MYC-binding lncRNA EPIC1 promotes AKT-mTORC1 signaling and Rapamycin resistance in breast and ovarian cancer

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AKT-mTORC1(mammalian target of rapamycin complex 1) signaling pathway plays a critical role in tumorigenesis and can be targeted by rapamycin. However, the underlying mechanism of how potential lncRNAs (long non-coding RNA) regulate the AKT-mTORC1 pathway remains unclear. *EPIC1* is a Myc-binding lncRNA, which has been previously demonstrated to be overexpressed in multiple cancer types. In a pathway analysis including 4962 cancer patients, we observed that lncRNA *EPIC1* expression is positively correlated with the AKT-mTORC1 signaling pathway in more than 10 cancer types, including breast and ovarian cancer. RNA-seq analysis of breast and ovarian cancer cells demonstrated that *EPIC1*-knockdown leads to the downregulation of genes in the AKT-mTORC1 signaling pathway. In MCF-7, OVAR4 and A2780cis cell lines, *EPIC1* knockdown and overexpression respectively inhibits and actives pAKT and the downstream phosphorylation levels of 4EBP1 and S6K. Further knockdown of Myc abolishes the *EPIC1*'s activation of AKT-mTORC1 signaling, suggesting *EPIC1* regulation of phosphorylation level of 4EBP1 and S6K depends on the expression of Myc. Moreover, *EPIC1* overexpressed MCF-7, A2780cis, and OVCAR4 cells treated with rapamycin showed significant decreasing in rapamycin mediated inhibition of p-S6K and p-S6 comparing with the control group. In addition, colony formation assay and MTT assay indicates that *EPIC1* overexpression leads to rapamycin resistance in breast and ovarian cancer cell lines. Our results demonstrated the Myc-

binding lncRNA *EPIC1* expression can activate the AKT-mTORC1 signaling pathway and lead to rapamycin resistance in breast and ovarian cancer.

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1.0 Introduction

1.1 The role of AKT-mTORC1 signaling pathway in cancer

AKT-mTORC1 (mammalian target of rapamycin) has been identified as a critical signaling pathway contributing to cancer initiation and progression. Two major downstream targets of mTORC1, 4EBP1 (eukaryotic initiation factor 4E binding protein 1) and S6K (ribosomal protein S6 kinase1) play critical roles in protein synthesis, ribosome biogenesis and lipid synthesis which are all related to cancer cell proliferation.[1-3]Besides AKT, ERK (extracellular-regulated kinase) has been demonstrated as another upstream regulator of mTORC1 and regulated its expressions and functions.[4-7] Recent studies showed that Myc, as a transcription factor, is a potential regulator of mTORC1 in multiple cancer types[8-11]. Moreover, the upregulation of Myc leads to drug resistance of mTORC1 inhibitors.[12] There are different types of AKT-mTORC1 inhibitors have been developed, and among these inhibitors, rapamycin has been known as a specific inhibitor of mTORC1.[13] Currently, rapamycin is a known drug targeting mTORC1 and showing anticancer effect in different cancer types;[14-17] including breast cancer.[18]

1.2 Introduction of lncRNA EPIC1

Long noncoding RNAs (lncRNAs) are RNA transcripts that are more than 200 nucleotides and do not translate into proteins.[19] Recent studies have shown that lncRNAs are involved in tumorigenesis of different cancer progression include cell proliferation, anti-apoptosis, motility by

interacting with chromatins, RNAs and proteins.[20, 21] Moreover, several lncRNAs have been reported as potential regulators of mTORC1, which correlate with cancer cell metabolism and energy stress.[22, 23] The nuclear lncRNA *EPIC1* (epigenetically-induced lncRNA1) has been shown to regulate cell proliferation through the cell cycle by interacting with transcription factor Myc in breast cancer.[24] However, the role of *EPIC1* in regulating the AKT-mTORC1 pathway and consequently rapamycin response is not clear.

1.3 Critical role of metabolism in cancer

Cancer cell metabolism has been identified playing an important role in tumorigenesis and cancer development. Manipulation of cancer cell metabolism will allow them to gain the building blocks contributing to cell proliferation and cancer progression. There are several hallmarks of cancer metabolism have been identified such as direct nutrient uptake of glucose and glutamine.[25] Another example is reprogramming of intracellular metabolism which also known as Warburg Effect. Cancer cells prefer to use a less efficient way to generate ATP but they could perform multiple times of glycolysis/TCA cycle processes which will provide cancer cells intermediates for biosynthesis and macromolecule productions.[25] The branch pathways such as pentose phosphate pathway, hexosamine biosynthesis will also contribute to the production of building blocks that are essential for cancer cells proliferation. Moreover, the changes of the metabolism of the tumor microenvironment can also lead to ineffective of immune system. Abundant glucose and glutamine uptake of cancer cells lead to essential nutrient deprivation of immune cells and cause suppression of CD8 effector T cell functions, increasing differentiations of Treg cells and immunosuppressive cells.[26] All these evidence showed us that targeting cancer cell metabolism

is a promising future direction to active immune cells at tumor microenvironment and to provide therapeutic opportunities for cancer.

1.4 Myc and related pathway regulate cell metabolism

Myc is a well-known oncogene and a critical transcription factor. Today's evidence suggest that Myc regulates its downstream target gene expression and promote cancer cells growth and tumorigenesis. Overexpression of Myc lead to increasing production of nucleic acids, proteins and lipids that support cancer cells' rapid proliferations. Through the upregulation of Myc target genes involved in metabolism, Myc directly contribute to the Warburg Effect of cancer cells and give the potential strength to reprogramming cancer cells metabolism. Recent studies showed that Myc downstream target genes involved in intermediary metabolism of glycolysis, lipid synthesis and glutaminolysis.[27] In details, publications have suggested that Myc played an important role in regulating Lactate Dehydrogenase A (LDHA), which converts pyruvate to lactate in glycolysis pathway. Other studies also showed Myc could regulate several critical intermediates involved in glycolysis such as glucose transporter 1 (GLUT1), hexokinase 2 (HK2), phosphofructokinase (PFKM), and enolase 1 (ENO1), which are all the limiting step enzymes that participate and highly upregualte in cancer cells metabolism.[28] Myc is also a regulator of mTORC1 signaling pathway which has been also established to promote cancer cell metabolism.[27]

1.5 Overview of thesis

In this study, we performed a comprehensive investigation into *EPIC1*'s regulation of the AKT-mTORC1 pathway using the genomics data from 4,962 tumor patients. The *EPIC1*'s regulation of the AKT-mTORC1 pathway in tumor samples was further confirmed by RNA-seq analysis in *EPIC1* knockdown cancer cell lines. We concluded that *EPIC1* can activate AKT-mTORC1 signaling pathway in breast and ovarian cancer cells through increasing the phosphorylation level of phspho-S6K, phospho-S6, and phospho-4EBP1. Further silencing of Myc rescues *EPIC1*'s activation of S6K, S6, and 4EBP1, suggesting *EPIC1*'s regulation of the AKT-mTROC1 pathway is dependent on Myc. Moreover, we have demonstrated that *EPIC1* overexpression leads to rapamycin resistance in both breast and ovarian cancer. In conclusion, our study revealed that *EPIC1* plays an essential role in the rapamycin resistance and AKT-mTROC1 signaling pathway by interacting with Myc in breast and ovarian cancers.

2.0 Experimental Section

2.1 Methods

2.1.1 Cell culture, stable cell line generation

Human breast cancer cell line MCF-7 was purchased from ATCC (American Type Culture Collection) and cultured with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and 1% penicillin-streptomycin. Human ovarian cancer cell line OVCAR4 was purchased from NIH/NCI and cultured with RPMI-1640 medium supplemented with 2 mM glutamine, 10% FBS, and 1% penicillin-streptomycin. Human ovarian cancer cell line A2780cis was purchased from ECACC (European Collection of Cell Cultures), supplied by Sigma-Aldrich and cultured with RPMI-1640 medium supplemented with 2 mM glutamine, 10% FBS and 1% penicillin-streptomycin. Stable cell lines were generated according to our previous publication.[24]

2.1.2 siRNA treatment and cloning

Cells in 6-well culture plates were transfected with 40 nM siRNA targeted *EPIC1*, Myc, and a control siRNA using Lipofectamine RNAiMAX reagent (ThermoFisher, 13778150). After 48 hr, cells were collected, and western blot analysis and quantitative real-time PCR (qRT-PCR) were performed. Full length of *EPIC1* expression plasmid was followed the previous description.[24]

2.1.3 SDA-PAGE and Western Blot

Cells were lysed in RIPA lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM PMSF, and 1x protease inhibitor cocktail). Total protein concentrations were measured with BCA protein assay kit (ThermoFisher, 23225). 20 µg of protein with 5x SDS sample buffer were loaded and then transferred to PVDF membranes (Bio-Rad, 162-0177). Membranes were blocked in 5% non-fat milk for 1 hr and then incubated in primary antibodies diluted 1:1000 at 4 ℃. ß-Actin antibody was purchased from Sigma-Aldrich (A5441). The following antibodies were purchased from Santa Cruz: p-ERK (sc-7383), total-ERK (sc-514302), PTEN (sc-7974). The rest of antibodies are purchased from Cell Signaling Technology: p-AKT (4060) phosphorylation site, total-AKT (4691), p-S6K (9234), total-S6K (2788), p-S6 (4858), total-S6 (2217), p-4EBP1 (2855), total 4EBP1 (9644), Myc (13987), Flag (14793). On the second day, membranes were incubated in horseradish peroxidase-conjugated secondary antibodies for 1hr (Anit-mouse IgG: ThermoFisher 31430; anti-rabbit IgG: ThermoFisher, 31460). Bands were detected by ECL (electrogenerated chemiluminescence) solution (ThermoFisher, 32106) on films.

2.1.4 Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using Trizol reagent. cDNAs were generated from 1 µg of RNA using cDNA Reverse Transcription Kit (Applied Biosystems, 4368813). qRT-PCR was performed with Power SYBR Green PCR Master Mix (Applied Biosystems, 4367659) on 6 Flex Real-Time PCR System (Applied Biosystems). Relative mRNA expression levels were normalized to

GAPDH. *GAPDH* primer sequences were used: forward: 5'-GGTGAAGGTCGGAGTCAACG-3'; and reverse: 5'-TGGGTGGAATCATATTGGAACA-3'. *EPIC1* primer sequences were used: forward: 5'-TATCCCTCAGAGCTCCTGCT-3'; and reverse: 5'-AGGCTGGCAAGTGTGAATCT-3'. *PTEN* primer sequences were used: forward: 5'-TGGATTCGACTTAGACTTGACCT-3'; and reverse: 5'-GGTGGGTTATGGTCTTCAAAAGG-3'.

2.1.5 RNA immunoprecipitation (RIP)

RIP followed the description of our previous publication.[24] Briefly, cells were collected and washed once with pre-chilled 1x PBS solution, then cells were treated for 10 minutes with 0.3% formaldehyde at room temperature on a rotor and add glycine solution to a final concentration of 125 mM to treat for another 5 minutes. Cells were pelleted and lysed with RIP buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 0.5 mM DTT, 1 mM PMSF, and 1 x protease inhibitor cocktail (Sigma, P8340)) and incubate on rotor for 20 minutes at 4 ℃. Cell lysates were incubated with Myc, MAX, and normal mouse or rabbit IgG used as a control overnight at 4 ℃. On the second day, prewashed Dynabeads Protein G (Invitrogen, 10004D) were added and incubated for 2 hr at 4 ℃. Finally, beads were washed six times with RIPA buffer. RNA was isolated with Trizol reagent, and qRT-PCR was performed.

2.1.6 Colony Formation Assay

10^4 cells stably overexpressing *EPIC1* or a control vector were seeded into 12-well culture plate and cultured overnight, then cells were treated with a serial of dilution of rapamycin (Selleckchem, S1039) ranging from 1.25, 10, 30, 100, to 200 nM, and DMSO was used as a vehicle control. Medium and drug were changed every two days. After 7 days, cells were washed once with PBS solution and fixed with 2% formaldehyde for 10 minutes at room temperature, and stained for 20 minutes in 0.01% crystal violet-25% methanol solution. Representative images were scanned and analyzed.

2.1.7 MTT Assay

5 x 10^3 cells were seed into 96-well culture plates and incubated overnight. Cells were treated as indicated with a serial of dilution of rapamycin (Selleckchem, S1039) ranging from 0.05, 0.25, 1.25, 10, 30, 100, 200 nM, and DMSO as a vehicle control. MCF-7 and OVCAR4 cells were treated for 72 hrs, and A2780cis cells were treated for 96 hr. Finally, cells were incubated for 4 hr with MTT solution (Biosynth, 1329524) and solubilizing solution (40% DMF, 16% SDS, 2% Acetic acid, PH4.7) were added to dissolve for overnight. The optical density of 570 nm and 637 nm were measured on the next day.

2.1.8 Computational Analysis

RNA-seq data of MCF-7 and A2780cis cells after *EPIC1* knockdown were profiled using the STAR-RSEM pipeline as in our previous study.[24, 29] To interpret the function of regulated

genes after *EPIC1* siRNA treatment, GSEA (version 2.2.0) was performed using the 50 cancer hallmark gene sets.[30],[31] The expression data for 4,962 patients for 20 cancer types were downloaded TCGA Pan-Cancer project (Data Freeze 1.3) processed as our previous publication.[24] To identify the pathways that are enriched with genes correlated with *EPIC1* expression in tumor samples, we performed a similar GSEA for each cancer type in the TCGA dataset. The False Discovery Rate (FDR) was used to show the significance of the enriched pathways. The Pearson and Spearman correlation was calculated between gene expressions, and significance was defined as $p < 0.05$.

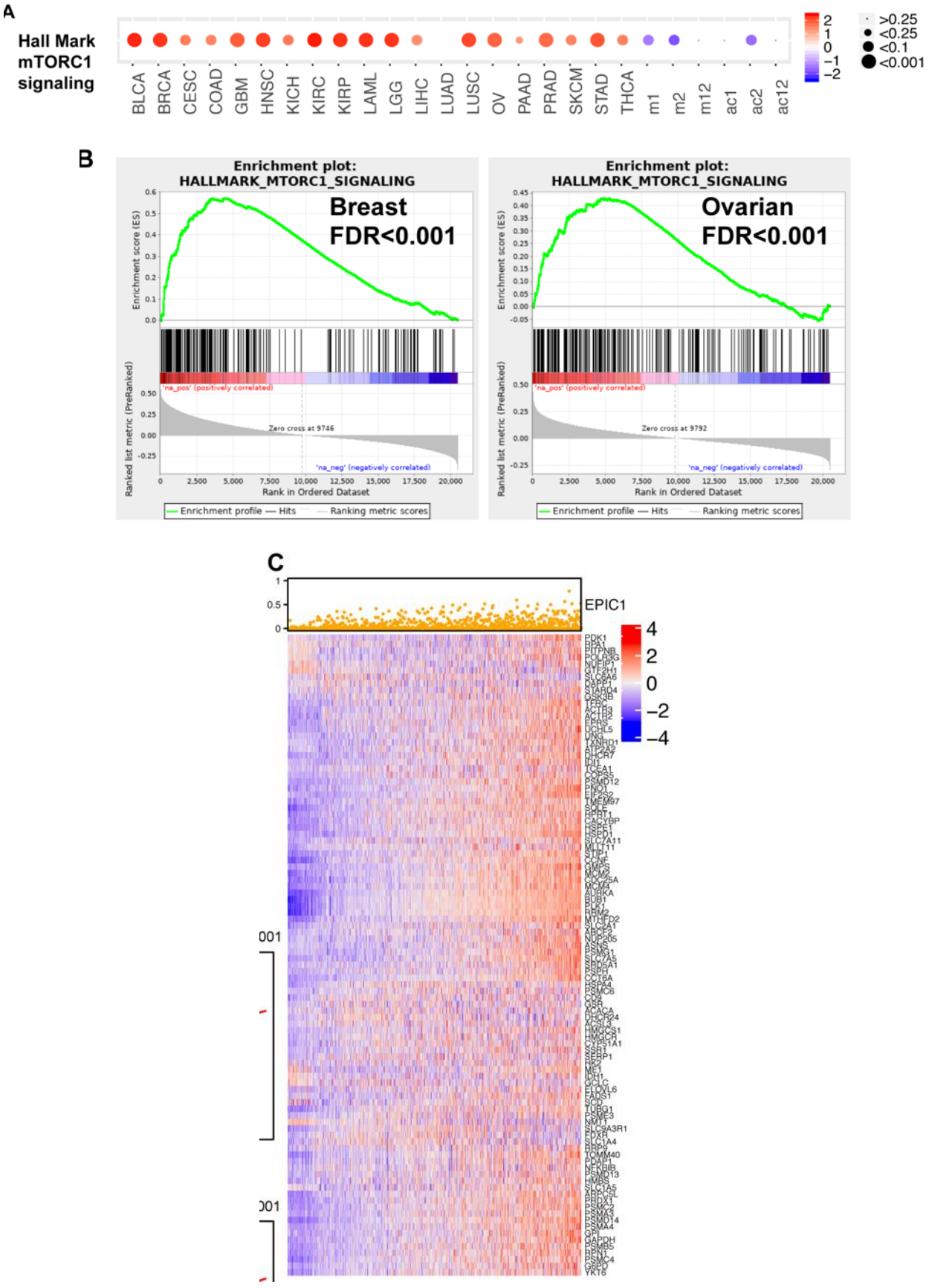
2.1.9 ChIP-seq

Use $1x10^7$ cells per sample cross-link with 1.42 % formaldehyde on shaker for 10 minutes at RT. Then quenched by adding 1.25M glycine and shake for another 5 minutes at RT. Cells were rinsed by pre-chilled PBS for two times. Harvested cells into 50 ml tubes and add lysis buffer (50 mM pH 7.5 Tris-HCl, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, and 1% Triton X-100) with proteasome inhibitor and sonicated to shear the chromatin to yield DNA fragment sizes 100-500bp. Then add Protein G beads that was pre-incubated with 30μg Myc antibodies for overnight at 4°C. Incubate beads and lysis for overnight at 4°C. On the next day, wash beads for six times used lysis buffer and elute the DNA from beads. Reversed cross-link protein and DNA at 65°C for overnight. On the second day, digest protein and RNA used RNase A and Proteinase K, respectively. Extracted DNA and run agarose gel to check the size of DNA fragments. Then performed library construction to enrich the DNA products. At last, send samples for sequencing.

3.0 Result

3.1 High expression level of EPIC1 is correlated with activated mTORC1 signaling pathway in multiple cancer types

To determine the function of lncRNA *EPIC1*, we first used Gene Set Enrichment Analysis (GSEA) to identify *EPIC1* related signaling pathways in 20 different cancer types. From the cancer patient RNA sequencing data, we found that the mTORC1 signaling pathway was significantly positively correlated with *EPIC1* expression in 12 out of 20 cancer types (**Figure 1A)** include breast **(Figure 1B left)** and ovarian cancer **(Figure 1B right)**. Meanwhile, in the cell line RNA-seq data, the mTORC1 signaling pathway was enriched with the under-expressed genes in the *EPIC1*-knockdown MCF-7 and A2780cis cell lines (**Figure 1A**). In the breast **(Figure 1C)** and ovarian **(Supplemental Figure 1A)** cancer patient samples, mTROC1 signaling core genes are highly positively correlated with *EPIC1* expression. For example, the expression of AURKA (serine/threonine-protein kinase 6), GSK3B (glycogen synthase kinase 3 beta), and PLK1 (polo-like kinase 1) were reported to regulate mTORC1 signaling pathway [32-34]. The expression of gene were positively associated with *EPIC1* expression showed in 927 breast and 264 ovarian cancer patients respectively **(Figure 1D and Supplemental Figure 1B**). These analyses suggested *EPIC1* may play a role in the activation of the mTORC1 signaling pathway in cancer.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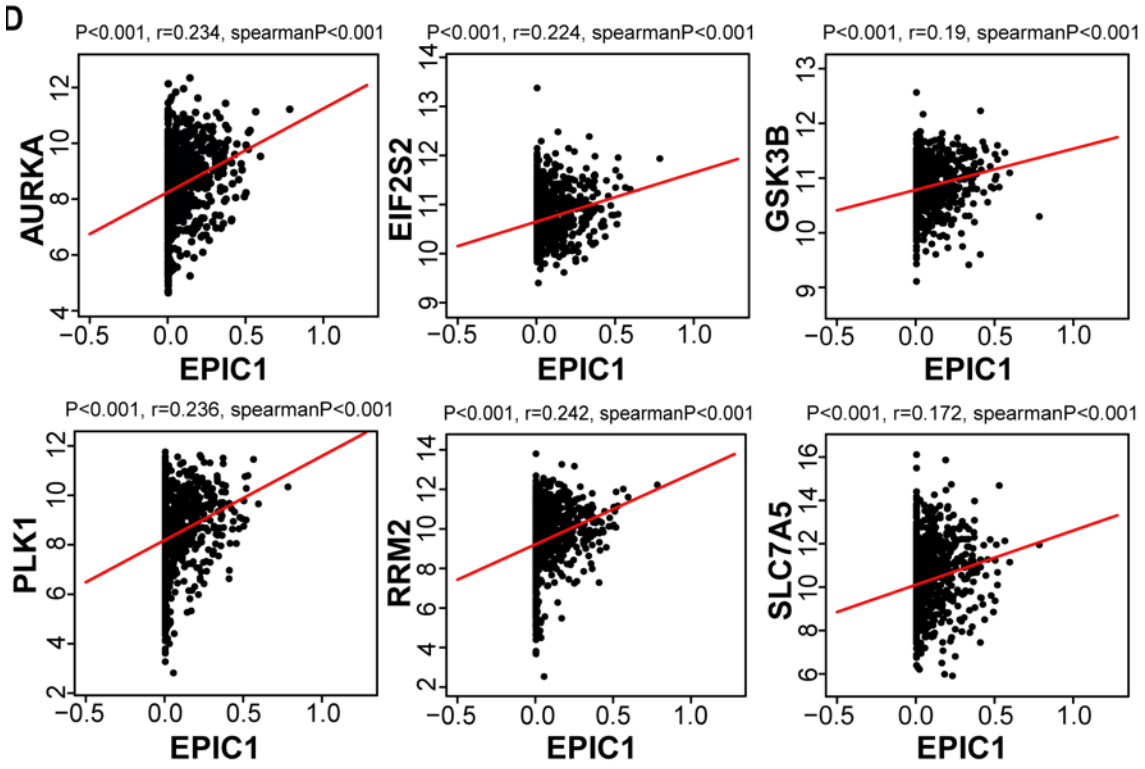
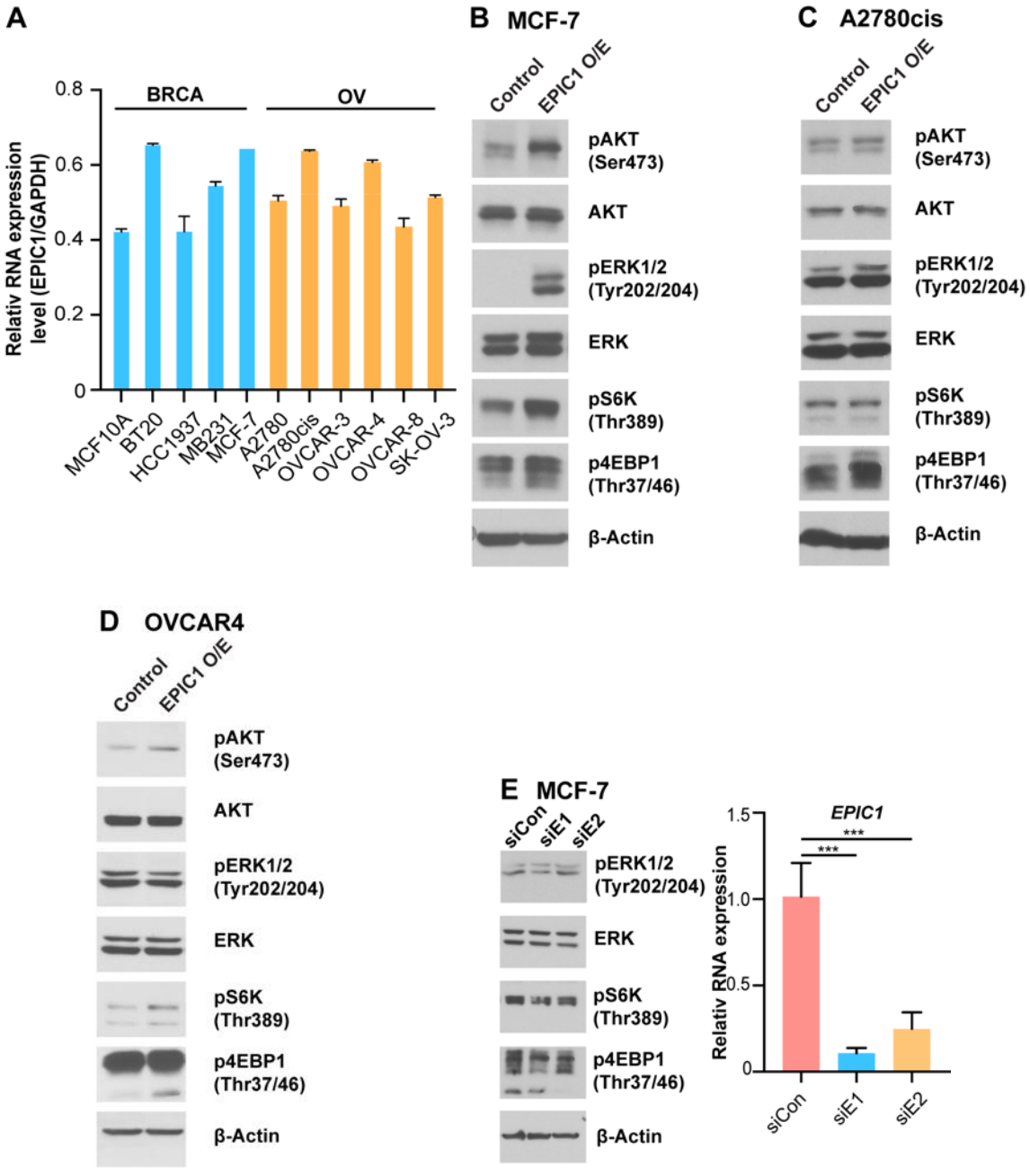


Figure 1. High expression level of EPIC1 is correlated with activated mTORC1 signaling pathway

A. Correlation between EPIC1 expression and mTORC1 signaling pathway by Gene set enrichment analysis (GSEA) in 20 cancer types and EPIC1 knockdown MCF-7 (m1, m2, m12), A2780cis cells (ac1, ac2, ac12). The heatmap indicates the GSEA scores. B. Association between the enrichment of mTORC1 signaling and EPIC1 expression in breast cancer patients (left) and ovarian cancer patients (right) by GSEA analysis (A). C. Expression analysis showing that EPIC1 expression is associated with hallmark mTORC1 genes (B) in 927 breast cancer patients. D. Correlation analysis between specific genes that regulate mTORC1 signaling pathway and EPIC1 expression in breast cancer patients.

3.2 EPIC1 promotes AKT-mTORC1 pathway through upregulating the phosphorylation level of 4EBP1 and S6K

To determine if *EPIC1* regulates the AKT-mTORC1 signaling pathway and its downstream targets, we first examined the relationship between *EPIC1* and phospho-AKT (p-AKT). Meanwhile, we also studied two downstream mTORC1 targets, phosphor-S6K (p-S6K) and phosphor-4EBP1 (p-4EBP1) in breast and ovarian cancer cell lines, which have relatively higher *EPIC1* expression levels identified by qRT-PCR (**Figure 2A**). We hypothesized that *EPIC1* might promote the mTORC1 signaling pathway through upregualte the phosphorylation level of AKT, S6K and 4EBP1. To test our hypothesis, we used a lentiviral expression system to generate *EPIC1* overexpression stable cell lines in MCF-7, OVCAR4, and A2780cis cells with the selection of Puromycin. Western Blot demonstrated that the *EPIC1* overexpressin increases expression level of p-AKT, p-S6K, and p-4EBP1 without affecting the total protein expression levels in these three cell lines (**Figure 2B, 2C, and 2D**). Next, we investigated the expression of p-ERK, which was known to be another upstream target of mTORC1 besides AKT;[4-7]. Our result showed that it has also been induced in *EPIC1* overexpressed cells (**Figure 2B, 2C, and 2D**). To further confirm the role of *EPIC1* in regulating the AKT-mTORC1 signaling pathway, we knockdown *EPIC1* by treating these three parental cell lines with *EPIC1* siRNAs. As shown in **Figure 2E,2F, and 2G**, *EPIC1* knockdown decreased the expression of p-ERK and two mTORC1 downstream targets p-S6K and p-4EBP1 without interfering the total S6K or 4EBP1 protein levels. The EPIC1 knockdown efficiency was confirmed by qRT-PCR (**Figure 2E, 2F, and 2G right**). These data suggested that *EPIC1* promotes the AKT-mTORC1 pathway through upregulating phosphorylation levels of AKT and mTROC1 downstream targets.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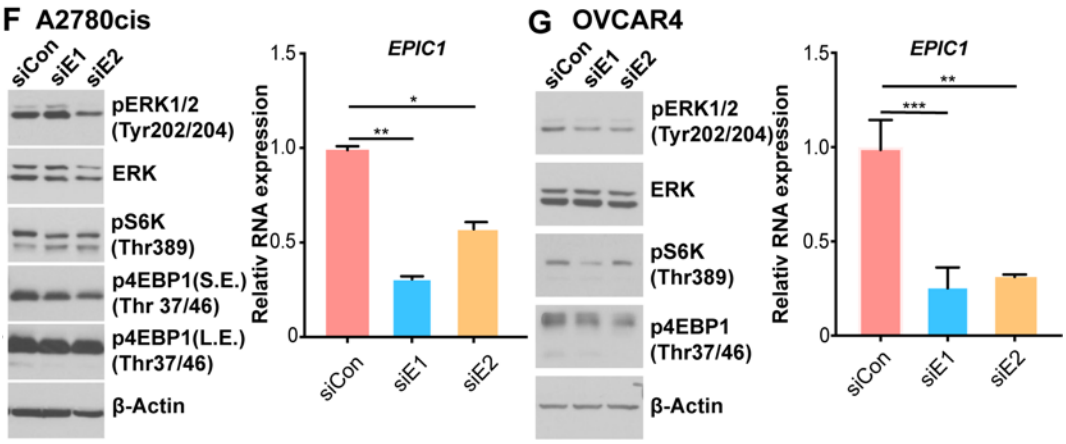


Figure 2. EPIC1 promotes AKT-mTORC1 signaling pathway through upregulating the phosphorylation level of 4EBP1 and S6K

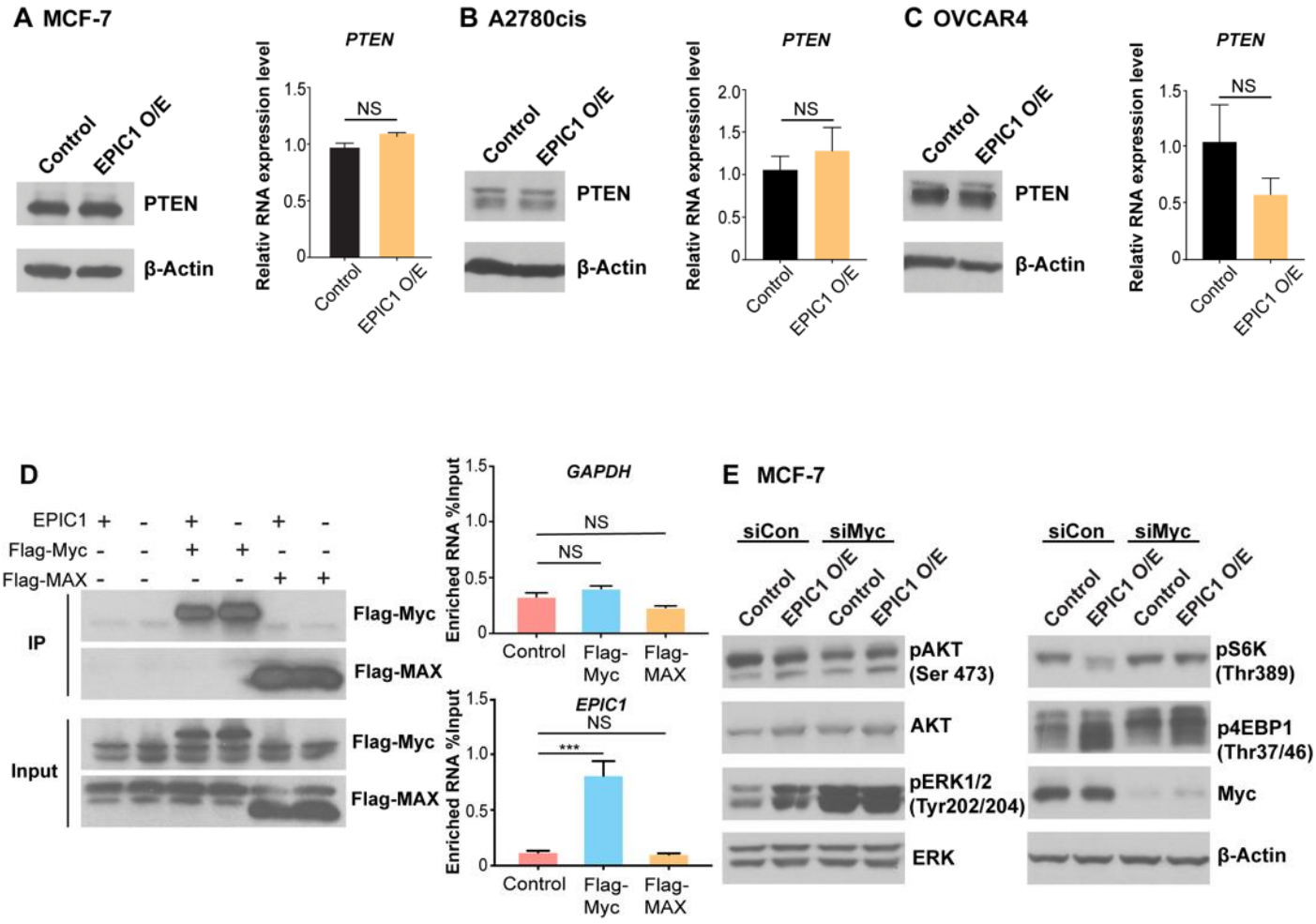
A. qRT-PCR analysis of EPIC1 level in breast and ovarian cancer cell lines. EPIC1 level was normalized to GAPDH. B to D. Western blot of effect on the AKT-mTORC1 signaling pathway in MCF-7 (B), A2780cis (C) and OVCAR4 (D) cells stably overexpressing EPIC1 and a control vector. E to G. Western blot of effect on the AKT-mTORC1 signaling pathway in MCF-7 (E), A2780cis (F) and OVCAR4 (G) cells treated with EPIC1 siRNA and a control siRNA. EPIC1 knockdown efficiency was also shown by qRT-PCR (right). Error bars indicate mean ± SD, n = 3 for technical replicates. *$p < 0.05$, **$p < 0.01$, and ***$p< 0.001$.

3.3 Activation of AKT-mTORC1 signaling pathway by EPIC1 is through the interaction with Myc

There are several critical proteins had been identified as directly regulators of AKT-mTORC1 pathway. Among them, PTEN is a well-known tumor suppressor gene which inhibit AKT-mTORC1 pathway through repressing AKT phosphorylation and activation.[35] We first examined if PTEN mediates *EPIC1*'s promotion of the AKT-mTORC1 signaling pathway.

Western blot data indicated there was no significant changing of PTEN at the protein level in MCF-7, A2780cis, OVCAR4 *EPIC1* overexpressed cells (**Figure 3A, 3B, and 3C left**). Furthermore, qRT-PCR showed comparable PTEN mRNA expression levels between control and *EPIC1* overexpression cells (**Figure 3A, 3B, and 3C right**). Our next question is to address whether lncRNA *EPIC1* promotes the AKT-mTORC1 pathway directly or through another potential protein that binds with *EPIC1*. Previous study had already found that *EPIC1* is located in the nucleus and binds with transcription factor Myc [24]. Since Myc has been reported as a potential regulator of AKT-mTORC1 pathway in previous studies, we reasoned that *EPIC1* may activate the AKT-mTORC1 signaling pathway through the interaction with Myc.[8, 9, 11, 12] In this end, we first confirmed the binding between *EPIC1* and Myc by performing RNA immunoprecipitation assay (RIP) in 293T cells. We have also included Flag-tagged MAX (Myc-associated protein X) which had been demonstrated as a Myc binding transcription factor.[36] Western Blot data showed Flag-Myc and Flag-MAX immunoprecipitation efficiency using Flag beads (**Figure 3D left**). qRT-PCR indicated that compared with control and Flag-MAX samples, only Flag-Myc could successfully pull down *EPIC1* after normalized with input which demonstrated directly binding between lncRNA EPIC1 and Myc (**Figure 3D right**). To further test our hypothesis, we used siRNA to knockdown Myc in MCF-7 control and *EPIC1* overexpressed cells. Western Blot data demonstrated that compared with siControl treatment, Myc knockdown abolished the *EPIC1*'s upregulation effect of p-AKT, p-ERK, and p-4EBP1 (**Figure 3E**). We have also performed the Myc knockdown experiment by treating siRNA in two ovarian cancer cell lines A2780cis (**Figure 3F**) and OVCAR4 (**Figure 3G**); we also found that knockdown Myc abolished *EPIC1*'s upregulation of p-AKT, p-ERK, p-S6K and p-4EBP1 which showed simiar outcomes with MCF-7 cellline. These data revealed that knockdown Myc can successfully

rescue the phosphorylation level of the AKT-mTORC1 signaling pathway and indicated *EPIC1* promotes the AKT-mTORC1 pathway through its interaction with Myc.



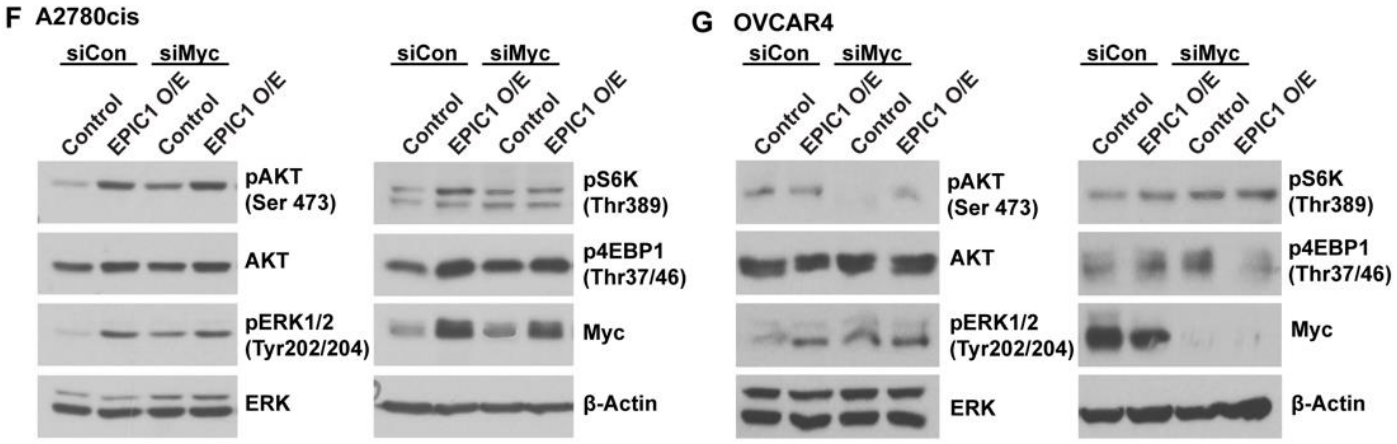


Figure 3. Activation of AKT-mTORC1 signaling pathway by EPIC1 is through the interaction with Myc

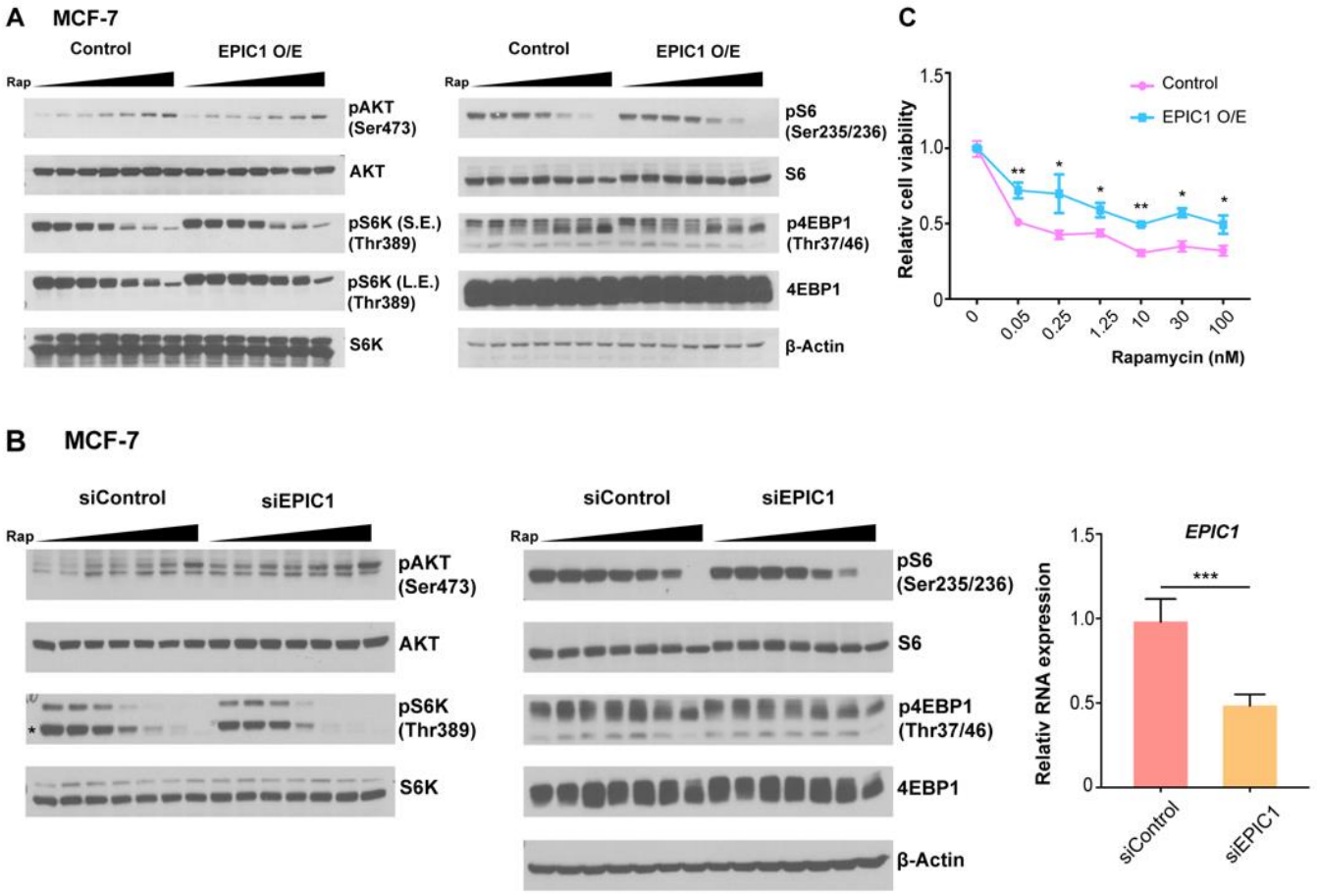
A to C. Western blot and qRT-PCR analysis of effect on PTEN protein and transcription level in MCF-7 (A), A2780cis (B), and OVCAR4 (C) cells stably overexpressing EPIC1 and a control vector. D. qRT-PCR analysis of EPIC1 enrichment by Flag-Myc, Flag-MAX, and control in 293T cells. Western blot of IP efficiency and input was also shown. E to G. Western-Blot of effect on the AKT-mTROC1 signaling pathway in MCF-7 (E), A2780cis (F), and OVCAR4 (G) stably overexpressing EPIC1 or a control vector after treatment with Myc siRNA and a control siRNA for 48 hours. Error bars indicate mean ± SD, n = 3 for technical replicates. *$p < 0.05$, **$p < 0.01$, and ***$p< 0.001$. NS, not significant.

3.4 Activation of AKT-mTORC1 by EPIC1 leads to rapamycin resistance

There are several clinical using drugs and inhibitors that had been reported to treat the AKT-mTORC1 signaling pathway.[12] Rapamycin, also named Sirolimus is a well-known Class I mTORC1 inhibitor; it was clinically used for treating breast cancer.[18] However, drug resistance of rapamycin and other types of mTOR inhibitors is a current challenge in treating cancer

patients.[37, 38] Based on our previous results, we hypothesized that *EPIC1* could lead to mTORC1 inhibitor resistance during the inhibition of AKT-mTORC1 signaling pathway. After treated with rapamycin in MCF-7 control and EPIC1 overexpression cells, western blots showed *EPIC1* overexpression cells had a less depleted expression of p-S6K and p-S6, which are the direct targets of rapamycin, compared with control cells at the same concentrations (**Figure 4A**). This result suggested that EPIC1 may play a critical role in rapamycin resistance. There was no significant difference at protein level of another mTORC1 downstream target p4EBP1, which has been identified by previous study that rapamycin has a strong effect on p-S6K, but a moderate to non-effect on p-4EBP1.[39] To further validate our hypothesis, we also used siRNA to knockdown *EPIC1* (**Figure 4B right**); we detected a more abolished signal at p-S6K and p-S6 expression level after siRNA treatment which suggested that inhibition of *EPIC1* lead to MCF-7 cells more sensitive to rapamycin treatment compared with cells treated with siControl (**Figure 4B left**). MTT experiment was performed by seeding 5,000 MCF-7 control and *EPIC1* overexpressed cells in 96-well plate and treated with different concentrations of rapamycin for 72 hr. Then, cell viability curve showed that *EPIC1* overexpressed cells had a higher IC50 and a more resistance rapamycin appearance (**Figure 4C**). Next, we also demonstrated colony formation assay by seeding MCF-7 control and *EPIC1* overexpressed cells in 12-well plates and treated with five different concentrations of rapamycin as indicated. Results showed that there were more colonies formed in *EPIC1* overexpressed cells compared with control cells (**Figure 4D**) treated at same concentrations. To further determine *EPIC1*'s regulation of rapamycin resistance in ovarian cancer cells, we treated different concentrations of rapamycin to two ovarian cancer cells. Western blot showed rapamycin resistance at p-S6K and p-S6K in A2780cis (**Figure 5A**) and OVCAR4 (**Figure 5B**) *EPIC1* overexpressed cells, respectively. MTT assay has also demonstrated a higher cell

viability in both *EPIC1* overexpression cells, which indicated rapamycin resistant phenomenon (**Figure 5C, 5D**). All these data suggested that the promotion of the AKT-mTORC1 pathway by *EPIC1* will lead to mTORC1 inhibitor-rapamycin resistance.



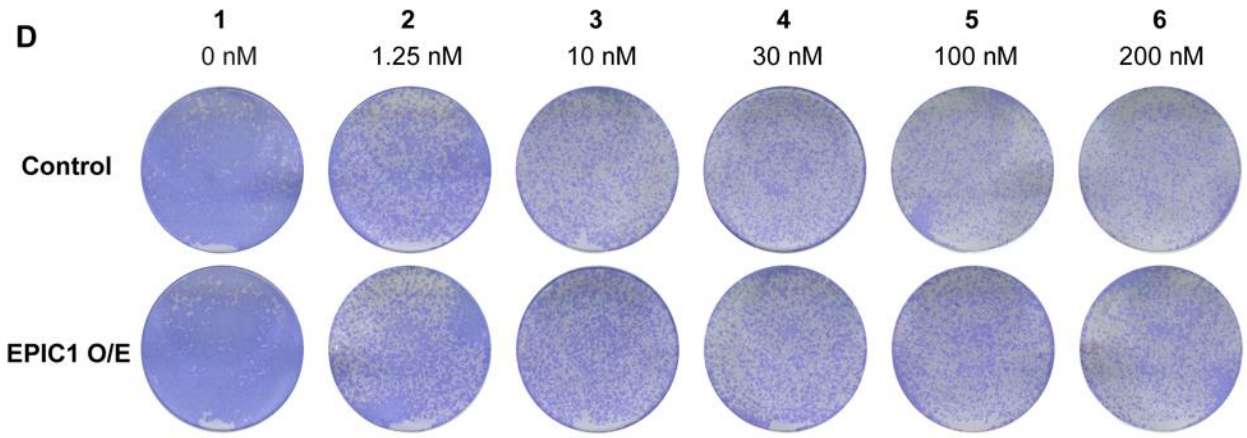
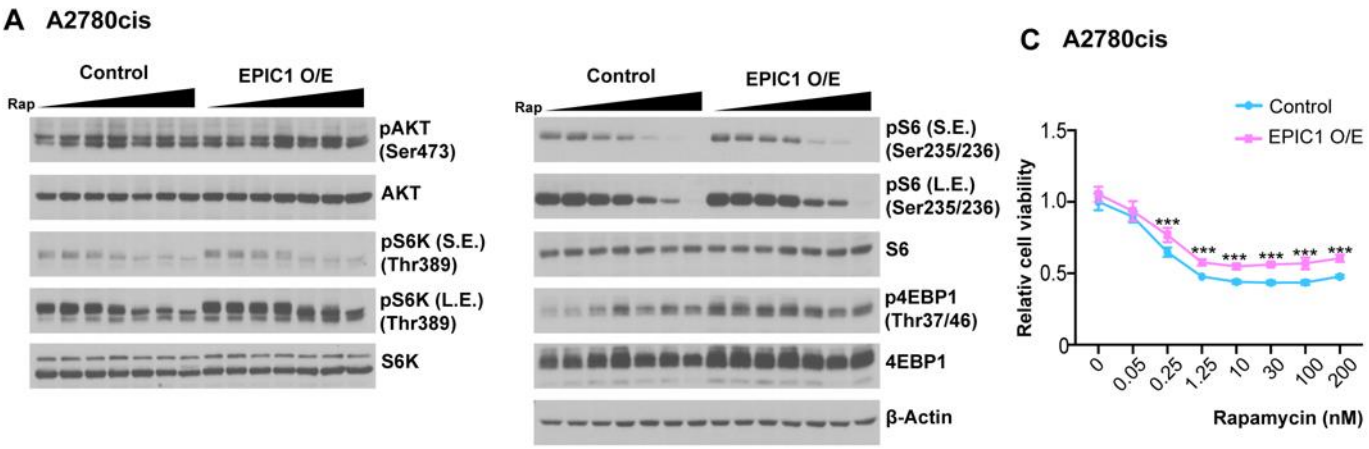


Figure 4. Activation of AKT-mTORC1 by EPIC1 leads to rapamycin resistance in MCF-7 cells

A. Western-Blot of effect on the AKT-mTROC1 signaling pathway in MCF-7 cells stably overexpressing EPIC1 or a control vector after treatment with a serial of rapamycin (Rap: 0, 0.05, 0.1, 0.2, 0.4, 1, 10 nM) 2 hrs. B. Western-Blot of effect on the AKT-mTROC1 signaling pathway in MCF-7 cells treated with a serial of rapamycin as above after transfected for 48 hr with EPIC1 siRNA or a control siRNA. EPIC1 knockdown efficiency was analyzed by qRT-PCR. C. Cell viability was detected by MTT assay after treatment with rapamycin as indicated for 72 hr in MCF-7 cells stably overexpressing EPIC1 or a control vector. D. Colony formation assay of MCF-7 cells stably overexpressing EPIC1 or a control vector after treatment for 7 days with rapamycin as indicated. Error bars indicate mean ± SD, n = 3 for technical replicates. *p < 0.05, **p < 0.01, and ***p< 0.001.



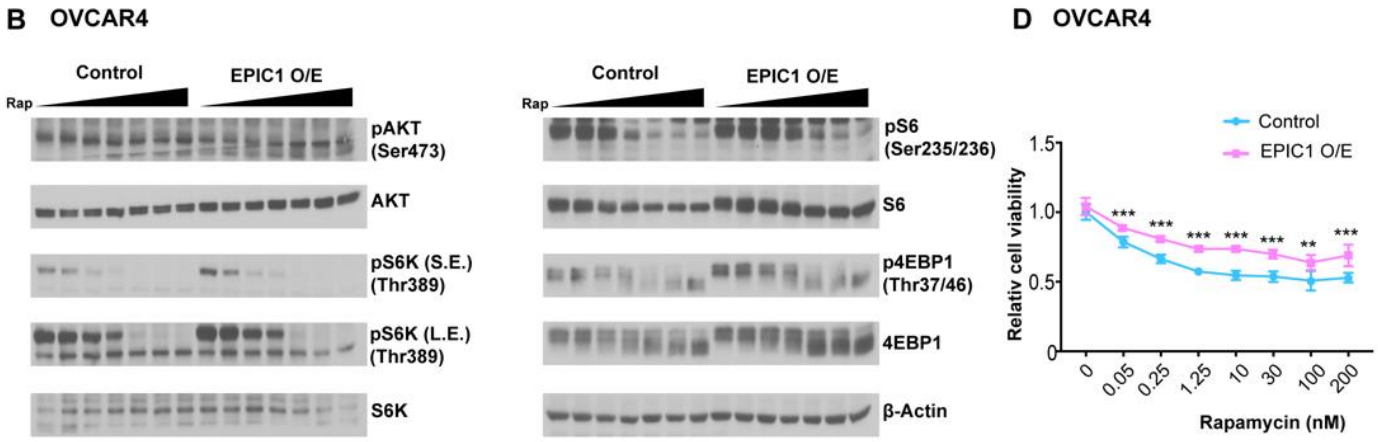


Figure 5. Activation of AKT-mTORC1 by EPIC1 leads to rapamycin resistance in A2780cis and OVCAR4 cells

A and B. Western-Blot of effect on the AKT-mTROC1 signaling pathway in A2780cis (A) and OVCAR4 (B) cells stably overexpressing EPIC1 or a control vector after treatment with a serial of rapamycin (Rap: 0, 0.05, 0.1, 0.2, 0.4, 1, 10 nM) 2 hrs. C and D. Cell viability was detected by MTT assay after treat with rapamycin for 72 hr and 96 hr in OVCAR4 and A2780cis cells stably overexpressing EPIC1 or control vector, respectively. Error bars indicate mean ± SD, n = 3 for technical replicates. *$p < 0.05$, **$p < 0.01$, and ***$p< 0.001$.

4.0 Discussion and future plan

In our study, we demonstrated that Myc binding lncRNA *EPIC1* is a potential regulator of the AKT-mTORC1 signaling pathway. *EPIC1* overexpression activated and upregulated phosphorylation level of crucial kinases involved in the AKT-mTORC1 signaling pathway; on the other hand, knockdown of *EPIC1* inhibited AKT-mTORC1signaling pathway. Moreover, our observation in breast cancer and ovarian cancer cell lines have been further confirmed in cancer patients. In 927 breast and 264 ovarian cancer patients, *the EPIC1* expression level was significantly correlated with genes involved in ribosome biogenesis and protein synthesis that are regulated by the AKT-mTORC1 signaling pathway. These observations suggested that *EPIC1* plays an essential role in AKT-mTORC1 mediated tumorigenesis. We have further shown that knockdown of Myc rescued kinases' upregulated phosphorylation level by *EPIC1*, suggested the regulation of AKT-mTORC1 by *EPIC1* is mediated through Myc protein. Finally, our study demonstrated that *EPIC1* overexpression leads to rapamycin resistance in breast and ovarian cancer cells, which provide a clinical biomarker for rapamycin resistance and potential drug target of cancer therapy. In previous studies, there are FDA approved and clinically used drugs target the AKT-mTORC1 signaling pathway to treat cancers.[13] Rapamycin, along with its derivatives and other mTOR inhibitors, has been demonstrated to have a therapeutic effect in cancer patients.[40] However, emerging evidence indicates that drug resistance has become a crucial problem in patients treated with rapamycin. The mechanisms of rapamycin resistance include activation of upstream regulators of the AKT-mTORC1 signaling pathway in different cancer types.[37, 38] Previous studies also showed that drug resistance exists in patients who treated with rapamycin derivatives and other types of mTOR inhibitors.[41, 42] To overcome mTOR inhibitors' drug

resistance, combination therapies have been examined in preclinical and clinical studies. For example, mTOR inhibitor RAD001 and hormone therapy drug Tamoxifen together can achieve a synergistic therapeutic effect in ovarian and breast cancers, which significantly decreases cell growth and enhance Tamoxifen derived apoptosis.[43] Another study demonstrated that a combination of mTOR inhibitor RAD001 and PI3K/mTOR dual kinase inhibitor NVP-BEZ235 synergistically inhibited growth of human non-small cell lung cancer cells. This combination therapy can also effectively overcome rapamycin-resistant cancer cells.[44] On the other hand, emerging evidence has shown that lncRNAs can also be targeted in tumor. In vivo preclinical studies have shown that drugs targeting-lncRNAs can therapeutically inhibit breast cancer metastasis.[45, 46] Another study in vitro and in vivo also demonstrated that targeting lncRNA can inhibit multiple melanoma cancer cell proliferation and trigger cell apoptosis.[47] These studies suggest that lncRNAs can be target therapeutically to inhibit its functions and potentially increase patients' survival and have higher prognostic rates. Together with our study, target lncRNA *EPIC1* and use mTOR inhibitors can be a future combination therapy treating breast and ovarian cancer patients. However, the clinically actionable biomarker that can robustly indicate rapamycin and other mTOR inhibitors' resistance in the tumor is missing. In recent publications, clinical studies have shown that lncRNAs could be potential biomarkers in poor prognostic cancers. For example, one clinical study demonstrated that colorectal cancer patients who have higher expression levels of lncRNA MALAT1 showed worse disease-free survival and overall survival compared with patients who have lower MALAT1 expression. This result suggested that MALAT1 may serve as a prognostic biomarker in colorectal cancer patients.[48] There is another preclinical study also revealed that expression of ten lncRNAs as potential candidates associated with stage progression in ovarian cancer patients, which suggested these lncRNAs as potential

prognostic biomarkers in ovarian cancer.[49] All these evidences together suggest that lncRNA *EPIC1* may be a future biomarker for predicting survival in breast and ovarian cancer patients. Meanwhile, we have not only identified a novel function of *EPIC1*; most importantly, the overexpression of *EPIC1* can serve as a clinically actionable biomarker in cancer patients.

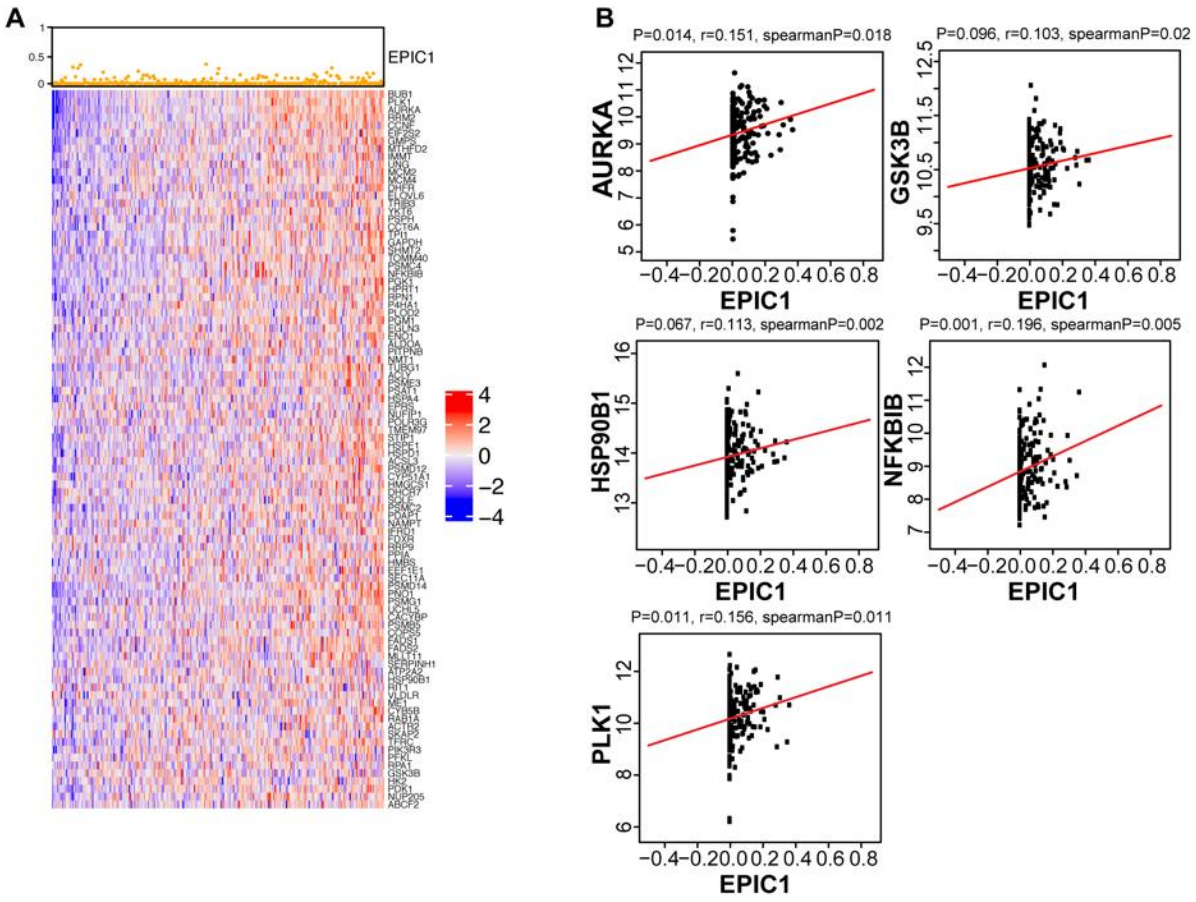
Transcription factor Myc is often expressed in cancers and increase upregulation of its downstream oncogenes, which relate to cell proliferation and tumor formation.[50] One recent study showed that Myc is a potential regulator of glycolysis and metabolic signaling pathway and it stimulated glutamine synthetase (GS) expression through demethylation of promoter region; inhibition of GS expression level decreased cell proliferation and cell survival.[51] Metabolic pathway plays an important role in activating T cells functions.[47,52] However, metabolic pathways have been manipulated and remolded in tumor microenvironment, which is a new challenge during the treatment of immunotherapy.[26, 53] AKT-mTORC1 signaling pathway can regulate tumor cell glycolysis and metabolic pathway in tumor microenvironment. Inhibition of AKT-mTORC1 lead to stimulation and activation of T cells, and further treat with antibodies to block immune co-inhibitory checkpoints such as PD-1, PD-L1 can improve efficacy of immunotherapy.[38] In summary, these studies suggest us that target lncRNA *EPIC1*, block AKT-mTORC1 signaling pathway by rapamycin or other mTOR inhibitors, and combine with treating immunotherapy together to cancer patients may be a potential combination therapy which benefits in clinical research.

We have previously performed Myc ChIP-seq and RNA-seq in control and *EPIC1* overexpression MCF-7 cell lines. We have identified that there are multiple genes, which are all involved in cell metabolism, in both sequencing results that are significantly upregulated in MCF-7 *EPIC1* overexpression cells. These studies suggest that lncRNA EPIC1 may potentially regulate

cancer cell metabolism through its interaction with Myc. As mentioned in the introduction, metabolism also could affect the efficiency of immune cells at tumor microenvironment. According to these results, we hypothesized that lncRNA *EPIC1* could suppress immune cells function through promoting cancer cell metabolism at tumor microenvironment. In the future, we will study more about how lncRNA *EPIC1* regulate cell metabolism and inhibit immune cell effects.

Appendix A Supplementary Materials

A.1 High expression level of EPIC1 is correlated with activated mTORC1 signaling pathway in ovarian cancer patients



A. Expression analysis showing that EPIC1 expression is associated with hallmark mTORC1 genes (Figure 1 B right) in 264 ovarian cancer patients. B. Correlation analysis between specific genes that regulate mTORC1 signaling pathway and EPIC1 expression in ovarian cancer patients.

Bibliography

[1] J.A. Engelman, Targeting PI3K signalling in cancer: opportunities, challenges and limitations, Nat Rev Cancer 9(8) (2009) 550-62.
[2] D.M. Sabatini, Twenty-five years of mTOR: Uncovering the link from nutrients to growth, Proc Natl Acad Sci U S A 114(45) (2017) 11818-11825.
[3] M. Laplante, D.M. Sabatini, mTOR signaling in growth control and disease, Cell 149(2) (2012) 274-93.
[4] X.G. Chen, F. Liu, X.F. Song, Z.H. Wang, Z.Q. Dong, Z.Q. Hu, R.Z. Lan, W. Guan, T.G. Zhou, X.M. Xu, H. Lei, Z.Q. Ye, E.J. Peng, L.H. Du, Q.Y. Zhuang, Rapamycin regulates Akt and ERK phosphorylation through mTORC1 and mTORC2 signaling pathways, Mol Carcinog 49(6) (2010) 603-10.
[5] J.N. Winter, T.E. Fox, M. Kester, L.S. Jefferson, S.R. Kimball, Phosphatidic acid mediates activation of mTORC1 through the ERK signaling pathway, Am J Physiol Cell Physiol 299(2) (2010) C335-44.
[6] J.N. Winter, L.S. Jefferson, S.R. Kimball, ERK and Akt signaling pathways function through parallel mechanisms to promote mTORC1 signaling, Am J Physiol Cell Physiol 300(5) (2011) C1172-80.
[7] E. Santini, M. Feyder, G. Gangarossa, H.S. Bateup, P. Greengard, G. Fisone, Dopamine- and cAMP-regulated phosphoprotein of 32-kDa (DARPP-32)-dependent activation of extracellular signal-regulated kinase (ERK) and mammalian target of rapamycin complex 1 (mTORC1) signaling in experimental parkinsonism, J Biol Chem 287(33) (2012) 27806-12.
[8] A.L. Hsieh, Z.E. Walton, B.J. Altman, Z.E. Stine, C.V. Dang, MYC and metabolism on the path to cancer, Semin Cell Dev Biol 43 (2015) 11-21.
[9] E.V. Schmidt, M.J. Ravitz, L. Chen, M. Lynch, Growth controls connect: interactions between c-myc and the tuberous sclerosis complex-mTOR pathway, Cell Cycle 8(9) (2009) 1344-51.
[10] C.J. Lin, A. Malina, J. Pelletier, c-Myc and eIF4F constitute a feedforward loop that regulates cell growth: implications for anticancer therapy, Cancer Res 69(19) (2009) 7491-4.
[11] R. Vartanian, J. Masri, J. Martin, C. Cloninger, B. Holmes, N. Artinian, A. Funk, T. Ruegg, J. Gera, AP-1 regulates cyclin D1 and c-MYC transcription in an AKT-dependent manner in response to mTOR inhibition: role of AIP4/Itch-mediated JUNB degradation, Mol Cancer Res 9(1) (2011) 115-30.
[12] J.F. Gera, I.K. Mellinghoff, Y. Shi, M.B. Rettig, C. Tran, J.H. Hsu, C.L. Sawyers, A.K. Lichtenstein, AKT activity determines sensitivity to mammalian target of rapamycin (mTOR) inhibitors by regulating cyclin D1 and c-myc expression, J Biol Chem 279(4) (2004) 2737-46.
[13] S.Y. Sun, mTOR kinase inhibitors as potential cancer therapeutic drugs, Cancer Lett 340(1) (2013) 1-8.

[14] W.C. Noh, W.H. Mondesire, J. Peng, W. Jian, H. Zhang, J. Dong, G.B. Mills, M.C. Hung, F. Meric-Bernstam, Determinants of rapamycin sensitivity in breast cancer cells, Clin Cancer Res 10(3) (2004) 1013-23.
[15] S. Vignot, S. Faivre, D. Aguirre, E. Raymond, mTOR-targeted therapy of cancer with rapamycin derivatives, Ann Oncol 16(4) (2005) 525-37.
[16] M. Hidalgo, E.K. Rowinsky, The rapamycin-sensitive signal transduction pathway as a target for cancer therapy, Oncogene 19(56) (2000) 6680-6.
[17] S. Chan, Targeting the mammalian target of rapamycin (mTOR): a new approach to treating cancer, Br J Cancer 91(8) (2004) 1420-4.
[18] S. Chan, M.E. Scheulen, S. Johnston, K. Mross, F. Cardoso, C. Dittrich, W. Eiermann, D. Hess, R. Morant, V. Semiglazov, M. Borner, M. Salzberg, V. Ostapenko, H.J. Illiger, D. Behringer, N. Bardy-Bouxin, J. Boni, S. Kong, M. Cincotta, L. Moore, Phase II study of temsirolimus (CCI-779), a novel inhibitor of mTOR, in heavily pretreated patients with locally advanced or metastatic breast cancer, J Clin Oncol 23(23) (2005) 5314-22.
[19] J.R. Evans, F.Y. Feng, A.M. Chinnaiyan, The bright side of dark matter: lncRNAs in cancer, J Clin Invest 126(8) (2016) 2775-82.
[20] M. Morlando, M. Ballarino, A. Fatica, Long Non-Coding RNAs: New Players in Hematopoiesis and Leukemia, Front Med (Lausanne) 2 (2015) 23.
[21] A.M. Schmitt, H.Y. Chang, Long Noncoding RNAs in Cancer Pathways, Cancer Cell 29(4) (2016) 452-463.
[22] X. Liu, Z.D. Xiao, L. Han, J. Zhang, S.W. Lee, W. Wang, H. Lee, L. Zhuang, J. Chen, H.K. Lin, J. Wang, H. Liang, B. Gan, LncRNA NBR2 engages a metabolic checkpoint by regulating AMPK under energy stress, Nat Cell Biol 18(4) (2016) 431-42.
[23] K. Yacqub-Usman, M.R. Pickard, G.T. Williams, Reciprocal regulation of GAS5 lncRNA levels and mTOR inhibitor action in prostate cancer cells, Prostate 75(7) (2015) 693-705.
[24] Z. Wang, B. Yang, M. Zhang, W. Guo, Z. Wu, Y. Wang, L. Jia, S. Li, N. Cancer Genome Atlas Research, W. Xie, D. Yang, lncRNA Epigenetic Landscape Analysis Identifies EPIC1 as an Oncogenic lncRNA that Interacts with MYC and Promotes Cell-Cycle Progression in Cancer, Cancer Cell 33(4) (2018) 706-720 e9.
[25] N.N. Pavlova, C.B. Thompson, The Emerging Hallmarks of Cancer Metabolism, Cell Metab 23(1) (2016) 27-47.
[26] T. Le Bourgeois, L. Strauss, H.I. Aksoylar, S. Daneshmandi, P. Seth, N. Patsoukis, V.A. Boussiotis, Targeting T Cell Metabolism for Improvement of Cancer Immunotherapy, Front Oncol 8 (2018) 237.
[27] Z.E. Stine, Z.E. Walton, B.J. Altman, A.L. Hsieh, C.V. Dang, MYC, Metabolism, and Cancer, Cancer Discov 5(10) (2015) 1024-39.
[28] D.M. Miller, S.D. Thomas, A. Islam, D. Muench, K. Sedoris, c-Myc and cancer metabolism, Clin Cancer Res 18(20) (2012) 5546-53.
[29] Y. Wang, Z. Wang, J. Xu, J. Li, S. Li, M. Zhang, D. Yang, Systematic identification of non-coding pharmacogenomic landscape in cancer, Nat Commun 9(1) (2018) 3192.
[30] A. Subramanian, P. Tamayo, V.K. Mootha, S. Mukherjee, B.L. Ebert, M.A. Gillette, A. Paulovich, S.L. Pomeroy, T.R. Golub, E.S. Lander, J.P. Mesirov, Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles, Proc Natl Acad Sci U S A 102(43) (2005) 15545-50.
[31] V.K. Mootha, C.M. Lindgren, K.F. Eriksson, A. Subramanian, S. Sihag, J. Lehar, P. Puigserver, E. Carlsson, M. Ridderstrale, E. Laurila, N. Houstis, M.J. Daly, N. Patterson, J.P.

Mesirov, T.R. Golub, P. Tamayo, B. Spiegelman, E.S. Lander, J.N. Hirschhorn, D. Altshuler, L.C. Groop, PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes, Nat Genet 34(3) (2003) 267-73.
[32] S.K. Pal, M. He, T. Tong, H. Wu, X. Liu, C. Lau, J.H. Wang, C. Warden, X. Wu, S. Signoretti, T.K. Choueiri, J.A. Karam, J.O. Jones, RNA-seq reveals aurora kinase-driven mTOR pathway activation in patients with sarcomatoid metastatic renal cell carcinoma, Mol Cancer Res 13(1) (2015) 130-7.
[33] X. Guo, W.D. Snider, B. Chen, GSK3beta regulates AKT-induced central nervous system axon regeneration via an eIF2Bepsilon-dependent, mTORC1-independent pathway, Elife 5 (2016) e11903.
[34] Z. Fu, D. Wen, The Emerging Role of Polo-Like Kinase 1 in Epithelial-Mesenchymal Transition and Tumor Metastasis, Cancers (Basel) 9(10) (2017).
[35] N.M. Davis, M. Sokolosky, K. Stadelman, S.L. Abrams, M. Libra, S. Candido, F. Nicoletti, J. Polesel, R. Maestro, A. D'Assoro, L. Drobot, D. Rakus, A. Gizak, P. Laidler, J. Dulinska-Litewka, J. Basecke, S. Mijatovic, D. Maksimovic-Ivanic, G. Montalto, M. Cervello, T.L. Fitzgerald, Z. Demidenko, A.M. Martelli, L. Cocco, L.S. Steelman, J.A. McCubrey, Deregulation of the EGFR/PI3K/PTEN/Akt/mTORC1 pathway in breast cancer: possibilities for therapeutic intervention, Oncotarget 5(13) (2014) 4603-50.
[36] E.M. Blackwood, R.N. Eisenman, Max: a helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with Myc, Science 251(4998) (1991) 1211-7.
[37] Y. Chen, Y. Zheng, D.A. Foster, Phospholipase D confers rapamycin resistance in human breast cancer cells, Oncogene 22(25) (2003) 3937-42.
[38] P.A. Gruppuso, J.M. Boylan, J.A. Sanders, The physiology and pathophysiology of rapamycin resistance: implications for cancer, Cell Cycle 10(7) (2011) 1050-8.
[39] A.Y. Choo, S.O. Yoon, S.G. Kim, P.P. Roux, J. Blenis, Rapamycin differentially inhibits S6Ks and 4E-BP1 to mediate cell-type-specific repression of mRNA translation, Proc Natl Acad Sci U S A 105(45) (2008) 17414-9.
[40] C. Delbaldo, S. Albert, C. Dreyer, M.P. Sablin, M. Serova, E. Raymond, S. Faivre, Predictive biomarkers for the activity of mammalian target of rapamycin (mTOR) inhibitors, Target Oncol 6(2) (2011) 119-24.
[41] J. Xie, X. Wang, C.G. Proud, mTOR inhibitors in cancer therapy, F1000Res 5 (2016).
[42] S. Huang, P.J. Houghton, Mechanisms of resistance to rapamycins, Drug Resist Updat 4(6) (2001) 378-91.
[43] O. Treeck, B. Wackwitz, U. Haus, O. Ortmann, Effects of a combined treatment with mTOR inhibitor RAD001 and tamoxifen in vitro on growth and apoptosis of human cancer cells, Gynecol Oncol 102(2) (2006) 292-9.
[44] C.X. Xu, Y. Li, P. Yue, T.K. Owonikoko, S.S. Ramalingam, F.R. Khuri, S.Y. Sun, The combination of RAD001 and NVP-BEZ235 exerts synergistic anticancer activity against non-small cell lung cancer in vitro and in vivo, PLoS One 6(6) (2011) e20899.
[45] Z. Xing, A. Lin, C. Li, K. Liang, S. Wang, Y. Liu, P.K. Park, L. Qin, Y. Wei, D.H. Hawke, M.C. Hung, C. Lin, L. Yang, lncRNA directs cooperative epigenetic regulation downstream of chemokine signals, Cell 159(5) (2014) 1110-1125.
[46] E.J. Richards, G. Zhang, Z.P. Li, J. Permuth-Wey, S. Challa, Y. Li, W. Kong, S. Dan, M.M. Bui, D. Coppola, W.M. Mao, T.A. Sellers, J.Q. Cheng, Long non-coding RNAs (LncRNA) regulated by transforming growth factor (TGF) beta: LncRNA-hit-mediated TGFbeta-induced epithelial to mesenchymal transition in mammary epithelia, J Biol Chem 290(11) (2015) 6857-67.

[47] N. Amodio, M.A. Stamato, G. Juli, E. Morelli, M. Fulciniti, M. Manzoni, E. Taiana, L. Agnelli, M.E.G. Cantafio, E. Romeo, L. Raimondi, D. Caracciolo, V. Zuccala, M. Rossi, A. Neri, N.C. Munshi, P. Tagliaferri, P. Tassone, Drugging the lncRNA MALAT1 via LNA gapmeR ASO inhibits gene expression of proteasome subunits and triggers anti-multiple myeloma activity, Leukemia 32(9) (2018) 1948-1957.
[48] H.T. Zheng, D.B. Shi, Y.W. Wang, X.X. Li, Y. Xu, P. Tripathi, W.L. Gu, G.X. Cai, S.J. Cai, High expression of lncRNA MALAT1 suggests a biomarker of poor prognosis in colorectal cancer, Int J Clin Exp Pathol 7(6) (2014) 3174-81.
[49] M. Zhou, X. Wang, H. Shi, L. Cheng, Z. Wang, H. Zhao, L. Yang, J. Sun, Characterization of long non-coding RNA-associated ceRNA network to reveal potential prognostic lncRNA biomarkers in human ovarian cancer, Oncotarget 7(11) (2016) 12598-611.
[50] M. Zhou, H. Zhao, Z. Wang, L. Cheng, L. Yang, H. Shi, H. Yang, J. Sun, Identification and validation of potential prognostic lncRNA biomarkers for predicting survival in patients with multiple myeloma, J Exp Clin Cancer Res 34 (2015) 102.
[51] A.J. Bott, I.C. Peng, Y. Fan, B. Faubert, L. Zhao, J. Li, S. Neidler, Y. Sun, N. Jaber, D. Krokowski, W. Lu, J.A. Pan, S. Powers, J. Rabinowitz, M. Hatzoglou, D.J. Murphy, R. Jones, S. Wu, G. Girnun, W.X. Zong, Oncogenic Myc Induces Expression of Glutamine Synthetase through Promoter Demethylation, Cell Metab 22(6) (2015) 1068-77.
[52] C.H. Chang, E.L. Pearce, Emerging concepts of T cell metabolism as a target of immunotherapy, Nat Immunol 17(4) (2016) 364-8.
[53] S. Kouidhi, F. Ben Ayed, A. Benammar Elgaaied, Targeting Tumor Metabolism: A New Challenge to Improve Immunotherapy, Front Immunol 9 (2018) 353.