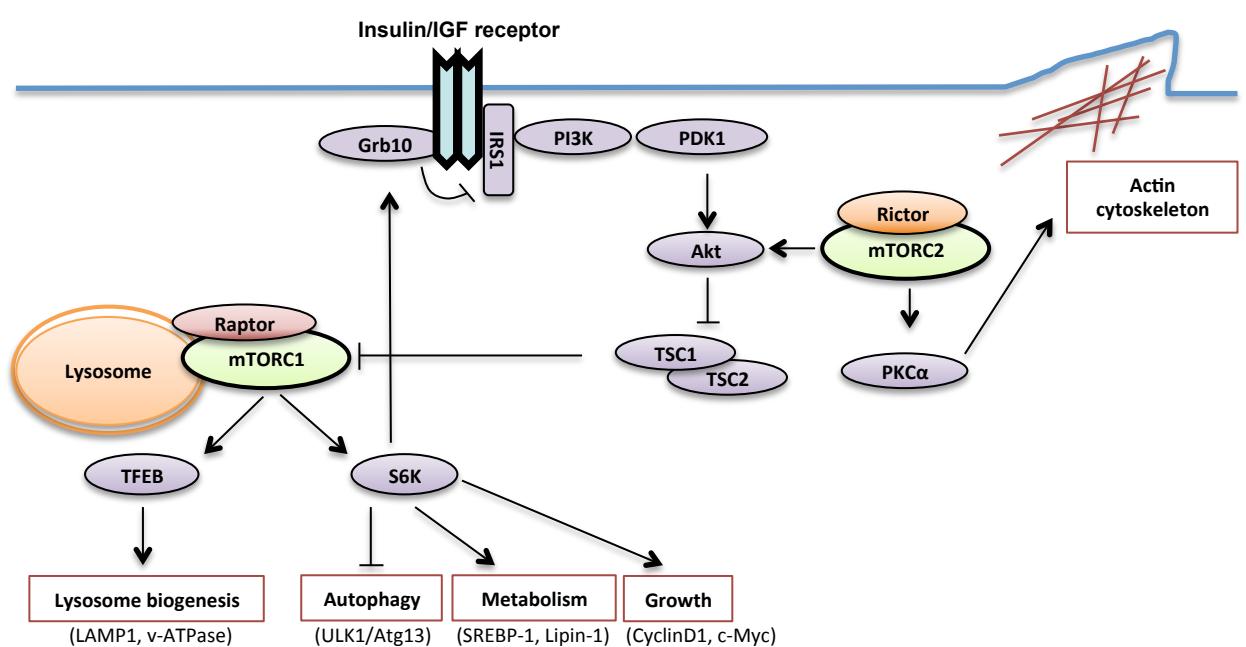
SGK1 was first identified because its mRNA expression was increased by glucocorticoid stimuli in rat mammary epithelial tumor cells (Con8.hd6) (Webster et al., 1993). Since then, it was shown that SGK1 expression is acutely regulated in response to stimulation with serum and hormones, such as glucocorticoid, aldosterone, and vasopressin (Verrey et al., 2003). A recent study demonstrated that multiple isoforms of SGK1 are produced as a result of differential use of translation initiation sites (Arteaga et al., 2007). The most intensively investigated isoform is isoform E (termed SGK1 long isoform in this study). Because of its high susceptibility for the ubiquitylation-proteasome system, SGK1 has a very short half-life. There are two types of proteasome degradation systems, namely, endoplasmic reticulum-associated protein degradation (ERAD)-dependent or -independent systems. In the ERAD-dependent system, the N-terminal 60-amino acid region of SGK1 is recognized by E3-ligases such as neural precursor cell expressed developmentally downregulated protein-4 (Nedd4-2) (Zhou et al., 2005), C-terminus of Hsc70 interacting protein (CHIP) (Belova et al., 2006), and synovial apoptosis inhibitor 1 (Synovioloin/HRD1) (Arteaga et al., 2006). In the ERAD-independent systems, however, Rictor interacts with Cullin-1, which acts as an E3-ligase for SGK1 (Gao et al., 2010). Although SGK1 regulates the survival signaling pathway, it also regulates presentation of ENaC (epithelial Na⁺

channel) in the cytoplasmic membrane (Debonneville et al., 2001). ENaC protein expression is regulated by endocytotic degradation mediated by ubiquitylation via Nedd4-2. SGK1 interacts with Nedd4-2 in a polyproline-tyrosine (PY) motif-dependent manner and phosphorylates Nedd4-2 on Ser422. Such phosphorylation suppresses the interaction between Nedd4-2 and ENaC, thereby retaining ENaC on the cell surface. As mentioned above, the functions of SGK1 isoform E have been well characterized, while the roles of the other isoforms are poorly characterized.

Akt and SGK1 directly phosphorylate FoxO transcriptional factors that control the expression of genes related to cell-cycle progression, oxidative resistance, and longevity. FoxO family proteins consist of four members (FoxO1, 3a, 4, and 6), of which FoxO1, 3a, and 4 are ubiquitously expressed in mammals to varying degrees (Greer et al., 2005). Notably, FoxO1 and FoxO3a are very similar and show high interspecies homology, and hence share post-translational modification sites and cis-binding elements in their promoters (TTGTTTAC) (Becker et al., 2010; Furuyama et al., 2000). Therefore, of the FoxO family proteins, the functions of FoxO1 and FoxO3a have been most extensively investigated. Although FoxO1 and 3a undergo multiple post-translational modifications, including phosphorylation, acetylation, methylation, and ubiquitination, nuclear translocation of FoxO3a is preferentially regulated by phosphorylation or acetylation. Activated Akt or SGK1 phosphorylates FoxO3a (Akt; Thr32/Ser252, SGK1; Ser314), which suppresses entry of FoxO3a into the nucleus (Brunet et al., 2001). By contrast, phosphorylation of FoxO3a by c-Jun N-terminal kinase (JNK) activated by pro-apoptotic signals promotes nuclear accumulation of FoxO3a (Wang et al., 2012). As a result, FoxO3a induces apoptosis by inducing expression of pro-apoptotic genes, such as BIM (Bcl-2 family member) (Barreyro et al., 2007). FoxO3a acetylation is mediated by CREB-binding protein (CBP)/p300 acetyltransferase, and promotes nuclear export of FoxO3a, leading to increased phosphorylation of Akt and SGK1 (van der Heide et al., 2005). This suggests that acetylation regulates the interaction between Akt/SGK1 and FoxO3a. By contrast, under oxidative stress conditions, deacetylation of

FoxO3a by sirtuin 1 (Sirt1) deacetylase activates FoxO3a and induces expression of stress-resistant genes, such as superoxide dismutase 2 (SOD2) (Giannakou et al., 2004; Pardo et al., 2011). Overall, the function and nuclear localization of FoxO3a is intricately regulated by multiple post-translational modifications according to cellular conditions. However, the regulatory mechanisms of FoxO3a function downstream of the mTOR pathway still remain to be elucidated. Furthermore, the mechanisms of transcriptional regulation of FoxO itself are largely unknown.

In this study, I aimed to elucidate the role of p18-mediated mTOR signaling in the regulation of cell proliferation. I first show that the specific inactivation of mTORC1 by depletion of p18 induces marked growth arrest at the G1 phase. To address the molecular mechanisms underlying this phenomenon, I investigated the effects of mTORC1 inactivation on various signaling components of the mTOR pathway. The results reveal that 1) mTORC1 inactivation breaks the S6K-mediated negative feedback regulation of growth factor signaling, thereby inducing hyperactivation of Akt; 2) mTORC1 inactivation enhances gene expression of Rictor, resulting in mTORC2 activation; 3) despite the hyperactivation of Akt, FoxO3a function is activated by increased protein expression of FoxO3a, increased accumulation of FoxO3a in the nucleus, and hypophosphorylation of FoxO3a at Ser314; 4) activation of FoxO3a is associated with upregulation of CDKIs, such as p27^{Kip1}; 5) FoxO3a expression is upregulated by the loss of p18 through suppression of DNA methylation at the enhancer region of the *FoxO3* gene; 6) expression of SGK1 short form is selectively downregulated by the loss of p18; 7) SGK1 is responsible for phosphorylation at Ser314, which is required for nuclear export of FoxO3a; 8) overexpression of SGK1 in p18KO cells promotes cell proliferation by inhibiting FoxO3a function; and 9) knockdown of SGK1 in wild-type cells attenuates cell proliferation by inducing CDKI. Taken together, the results suggest that mTORC1, in coordination with mTORC2, controls cell proliferation by regulating both FoxO3a gene expression and SGK1-mediated phosphorylation of the FoxO3a protein at Ser314.

A**B****C****Figure. General Int.**

(A) Mammalian target of rapamycin (mTOR) complex 1 (mTORC1) and complex 2 (mTORC2) components. (B) The activation mechanism of mTORC1 on the lysosomal membrane. (C) Model of the mTOR signaling network.

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Abstract

The mechanistic target of rapamycin (mTOR) functions as a component of two large complexes, mTORC1 and mTORC2, which play crucial roles in regulating cell growth and homeostasis. However, the molecular mechanisms by which mTOR controls cell proliferation remain elusive. Here we show that FoxO3a transcription factor is coordinately regulated by mTORC1 and mTORC2, and plays a crucial role in controlling cell proliferation. To dissect mTOR signaling, we specifically inactivated mTORC1 by depleting p18, an essential anchor of mTORC1 on lysosomes. mTORC1 inactivation caused a marked retardation of cell proliferation, mediated by induction of expression of cyclin-dependent kinase inhibitors (CDKIs), despite the robust activation of mTORC2 and Akt due to the abrogation of negative-feedback inhibition. Akt promotes cell proliferation by inhibiting the nuclear function of FoxO3a that activates CDKI gene expression. However, mTORC1 inactivation upregulated FoxO3a function by alleviating epigenetic suppression of FoxO3a gene expression and inducing hypophosphorylation at Ser314 on FoxO3a protein, a site critical for nuclear export, resulting in accumulation of FoxO3a in the nucleus. Furthermore, mTORC1 inactivation induced downregulation of serum- and glucocorticoid-inducible kinase 1 (SGK1), the kinase responsible for Ser314 phosphorylation. Expression of FoxO3a mutated at Ser314 in wild-type cells suppressed cell proliferation by inducing CDKI expression. Overexpression of SGK1 in p18-deficient cells suppressed CDKI expression, whereas knockdown of SGK1 in wild-type cells induced CDKI expression, resulting in suppression of cell proliferation. These results suggest that mTORC1, in coordination with mTORC2, controls cell proliferation via FoxO3a by regulating both FoxO3a gene expression and SGK1-mediated phosphorylation of FoxO3a protein at Ser314.

Introduction

The mechanistic (or ‘mammalian’) target of rapamycin (mTOR) is a Ser/Thr kinase that regulates key cellular functions related to the promotion of cell growth and metabolism (Laplante et al., 2012). mTOR kinase functions as a component of two large complexes, mTORC1 and mTORC2, each of which contains specific regulatory proteins: mTORC1 contains Raptor (Hara et al., 2002) and PRAS40 (Sancak et al., 2007), whereas mTORC2 contains Rictor (Sarbassov et al., 2004), mSin1 (Jacinto et al., 2004), and Protor (Pearce et al., 2007). mTORC1 is preferentially inhibited by the macrolide rapamycin via an interaction with FKBP12, although the mechanism remains unclear (Sabatini et al., 1994; Sarbassov et al., 2006). The functions and regulation of mTORC1 have been better characterized than those of mTORC2 (Laplante et al., 2013).

mTORC1 activity is regulated by growth factors and nutrients. Activation of receptor tyrosine kinases by growth factors, such as insulin and insulin-like growth factor, stimulates Akt kinase (also known as “protein kinase B”, PKB) via activation of phosphoinositide 3-kinase (PI3K). Activated Akt phosphorylates and inactivates TSC1/2, a GTPase-activating protein (GAP) for the Rheb GTPases, resulting in activation of mTORC1 (Inoki et al., 2002). Activated mTORC1 phosphorylates eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) and S6 kinase 1 (S6K1), thereby promoting protein synthesis (Blommaart et al., 1995; Hara et al., 1998). mTORC1 also promotes lipid biogenesis and metabolism, and suppresses autophagy by regulating several other downstream effectors, such as TFEB, SREBP-1, HIF1 α , and ULK-Atg13 (Laplante et al., 2009; 2013). The activation of mTORC1 also leads to the inactivation of growth-factor signaling by closing the negative-feedback loop mediated by S6K1 (Um et al., 2004), mTORC1 (Tzatsos et al., 2006), and Grb10 (Hsu et al., 2011; Yu et al., 2011).

The activation of mTORC1 by nutrients is achieved on the surface of lysosomes (Bar-Peled et al., 2012; Sancak et al., 2010). Amino acids supplied to starved cells are sensed by v-ATPase on lysosomes, resulting in activation of Rag GTPase via Ragulator, a lysosomal scaffold protein complex with guanine nucleotide exchange factor (GEF) activity (Bar-Peled et al., 2012).

Activated Rag GTPase recruits and activates mTORC1 at the lysosomal surface via Rheb (Sancak et al., 2008). The Ragulator complex consists of five small proteins: p18, p14, MP1, HBXIP, and C7orf59. One of these, p18, has a fatty-acyl modification and serves as an essential anchor of the complex to the lysosomal membrane. We previously identified p18 as a membrane anchor of the p14/MP1 complex on late endosomes/lysosomes (Nada et al., 2009), and subsequently showed that p18 plays a crucial role in regulating mTORC1 function in lysosome biogenesis and maturation processes (Soma-Nagae et al., 2013; Takahashi et al., 2012).

Although the functions and regulatory mechanisms of mTORC2 remain unclear, interplay between the mTORC1 and mTORC2 pathways is crucial for control of cell proliferation and homeostasis. When cells are stimulated by growth factors, mTORC2 phosphorylates Akt at a specific site to facilitate its full activation by 3-phosphoinositide-dependent protein kinase 1 (PDK1) (Sarbassov et al., 2005). Activated Akt contributes to activation of mTORC1 via TSC1/2, and also directly promotes cell growth by suppressing gene expression of cyclin-dependent kinase inhibitors (CDKIs), e.g., p27^{Kip1} and p21^{Cip1}, and pro-apoptotic molecules, e.g., Bcl2-family proteins and Fas ligand (Zhang et al., 2011). This Akt-dependent survival function is mediated by the Fork head box O (FoxO) family of transcription factors, which consists of FoxO1, 3, 4, and 6 (Tzivion et al., 2011). FoxO proteins function as key downstream effectors of growth-factor receptors, and are involved in the regulation of diverse cellular processes, including cell proliferation, apoptosis, longevity, cancer, and the cell cycle. Akt suppresses FoxO protein function by phosphorylating the transcription factor at critical sites required for export from the nucleus and degradation (Tzivion et al., 2011). These observations indicate that mTOR signaling regulates cell growth and homeostasis by coordinating the interplay between mTORC1, mTORC2, Akt, and FoxO proteins, although the underlying molecular mechanisms remain to be clarified (Hay, 2011).

Previously, we showed that ablation of p18 induced dramatic growth retardation even under nutrient-rich conditions (Nada et al., 2009), indicating that mTORC1 plays crucial roles in controlling cell proliferation. However, the signaling pathways leading to growth arrest remain

unknown. To address this issue, we analyzed the molecular circuits controlled by mTORC1 using p18-deficient cells. Because chronic treatment with rapamycin disrupts mTORC2 action in some cell types (Sarbassov et al., 2006), p18-deficient cells are useful for dissecting the specifically, because the function of other components of the mTOR complexes are not affected in these cells. In this study, we found that inactivation of mTORC1 promoted the nuclear function of FoxO3a by activating its gene expression through an epigenetic mechanism, as well as by suppressing phosphorylation of FoxO3a at Ser314, a site required for nuclear export, which is mediated by serum- and glucocorticoid- inducible kinase 1 (SGK1). Our findings suggest that mTORC1 coordinates with mTORC2 to control cell proliferation by regulating the nuclear function of FoxO3a.

Materials and Methods

Reagents and antibodies.

The following reagents and antibodies were obtained commercially: MG-132 (Calbiochem), anti-Akt1, anti-phospho-Akt1 (Thr308, Ser473), anti-p44/42 (Erk1/2), anti-phospho-p44/42 (Erk1/2) (Thr202/Tyr204), anti-MEK1/2, anti-phospho-MEK1/2 (Ser 217/221), anti-mTOR, anti-phospho-mTOR (Ser2448), anti-p70S6K, anti-phospho-p70S6K (Thr389), anti-Rictor, anti-Raptor, anti-PRAS40, anti-phospho-PRAS40 (Thr246), anti-4E-BP1, anti-phospho-4E-BP1 (Thr 70), anti-FoxO1, anti-phospho-FoxO1 (Thr24 and Ser256), anti-FoxO3a, anti-phospho-FoxO3a (Thr32 and Ser252), anti-TSC2, anti-phospho-TSC2 (Thr1462), anti-SGK1, anti-SGK3 (Cell Signaling Technology), anti-p21^{Cip1} (Santa Cruz Biotechnology); anti-p27^{Kip1} (BD Biosciences), anti-cyclin D1 (MBL), and anti-β-tubulin (Sigma). Anti-phospho-FoxO3a Ser314 antibodies were a generous gift from Dr. Michael E. Greenberg (Harvard Medical School, Boston).

Cell culture.

p18^{fl/fl} mouse embryonic fibroblasts (MEFs) were established from a *p18*-floxed mouse (Soma-Nagae et al., 2013). *p18^{-/-}* cell lines were established by transfecting *p18^{fl/fl}* MEFs with an integrase-defective Cre vector, a gift from Dr. Masahito Ikawa. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, and grown at 37°C in a humidified atmosphere containing 5% CO₂.

Growth assay and flow cytometry analysis.

For cell-growth assays, cells were seeded in 96-well plates (500 cells/well). Cell growth was measured using the WST-1 Cell Proliferation Reagent (Roche) at the indicated time points. For FACS analysis, cells were plated onto 60-mm dishes at a density of 8 × 10⁵ cells per dish. After 24 h, cells were harvested and stained with propidium iodide using the Cycle TEST™ PLUS

DNA Reagent Kit, and acquisition was carried out on a FACScan flow cytometer (Becton Dickinson).

Expression constructs.

All gene-transfer experiments were carried out using the pCX4 series of retroviral vectors (Akagi et al., 2003). cDNA constructs were generated by the polymerase chain reaction (PCR) using mouse cDNA as a template. The p18ΔN5-CAAX mutant was generated by deleting the five N-terminal amino acids of p18 and adding the K-Ras CAAX motif (KHKEKMSKDGKKKKKSCKCVIM) to the C-terminus. FoxO3a and the short and long forms of SGK1 were cloned into a vector containing a hemagglutinin (HA) tag on the 5' side of the cloning site. FoxO3a point mutants were generated by PCR-based mutagenesis. Mouse SGK1 lentiviral shRNA duplexes and non-target shRNA control (SHC202) were purchased from Sigma. The Mouse SGK1-shRNA sequences used was CGGCTGAGATGTACGACAATA.

Real-time PCR analysis.

Total RNA was extracted using Sepasol®-RNA Super G (Nacalai Tesque), and then reverse transcribed by extension with random hexamer primers, using the Transcriptor First Strand cDNA Synthesis Kit (Roche). qRT-PCR was performed using THUNDERBIRD® SYBR® qPCR Mix (TOYOBO) with the following primers: β -tubulin (forward: 5'-TATGTACCTCGGCCATCC-3' and reverse: 5'-TTATTCCTGCACCACTCTGG-3'), FoxO3a (forward: 5'-GATAAGGGCGACAGCAACAG and reverse: 5'-CATTCTGAACGCGCATGA-3'), FoxO1 (forward: 5'-CTTCAAGGATAAGGGCGACA-3' and 5'-GACAGATTGTGGCGAATTGA-3'), Rictor (forward: 5'-CACAGCCCTTCGCTTAGTC and reverse: 5'-GTTCACAGATGATGGCGATG-3'), and p27^{Kip1} (forward 5'-GTTAGCGGAGGCAGTGTCCA-3' and reverse 5'-TCTGTTCTGTTGGCCCTTT-3').

Immunofluorescence.

Cells were plated onto fibronectin-coated glass coverslips in 24-well culture plates. The cultures were rinsed once with cold phosphate-buffered saline (PBS), and then fixed for 30 min with 4% paraformaldehyde in PBS. After three rinses with IF Wash buffer (0.005% saponin in PBS), cells were permeabilized for 30 min with 0.25% saponin in PBS, and then blocked for 30 min with Blocking buffer (0.1% saponin, 1% BSA in PBS). The blocked coverslips were incubated overnight at 4°C with primary antibodies in Blocking buffer, rinsed three times with Wash buffer, and incubated for 3 h with secondary antibodies in Blocking buffer. The washed coverslips were mounted on glass slides using Prolong Gold (Invitrogen). Fluorescence was observed using an Olympus IX81 confocal microscope controlled by the FluoView FV1000 software. The intensity of the signal for FoxO3a was determined by using MetaMorph software (Universa; Imaging).

Western blotting.

Cells were washed with PBS and lysed in cell lysis buffer (0.1% SDS, 0.1% Sodium deoxycholate, 25 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% NP-40, 50 mM NaF, 1 mM Na₃VO₄, and protease inhibitor cocktail [Nacalai Tesque]) on ice. Equal amounts of total protein were separated by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were blocked and incubated with primary antibodies, followed by incubation with HRP-conjugated secondary antibodies. Signals from immunopositive bands were visualized on X-ray film using Immuno Star Zeta (Wako, Tokyo, Japan). Representative blots obtained from at least three independent experiments are shown. Subcellular fractionation was performed according to a previously described method (Roczniai-Ferguson et al., 2012). Cells were plated on 100-mm dishes at a density of 5×10^5 cells per dish. After 2 days, the cells were harvested and separated into cytoplasm and nuclear fractions.

Bisulfite sequencing.

Sodium bisulfite modification of genomic DNA was conducted using the EpiTect® Bisulfite Kit (QIAGEN). Bisulfite-treated DNA was used as the template for PCR using the following primers: 5'-GGTTTGAAATTAAGGAAATGTT-3' and 5'-ACTCCTCTACCAACCCTCTAAAC-3'. Amplified products were subcloned using the TOPO-TA cloning system (Invitrogen). Plasmid DNAs from ten insert-positive clones were isolated and sequenced.

Reporter constructs and luciferase assay.

The mouse *Foxo3* intronic region (Full, +1928/+2914; Del-1, +2145/+2914; Del-2, +2245/+2914; Del-3, +2381/+2914) was amplified by PCR from the RPCI-23 C57BL/6J Mouse BAC Library (BAC/PAC Resources), and then subcloned into the luciferase reporter plasmid pGN-P2 (Toyo Ink, Tokyo, Japan). For luciferase assays, cells were co-transfected with an intronic-region construct along with the control plasmid pRL-TK (Toyo Ink). Cells were harvested 24 h after transfection, and luciferase activities were measured using the PicaGene Dual Sea Pansy Luminescence Kit (Toyo Ink).

Results

Inactivation of mTORC1 by the loss of p18 causes growth arrest.

To dissect the role of lysosomal mTORC1 in cell-cycle progression, we generated p18-knockout cells (p18KO) from *p18*^{fl/fl} MEFs (Soma-Nagae et al., 2013) using an *in vitro* Cre-loxP system. We also prepared a revertant cell line that re-expresses p18 in p18KO cells (p18Rev), for use as a control. Furthermore, to verify the significance of lysosomal localization of mTORC1, we introduced a mutant p18 that lacks the five N-terminal amino acids required for lysosomal localization, but contains the K-Ras CAAX motif at the C-terminus (p18NΔ5-CAAX), into p18KO cells (Figure 1A). In WT cells, mTOR was localized to perinuclear Lamp1-positive lysosomes, whereas in p18KO cells it was diffusely distributed in the cytoplasm (Figure 1B). Re-expressed p18 was widely colocalized with mTOR on lysosomes. By contrast, p18NΔ5-CAAX was distributed in the cytoplasm and on the plasma membrane, resulting in delocalization of mTOR from lysosomes (Figure 1B). These observations confirm that p18 is required for lysosomal localization of mTOR.

The activity of mTORC1 was assessed by determining phosphorylation of two mTORC1 substrates, p70S6K1 and 4E-BP1 (Figure 1C). mTORC1 activity was strongly inhibited by delocalization of mTOR from lysosomes, although the expression of mTOR and Raptor was unaffected. As a consequence, protein expression of growth-promoting cyclin D1 was dramatically suppressed (Figure 1C). By contrast, the loss of lysosomal p18 did not significantly affect the activity of the MAP kinase pathway (Figure 1, right panel), suggesting that Ragulator does not play a prominent role as a scaffold for MEK1 (Teis et al., 2006; Teis et al., 2002), at least in these cells. These results demonstrate that lysosomal p18 is primarily involved in mTORC1 activation on lysosomes.

We then examined the effects of mTORC1 inactivation on cell proliferation, using the WST-1 assay (Figure 2A). p18KO and p18NΔ5-CAAX cells exhibited a dramatic reduction in cell proliferation rates relative to WT and p18Rev cells. Flow cytometry analysis of p18KO and

WT cells revealed that a significant proportion of p18KO cells were arrested in G1-phase (Figures 2B and 2C). These findings demonstrate that induction of cell-cycle entry by p18-mediated activation of lysosomal mTORC1 is required for promotion of cell proliferation.

Inactivation of mTORC1 upregulates mTORC2 and FoxO3a.

To address the molecular mechanisms by which lysosomal mTORC1 regulates cell proliferation, we investigated changes in signaling molecules upstream and downstream of the mTOR pathway (Figure 3A). Western-blot analyses of total cell lysates revealed that expression levels of mTOR and Raptor were not significantly influenced by inactivation of mTORC1, whereas protein and mRNA levels of Rictor were significantly upregulated (Figures 3A and B). Consistent with this, phosphorylation of Akt at Ser473, a site specifically phosphorylated by mTORC2 (Sarbassov et al., 2005), was elevated, indicating that mTORC2 is activated by mTORC1 inactivation. Activated S6K1 phosphorylates Grb10, which mediates negative-feedback regulation of growth-factor signaling (Hsu et al., 2011). Indeed, inactivation of S6K1 by mTORC1 inactivation induced a mobility shift of Grb10, indicating dephosphorylation of this protein. Consequently, phosphorylation of Akt at Thr308, a PDK1 phosphorylation site (Stokoe et al., 1997), was elevated, probably due to activation of PI3K. Although the reason remains unknown, the Grb10 expression level was slightly decreased in p18Rev cells compared with WT cells. However, the phosphorylation level of Akt Thr308 in these cells was almost comparable with that in WT cells, suggesting that perturbation of Grb10 expression had no significant effect on the Akt pathway. Activation of Akt was further confirmed by elevated phosphorylation of an Akt substrate, TSC2. These results suggest that inactivation of mTORC1 strongly activated Akt via activation of mTORC2 and abrogation of the negative-feedback inhibition mediated by Grb10. Activated Akt phosphorylates FoxO transcription factors and suppresses their nuclear function (Tzivion et al., 2011). In p18KO cells, the levels of FoxO3a protein and mRNA were significantly upregulated (Figures 3A and B). Although the levels of FoxO1 protein, a close relative of FoxO3a, appeared

unchanged (Figure 3A). Furthermore, both FoxO3a and FoxO1 proteins exhibited apparent gel-mobility shifts (Figure 3A), as discussed further below. These findings suggest that the expression and modification of FoxO transcription factors are regulated via the p18-mTORC1 pathway. Based on the observations that FoxO3a protein is more markedly upregulated in p18KO cells than FoxO1 and that FoxO3a is widely expressed in various tissues (Greer et al., 2005), we hereafter focused on the regulatory mechanism for FoxO3a function.

Because the upregulation of FoxO3a was a chronic event, we investigated the role of the epigenetic mechanisms for FoxO3a upregulation in p18KO cells. Treatment of WT cells with 5-aza-deoxycytidine (5-Aza), an inhibitor of DNA methyltransferase, significantly upregulated the transcription of FoxO3a; trichostatin A (TSA), an inhibitor of histone deacetylase, also upregulated *Foxo3* transcription, albeit to a lesser extent (Figure 4A). This observation suggests that the expression of the *Foxo3* gene is affected by its DNA methylation status. Bisulfite sequencing analyses of *Foxo3* CpG islands revealed that DNA demethylation of a specific region at the 5' end of the second intron occurred specifically in p18KO cells. Luciferase reporter assays revealed that the enhancer activity was located close to the 3' end of the methylated region (Figure 4C). Thus, it is possible that DNA methylation in the adjacent region would interfere with enhancer activity, and hence inactivates the expression of *Foxo3* gene. These observations suggest that mTORC1 is involved in the regulation of gene expression by controlling the DNA methylation status of the *Foxo3* gene.

Inactivation of mTORC1 induces nuclear accumulation of FoxO3a.

As mentioned above, we noticed that mTORC1 inactivation caused mobility shifts and hyperphosphorylation of Akt phosphorylation sites in FoxO3a and FoxO1 proteins (Figures 5A and 5B). Akt phosphorylates and inactivates FoxO proteins by causing them to translocate from the nucleus to the cytoplasm, which results in the promotion of cell proliferation via the suppression of the expression of CDKIs and pro-apoptotic molecules (Zhang et al., 2011).

Surprisingly, however, expression of two CDKIs, p27^{Kip1} and p21^{Cip1}, was increased by Akt-mediated FoxO3a phosphorylation (Figure 5A).

To resolve this apparent discrepancy, we investigated the phosphorylation status at Ser314, a site that is preferentially phosphorylated by SGK1, an Akt-related kinase that phosphorylates FoxO3a (Brunet et al., 2001) (Figure 5B). Western-blot analysis using a site-specific antibody revealed that phosphorylation at Ser314 was decreased by mTORC1 inactivation, despite upregulation of FoxO3a protein expression (Figure 5A). To further confirm the reduced phosphorylation at Ser314, we overexpressed several point mutants of FoxO3a in WT cells and analyzed their mobility shifts (Figure 5C). Mutants lacking Akt phosphorylation sites (T32A and S252A) exhibited only moderate mobility shifts, whereas a mutant lacking the SGK1 site (S314A) exhibited a more dramatic mobility shift. Mutation at sites phosphorylated by MST1 or IKK β did not affect mobility. These results demonstrate that the mobility shift of FoxO3a observed in mTORC1-inactivated cells was due to reduced phosphorylation at Ser314. Consistent with this, we found that protein expression of SGK1, but not SGK3, was markedly suppressed by mTORC1 inactivation, although SGK1 mRNA levels were unaffected (Figures 5A and 5D). As described below, SGK1 is expressed as several isoforms with distinct N-termini, generated by translation initiation at alternative sites (Arteaga et al., 2007). The expression of the shortest form was clearly downregulated. These findings suggest that phosphorylation at Ser314, potentially mediated by SGK1, is crucial for the regulation of FoxO3a function.

To elucidate the contribution of mTORC1-mediated modification to the function of FoxO3a, we examined nuclear localization of FoxO3a in p18KO and WT cells. Subcellular fractionation analysis revealed that p18KO cells accumulated more nuclear FoxO3a protein, which lacks Ser314 phosphorylation, than WT cells (Figure 5E). Quantitative immunofluorescence analysis also revealed that FoxO3a was significantly accumulated in the nucleus of p18 KO cells (Figure 5F). Accumulation of CDKIs in the nucleus was detected in p18KO cells. SGK1 was more abundantly localized to the nucleus in WT cells than in p18KO cells, indicating that it facilitates

nuclear export of FoxO3a by phosphorylating Ser314. These observations suggest that inefficient hypophosphorylation at Ser314 is involved in nuclear accumulation of FoxO3a in p18KO cells.

Hypophosphorylation of Ser314 promotes nuclear entry of FoxO3a.

To examine the role of Ser314 phosphorylation in the regulation of cell proliferation, we generated WT cell lines that stably overexpressed wild-type FoxO3a, the S314A mutant (S314A), or a mutant with triple alanine replacements at T32, S252, and S314 (3A) (Figure 6A). Immunofluorescence analysis revealed that whereas WT FoxO3a was mostly distributed in the cytoplasm, the S314A mutant was localized to both the cytoplasm and the nucleus (Figure 6B). This distribution pattern of the S314A mutant was consistent with that of FoxO3a in p18KO cells (Figure 5F), indicating that hypophosphorylation of Ser314 allows nuclear entry of FoxO3a even though other sites are hyperphosphorylated (Figure 6C). The 3A mutant was predominantly localized to the nucleus (Figure 6B), which supports the results of previous observation showing that Akt-mediated phosphorylation contributes to the nucleus export of FoxO3a (Greer et al., 2005). In both case, the nuclear localization of FoxO3a mutants strongly suppressed cell proliferation (Figure 6D). Western-blot and real-time PCR analyses confirmed that expression of the S314A and 3A mutants resulted in elevated protein and mRNA levels of p27^{Kip1}, and these levels were inversely correlated with cyclin D1 expression (Figures 6C and 6E). These findings suggest that the phosphorylation status of Ser314 is crucial for the regulation of the nuclear function of FoxO3a.

SGK1 is involved in the regulation of cell proliferation via the FoxO3a-CDKI axis.

Finally, we examined the role of SGK1 in the regulation of the nuclear function of FoxO3a. SGK1 has a very short half-life (< 30 min) and is tightly regulated by the ubiquitin-proteasome system (Belova et al., 2006; Gao et al., 2010; Zhou et al., 2005). Indeed, treatment with the proteasome inhibitor MG132 caused dramatic upregulation of SGK1 protein to a similar extent in both p18KO and WT cells (Figure 7A), indicating that synthesis of SGK1 protein is not affected

by mTORC1 inactivation. In sharp contrast to the case of SGK1, expression of Akt was not affected by MG132 treatment. Upregulation of SGK1 by MG132 induced a mobility shift of FoxO3a and increased the phosphorylation of Ser314 in FoxO3a, supporting the idea that SGK1 is involved in FoxO3a Ser314 phosphorylation. These results, together with the observation that SGK1 mRNA expression was unaffected (data not shown), suggest that mTORC1 inactivation affects SGK1 protein stability.

As noted above, SGK1 is expressed as isoforms with different N-termini owing to translation initiation at alternative sites (Arteaga et al., 2007) (Figure 7B). Western-blot analysis revealed multiple bands corresponding to SGK1; of these, the shortest form was clearly downregulated by mTORC1 inactivation (Figures 5A and 7C). Based on the molecular sizes, the largest and smallest bands correspond to the full-length (isoform e) and the shortest (isoform b) isoforms, respectively (Figure 7B). To discriminate the functions of these isoforms, the corresponding cDNAs were transiently transfected into p18KO cells. The long form was predominantly localized in the cytoplasm, whereas the short isoform was evenly distributed between the cytoplasm and the nucleus (Figure 7B), indicating that the short isoform can function in the nucleus to promote nuclear export of FoxO3a. We then assessed the function of the short isoform (SGK1 SI) by stably overexpressing it in p18KO cells (Figure 7C). Expression of SGK1 SI significantly suppressed p27^{Kip1} expression to a level comparable with that in WT cells (Figure 7D). Accordingly, cell proliferation was promoted by SGK1 SI expression, although the effect was not large (Figure 7E). On the other hand, shRNA-mediated knockdown of SGK1 promoted p27^{Kip1} expression (Figures 7F and 7G), resulting in significant suppression of cell proliferation (Figure 7H). These effects were rescued by re-expression of an shRNA-resistant SGK1 SI (Figures 7F-7H). These results suggest that the short SGK1 isoform contributes to the regulation of cell proliferation via the FoxO3a-CDKI axis.

Discussion

To dissect the function of mTOR in the regulation of cell proliferation, we investigated the effects of specific inactivation of mTORC1 using p18-deficient cells. Based on our findings, together with those of previous studies, we propose a hypothetical model for mTORC1 function (Figure 8). In WT cells, active mTORC1 promotes protein synthesis to support cell growth, and maintains the activity of Akt at a resting level through negative-feedback regulation via Grb10 (Hsu et al., 2011). Akt and SGK1 activated by mTORC2 (García-Martínez et al., 2008) coordinately phosphorylate FoxO3a at Thr32/Ser252 and Ser314, respectively (Brunet et al., 2001). These modifications result in translocation of FoxO3a from the nucleus, thereby inhibiting expression of CDKIs and ultimately promoting cell proliferation. According to this model, mTORC2 must be constitutively active at an appropriate level to continuously promote cell proliferation. By contrast, when mTORC1 is inactivated, mTORC2 is activated by the induction of Rictor, potentially through the loss of negative-feedback inhibition of the mTORC1 pathway (Chen et al., 2010). mTORC2 then induces Akt hyperactivation, resulting in elevated phosphorylation of FoxO3a at Thr32 and Ser252. The induction of Rictor also facilitates ubiquitylation-mediated destruction of SGK1 (Gao et al., 2010). Consequently, phosphorylation of FoxO3a at Ser314 is reduced, and FoxO3a retained in the nucleus activates expression of CDKIs, thereby inducing growth arrest. In addition, mTORC1 inactivation alleviates the epigenetic suppression of *Foxo3* gene expression, further enhancing the accumulation of FoxO3a in the nucleus. This model highlights the crucial role of the coordinated action of mTORC1 and mTORC2 in the regulation of cell proliferation via FoxO3a. When mTORC1 is inactivated under physiological conditions, such as starvation, activation of FoxO3a contributes to growth arrest; this process is critical for the survival of cells that are undergoing autophagy to recycle materials and obtain energy. Because we observed that expression and phosphorylation status of FoxO1 were significantly affected by mTORC1 inactivation, it is possible that other FoxO family members also contribute to the growth control via the mTOR pathway.

Previously, we identified p18 as an essential membrane anchor of the p14/MP1 complex, which was isolated as a scaffold for the MAP kinase pathway (Nada et al., 2009; Teis et al., 2006; Teis et al., 2002). However, our analyses in this study using the newly developed p18-deficient fibroblasts, demonstrated that loss of p18 exerts no significant effects on the MAP kinase pathway, suggesting that the role of p14/MP1 is cell context-dependent. In our system, mTORC1 activity is exclusively dependent on the presence of p18 on lysosomes. Therefore, we used this new system to dissect mTORC1-specific functions in the mTOR pathway. The most intriguing finding was that cell proliferation was dramatically suppressed, even though Akt activity was substantially upregulated and FoxO3a was hyperphosphorylated. This apparent discrepancy was resolved by our finding that the reduction in phosphorylation of FoxO3a at Ser314 occurred concomitantly with the downregulation of SGK1 expression.

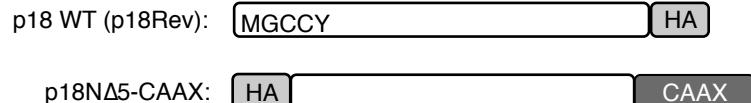
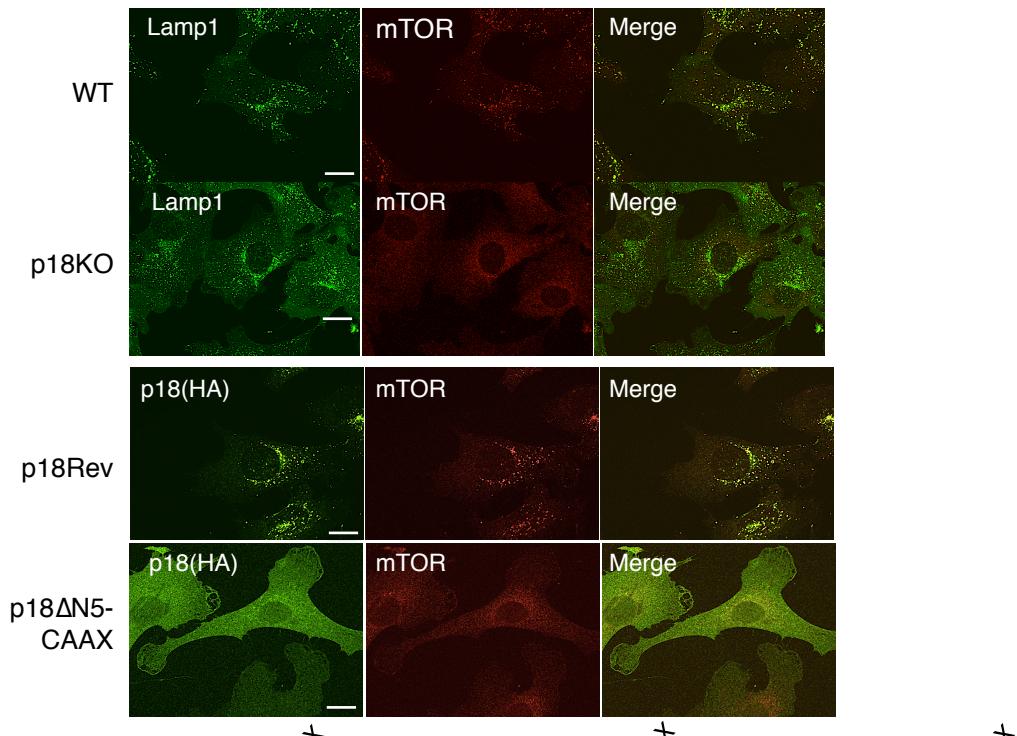
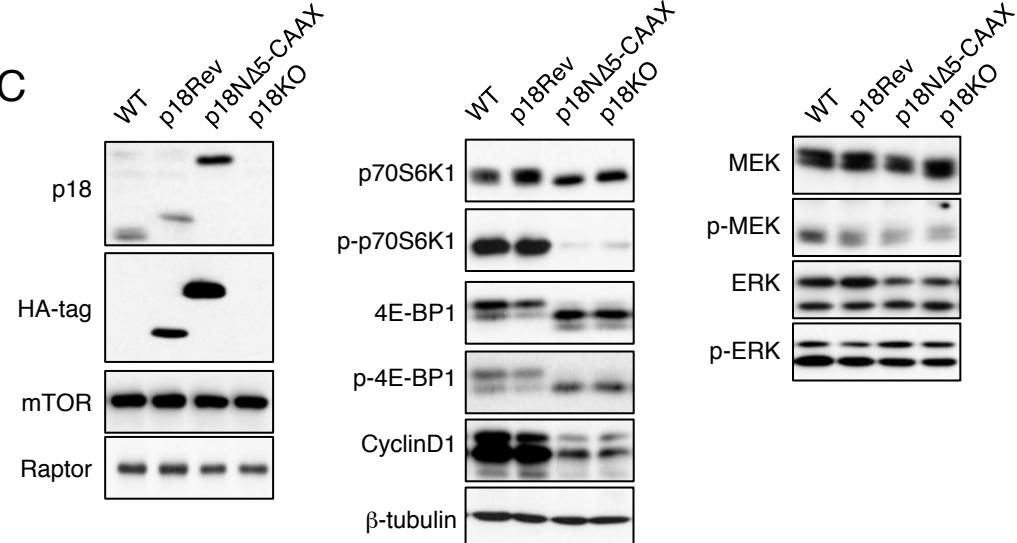
SGK1 is a member of the SGK family, which consists of SGK1, 2, and 3 (Lang et al., 2006). SGK1 is rapidly regulated at the transcriptional level, and by posttranslational modifications, such as phosphorylation and/or ubiquitylation (Loffing et al., 2006). Like Akt, SGKs are activated by phosphorylation via PDK1 (Kobayashi et al., 1999) and mTORC2 (García-Martínez et al., 2008) in response to growth factors; when activated, they phosphorylate various regulatory proteins that control cellular processes such as ion transport in epithelia (Loffing et al., 2006) and cell growth (Brunet et al., 2001). SGK1 is expressed as multiple isoforms with different N-termini due to translation initiation at alternative sites; these isoforms have different subcellular localizations, functions, and turnover rates (Arteaga et al., 2007). In this study, we found that expression of the shortest form of SGK1 was most clearly downregulated by mTORC1 inactivation. Because the shortest form can be imported into the nucleus, it is likely that this form is preferentially involved in the nuclear export of FoxO3a. We also observed that inhibition of the proteasome dramatically induced accumulation of SGK1, but not Akt, confirming that SGK1 is tightly regulated by the ubiquitin-proteasome system. SGK1 is degraded by several E3 ligases, including Rictor/cullin-1 (Gao et al., 2010), ERAD systems such as Nedd4-2 (Zhou et al., 2005), and CHIP (Belova et al.,

2006). Because Rictor is upregulated by mTORC1 inactivation, it is possible that SGK1 is downregulated via the Rictor/cullin-1 E3 ligases when mTORC1 is inactivated, although the potential contribution of other systems cannot be excluded.

We observed that overexpression of a FoxO3a mutant that lacks Ser314 strongly suppressed proliferation of WT cells, demonstrating the crucial role of Ser314 phosphorylation in regulating FoxO3a function in cell proliferation. Overexpression of SGK1 in p18KO cells suppressed the expression of CDKI and promoted cell proliferation, whereas SGK1 knockdown in WT cells induced CDKI expression and suppressed cell proliferation. These results support the idea that SGK1 contributes to the regulation of cell proliferation by phosphorylating FoxO3a. However, these effects of SGK1 expression and knockdown on cell proliferation were relatively moderate compared to the effects of the FoxO3a mutants. Therefore, it is possible although SGK1 takes part in the regulation of FoxO3a, other kinase(s) and/or phosphatase(s), such as PP2A (Hales et al., 2014), are also required to fully control the phosphorylation status of FoxO3a. Future studies will be directed toward identifying the kinase(s) and/or phosphatase(s) involved will be necessary. Furthermore, the potential contribution of other types of modifications, such as acetylation (Brunet et al., 2004), and methylation (Xie et al., 2012; Yamagata et al., 2008), will also need to be examined to elucidate the full regulatory mechanism of FoxO3a.

In this study, we found that expression of FoxO3a is regulated by DNA methylation, at a site adjacent to its enhancer region, in a manner that depends on the p18-mTORC1 pathway. To our knowledge, this is the first report to show a functional link between DNA methylation and mTOR signaling. It is possible that mTORC1 signaling regulates the expression of a wider range of genes via epigenetic mechanisms by regulating transcriptional cofactors and enzymes involved in DNA methylation. Because environmental changes, such as starvation, have been implicated in epigenetic changes (Pozharny et al., 2010), mTORC1 may also affect DNA methylation status by regulating metabolic pathways that supply donor molecules for DNA methylation reactions. Future studies should address the underlying mechanisms.

Given that SGK1 plays a role in controlling cell proliferation by suppressing FoxO3a function, deregulation of SGK function may be involved in tumor growth. Indeed, several reports have described the contribution of SGK1 and/or SGK3 to cancer progression (Abbruzzese et al., 2012; Bruhn et al., 2010; 2013; Lang et al., 2006; Lang et al., 2010; Sommer et al., 2013; Szmulewitz et al., 2012), and a recent report identified a subset of breast-cancer cell lines that are intrinsically resistant to Akt inhibition due to constitutive upregulation of SGK1 (Moniz et al., 2013; Sommer et al., 2013). These observations suggest that SGK might represent a promising therapeutic target in a wide range of cancers in which growth-factor signaling is upregulated, e.g., through mutations in the PI3K pathway.

A**B****C****Figure 1. Lysosomal localization is required for mTORC1 activation**

(A) Schematic structures of HA-tagged wild-type p18 (p18Rev) and a p18 mutant targeted to the plasma membrane (p18 Δ 5-CAAX). **(B)** Immunofluorescence analyses of localizations of p18 and mTOR. WT and p18KO cells were stained for Lamp1 (green), a lysosome marker, and mTOR (red) (upper panels). p18Rev and p18 Δ 5-CAAX cells were stained for p18 (HA) and mTOR (lower panels). Scale bars: 10 μ m. **(C)** Western-blot analyses to detect the indicated signaling molecules, using total cell lysates from WT, p18Rev, p18 Δ 5-CAAX, and p18KO cells.

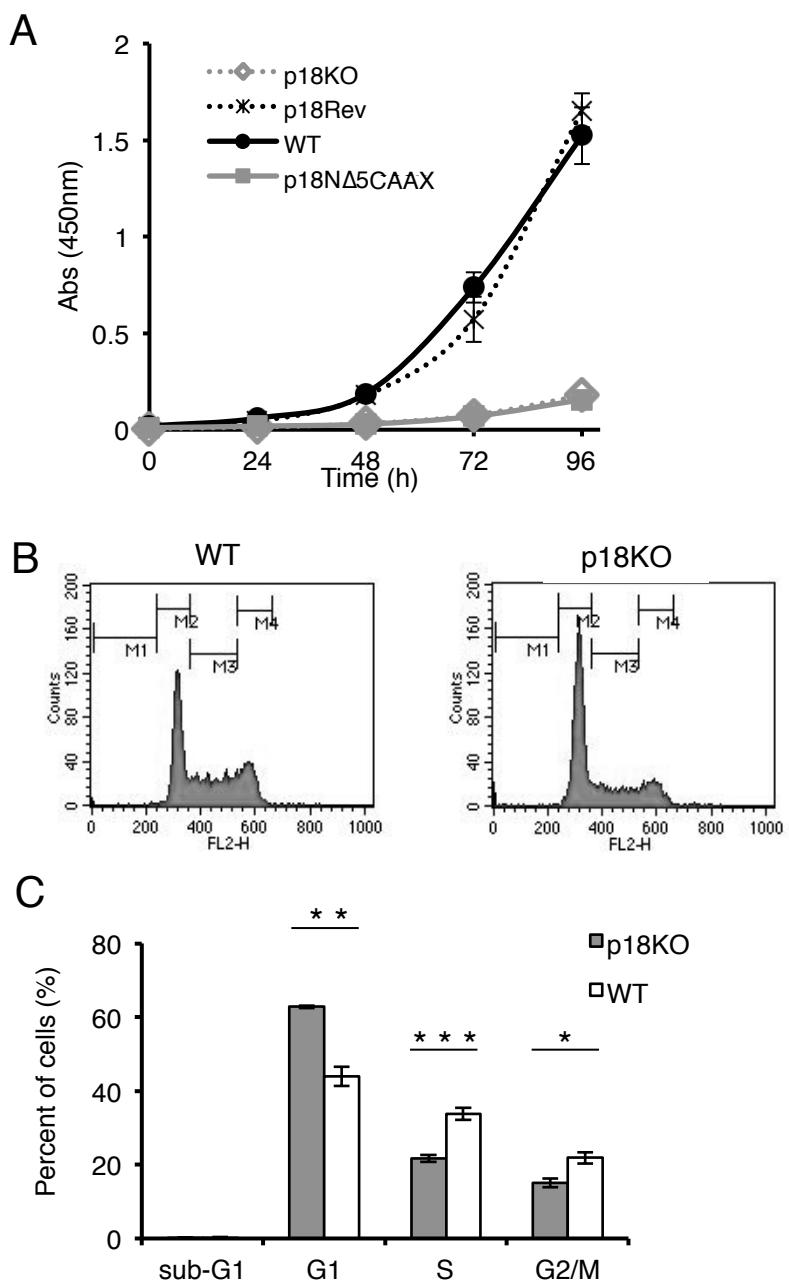


Figure 2. Inactivation of lysosomal mTORC1 causes growth arrest

(A) Cell proliferation of WT, p18Rev, p18NΔ5-CAAX, and p18KO cells analyzed by the WST-1 growth assay over the indicated time course. (B) WT and p18KO cells were analyzed for DNA content by flow cytometry. M1-4 indicates the following areas: M1; sub-G1, M2; G1, M3; S, M4; G2/M. (C) Quantitative data from FACS analysis. Means \pm SD were obtained from three independent assays. *** P < 0.001, ** P < 0.01, and * P < 0.05 (Student's *t*-test).

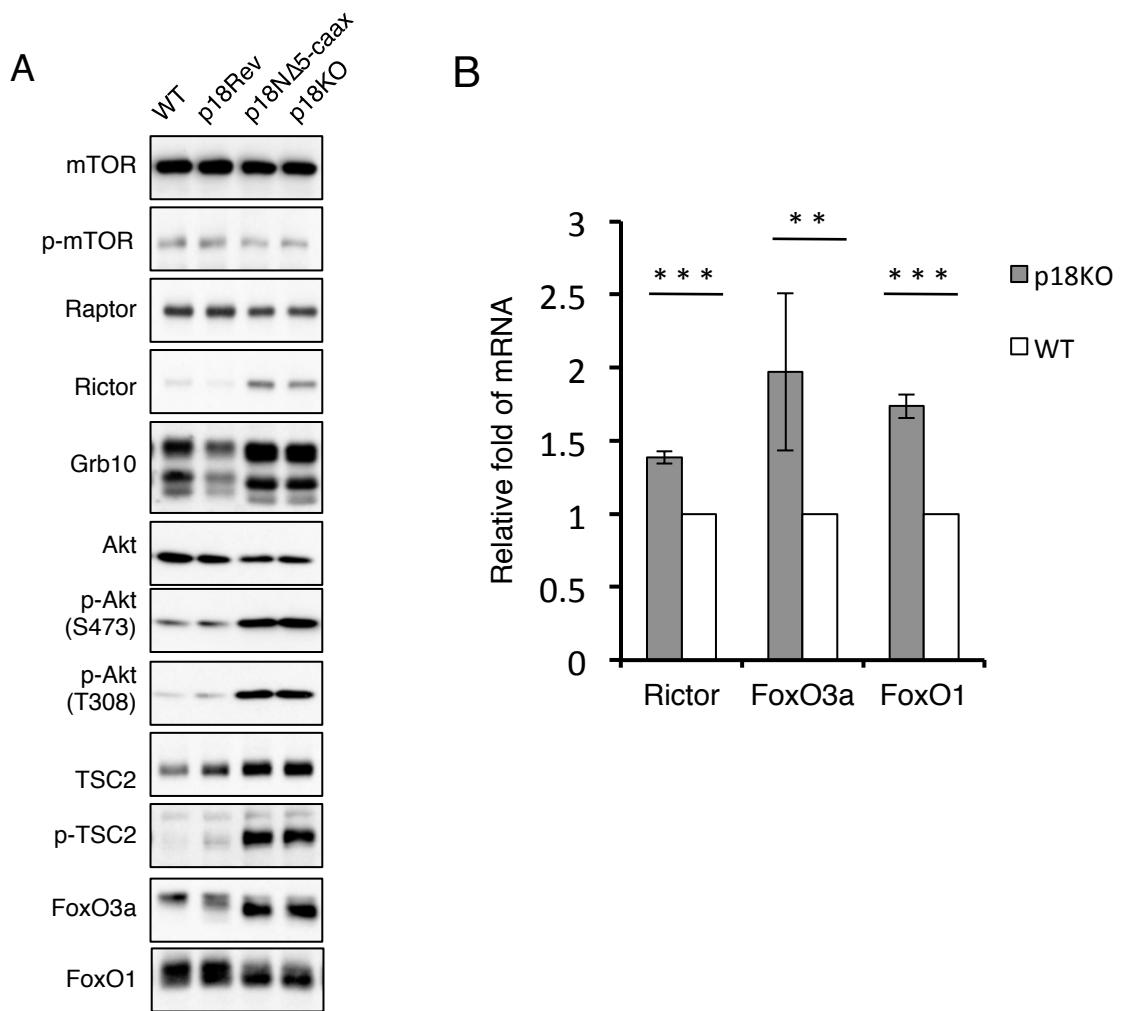


Figure 3. Inactivation of mTORC1 upregulates mTORC2 and FoxO3a

(A) Western-blot analyses to detect the indicated signaling molecules, using total cell lysates from WT, p18Rev, p18NΔ5-CAAX, and p18KO cells. **(B)** Expression levels of mRNAs encoding Rictor, FoxO3a and FoxO1 in p18KO and WT cells were determined by quantitative real-time PCR. Means \pm SD were obtained from three independent assays. *** $P < 0.001$ and ** $P < 0.01$ (Student's *t*-test).

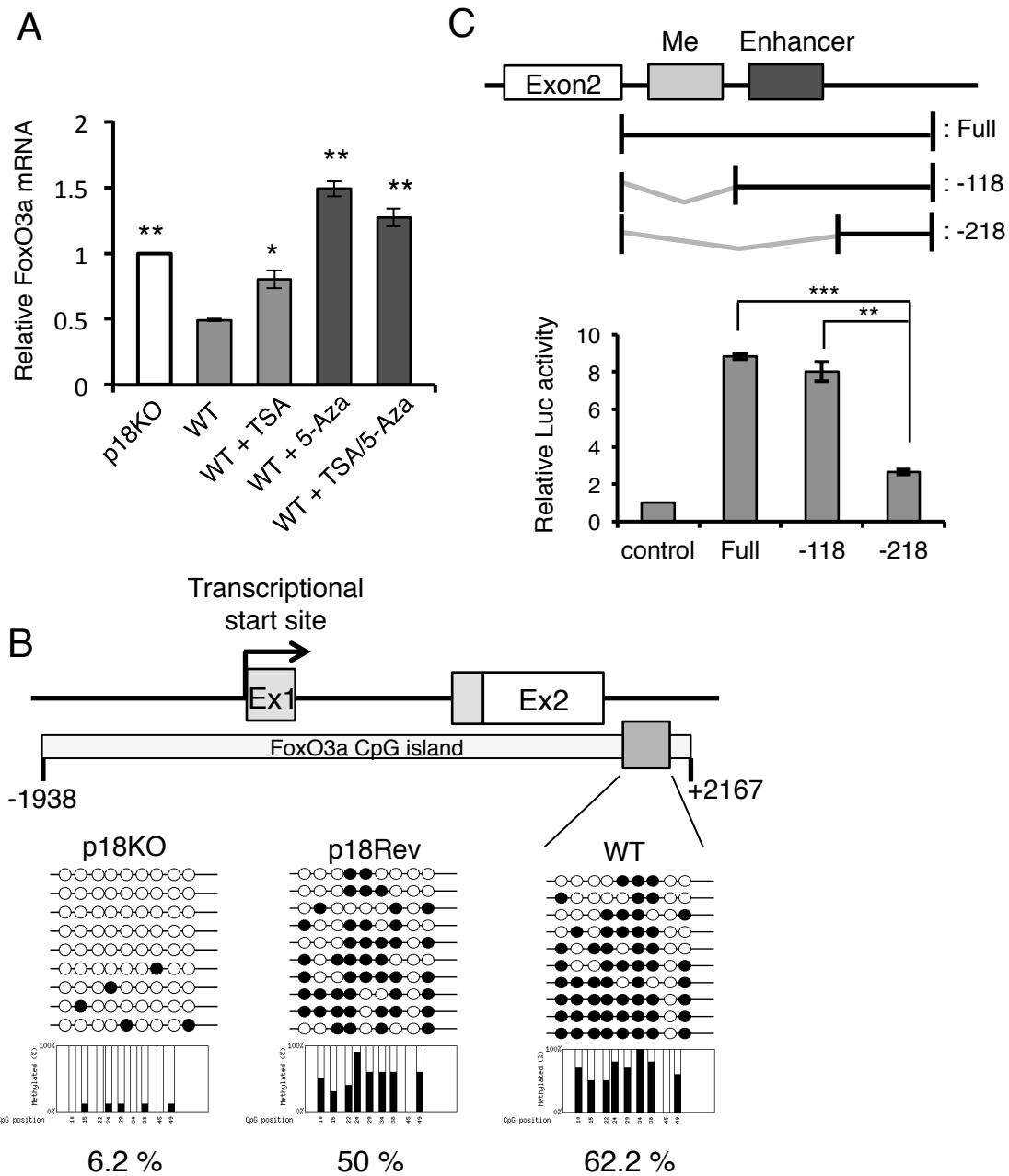


Figure 4. Expression of FoxO3a is regulated by DNA methylation

(A) WT cells were treated with trichostatin A (TSA), 5-aza-deoxycytidine (5-aza-dCA), or a combination of TSA and 5-aza-dCA for 48 h, and FoxO3a mRNA levels were determined by quantitative real-time PCR. Fold change in mRNA levels was calculated after normalization against β -tubulin mRNA (internal control). ** $P < 0.01$ and * $P < 0.05$ (Student's t -test). (B) DNA methylation status of *FoxO3* CpG islands in p18KO, p18Rev, and WT cells was analyzed by bisulfite sequencing. Methylation status in the gray boxed region was determined for ten clones obtained from each cell line. Individual clones are indicated by lines with circles; methylated and non-methylated cytosines are indicated by closed and open circles, respectively. (C) A series of fragments from the intronic region of the mouse FoxO3a gene were subcloned upstream of a luciferase reporter gene. Each construct was transfected into MEFs, and luciferase activity was measured and normalized against pRLTK activity. Normalized luciferase activity is expressed as means \pm SD ($n=3$). *** $P < 0.001$ and ** $P < 0.01$ (Student's t -test).

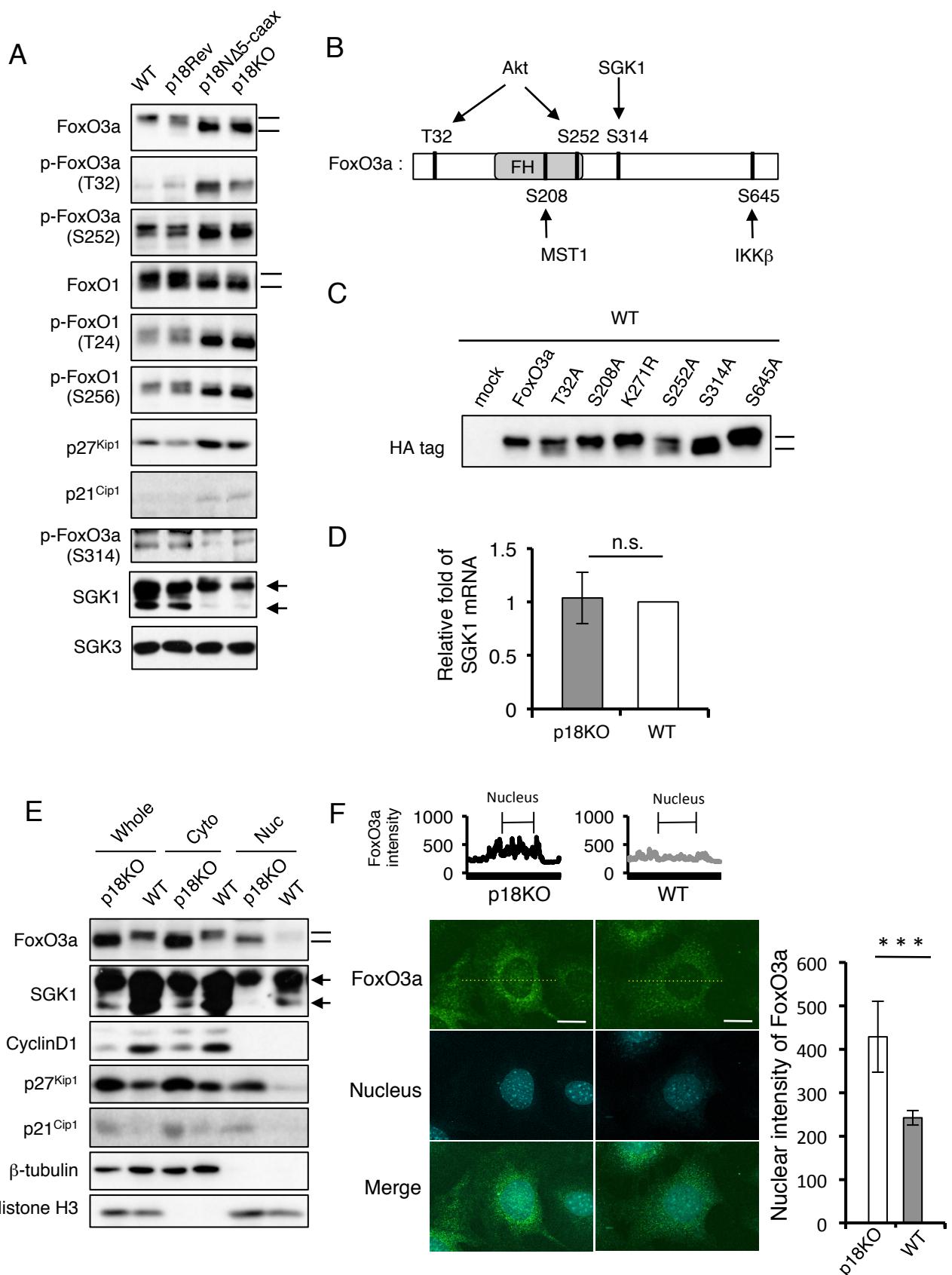
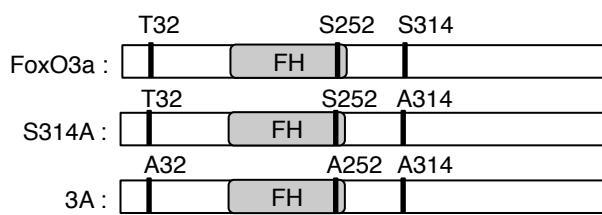


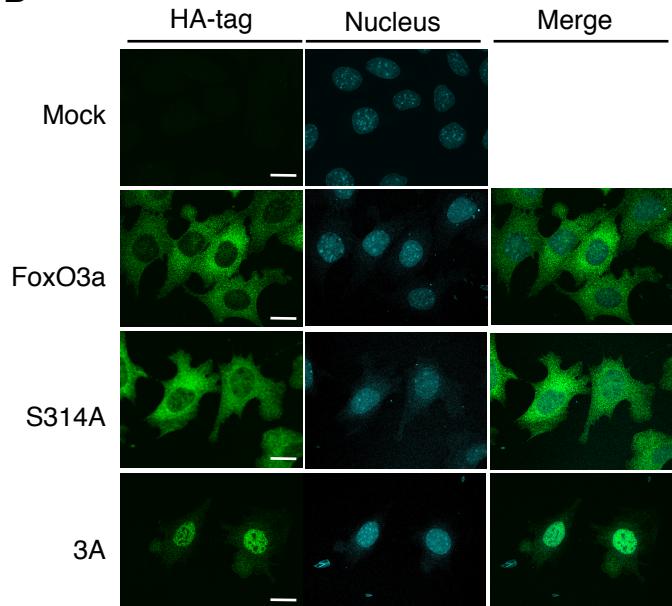
Figure 5. Inactivation of mTORC1 induces nuclear accumulation of FoxO3a

(A) Western-blot analyses to detect the indicated signaling molecules, using total cell lysates from WT, p18Rev, p18N Δ 5-CAAX, and p18KO cells. The panels for FoxO3a and FoxO1 are the same panels used in Figure 3A. Mobility shifts of FoxO proteins are shown by bars. Locations of bands corresponding to long and short forms of SGK1 are indicated by arrows. **(B)** Schematic structure of FoxO3a. Sites of phosphorylation by the indicated kinases are shown. FH: Fork head domain. **(C)** HA-tagged FoxO3a constructs with point mutations at the indicated amino-acid positions were transiently expressed in WT cells, and their mobility shifts (indicated by bars) were analyzed by Western blotting. **(D)** Expression level of mRNA encoding SGK1 in p18KO and WT cells were determined by quantitative real-time PCR. Means \pm SD were obtained from three independent assays. n.s.; not significant (Student's *t*-test). **(E)** Whole-cell lysates from p18KO and WT cells were separated into cytoplasmic and nuclear fractions, and the indicated proteins were detected by Western-blot analyses. β -tubulin and histone H3 represent control proteins for the cytoplasmic and nuclear fractions, respectively. **(F)** Immunofluorescence analysis for FoxO3a in p18KO and WT cells. Nuclei were visualized with propidium iodide (PI). Merged images are also shown. Scale bars: 10 μ m. Upper graphs show the intensity of signals for FoxO3a obtained by scanning the yellow dot lines. Right graph shows the statistic data from 15 cells. ****P* < 0.001 (Student's *t*-test).

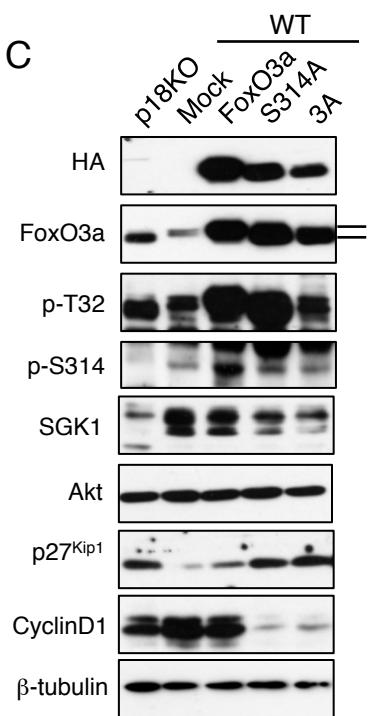
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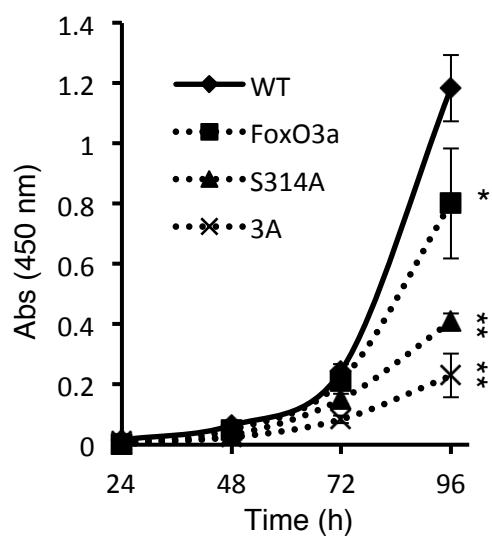
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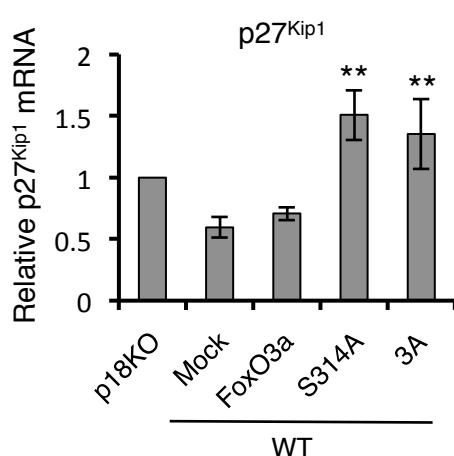


Figure 6. Nuclear localization of FoxO3a suppresses cell proliferation

(A) Schematic structures of WT FoxO3a, FoxO3a S314A mutant (S314A), and FoxO3a T32/S252/S314A triple mutant (3A). **(B)** Immunofluorescence analyses to determine localizations of HA-tagged WT FoxO3a, S314A, and 3A cells to determine FoxO3a (HA) localization. Merged images with PI staining are shown. Scale bars: 10 μ m. **(C)** Western-blot analyses for the indicated molecules in p18KO cells, mock-treated WT cells, and WT cells expressing FoxO3a, S314A, or 3A. **(D)** Cell proliferation of WT cells and WT cells expressing WT FoxO3a, S314A, or 3A was analyzed by the WST-1 growth assay over the indicated time course. Means \pm SD were obtained from three independent assays. ** P < 0.01 and * P < 0.05 (Student's *t*-test). **(E)** Expression level of p27^{Kip1} mRNA in cells used in (C) was determined by quantitative real-time PCR. Means \pm SD were obtained from three independent assays. ** P < 0.01, (Student's *t*-test).

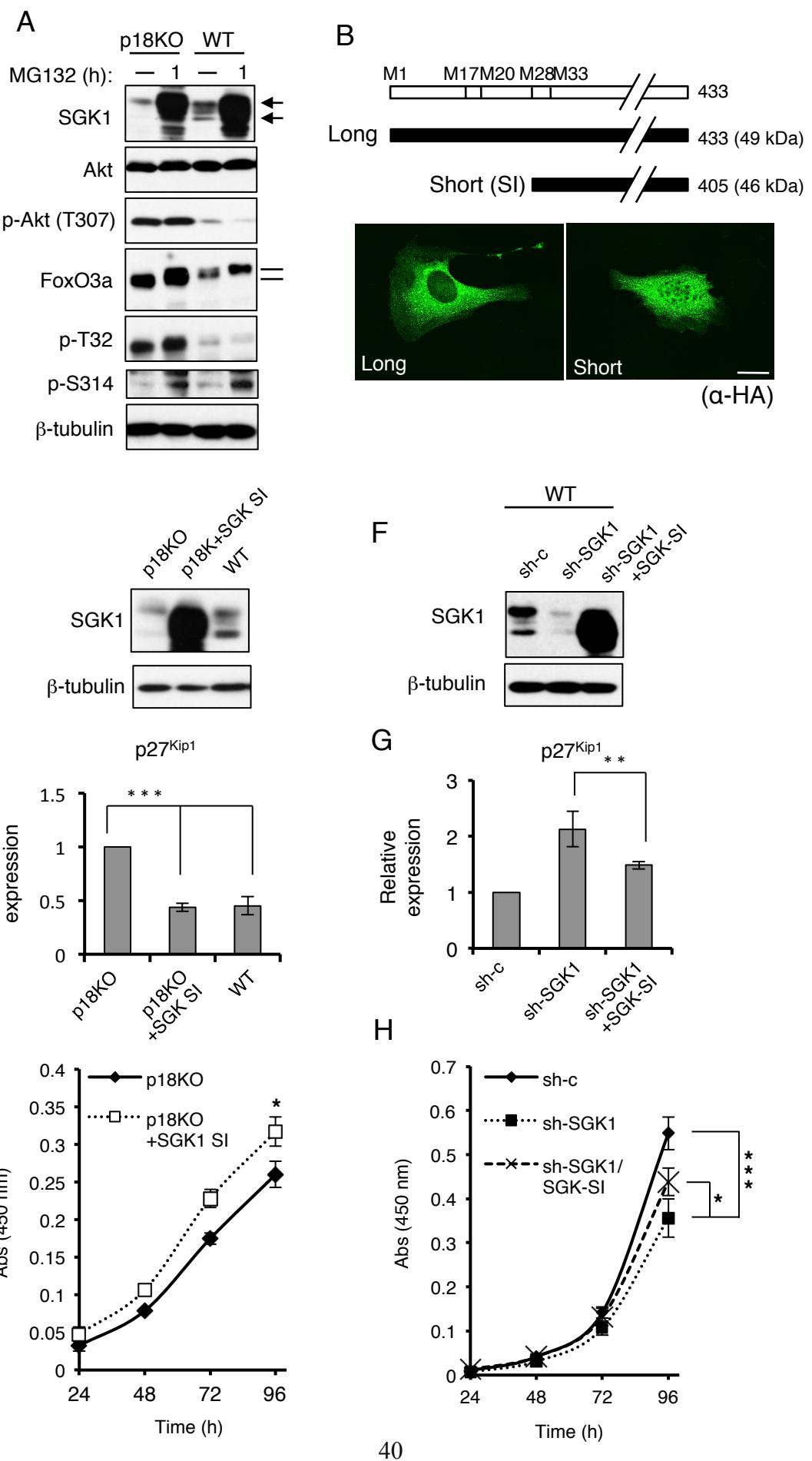


Figure 7. Phosphorylation of FoxO3a Ser314 is mediated by a SGK1 isoform that functions in the nucleus

(A) p18KO and WT cells were treated with MG132 for 1 h, and whole-cell lysates were subjected to Western-blot analyses to detect the indicated molecules. Bands corresponding to long and short forms of SGK1 are indicated by arrows. Bars indicate the mobility shift of FoxO3a **(B)** Schematic structures of long and short forms of SGK1 (upper). Immunofluorescence staining to detect HA-tagged long and short forms of SGK1 (lower). Scale bars: 10 μ m. **(C)** Western-blot analyses of SGK1 and β -tubulin in p18KO cells, p18KO cells expressing SGK1 SI, and WT cells. Bands corresponding to long and short forms of SGK1 are indicated by arrows. **(D)** Expression of p27^{Kip1} mRNA in cells used in (C) was determined by quantitative real-time PCR. Means \pm SD were obtained from three independent assays. *** P < 0.001 (Student's *t*-test). **(E)** Cell proliferation of p18KO cells and p18KO cells expressing SGK1 SI was analyzed by the WST-1 growth assay over the indicated time course. Means \pm SD were obtained from three independent assays. * P < 0.05 (Student's *t*-test). **(F)** Western-blot analyses of SGK1 and β -tubulin in WT cells expressing control shRNA (sh-c), SGK1 shRNA (sh-SGK1), or SGK1 shRNA plus sh-resistant SGK1 SI cDNA (sh-SGK1 + SGK1 SI) Bands corresponding to long and short forms of SGK1 are indicated by arrows. **(G)** Expression of p27^{Kip1} mRNA in cells used in (F) was determined by quantitative real-time PCR. Means \pm SD were obtained from three independent assays. ** P < 0.01 (Student's *t*-test). **(H)** Cell proliferation of cells used in (F) was analyzed by the WST-1 growth assay for the indicated time course. Means \pm SD were obtained from three independent assays. *** P < 0.001 and * P < 0.05 (Student's *t*-test).

Figure 8

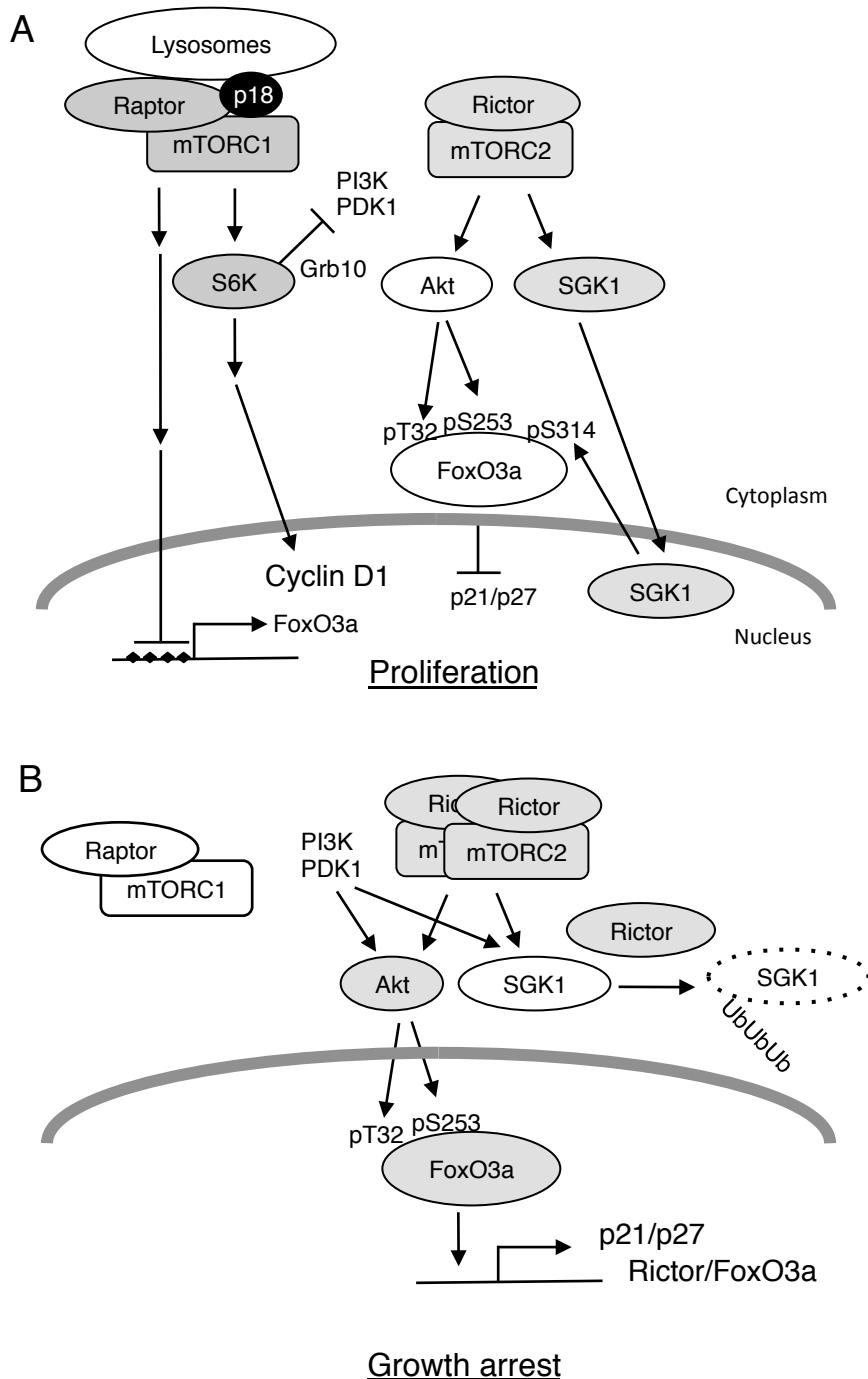


Figure 8. Schematic model of the function of mTOR pathway in the regulation of cell proliferation

(A) When mTORC1 is active. (B) When mTORC1 is inactive.

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Acknowledgements

This study was carried out at Department of Oncogene Research, Research Institute for Microbial Diseases, Osaka University.

I would like to express my deep gratitude to Prof. Masato Okada for his invaluable advices, discussion, and empathetic encouragement. I am especially grateful to Dr. Shigeyuki Nada and Dr. Chitose Oneyama (Osaka University) for their helpful advices and sincere encouragements.

I would like to thank my collaborators, Prof. Shoji Tajima and Dr. Hironobu Kimura (Osaka University) for epigenetic analysis, Dr. Yusuke Takahashi (Sysmex Corporation) and Ms. Ayaka Kitamura (Osaka University) for helpful discussion and technical advices and critical reading of the manuscript.

I would like to express my thanks to Prof. Kiyotoshi Sekiguchi (Osaka University) and Prof. Kazuaki Yoshikawa (Osaka University) for review of this thesis, Prof. Masahito Ikawa (Osaka University) for discussion and the kind gift of the Lentiviral-integrate defective Cre vector, Prof. Takashi Akagi for the kind gift of the pCX4 retroviral vector and Prof. Michael E. Greenberg for the kind gift of the phospho-FoxO3a Ser314 antibody.

Finally, I am deeply grateful to my family and all members of Department of Research for hearty help and encouragement.

Shunsuke Mori

January 2014

List of publications

Mori S, Nada S, Kimura H, Tajima S, Takahashi Y, Kitamura A, Oneyama C, and Okada M (2014) The mTOR pathway controls cell proliferation by regulating the FoxO3a transcriptional factor via SGK1 kinase. *Plos one* (in press)

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