npg

ORIGINAL ARTICLE

Glycogen synthase kinase-3β positively regulates protein synthesis and cell proliferation through the regulation of translation initiation factor 4E-binding protein 1

S Shin[1], L Wolgamott[1], J Tcherkezian[2], S Vallabhapurapu[1], Y Yu[3], PP Roux[2] and S-O Yoon[1]

Protein synthesis has a key role in the control of cell proliferation, and its deregulation is associated with pathological conditions, notably cancer. Rapamycin, an inhibitor of mammalian target of rapamycin complex 1 (mTORC1), was known to inhibit protein synthesis. However, it does not substantially inhibit protein synthesis and cell proliferation in many cancer types. We were interested in finding a novel target in rapamycin-resistant cancer. The rate-limiting factor for translation is eukaryotic translation initiation factor 4E (eIF4E), which is negatively regulated by eIF4E-binding protein 1 (4E-BP1). Here, we provide evidence that glycogen synthase kinase (GSK)-3β promotes cell proliferation through positive regulation of protein synthesis. We found that GSK-3β phosphorylates and inactivates 4E-BP1, thereby increasing eIF4E-dependent protein synthesis. Considering the clinical relevance of pathways regulating protein synthesis, our study provides a promising new strategy and target for cancer therapy.

INTRODUCTION

GSK-3 has important roles in cell metabolism and neurodevelopmental processes. Therefore, dysregulation of GSK-3 is involved in type 2 diabetes and neurological disorders.[1,2] However, the role of GSK-3 in cancer is controversial. Early studies suggested that GSK-3 was a negative regulator of cancer cell proliferation.[3] Therefore, use of GSK-3 inhibitors has been questioned because of a concern that inhibition of GSK-3 may promote oncogenesis by activating pathways stimulating cell proliferation.[4] However, no direct in vivo evidence has indicated that GSK-3 inhibitors promote tumor development.[4–6] Moreover, Wang et al.[7,8] recently demonstrated that a GSK-3 inhibitor prolonged the survival of animals with leukemia. In addition, growing evidence supports the idea that GSK-3 has a positive role in cancer cell proliferation. For example, GSK-3β overexpression and high GSK-3 activity has been found in many cancers,[3,6] and GSK-3 inhibition reduced the survival of various cancer cell lines and predisposed them to undergo apoptosis.[6] Several reports indicate certain pathways in GSK-3β's role in tumorigenesis and tumor progression. For example, GSK-3β regulates nuclear factor-κB (NF-κB), Akt, Notch signaling, p53, the oncoprotein Maf and the homeobox gene.[7,9–15] Therefore, the overall conclusion from these recent studies is that GSK-3 is a positive regulator of cancer cell proliferation and survival and that GSK-3 activity promotes tumorigenesis and tumor progression.[3,6,16]

We recently showed that GSK-3 positively regulated the 70-kDa ribosomal protein S6 Kinase 1 (S6K1).[9] S6K1 is a well-known substrate of mTORC1. Rapamycin, an mTORC1 inhibitor, completely inhibits the activity of S6K1. In many breast cancer cell lines, rapamycin decreases cell proliferation through inhibition of S6K1. However, some breast cancer cell lines such as triple-negative breast cancer (ER −, PR − and HER2 −), are resistant to

rapamycin, and rapamycin does not suppress proliferation of these cells despite effective S6K1 inhibition.[17,18] This suggests that S6K1 is not in the primary proliferation-regulating pathway in these cells. We showed that GSK-3 inhibition or rapamycin decreased proliferation of rapamycin-sensitive breast cancer cells through inhibition of S6K1.[9] We then asked whether GSK-3 could regulate the proliferation of rapamycin-resistant breast cancer cells. We found that proliferation of rapamycin-resistant breast cancer cells was markedly decreased by GSK-3 inhibition. These results suggested that mechanisms other than S6K1 regulation underlie the positive role of GSK-3 in the proliferation of breast cancer cells, especially rapamycin-resistant cancer cells. In this report, we show that GSK-3 positively regulates breast cancer cell proliferation by controlling protein synthesis.
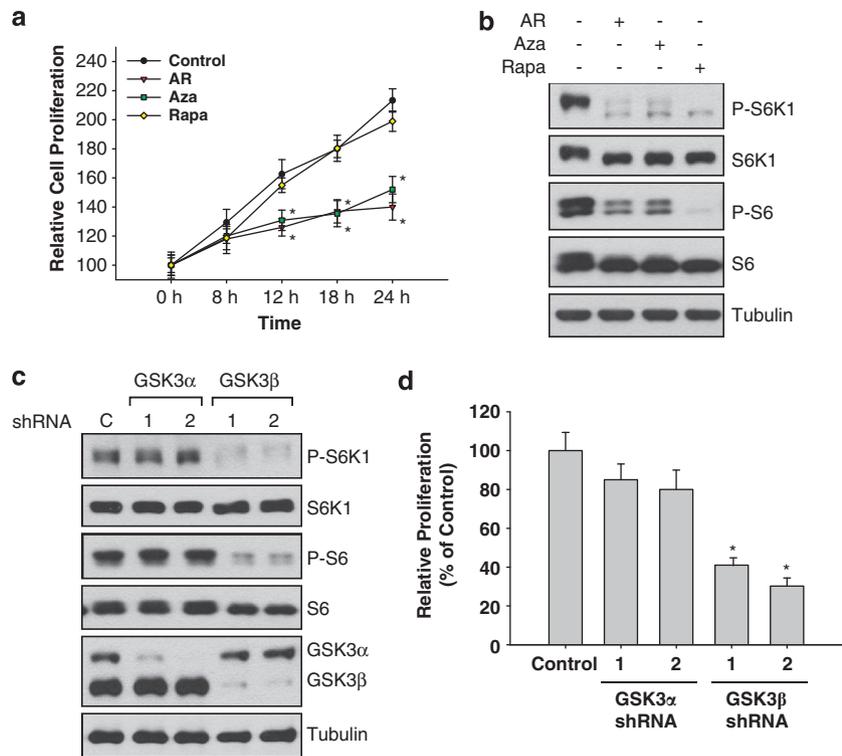
RESULTS

GSK-3 positively regulates cell proliferation

We first asked whether GSK-3 could regulate the proliferation of rapamycin-resistant breast cancer cells in which complete inhibition of S6K1 activity by rapamycin does not affect cell proliferation. For this, we used the rapamycin-resistant breast cancer cell line, HCC1806 and two specific inhibitors of GSK-3, AR-A014418 and 1-Azakenpaullone. As expected, these GSK-3 inhibitors were effective in regulating a well-known GSK-3 substrate, glycogen synthase (GS) (Supplementary Figure S1A). Cells were treated with the GSK-3 inhibitors or rapamycin, and cell proliferation was determined. Unlike rapamycin, the GSK-3 inhibitors suppressed cell proliferation (Figure 1a), even though all three compounds reduced S6K1 phosphorylation (Figure 1b). To examine the possibility that the decreased cell proliferation

[1]Department of Cancer and Cell Biology, University of Cincinnati College of Medicine, Cincinnati, OH, USA; [2]Department of Pathology and Cell Biology, Institute for Research in Immunology and Cancer, Université de Montréal, Montréal, Quebec, Canada and [3]Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, TX, USA. Correspondence: Dr S-O Yoon, Department of Cancer and Cell Biology, University of Cincinnati College of Medicine, 3125 Eden Avenue, Cincinnati, OH 45267, USA.
E-mail: yoonsh@ucmail.uc.edu

Figure 1. GSK-3β positively regulates cell proliferation. Data are representative of at least three independent experiments. Where applicable, data are the means ± s.e.m. of three separate experiments performed in triplicate. Results were statistically significant (*$P < 0.01$) as assessed by using the Student's t-test. (**a**) HCC1806 cells growing in the presence of serum were treated with AR-A014418 (20 μM), 1-Azakenpaullone (30 μM), or rapamycin (20 nM) for the indicated time after which cell numbers were counted. (**b**) Immunoblot analysis was performed on HCC1806 cells treated with AR-A014418 (20 μM), 1-Azakenpaullone (30 μM), or rapamycin (20 nM) for 24 h. (**c, d**) Stable HCC1806 cells with GSK-3α or GSK-3β knockdown were generated. Immunoblot analysis was performed (**c**) and the rate of proliferation was measured by counting cell numbers (**d**).

might be due to cell death, we measured cell death. As shown in Supplementary Figure S1B, cell death rates were between 4 and 7% in control cells and cells treated with GSK-3 inhibitors, suggesting that there is not much difference in cell-survival rates between these groups. To determine if decreased cell proliferation by GSK-3 inhibitors was a general phenomenon, we repeated the experiment with a different rapamycin-resistant breast cancer cell line, AU565. GSK-3 inhibitors decreased GS phosphorylation (Supplementary Figure S1A), S6K1 phosphorylation (Supplementary Figure S1C) and cell proliferation (Supplementary Figure S1D). Rapamycin also blocked S6K1 phosphorylation (Supplementary Figure S1C) but did not change the cell proliferation rate (Supplementary Figure S1D). We also screened and used rapamycin-sensitive breast cancer cell lines, HCC1937 and SUM159. GSK-3 inhibitors decreased phosphorylation of GS (Supplementary Figure S1A). In these cell lines, rapamycin and GSK-3 inhibitors decreased S6K1 phosphorylation (Supplementary Figure S1C) and cell proliferation (Supplementary Figure S1D). These results suggest that GSK-3 can positively regulate cell proliferation independent of rapamycin sensitivity.

Mammalian GSK-3 has two isoforms, GSK-3α and GSK-3β. To determine which isoform of GSK-3 regulates cell proliferation, we generated stable GSK-3α and GSK-3β knockdown cells. As shown in Figure 1c, GSK-3β knockdown decreased S6K1 activity dramatically. We next measured cell proliferation. The GSK-3α knockdown had only a moderate effect on cell proliferation, but knockdown of GSK-3β profoundly decreased cell proliferation (Figure 1d), suggesting that GSK-3β is a major positive regulator of cell proliferation. We observed the same results in other cell lines (Supplementary Figures S1E and S1F).

To further study the role of GSK-3 in cell proliferation, we used three-dimensional (3D) culture systems. Compared to control or rapamycin-treated cells, HCC1806 cells treated with GSK-3 inhibitors formed acini approximately three-fold smaller than those formed by control cells (Supplementary Figure S2A). In all cell lines used, we observed the same results: cells treated with GSK-3 inhibitors showed smaller colony size irrespective of rapamycin sensitivity (Supplementary Figure S2A). We measured the cell proliferation rate and found that GSK-3 inhibitors reduced cell proliferation in 3D culture systems (Supplementary Figure S2B). We also found that knockdown of GSK-3β, but not GSK-3α, inhibited the increase in acini size (Supplementary Figure S2C) and cell proliferation (Supplementary Figure S2D). Taken together, these results suggest that GSK-3β positively regulates cell proliferation.

To determine the function of GSK-3 in tumor growth *in vivo*, we used a mouse xenograft tumor model. After tumors were established, mice were treated with AR-A014418. All mice tolerated the inhibitor well. No significant adverse events or marked difference in mean body weight was observed during the treatment (Figure 2a). GSK-3 inhibitor treatment significantly reduced tumor volume compared with control (Figures 2b and c). We next used stable control or GSK-3β knockdown cells. As shown in Figures 2d and e, GSK-3β knockdown cells showed a dramatic reduction in tumor growth. These results suggest that GSK-3 is an important regulator of tumor growth *in vivo*.

GSK-3β is a positive regulator of protein synthesis
We were curious about the mechanisms by which GSK-3β positively regulated cell proliferation. Protein synthesis has a key
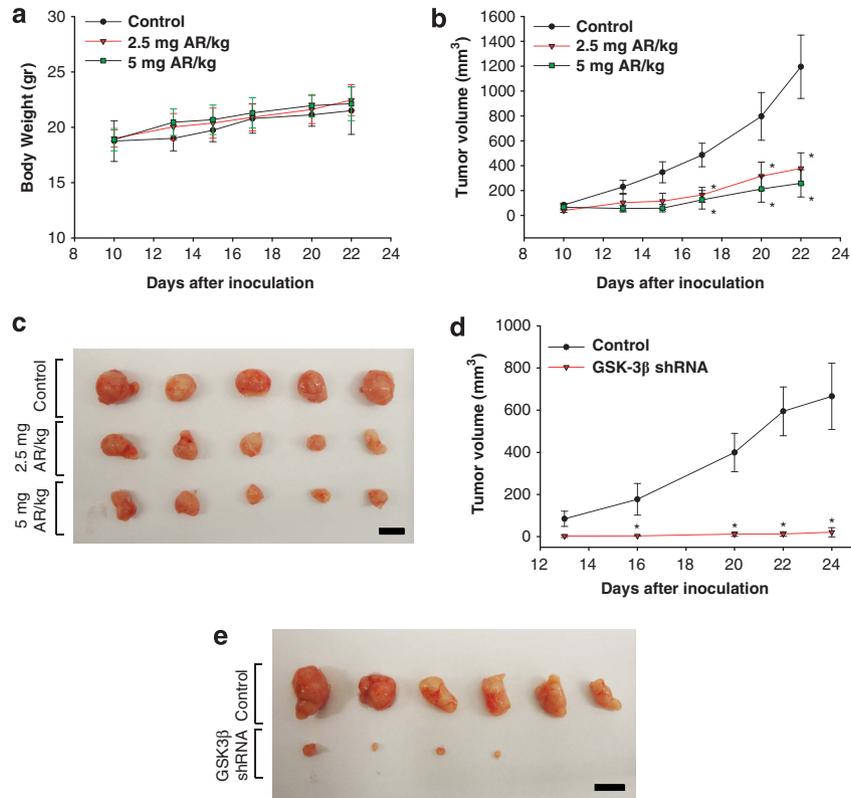
Figure 2. GSK-3β positively regulates cell proliferation *in vivo*. (**a**–**c**) HCC1806 cells were injected subcutaneously into nude mice. When the tumors were palpable, mice were grouped into three and treated with DMSO or AR-A014418 (2.5 mg kg^{-1} or 5 mg per kg body weight) as described in Materials and methods. Mouse body weight (**a**) and tumor growth was measured (*$P < 0.01$) (**b**), and images were taken with tumors isolated from the mice at the end of treatment (**c**). Scale bar, 1 cm. (**d**, **e**) Stable HCC1806 control or GSK-3β knockdown cells were injected into mice. Tumor growth was monitored (*$P < 0.01$) (**d**) and images were taken with tumors isolated from the mice (**e**). Scale bar, 1 cm.

role in the control of tumor cell proliferation.[19] To determine whether GSK-3β regulates protein synthesis, we examined polysome assembly. Notably, we found that treatment of cells with GSK-3 inhibitor (Figure 3a) or knockdown of GSK-3β (Figure 3b) significantly decreased polysome assembly, suggesting that GSK-3β has a major role in regulating mRNA translation. Next, we measured nascent protein synthesis with an amino-acid analog that incorporates into proteins during active protein synthesis. As shown in Figure 3c, GSK-3 inhibitors decreased protein synthesis.

In eukaryotic cells, the majority of mRNA translation is cap-dependent, meaning that it relies on a complex of proteins that assemble at the 7-methylguanosine cap at the 5′ end of mRNA.[19] We found that treatment of cells with GSK-3 inhibitors inhibited the cap-dependent translation rate (Figure 3d). To further explore the function of GSK-3β in cap-dependent translation, we used 7-methyl GTP sepharose beads in which 7-methyl GTP mimics the cap at the 5′ end of mRNA. It is known that an eIF4F complex (eIF4E, eIF4A and eIF4G) binds to the cap of mRNA and initiates cap-dependent translation. Formation of the eIF4F complex is dependent on the bioavailability of eIF4E that is negatively regulated by its binding partner, 4E-BP1.[19] The binding of 4E-BP1 to eIF4E depends on 4E-BP1 phosphorylation. The unphosphorylated or hypophosphorylated form of 4E-BP1 is active and binds to eIF4E, which inhibits eIF4E. Phosphorylation of 4E-BP1 inhibits its activity, and thereby relieves its binding to eIF4E, which allows eIF4E to initiate translation. To determine whether GSK-3β regulated mRNA cap complex formation, we performed precipitations using 7-methyl GTP sepharose beads. As shown in Figure 3e, the amount of 7-methyl GTP-bound eIF4E was

nearly unchanged after treatment with GSK-3 inhibitors. However, unlike rapamycin, GSK-3 inhibition resulted in a dramatic increase in 4E-BP1 binding to 7-methyl GTP. eIF4G interacts with eIF3, a scaffold of the translation initiation complex, and other components of the eIF4F complex such as eIF4E and eIF4A.[20] Therefore, eIF4G competes with 4E-BP1 to bind eIF4E. As GSK-3 inhibition resulted in increased interaction of 4E-BP1 to eIF4E, we were interested in determining whether GSK-3 inhibition could decrease the interaction between eIF4G and eIF4E. As shown in Figure 3e, GSK-3 inhibition decreased the interaction between eIF4G and eIF4E. Next, we used different cell lines and performed a cap-binding assay. As shown in Supplementary Figure S3A–S3C, GSK-3 inhibition increased binding of 4E-BP1 to eIF4E. We also found that eIF4E-bound 4E-BP1 levels were increased in GSK-3β knockdown cells, while interaction of eIF4G and eIF4E was decreased in these cells (Figure 3f).
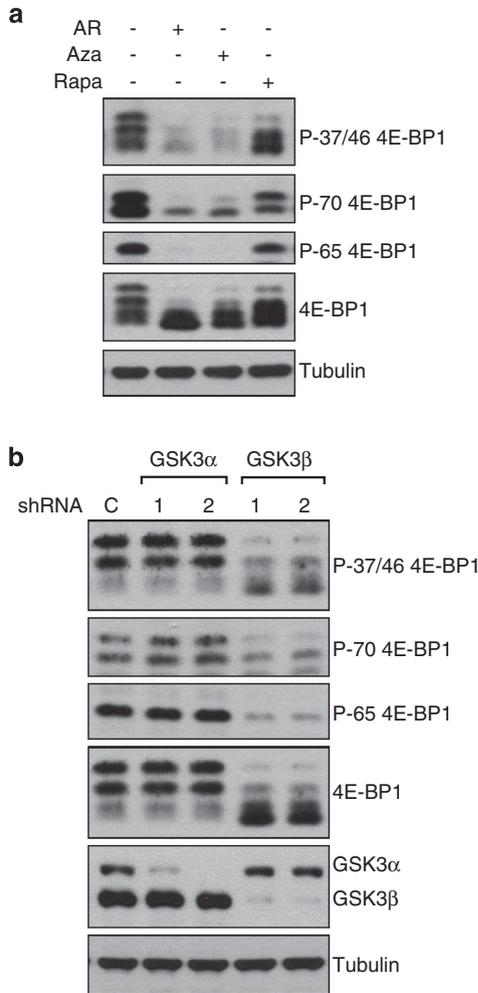
GSK-3β regulates 4E-BP1 phosphorylation

We next studied, in detail, the relationship between GSK-3β and 4E-BP1. 4E-BP1 has multiple important phosphorylation sites. Thr37/Thr46, Thr70 and Ser65 all have key roles in regulating 4E-BP1 activity. It is known that Thr37/Thr46 must be phosphorylated first. Subsequently, Ser65 is phosphorylated, and then 4E-BP1 becomes inactive and dissociates from eIF4E.[21] The phosphorylation of Thr70 is complicated and controversial.[22,23] We first measured phosphorylation of these sites after treatment with GSK-3 inhibitors or rapamycin. Interestingly, we found that GSK-3 inhibitors profoundly decreased phosphorylation at all of these sites, but rapamycin did not (Figure 4a). We used different
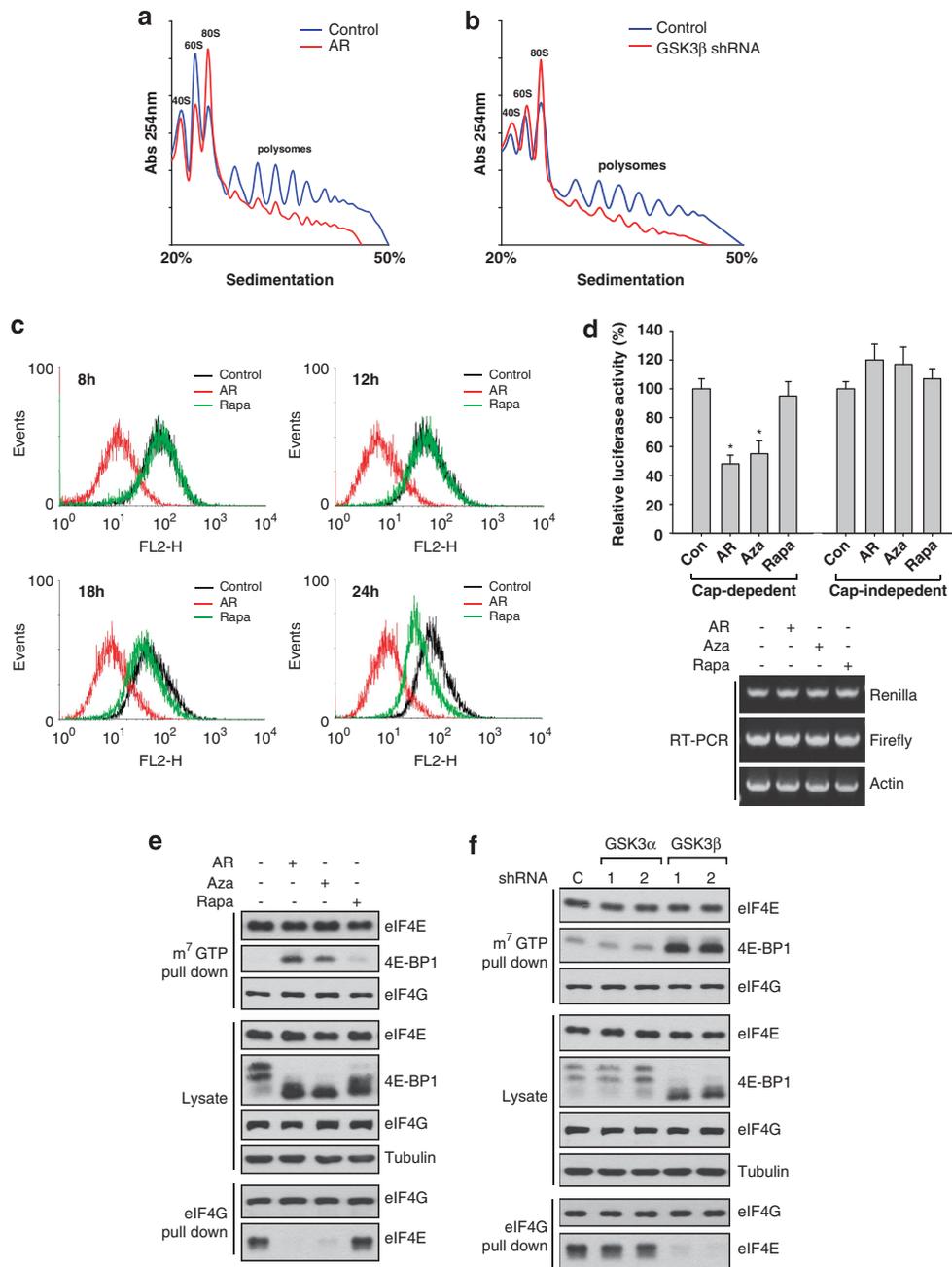
Figure 3. GSK-3β positively regulates protein synthesis. Data are representative of at least three independent experiments. Where applicable, data are the means ± s.e.m. of three separate experiments performed in triplicate. Results were statistically significant (*$P < 0.01$) as assessed by using the Student's t-test. (a) HCC1806 cells growing in the presence of serum were treated with AR-A014418 (25 μM) for 24 h, after which polysomal fractionation was performed. (b) Polysomal fractionation was performed using stable control or GSK-3β knockdown cells. (c) Nascent protein synthesis was measured in a time-dependent manner with GSK-3 inhibitor and rapamycin as described in Materials and methods. (d) HCC1806 cells were transfected with the bicistronic reporter plasmid pRMF to measure cap-dependent or -independent translation. Forty-eight hours after transfection, cells were treated with GSK-3 inhibitors or rapamycin for 24 h. Luciferase activity was measured and normalized by mRNA. (e) HCC1806 cells growing in the presence of serum were treated with AR-A014418 (20 μM), 1-azakenpaullone (30 μM), or rapamycin (20 nM) for 24 h. 4E-BP1 cap-binding activity was measured using 7-methyl GTP sepharose. For eIF4G pull-down, cell lysate was immunoprecipitated with anti-eIF4G antibodies and subjected to immunoblot analysis. (f) GSK-3α or GSK-3β knockdown HCC1806 cells were lysed and cap-binding activity was measured or immunoprecipitated with anti-eIF4G antibodies.

breast cancer cell lines (Supplementary Figures S4A–C), MCF-10A (normal breast cells) (Supplementary Figure S4D) and UB (normal ureteric bud cell) (Supplementary Figure S4E), and determined that GSK-3 regulated 4E-BP1 phosphorylation, while rapamycin did not, in rapamycin-resistant cells. We also found that GSK-3β knockdown decreased phosphorylation at all of the sites, whereas

GSK-3α knockdown did not have a significant effect (Figure 4b). We examined this effect using 3D culture as well. Treatment with GSK-3 inhibitors for 24 h decreased phosphorylation at these sites, but rapamycin did not (Supplementary Figure S4F). Taken together, these results suggest that GSK-3β is a positive regulator of 4E-BP1 phosphorylation.

a

AR   − + − −
Aza   − − + −
Rapa − − − +

P-37/46 4E-BP1

P-70 4E-BP1

P-65 4E-BP1

4E-BP1

Tubulin

b

           GSK3α   GSK3β

shRNA C   1 2   1 2

P-37/46 4E-BP1

P-70 4E-BP1

P-65 4E-BP1

4E-BP1

GSK3α

GSK3β

Tubulin

Figure 4. GSK-3β positively regulates 4E-BP1 phosphorylation. Data are representative of at least three independent experiments. (**a**) HCC1806 cells growing in the presence of serum were treated with GSK-3 inhibitors or rapamycin for 24 h, lysed and analyzed by immunoblot. (**b**) GSK-3α or GSK-3β knockdown HCC1806 cells were lysed and immunoblot analysis was performed.

GSK-3β regulates 4E-BP1 phosphorylation at Thr37/Thr46 independent of known upstream kinases of 4E-BP1

After finding that GSK-3β regulated phosphorylation of 4E-BP1, we were curious about the mechanism of this regulation. We first examined the phosphorylation of Akt and ERK, the well-known upstream kinases responsible for 4E-BP1 phosphorylation. As shown in Figure 5a, GSK-3 inhibitors did not affect phosphorylation of Akt and ERK. Neither GSK-3α knockdown nor GSK-3β knockdown resulted in any change in phospho-Akt and phospho-ERK levels (Figure 5b). Next, we knocked down raptor, an essential component of mTORC1, and determined 4E-BP1 phosphorylation. As shown in Figure 5c, raptor knockdown did not decrease 4E-BP1 phosphorylation, which suggests that mTORC1 may not be involved in 4E-BP1 phosphorylation at Thr37/46. However, GSK-3 inhibitors still decreased 4E-BP1 phosphorylation in raptor knock-down cells. These results suggest that regulation of 4E-BP1 by GSK-3β is not dependent on these known upstream regulators of 4E-BP1 phosphorylation. It is known that T37/T46 phosphorylation is required for S65 phosphorylation, and we found that GSK-3β markedly affected T37/T46 and S65 phosphorylation (Figure 4). In addition, it is also known that T37/T46 phosphorylation is constitutive irrespective of growth factor withdrawal[24,25] and,

unlike other kinases, GSK-3β is also active in the absence of growth factors.[4,26] Thus, we hypothesized that GSK-3β might regulate 4E-BP1 activity through T37/T46 phosphorylation. Before testing this idea, we first determined whether T37/T46 phosphorylation indeed affected the phosphorylation of S65. For this, we expressed a 4E-BP1 T37A/T46A mutant and examined S65 phosphorylation. As shown in Supplementary Figure S5A, wild-type 4E-BP1 showed S65 phosphorylation, but the T37A/T46A mutant did not show any increase in phosphorylation at S65. Next, we starved cells by incubation in serum-free medium and found that there was little decrease in total T37/46 phosphorylation, although there was a band shift. However, serum starvation decreased the levels of S65 phosphorylation (Supplementary Figure S5B). Next, we examined the effect of T37/46 on polysome assembly. As shown in Figure 5d, T37/46A mutant-expressing cells showed decreased polysome assembly, suggesting that phosphorylation of T37/46 is important for mRNA translation. We next examined the function of 4E-BP1 on tumor growth in vivo. As shown in Figures 5e and f, the T37/46A mutation profoundly inhibited tumor growth in vivo.

GSK-3β directly phosphorylates 4E-BP1 at Thr37/Thr46, which requires phosphorylation at priming sites

We next tested our hypothesis that GSK-3β regulates 4E-BP1 phosphorylation through the regulation of T37/46 phosphorylation. First, we focused on the identical amino acid sequence around T37 and T46: [37]TPGGT[41] and [46]TPGGT[50]. T37 and T46 are each N-terminal to prolines (P), which are possible phosphorylation sites for proline-directed kinases that phosphorylate serine or threonine residues preceding prolines (Ser/Thr-Pro).[27] Interestingly, GSK-3β is also a proline-directed kinase and many GSK-3β substrates have S^1/T^1-P-X-X-S^2/T^2 motifs in which the first S^1 or T^1 is phosphorylated by GSK-3β[27] (Figure 6a). Both T37 and T46 in 4E-BP1 have T-P-X-X-T motifs, consistent with other GSK-3β substrates. This suggests that T37 and T46 could be directly regulated by GSK-3β. To test this, we asked whether other known proline-directed kinases could regulate phosphorylation of T37/46. We found that only a GSK-3 inhibitor altered T37/T46 phosphorylation, and inhibitors of other proline-directed kinases did not affect the phosphorylation of T37/46 (Figure 6b). Therefore, we wondered if GSK-3 directly phosphorylates 4E-BP1 at T37/46. By using recombinant GSK-3β and purified 4E-BP1, we performed an in vitro kinase assay in which we examined phosphorylation of T37/T46. As shown in Figure 6c, GSK-3β increased phosphorylation at these sites, demonstrating that GSK-3β is a kinase for 4E-BP1 phosphorylation at T37/46 in vitro. We also measured phosphorylation of 4E-BP1 at S65 by GSK-3β, but we could not see the increase (Supplementary Figure S6A).

GSK-3β usually depends on a priming-site phosphorylation. Many GSK-3β substrates have a S^1/T^1-P-X-X-S^2/T^2 motif, where the first S^1 or T^1 is phosphorylated by GSK-3β and the second S^2 or T^2 is the priming site that facilitates the phosphorylation of the first S^1 or T^1 by GSK-3.[28,29] We wondered if T41 ([37]TPGGT[41]) and T50 ([46]TPGGT[50]) were required for the phosphorylation of T37/T46. By using mass spectrometry analysis, we found that both T41 and T50 were phosphorylated (Supplementary Figure S6B).[30] We also used Cell Signaling Technology PhosphositePlus, a phosphorylation site database, and confirmed the phosphorylation of 4E-BP1 at T41 and T50. To test the idea, we purified T41A/T50A 4E-BP1 mutants and performed a kinase assay using recombinant GSK-3β. The phosphorylation of T37/T46 in these mutants was only slightly affected by GSK-3β, compared with wild-type 4E-BP1 (Figure 6d). As mTORC1 is known to phosphorylate 4E-BP1 in vitro, we determined whether mTORC1 could phosphorylate T41/50A mutant 4E-BP1 at T37/46. As shown in Supplementary Figure S6C, mTORC1 increased phosphorylation of T37/46 in vitro. We also expressed these mutants in cells and examined the
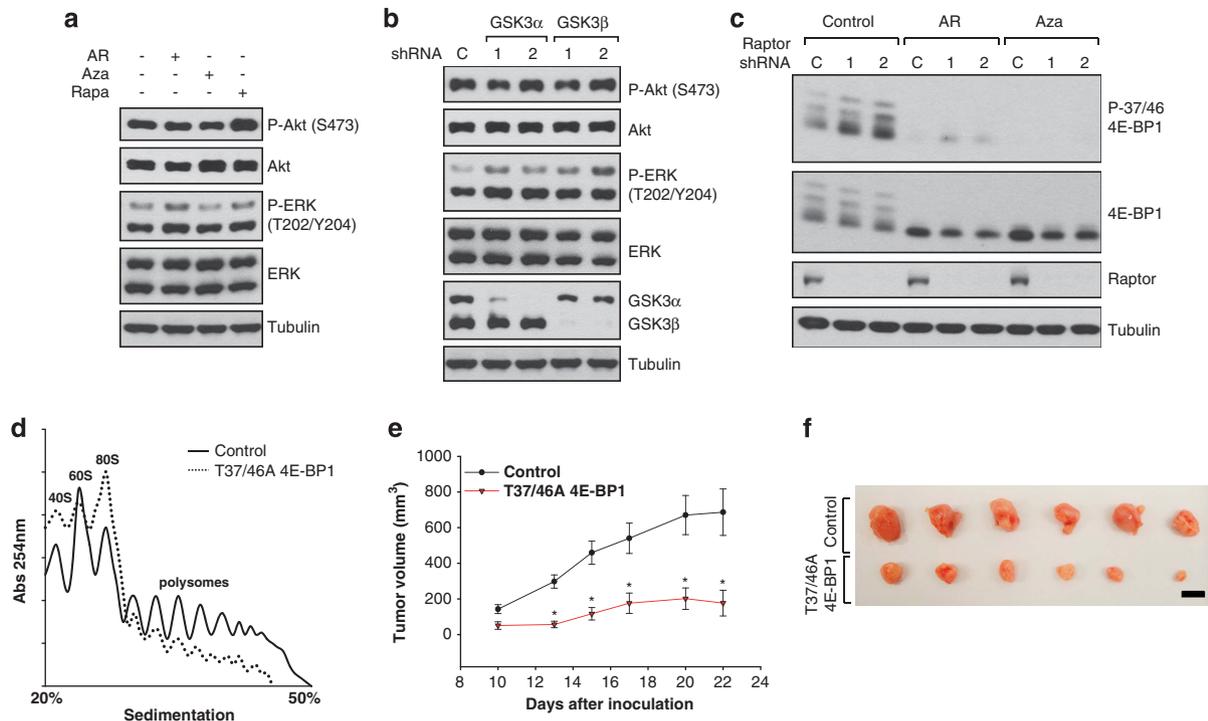
Figure 5. T37/46 phosphorylation is important for protein synthesis and tumor growth. Data are representative of at least three independent experiments. (**a**) HCC1806 cells growing in the presence of serum were treated with GSK-3 inhibitors or rapamycin for 24 h, lysed and then analyzed by immunoblot. (**b**) HCC1806 cells with GSK-3α or GSK-3β knockdown were lysed and immunoblot analysis was performed. (**c**) HCC1806 cells with raptor knockdown were treated or untreated with GSK-3 inhibitors, and immunoblot analysis was performed. (**d**) Polysomal fractionation was performed using stable control or 4E-BP1 T37/46A mutant-expressing cells. (**e, f**) Stable HCC1806 control or cells stably expressing T37/46A mutant were injected into mice. Tumor growth was monitored (*$P < 0.01$) (**e**) and images were taken with tumors isolated from the mice (**f**). Scale bar, 1 cm.

phosphorylation of 4E-BP1. The dominant-negative mutation T41A/T50A dramatically decreased the phosphorylation of T37/T46 and S65 (Figure 6e). We next examined the proliferation of cells stably expressing the T37/46A or T41/50A mutants. As shown in Figure 6f, both mutants decreased cell proliferation. Considering that T37/46A and T41/50A mutant expression levels are low compared with WT 4E-BP1 (Figure 6e and Supplementary Figure S5A), dramatic inhibition of cell proliferation by these mutants with this lower expression suggests that both mutants are strong negative regulators of cell proliferation.

*GSK-3 positively regulates cell proliferation through inactivation of 4E-BP1*

We were interested in determining if GSK-3 regulated cell proliferation through 4E-BP1 phosphorylation. We first examined whether GSK-3 inhibitors or rapamycin affect cell proliferation and 4E-BP1 phosphorylation in 3D culture systems. As shown in Supplementary Figure S7A, long-term treatment with GSK-3 inhibitors profoundly inhibited increase of acini size and T37/T46 phosphorylation. However, rapamycin did not show any dramatic effect. We next used GSK-3α or GSK-3β knockdown cells and found that GSK-3β knockdown also showed the same pattern as GSK-3 inhibitors (Supplementary Figure S7B). As GSK-3 inhibition prevented dissociation of 4E-BP1 and eIF4E, we hypothesized that GSK-3 inhibition would not affect 4E-BP1-dependent eIF4E activity, translation and cell proliferation in the presence of very low 4E-BP1 levels. To test this idea, we made stable 4E-BP1 knockdown cells (Figure 7a) and compared cap-dependent translation in these cells to cells with normal 4E-BP1 levels. Unlike the control cells, GSK-3 inhibitor treatment did not dramatically decrease cap-dependent translation in stable 4E-BP1 knockdown cells

(Figure 7b). We next examined the proliferation of 4E-BP1 knockdown cells in the presence or absence of GSK-3 inhibitors. GSK-3 inhibitors decreased control cell proliferation (Figure 7c), but did not have much effect on the proliferation of 4E-BP1 knockdown cells. Similar results were seen in 3D culture; decreased acini size by GSK-3 inhibitors was also recovered in 4E-BP1 knockdown cells (Supplementary Figures S7C and S7D).

DISCUSSION

GSK-3 has paradoxical roles in cancer. On one hand, GSK-3 can act as a tumor promoter that potentiates tumor growth. On the other hand, it can function as a tumor suppressor that inhibits tumor cell survival. Nonetheless, many other molecules have similar dual roles in tumor initiation and progression. For example, Notch signaling is often considered a proto-oncogene because of its role as the main trigger of T-cell acute lymphoblastic leukemia. However, recent evidence unexpectedly showed that Notch signaling can also have a potent tumor-suppressor function in both solid tumors and hematological malignancies.[31] GSK-3 inhibitors have emerged as promising drugs to treat type 2 diabetes and neurological disorders. However, these diseases are also associated with increased cancer incidence and progression.[32–34] If GSK-3 inhibitors are used therapeutically to treat type 2 diabetes and neurological disorders, it will be important to understand how the tumor-promoter function of GSK-3 is counterbalanced by its tumor-suppressor function. GSK-3 inhibitors could block either tumor-suppressive or tumor-promoting activity, and it will be important to determine which functions predominate in cancer.[35]

Our results suggest that targeting GSK-3 may be another way to inhibit the proliferation of cancer cells irrespective of rapamycin
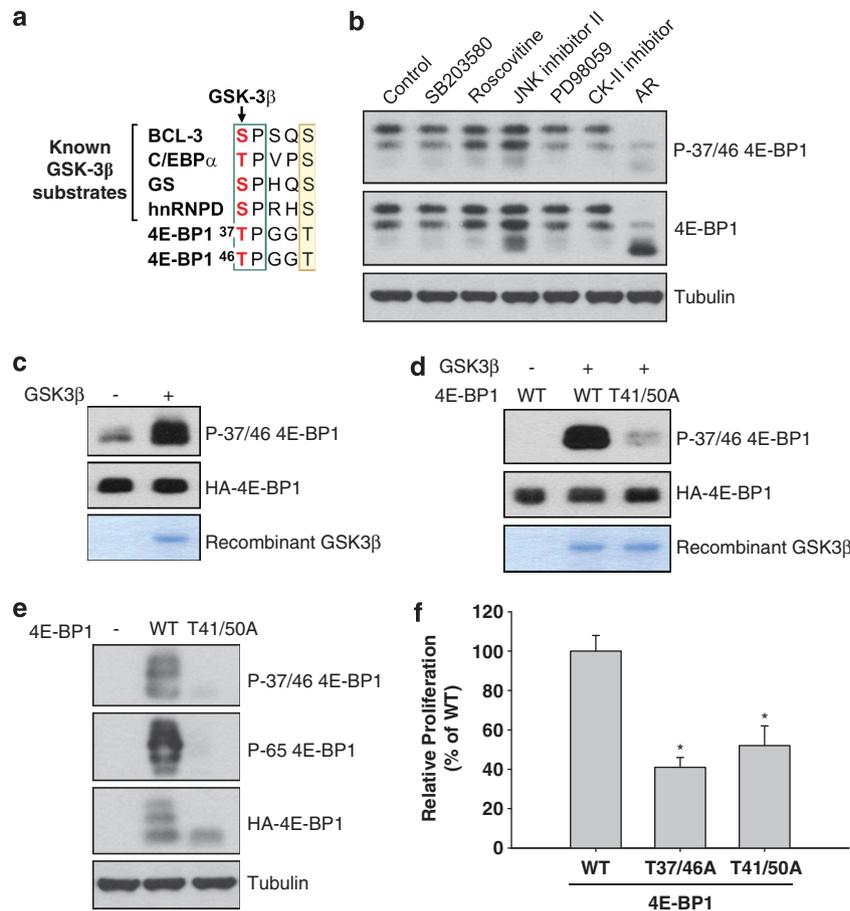
Figure 6. GSK-3β directly phosphorylates 4E-BP1 at T37/T46. Data are representative of at least three independent experiments. (**a**) Comparison of GSK-3 substrate phosphorylation sites and 4E-BP1 sequence. (**b**) HCC1806 cells were treated with inhibitors of proline-directed kinases, 20 μm SB203580 (p38 inhibitor), 20 μm Roscovitine (CDK inhibitor), 20 μm JNK inhibitor, 20 μm PD98059 (MEK/ERK inhibitor), 40 μm casein kinase II inhibitor and 20 μm AR-A014418 (GSK-3 inhibitor) for 24 h. After cell lysis, immunoblot analysis was performed. (**c, d**) *In vitro* phosphotransferase assays were performed by using recombinant GSK-3β (NEB) and immunopurified HA-4E-BP1 (**c**) or HA-4E-BP1 T41A/T50A mutant (**d**). T37/T46 phosphorylation was measured by immunoblot analysis. (**e**) Stable 4E-BP1 WT or T41A/T50A mutant cells growing in the presence of serum were lysed and immunoblot analysis was performed. (**f**) Stable 4E-BP1 WT, T37A/T46A, or T41A/T50A mutant cells growing in the presence of serum and the rate of proliferation was measured by counting cell numbers. Data are the means ± s.e.m. of three separate experiments performed in triplicate. Results were statistically significant (*P < 0.01) as assessed by using the Student's *t*-test.

sensitivity. It has been shown that rapamycin and its analogs are having disappointingly mild effects on many cancers, such as breast cancer. One of the reasons for the weak effect that we and others have identified is that mTORC1 inhibition leads to feedback activation of the cell-survival pathway.[30,36] In addition, we and others found that rapamycin does not block 4E-BP1 phosphorylation and mRNA translation in many cell types.[37,38] In rapamycin-resistant cancer cells, 4E-BP1 is a key determinant of cell proliferation and cancer progression, while S6K1 is dispensable for these functions.[39,40] These mechanisms partly explain why rapamycin alone does not exert a strong anti-proliferative effect on cancer cells. To overcome rapamycin's weaker effect and incomplete inhibition of mTORC1, ATP-competitive mTOR kinase inhibitors to block both mTORC1 and mTORC2, and dual-specificity inhibitors to block PI3-Kinase and mTOR have been developed. Although these inhibitors show a stronger effect than rapamycin alone on tumor progression, recent studies using a panel of colorectal cancer cell lines show that still 40% of the cell lines are resistant to these inhibitors and this resistance is correlated with inability of these inhibitors to block 4E-BP1 phosphorylation at T37/46.[39] In light of this, we were interested in finding the unidentified 4E-BP1 kinase. Recently, it was reported that 4E-BP1 interacts with GSK-3β.[41] Here, we show

that GSK-3β positively regulates 4E-BP1 phosphorylation and protein synthesis, which is consistent with other current reports that GSK-3 is a tumor promoter and positively regulates cancer cell growth and proliferation.[3,4,6,16] Although we determined that GSK-3 regulates the phosphorylation of Thr37/46, it is also possible that GSK-3 could regulate the phosphorylation of other sites (S65 and/or T70) in some cancer cells. It is known that S65 is regulated by mTORC1,[42] which is mainly regulated by the Akt pathway.[43] We showed that GSK-3 regulated Akt activity in some cancer cells,[9] and a recent kinome-wide screen demonstrated that GSK-3 is a positive regulator of Akt in breast cancer cell lines.[15] It is also known that p53 activation results in rapid dephosphorylation of 4E-BP1 and inhibition of translation initiation.[44] GSK-3 can inactivate p53,[13,45] which may lead to increases in the phosphorylation of 4E-BP1 and protein synthesis. Therefore, GSK-3 may act through several pathways that can regulate 4E-BP1 and protein synthesis depending on cellular context.

Several AGC kinases, which include S6K1 and Akt, contain an important phosphorylation site, the turn motif, which has a Ser/Thr-Pro sequence that stabilizes the active conformation of the kinase.[42] We recently reported that GSK-3β, a proline-directed kinase, positively regulates S6K1 activity by modulating turn motif [371]SPDDS[375] phosphorylation at S371.[9] Interestingly, 4E-BP1 has
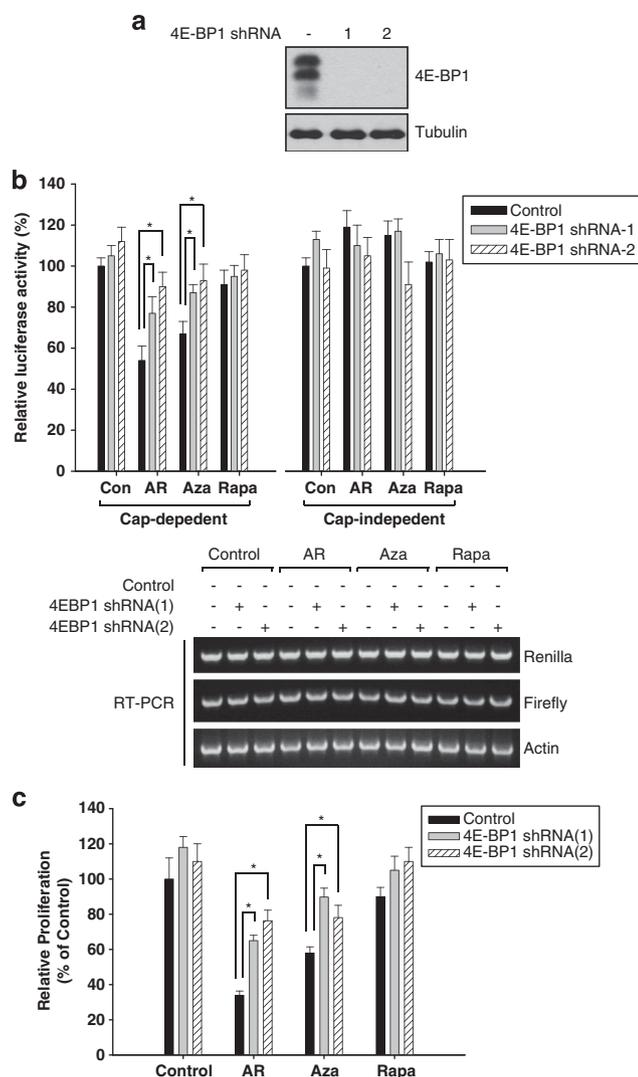
Figure 7. GSK-3β positively regulates cell proliferation by inhibiting 4E-BP1. Data are representative of at least three independent experiments. Where applicable, data are the means ± s.e.m. of three separate experiments performed in triplicate. Results were statistically significant (*$P < 0.01$) as assessed by using the Student's t-test. (**a**) Immunoblot analysis was performed on 4E-BP1 knockdown HCC1806 cells. (**b**) HCC1806 cells stably expressing control or 4E-BP1 shRNA were transfected with bicistronic reporter plasmid pRMF for 48 h. Cells were then treated with GSK-3 inhibitors for 24 h and lysed. Luciferase activity was measured and normalized by mRNA. (**c**) Stable control or 4E-BP1 knockdown HCC1806 cells were treated with GSK-3 inhibitors for 2 days and the rate of proliferation was measured by determining cell numbers.

the same turn motif-like sequences (^{37}TPGGT^{41} and ^{46}TPGGT^{50}), which also function like the turn motif in S6K1.[42] That is, both motifs include the S/T-P-X-X-S/T sequence (Figure 6a), and like S6K1 turn-motif phosphorylation (S371), the motifs of 4E-BP1 (T37/46) are constitutively phosphorylated irrespective of the presence of serum.[24,25] Moreover, turn-motif phosphorylation in S6K1 is essential for subsequent hydrophobic-motif phosphorylation (T389) and S6K1 activity, and phosphorylation of turn motif-like T37/46 residues in 4E-BP1 is essential for subsequent phosphorylation at S65 and the ensuing inactivation of 4E-BP1. The turn-motif phosphorylation mechanism is poorly characterized, but recently, it was shown that mTOR complex 2 (mTORC2) regulates phosphorylation of the turn motif in Akt

cotranslationally.[46] A recent study suggests that turn motif-site phosphorylation of S6K1 at S371 may occur cotranslationally.[47] For some members of the AGC kinase group, the turn-motif site is constitutively phosphorylated and this phosphorylation occurs during or immediately after mRNA translation.[42] In addition, dephosphorylation of these turn-motif sites requires a longer time than that of the hydrophobic motifs.[42] Considering the similarities in sequence and function between the S6K1 and 4E-BP1 motifs, it will be interesting to determine whether GSK-3β regulates phosphorylation of 4E-BP1 at turn motif-like sequences during or immediately after mRNA translation.

In summary, we have provided strong evidence that GSK-3β positively regulates 4E-BP1 phosphorylation thereby regulating 4E-BP1 activity, protein synthesis and cell proliferation. However, 4E-BP1 knockdown did not completely restore cap-dependent translation and cell proliferation suppressed by GSK-3 inhibition (Figure 7 and Supplementary Figure S7). This suggests that GSK-3β may regulate other molecules involved in translation and proliferation in cancer. In support of this, GSK-3β is also known to modulate NF-κB, Akt, Notch signaling, p53, the oncoprotein Maf and the homeobox gene to positively regulate cancer cell proliferation and tumor progression.[7,9–15] Many studies have shown that GSK-3 is highly active and overexpressed in many cancers.[3,48–50] Moreover, it has been shown that GSK-3 remains highly active in most cancers that have highly active Akt.[48,49,51] Therefore, for reasons that are not clear, Akt activation and GSK-3 inhibition are not always correlated in human cancers, and a pool of GSK-3 remains active in cancers and cells growing in the presence of serum.[9] Therefore, targeting GSK-3 alone or targeting both GSK-3 and mTORC1 may be effective in inhibiting cancer cell growth and proliferation.

MATERIALS AND METHODS

Cells and reagents
HCC1806, HCC1937, AU565, SUM-159-PT, MCF-10A and UB cells were cultured as previously described.[9,52,53] All phospho- or non-phospho-4E-BP1, S6K1, Akt, ERK, S6, eIF4G, eIF4E, tubulin and HA antibodies were purchased from Cell Signaling (Danvers, MA, USA). 1-Azakenpaullone was from Enzo Life Sciences (Farmingdale, NY, USA). AR-A014418 (GSK-3β inhibitor VIII), roscovitine, JNK inhibitor II, casein kinase 2 inhibitor, PD98059, SB203580, LY294002, and rapamycin were obtained from Calbiochem (Darmstadt, Germany). Recombinant GSK-3β and λ-phosphatase were obtained from NEB (Ipswich, MA, USA). Methyl-7-GTP-sepharose beads were purchased from GE Healthcare (Pittsburgh, PA, USA). An HA antibody immobilized onto Sepharose matrix was obtained from Covance (Princeton, NJ, USA).

Plasmids
Lentiviral packaging and envelope plasmids were a generous gift from Dr Andrew L Kung (Dana-Farber Cancer Institute, MA, USA) and Dr David Baltimore (California Institute of Technology, CA, USA). The bicistronic reporter plasmid pRMF and HA-Raptor were kindly provided by Dr Anne E Willis (University of Leicester, UK) and Dr Do-Hyung Kim (University of Minnesota), respectively. Lentiviral GSK-3α, GSK-3β, and 4E-BP1 shRNA plasmids were purchased from Open Biosystems (Waltham, MA, USA). For pcDNA3-HA-4E-BP1 WT and pLEX-MCS-HA-4E-BP1 WT constructs, a human 4E-BP1 plasmid was purchased from Open Biosystems and subcloned into pcDNA3-HA or pLEX-MCS-HA plasmids. The point mutation of 4E-BP1 was generated by using the Expand Long Template PCR System (Roche, South San Francisco, CA, USA) and sequences were verified.

Generation of stable knockdown and overexpression cells
Stable cells were generated as described previously.[9]

Polysomal fractionation
Polysome fractionation was performed as described previously.[54]

Cell death assays

The sub-G1 cell population was measured using flow cytometry as described previously.[55]

Protein synthesis assay

To determine nascent protein synthesis, Click-iT AHA (L-azidohomoalaine), Alexa Fluor 488 alkyne, and Click-iT cell reaction buffer kit were purchased from Invitrogen (Grand Island, NY, USA) and used according to the manufacturer's protocol.

Animal studies

For GSK-3 inhibitor treatment studies, HCC1806 cells (1×10^6 cells) were injected subcutaneously into 5–6-week-old female nu/nu mice (Harlan Laboratories, Indianapolis, IN, USA). After subcutaneous tumors were formed, mice were randomly divided into three groups for intraperitoneal injection three times a week with DMSO (a diluent of AR-A014418) or two different concentration of AR-A014418 (2.5 mg kg^{-1} or 5 mg kg^{-1} body weight) based on previous reports.[56,57] For another study, to determine the effect of GSK-3β or 4E-BP1 mutant T37/46A on tumor growth, stable cells were injected subcutaneously into 5–6-week-old female nu/nu mice and tumor diameter was measured.

mTORC1 kinase assay

mTORC1 kinase assay using 4E-BP1 as a substrate was performed as described previously.[58]

Three-dimensional cell culture

The 3D culture of cells in Matrigel (BD Biosciences, San Jose, CA, USA) was carried out as previously described.[59,60]

GSK-3β kinase assays

For the GSK-3β kinase assay using 4E-BP1 as the substrate, HEK293 cells were transfected with pcDNA3-HA-4E-BP1 for 2 days and treated for 2 h with LY294002, a PI3-kinase pathway inhibitor. Cells were lysed and immunoprecipitation was performed using an HA antibody immobilized onto sepharose matrix. Beads were washed twice in lysis buffer and three times in λ-phosphatase buffer, and incubated with λ-phosphatase for 1.5 h at 37 °C. Beads were then washed three times with GSK-3 reaction buffer (NEB). Kinase assays were performed for 1 h at 30 °C in GSK-3 reaction buffer containing 500 μM ATP, with recombinant GSK-3β (NEB) and immunoprecipitated HA-4E-BP1 as the substrate. The reaction products were subjected to SDS–PAGE and immunoblot analysis was performed using an anti-phospho-T37/T46 4E-BP1 antibody. For the GSK-3β kinase assay using 4E-BP1 WT and T41A/T50A mutant, the same method was used except that kinase assays were performed for 20 min at 30 °C.

Cap-binding assay and bicistronic luciferase assay

Cap-binding assay and bicistronic luciferase assay were performed as described previously.[54]

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1 Lee J, Kim MS. The role of GSK3 in glucose homeostasis and the development of insulin resistance. *Diabetes Res Clin Pract* 2007; **77**(Suppl 1): S49–S57.

2 Hur EM, Zhou FQ. GSK3 signalling in neural development. *Nat Rev Neurosci* 2010; **11**: 539–551.

3 Luo J. Glycogen synthase kinase 3beta (GSK3beta) in tumorigenesis and cancer chemotherapy. *Cancer Lett* 2009; **273**: 194–200.

4 Patel S, Woodgett J. Glycogen synthase kinase-3 and cancer: good cop, bad cop? *Cancer Cell* 2008; **14**: 351–353.

5 Manoukian AS, Woodgett JR. Role of glycogen synthase kinase-3 in cancer: regulation by Wnts and other signaling pathways. *Adv Cancer Res* 2002; **84**: 203–229.

6 Miyashita K, Nakada M, Shakoori A, Ishigaki Y, Shimasaki T, Motoo Y et al. An emerging strategy for cancer treatment targeting aberrant glycogen synthase kinase 3beta. *Anticancer Agents Med Chem* 2009; **9**: 1114–1122.

7 Wang Z, Iwasaki M, Ficara F, Lin C, Matheny C, Wong SH et al. GSK-3 promotes conditional association of CREB and its coactivators with MEIS1 to facilitate HOX-mediated transcription and oncogenesis. *Cancer Cell* 2010; **17**: 597–608.

8 Wang Z, Smith KS, Murphy M, Piloto O, Somervaille TC, Cleary ML. Glycogen synthase kinase 3 in MLL leukaemia maintenance and targeted therapy. *Nature* 2008; **455**: 1205–1209.

9 Shin S, Wolgamott L, Yu Y, Blenis J, Yoon SO. Glycogen synthase kinase (GSK)-3 promotes p70 ribosomal protein S6 kinase (p70S6K) activity and cell proliferation. *Proc Natl Acad Sci USA* 2011; **108**: E1204–E1213.

10 Ougolkov AV, Bone ND, Fernandez-Zapico ME, Kay NE, Billadeau DD. Inhibition of glycogen synthase kinase-3 activity leads to epigenetic silencing of nuclear factor kappaB target genes and induction of apoptosis in chronic lymphocytic leukemia B cells. *Blood* 2007; **110**: 735–742.

11 Hoeflich KP, Luo J, Rubie EA, Tsao MS, Jin O, Woodgett JR. Requirement for glycogen synthase kinase-3beta in cell survival and NF-kappaB activation. *Nature* 2000; **406**: 86–90.

12 Foltz DR, Santiago MC, Berechid BE, Nye JS. Glycogen synthase kinase-3beta modulates notch signaling and stability. *Curr Biol* 2002; **12**: 1006–1011.

13 Qu L, Huang S, Baltzis D, Rivas-Estilla AM, Pluquet O, Hatzoglou M et al. Endoplasmic reticulum stress induces p53 cytoplasmic localization and prevents p53-dependent apoptosis by a pathway involving glycogen synthase kinase-3beta. *Genes Dev* 2004; **18**: 261–277.

14 Rocques N, Abou Zeid N, Sii-Felice K, Lecoin L, Felder-Schmittbuhl MP, Eychene A et al. GSK-3-mediated phosphorylation enhances Maf-transforming activity. *Mol Cell* 2007; **28**: 584–597.

15 Lu Y, Muller M, Smith D, Dutta B, Komurov K, Iadevaia S et al. Kinome siRNA-phosphoproteomic screen identifies networks regulating AKT signaling. *Oncogene* 2011; **30**: 4567–4577.

16 Ougolkov AV, Billadeau DD. Targeting GSK-3: a promising approach for cancer therapy? *Future Oncol* 2006; **2**: 91–100.

17 Liu T, Yacoub R, Taliaferro-Smith LD, Sun SY, Graham TR, Dolan R et al. Combinatorial effects of lapatinib and rapamycin in triple-negative breast cancer cells. *Mol Cancer Ther* 2011; **10**: 1460–1469.

18 Zeng Q, Yang Z, Gao YJ, Yuan H, Cui K, Shi Y et al. Treating triple-negative breast cancer by a combination of rapamycin and cyclophosphamide: an in vivo bio-luminescence imaging study. *Eur J Cancer* 2010; **46**: 1132–1143.

19 Blagden SP, Willis AE. The biological and therapeutic relevance of mRNA translation in cancer. *Nat Rev Clin Oncol* 2011; **8**: 280–291.

20 Mir MA, Panganiban AT. A protein that replaces the entire cellular eIF4F complex. *EMBO J* 2008; **27**: 3129–3139.

21 Gingras AC, Gygi SP, Raught B, Polakiewicz RD, Abraham RT, Hoekstra MF et al. Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism. *Genes Dev* 1999; **13**: 1422–1437.

22 Ayuso MI, Hernandez-Jimenez M, Martin ME, Salinas M, Alcazar A. New hierarchical phosphorylation pathway of the translational repressor eIF4E-binding protein 1 (4E-BP1) in ischemia-reperfusion stress. *J Biol Chem* 2010; **285**: 34355–34363.

23 Wang X, Proud CG. Methods for studying signal-dependent regulation of translation factor activity. *Methods Enzymol* 2007; **431**: 113–142.

24 Gingras AC, Raught B, Gygi SP, Niedzwiecka A, Miron M, Burley SK et al. Hierarchical phosphorylation of the translation inhibitor 4E-BP1. *Genes Dev* 2001; **15**: 2852–2864.

25 Wang X, Li W, Parra JL, Beugnet A, Proud CG. The C terminus of initiation factor 4E-binding protein 1 contains multiple regulatory features that influence its function and phosphorylation. *Mol Cell Biol* 2003; **23**: 1546–1557.

26 Tullai JW, Graham JR, Cooper GM. A GSK-3-mediated transcriptional network maintains repression of immediate early genes in quiescent cells. *Cell Cycle* 2011; **10**: 3072–3077.

27 Hardt SE, Sadoshima J. Glycogen synthase kinase-3beta: a novel regulator of cardiac hypertrophy and development. *Circ Res* 2002; **90**: 1055–1063.

28 Eldar-Finkelman H. Glycogen synthase kinase 3: an emerging therapeutic target. *Trends Mol Med* 2002; **8**: 126–132.

29 Doble BW, Woodgett JR. GSK-3: tricks of the trade for a multi-tasking kinase. *J Cell Sci* 2003; **116**: 1175–1186.

30 Yu Y, Yoon SO, Poulogiannis G, Yang Q, Ma XM, Villen J et al. Phosphoproteomic analysis identifies Grb10 as an mTORC1 substrate that negatively regulates insulin signaling. *Science* 2011; **332**: 1322–1326.

31 Lobry C, Oh P, Aifantis I. Oncogenic and tumor suppressor functions of Notch in cancer: it's NOTCH what you think. *J Exp Med* 2011; **208**: 1931–1935.

32 Tabares-Seisdedos R, Dumont N, Baudot A, Valderas JM, Climent J, Valencia A et al. No paradox, no progress: inverse cancer comorbidity in people with other complex diseases. *Lancet Oncol* 2011; **12**: 604–608.

33 Barone BB, Yeh HC, Snyder CF, Peairs KS, Stein KB, Derr RL et al. Long-term all-cause mortality in cancer patients with preexisting diabetes mellitus: a systematic review and meta-analysis. *JAMA* 2008; **300**: 2754–2764.

34 Wolf I, Sadetzki S, Catane R, Karasik A, Kaufman B. Diabetes mellitus and breast cancer. *Lancet Oncol* 2005; **6**: 103–111.

35 Birch NW, Zeleznik-Le NJ. Glycogen synthase kinase-3 and leukemia: restoring the balance. *Cancer Cell* 2010; **17**: 529–531.

36 Um SH, Frigerio F, Watanabe M, Picard F, Joaquin M, Sticker M et al. Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. *Nature* 2004; **431**: 200–205.

37 Jiang YP, Ballou LM, Lin RZ. Rapamycin-insensitive regulation of 4e-BP1 in regenerating rat liver. *J Biol Chem* 2001; **276**: 10943–10951.

38 Choo AY, Yoon SO, Kim SG, Roux PP, Blenis J. Rapamycin differentially inhibits S6Ks and 4E-BP1 to mediate cell-type-specific repression of mRNA translation. *Proc Natl Acad Sci USA* 2008; **105**: 17414–17419.

39 Zhang Y, Zheng XF. mTOR-independent 4E-BP1 phosphorylation is associated with cancer resistance to mTOR kinase inhibitors. *Cell Cycle* 2012; **11**: 594–603.

40 Hsieh AC, Costa M, Zollo O, Davis C, Feldman ME, Testa JR et al. Genetic dissection of the oncogenic mTOR pathway reveals druggable addiction to translational control via 4EBP-eIF4E. *Cancer Cell* 2010; **17**: 249–261.

41 Vinayagam A, Stelzl U, Foulle R, Plassmann S, Zenkner M, Timm J et al. A directed protein interaction network for investigating intracellular signal transduction. *Sci Signal* 2011; **4**: rs8.

42 Alessi DR, Pearce LR, Garcia-Martinez JM. New insights into mTOR signaling: mTORC2 and beyond. *Sci Signal* 2009; **2**: pe27.

43 Efeyan A, Sabatini DM. mTOR and cancer: many loops in one pathway. *Curr Opin Cell Biol* 2010; **22**: 169–176.

44 Horton LE, Bushell M, Barth-Baus D, Tilleray VJ, Clemens MJ, Hensold JO. p53 activation results in rapid dephosphorylation of the eIF4E-binding protein 4E-BP1, inhibition of ribosomal protein S6 kinase and inhibition of translation initiation. *Oncogene* 2002; **21**: 5325–5334.

45 Kulikov R, Boehme KA, Blattner C. Glycogen synthase kinase 3-dependent phosphorylation of Mdm2 regulates p53 abundance. *Mol Cell Biol* 2005; **25**: 7170–7180.

46 Oh WJ, Wu CC, Kim SJ, Facchinetti V, Julien LA, Finlan M et al. mTORC2 can associate with ribosomes to promote cotranslational phosphorylation and stability of nascent Akt polypeptide. *EMBO J* 2010; **29**: 3939–3951.

47 Keshwani MM, von Daake S, Newton AC, Harris TK, Taylor SS. Hydrophobic motif phosphorylation is not required for activation loop phosphorylation of p70 ribosomal protein S6 kinase 1 (S6K1). *J Biol Chem* 2011; **286**: 23552–23558.

48 Kim GP, Billadeau DD. GSK-3β inhibition in pancreatic cancer. In: Lowy AM, Leach SD, Philip PA (eds). *Pancreatic Cancer*. Springer: US, 2008, pp 635–646.

49 Ougolkov AV, Fernandez-Zapico ME, Savoy DN, Urrutia RA, Billadeau DD. Glycogen synthase kinase-3beta participates in nuclear factor kappaB-mediated gene transcription and cell survival in pancreatic cancer cells. *Cancer Res* 2005; **65**: 2076–2081.

50 Mishra R. Glycogen synthase kinase 3 beta: can it be a target for oral cancer. *Mol Cancer* 2010; **9**: 144.

51 Shakoori A, Ougolkov A, Yu ZW, Zhang B, Modarressi MH, Billadeau DD et al. Deregulated GSK3beta activity in colorectal cancer: its association with tumor cell survival and proliferation. *Biochem Biophys Res Commun* 2005; **334**: 1365–1373.

52 Shin S, Dimitri CA, Yoon SO, Dowdle W, Blenis J. ERK2 but not ERK1 induces epithelial-to-mesenchymal transformation via DEF motif-dependent signaling events. *Mol Cell* 2010; **38**: 114–127.

53 Neve RM, Chin K, Fridlyand J, Yeh J, Baehner FL, Fevr T et al. A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. *Cancer Cell* 2006; **10**: 515–527.

54 Roux PP, Shahbazian D, Vu H, Holz MK, Cohen MS, Taunton J et al. RAS/ERK signaling promotes site-specific ribosomal protein S6 phosphorylation via RSK and stimulates cap-dependent translation. *J Biol Chem* 2007; **282**: 14056–14064.

55 Chen Y, Azad MB, Gibson SB. Methods for detecting autophagy and determining autophagy-induced cell death. *Can J Physiol Pharmacol* 2010; **88**: 285–295.

56 Shakoori A, Mai W, Miyashita K, Yasumoto K, Takahashi Y, Ooi A et al. Inhibition of GSK-3 beta activity attenuates proliferation of human colon cancer cells in rodents. *Cancer Sci* 2007; **98**: 1388–1393.

57 Mai W, Kawakami K, Shakoori A, Kyo S, Miyashita K, Yokoi K et al. Deregulated GSK3(beta) sustains gastrointestinal cancer cells survival by modulating human telomerase reverse transcriptase and telomerase. *Clin Cancer Res* 2009; **15**: 6810–6819.

58 Ikenoue T, Hong S, Inoki K. Monitoring mammalian target of rapamycin (mTOR) activity. *Methods Enzymol* 2009; **452**: 165–180.

59 Kenny PA, Lee GY, Myers CA, Neve RM, Semeiks JR, Spellman PT et al. The morphologies of breast cancer cell lines in three-dimensional assays correlate with their profiles of gene expression. *Mol Oncol* 2007; **1**: 84–96.

60 Lee GY, Kenny PA, Lee EH, Bissell MJ. Three-dimensional culture models of normal and malignant breast epithelial cells. *Nat Methods* 2007; **4**: 359–365.

Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)