**Therapeutics, Targets, and Chemical Biology**

**Maintaining Glycogen Synthase Kinase-3 Activity Is Critical for mTOR Kinase Inhibitors to Inhibit Cancer Cell Growth**

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**Abstract**

mTOR kinase inhibitors that target both mTORC1 and mTORC2 are being evaluated in cancer clinical trials. Here, we report that glycogen synthase kinase-3 (GSK3) is a critical determinant for the therapeutic response to this class of experimental drugs. Pharmacologic inhibition of GSK3 antagonized their suppressive effects on the growth of cancer cells similarly to genetic attenuation of GSK3. Conversely, expression of a constitutively activated form of GSK3β sensitized cancer cells to mTOR inhibition. Consistent with these findings, higher basal levels of GSK3 activity in a panel of human lung cancer cell lines correlated with more efficacious responses. Mechanistic investigations showed that mTOR kinase inhibitors reduced cyclin D1 levels in a GSK3β-dependent manner, independent of their effects on suppressing mTORC1 signaling and cap binding. Notably, selective inhibition of mTORC2 triggered proteasome-mediated cyclin D1 degradation, suggesting that mTORC2 blockade is responsible for GSK3-dependent reduction of cyclin D1. Silencing expression of the ubiquitin E3 ligase FBX4 rescued this reduction, implicating FBX4 in mediating this effect of mTOR inhibition. Together, our findings define a novel mechanism by which mTORC2 promotes cell growth, with potential implications for understanding the clinical action of mTOR kinase inhibitors. *Cancer Res; 74(9); 1–14. ©2014 AACR.*

**Introduction**

The mTOR, a serine–threonine protein kinase related tightly to the family of the phosphoinositide 3-kinase–related kinases, exerts different biologic functions primarily through forming two complexes with the essential partner protein raptor (mTOR complex 1; mTORC1) and rictor (mTOR complex 2; mTORC2; refs. 1, 2). Compared with the mTORC1, which is involved in regulation of many key cellular processes, including cell growth and metabolism primarily via regulating cap-dependent protein translation initiation, relatively little is known about the biologic functions of the mTORC2 other than its regulation of cytoskeleton and Akt-mediated cell survival (2). Nonetheless, mTOR signaling is dysregulated in various types of human cancers and, hence, has emerged as an attractive cancer therapeutic target (3).

The conventional mTOR inhibitors, rapamycin and its analogs (rapalogs), are specific allosteric inhibitors of mTORC1 with weak activity against mTORC2. Although some rapalogs (e.g., everolimus) are approved by the U.S. Food and Drug Administration for the treatment of advanced renal cell cancer and pancreatic neuroendocrine tumors, the single-agent activity of rapalogs in most other tumor types has been modest at best (4). Hence, great efforts have been made to identify novel mTOR inhibitors that suppress both mTORC1 and mTORC2 activity. As a result, several ATP-competitive inhibitors of mTOR kinase, including INK128 and PP242, have been developed and are being tested in clinical trials (5, 6). These mTOR kinase inhibitors (TORKinibs) in general more dramatically inhibit protein synthesis, suppress Akt phosphorylation, and induce G1 arrest and/or apoptosis in some cancer cells than the conventional allosteric mTOR inhibitor, rapamycin (7–10). A robust *in vivo* anticancer activity of these inhibitors against certain types of cancers was also observed (8, 11, 12). Some TORKinibs have been tested in clinical trials (5, 6). Therefore, these TORKinibs not only represent novel potential cancer therapeutic agents, but also are valuable research tools for understanding the biology of mTORCs.

Glycogen synthase kinase-3 (GSK3) is a ubiquitous serine/threonine kinase that is present in mammals in two isoforms: α and β (13). GSK3 was initially identified as an enzyme involved in the regulation of glycogen metabolism. Increasing evidence during the past decades indicates that GSK3 has a key role in regulating a diverse range of cellular functions, including cell survival and death (13). Thus, GSK3 inhibition has been considered an attractive therapeutic strategy for certain diseases such as diabetes, neurodegenerative diseases, and mental disorders (14, 15). GSK3 has been implicated in the regulation of oncogenesis with complex patterns: It acts paradoxically as a tumor suppressor in some cancer types while potentiating...
growth of cancer cells in others (16, 17). One well-known important cancer-related function of GSK3 is to positively regulate proteasomal degradation of several oncogenic proteins such as c-Myc, c-Jun, cyclin E, McI-1, and cyclin D (18–20). For example, GSK3-dependent cyclin D1 phosphorylation is required for cyclin D1 degradation mediated by the E3 ubiquitin ligase FBX4 (20, 21).

It has been suggested that GSK3 can inhibit the mTOR pathway by phosphorylating TSC2 in a manner dependent on AMPK-priming phosphorylation (22). A recent study has shown that GSK3 phosphorylates the turn motif of p70S6K and cooperates with mTOR to control the activity of p70S6K and cell proliferation (23), thus providing a rationale for cotargeting mTOR and GSK3 to treat diseases such as cancer. In a longstanding effort to identify strategies or agents that can potentially enhance the therapeutic efficacy of mTOR inhibitors in cancer therapy, we unexpectedly found that the activity of GSK3 is crucial for TORKinibs to exert their inhibitory effects on the growth of cancer cells. Thus, this work has focused on demonstrating the impact of GSK3 on the therapeutic activity of TORKinibs against cancer cells and on understanding the underlying mechanisms.

Materials and Methods

Reagents

PP242, INK128, and AZD8055 were purchased from Active Biochem. Torin 1 was purchased from Tocris. The GSK3 inhibitor SB216763, the proteasome inhibitor MG132, and the protein synthesis inhibitor cycloheximide (CHX) were purchased from Sigma Chemical Co. The NEDD8-activating enzyme inhibitor MLN4924 was provided by Millennium Pharmaceuticals, Inc. Cyclin D1, p-GSK3β/β (S21/9), p-AKT (S473), AKT, p-S6 (S235/236), and S6 antibodies were purchased from Cell Signaling Technology, Inc. GSK3α/β antibody was purchased from Upstate/EMD Millipore. Polyclonal rictor and raptor antibodies were purchased from Bethyl Laboratories, Inc. Both polyclonal and monoclonal actin antibodies were purchased from Sigma Chemical Co. Myc-tagged constitutively active form of GSK3β (GSK3βCA; ref. 24) was provided by Dr. Binhua P. Zhou (The University of Kentucky, College of Medicine, Lexington, Kentucky). Flag-cyclin D1 expression plasmid was provided by Dr. Alan Diehl (Abramson Family Cancer Research Institute, University of Pennsylvania, Philadelphia, PA). Myc-Rictor and HA-raptor expression plasmids were purchased from Addgene.

Cell lines and cell culture

Human non–small cell lung cancer (NSCLC) cell lines used in this study and H157-scrable, H157-shRaptor, and H157-shRictor stable cell lines were described in our previous work (25). Wild-type (WT), GSK3α/β, and GSK3β/β murine embryonic fibroblasts (MEF) were generously provided by Dr. Jim Woodgett (Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada). HEK-293T cells were provided by Keqiang Ye (Emory University, Atlanta, GA). Except for H157 and A549 cells, which were authenticated by Geneticia DNA Laboratories, Inc., through analyzing short tandem repeat DNA profile, other cell lines have not been authenticated. These cell lines were cultured in RPMI-1640 or Dulbecco’s Modified Eagle Medium containing 5% fetal calf serum at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Plasmid transfection was conducted in H1299 or HEK-293T cells largely because of their high transfection efficiency.

Cell growth assay

Cells were seeded in 96-well cell culture plates and treated the next day with the given agents. Viable cell numbers were determined using a sulforhodamine B (SRB) assay as described previously (26). Combination index (CI) for drug interaction (e.g., synergy) was calculated using the Compusyn software (ComboSyn, Inc.). The IC₅₀, which represents the concentration required for 50% growth inhibition, was estimated from a concentration-dependent growth curve.

Cell-cycle analysis

Cells were harvested after a given treatment and stained with propidium iodide for cell-cycle analysis as described previously (27).

Colonies formation assay

The effects of the given drugs on colony formation on plates were measured as previously described (28).

Western blot analysis

Preparation of whole-cell protein lysates and Western blot analysis was performed as described previously (28).

Gene knockdown by siRNA or small hairpin RNA

Rictor #1 (5′-AAGCAGCCCTTGAACTGTTA-3′), rictor #2 (5′-AACTTGGAAGAATCTGTATC-3′), raptor #1 (5′-AAGGCTAGTCTGGTTTCAAT-3′), raptor #2 (5′-AAGGACACGCGCACAAGTAC-3′), GSK-3α (5′-AATGTGATGGCAATGGCT-3′), and GSK-3β (5′-AAGGATATCCACCCCTGCTAC-3′) siRNAs were synthesized by Qiagen. GSK3α/β siRNA (#6301) were purchased from Cell Signaling Technology, Inc. The nonsilencing control siRNA duplexes were designed previously (29). Transfection of these siRNA duplexes was conducted in 6-well plates using the Lipofectamine 2000 transfection reagent (Invitrogen) following the manufacturer’s manual. Short hairpin RNA (shRNA) sets in lentiviral pLKO.1 vector for FBX4 were purchased from Open Biosystems and used as the manufacturer instructed.

m7GTP pull-down for analysis of the elf4f complex

The elf4f complex in cell extracts was detected using affinity chromatography m7GTP-sepharose as described previously (30).

Immunoprecipitation for detection of cyclin D1 ubiquitination

H1299 cells were cotransfected with HA-ubiquitin only or with HA-ubiquitin plus Flag-cyclin D1 plasmids using Lipofectamine 2000 transfection reagent (Invitrogen) based on the manufacturer’s instructions. After 24 hours, the cells were treated with INK128 or INK128 plus MG132 for 4 hours. Cells
were collected and lysed for immunoprecipitation using cyclin D1 antibody (Santa Cruz Biotechnology, Inc.) or Flag M2 monoclonal antibody (Sigma) as previously described (31), followed by detection of ubiquitinated cyclin D1 with Western blot analysis using anti-HA (Abgent) or antiubiquitin (Cell Signaling Technology, Inc.) antibody.

**Reverse transcription PCR to detect FBX4 mRNA expression**

Total cellular RNA was isolated from the given cell line with TRIzol (Sigma Chemical Co.). Reverse transcription was performed with the iScript Select cDNA Synthesis Kit (Bio-Rad), followed with PCR using primers as follows: FBX4 (5'-AGCCGTACAGTGATTCC-3', forward) and (5'-CCAAA-GGCTTGATCTGTACAT-3', reverse). Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control.

**Lung cancer xenografts and treatments**

Lung cancer xenograft experiments were approved by the Institutional Animal Care and Use Committee of Emory University and conducted as previously described (25). Five- to 6-week-old (about 20 g of body weight) female athymic (nu/nu) mice were ordered from Harlan. Mice (n = 5 or 6/group) received the following treatments: vehicle control, INK128 formulated in 5% polyvinylpropylene/15% N-methyl-2-pyrrolidone/80% water (1 mg/kg/d, oral gavage; ref. 32), SB216763 in dimethyl sulfoxide (DMSO; 5 mg/kg/d, i.p.; ref. 33), and the combination of INK128 and SB216763.

**Immunohistochemistry**

Immunohistochemistry (IHC) on formalin-fixed and paraffin-embedded human NSCLC tissues collected by the Emory University/Winship Cancer Institute human tissue procurement services core was performed using Cytomation EnVision + Dual Link System-HPR (DAKO; Dako North America, Inc.) following the standard manufacturer’s protocol. Tissues were incubated overnight at 4°C with the primary antibody against p-GSK3α/β (S21/9; Cell Signaling Technology, Inc.) at 1:75 dilution. p-GSK3α/β staining was scored as negative (<10% staining) and positive (≥10% staining) staining, respectively.

**Statistical analysis**

The statistical significance of differences between two groups was analyzed with two-sided unpaired Student t tests when the variances were equal or with the Welch corrected t test when the variances were not equal by use of GraphPad InStat 3 software (GraphPad Software). Data were examined as suggested by the same software to verify that the assumptions for use of the t tests held. Results were considered to be statistically significant at P < 0.05.

**Results**

**GSK3 is required for the growth-inhibitory effects of INK128 and other TORKinibs in NSCLC cells**

INK128 is a representative TORKinib and is currently under clinical testing (5, 6). In the presence of the GSK3 inhibitor SB216763, we found that the growth suppressive effects of INK128 were substantially attenuated in several NSCLC cell lines (Fig. 1A). Using 7.5 μmol/L SB216763 as an example, the CIs in every tested cell line were far greater than 1 (Supplementary Fig. S1), indicating antagonistic effects. In a long-term colony formation assay that allows us to repeat the treatments, we also found that INK128 alone had greater inhibitory effect than the combination of SB216763 and INK128 on the growth of NSCLC colonies (Fig. 2A), further confirming the antagonistic effect. In our tested cell lines, INK128 at the tested concentration (e.g., 100 nmol/L) primarily induced G1 arrest; however, this effect was abrogated in the presence of SB216763 (Fig. 1B). Furthermore, we tested the effects of SB216763 on the growth-inhibitory effects of other TORKinibs, including AZD8055, Torin 1, and PP242. Consistently, the presence of SB216763 significantly attenuated the growth-inhibitory effects of all TORKinibs tested in both H460 and H292 cells (Fig. 1C and D). Collectively, these results strongly suggest that GSK3 inhibition impairs the ability of TORKinibs to suppress cancer cell growth.

To validate our above finding in vivo, we conducted two human NSCLC xenograft (H460 and A549) experiments in nude mice. In both models, INK128 alone at 1 mg/kg (orally) effectively inhibited the growth of xenografts. SB216763 at 5 mg/kg (i.p.) did not significantly inhibit the growth of xenografts. The combination of INK128 and SB216763 treatment was significantly less effective than INK128 alone in inhibiting the growth of xenografts as measured by both tumor size (Fig. 2B) and tumor weight (Fig. 2C). Moreover, the combination treatment slightly reduced the body weights of mice in comparison with other treatment groups (Supplementary Fig. S2). Hence, it is clear that the presence of SB216763 attenuates the anticancer efficacy of INK128 in vivo.

To ensure that SB216763 indeed antagonizes growth-inhibitory effects of TORKinibs through GSK3 inhibition, we used specific genetic inhibition of GSK3, including gene knockdown and knockout, and then examined the impact of these manipulations on cell responses to TORKinibs. siRNA-mediated knockdown of GSK3α or GSK3β was confirmed with Western blotting (Fig. 3A, left). In a 3-day SRB assay, knockdown of either GSK3α or GSK3β significantly reduced cell sensitivity to INK128 and to another mTORkinib, PP242 (Fig. 3A, right). Similar results were also generated with another siRNA that knocks down the expression of both GSK3α and GSK3β (Fig. 3B). Consistently, GSK3α-KO or GSK3β-KO MEFs were significantly less sensitive to both INK128 and PP242 in comparison with WT MEFs (Fig. 3C). Together, these data further support the notion that GSK3 inhibition impairs cell responses to TORKinibs.

Furthermore, we asked whether GSK3 activation could increase cell sensitivity to INK128. To this end, we transfected a constitutively active form of GSK3β (GSK3βCA) into H1299 cells and then analyzed its impact on cell response to INK128. Compared with vector control-transfected cells, GSK3βCA-transfected cells were more sensitive to INK128 treatment (Fig. 3D). Thus, it seems that increased GSK3 activation, in contrast with GSK3 inhibition, sensitizes cells to INK128. Taking the above results together, we conclude that GSK3 activity is required for the growth-inhibitory effects of INK128 and other TORKinibs, at least in NSCLC cells.
Basal levels of GSK3 activity are significantly associated with cell sensitivity to INK128

We next determined whether basal levels of GSK3 activity are associated with cell sensitivity to TOBkinins. Here, we detected basal levels of p-GSK3 as an indication of inactivated or low GSK3 activity in a panel of NSCLC cell lines by Western blotting (Fig. 4A). These cell lines possessed different sensitivities to INK128 as determined in a 3-day growth-inhibitory assay (Fig. 4B). Correlation analysis showed that high p-GSK3 levels were significantly associated with high IC_{50} values of INK128 against these cells (r = 0.689, P = 0.0064; Fig. 4C), implying that GSK3 activity is associated with cell sensitivity to INK128.

To evaluate the prevalence of GSK3 activity in human NSCLC tissues, we further conducted IHC to detect p-GSK3 in 50 cases of human NSCLC specimens. We found that there were 41 cases positive for p-GSK3 staining (82%) and only 9 cases negative for p-GSK3 staining (18%; Fig. 4D). Hence, these data suggest that the majority of NSCLC tissues possess low GSK3 activity.
INK128 and other TORKinibs decrease cyclin D1 levels through a GSK3-mediated mechanism, which is not associated with their ability to inhibit mTOR signaling and eIF4F complex formation.

To understand the mechanism by which GSK3 activity regulates cell response to INK128, we first determined whether GSK3 inhibition interferes with the ability of INK128 to suppress mTOR signaling and cap-dependent translation. In different NSCLC cell lines, we found that INK128 was equally effective in decreasing the levels of p-4EBP1 and p-S6, two well-known readouts of the mTORC1, both in the absence and presence of SB216763. After 9 days, the plates were stained for the formation of cell colonies with crystal violet dye and pictured using a digital camera. B and C, both A549 and H460 xenografts were treated as described in Materials and Methods. Tumor sizes (A) and mouse body weights (C) were measured once every 2 days. Each measurement is a mean ± SE (n=6 or 5). At the end of the experiment, tumor xenografts were removed and weighed (B). * P < 0.05, ** P < 0.01, *** P < 0.001 compared with the INK128 group alone using the Student t test.

Figure 2. The presence of SB216763 impairs the inhibitory effects of INK128 on the colony formation of cancer cells in dishes (A) and the growth of NSCLC xenografts in nude mice (B and C). A, the indicated cell lines were seeded in 12-well plates at a density of approximately 400 cells per well. On the second day, the cells were treated with DMSO, 25 nmol/L INK128, 7.5 μmol/L SB216763, or INK128 plus SB216763. After 9 days, the plates were stained for the formation of cell colonies with crystal violet dye and pictured using a digital camera. B and C, both A549 and H460 xenografts were treated as described in Materials and Methods. Tumor sizes (A) and mouse body weights (C) were measured once every 2 days. Each measurement is a mean ± SE (n=6 or 5). At the end of the experiment, tumor xenografts were removed and weighed (B). * P < 0.05, ** P < 0.01, *** P < 0.001 compared with the INK128 group alone using the Student t test.

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Figure 3. Knockdown (A and B) or knockout of GSK3 (C) and enforced expression of a constitutively activated form of GSK3β (D) alters cell responses to mTORK inhibitors. A and B, H292 cells were transfected with control (Ctrl), GSK3α, GSK3β, or GSK3α/β siRNA for 24 hours and then reseeded in 96-well plates. After 48 hours, the cells were exposed to different concentrations of INK128 or PP242 as indicated for an additional 3 days. Cell numbers were estimated with the SRB assay. GSK3 knockdown effects at 48 hours or the indicated times after transfection were detected with Western blotting. C, the indicated MEFs were seeded in 96-well plates and the next day treated with different concentrations of INK128 or PP242 as indicated. After 3 days, cell numbers were estimated with the SRB assay. D, H1299 cells were transfected with empty vector or expression plasmid carrying GSK3βCA and after 24 hours, were reseeded in 96-well plates. After 48 hours, the cells were exposed to different concentrations of INK128 as indicated for an additional 3 days. Cell numbers were estimated with the SRB assay. GSK3βCA expression was confirmed with Western blotting (top). Data, means of four replicate determinations; bars, ± SDs. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001 compared with its matched control using the Student t test.
INK128 on cap-binding of the eIF4F complex in the absence and presence of SB216763. Again, INK128 effectively reduced the amounts of eIF4G bound to eIF4E with increased amounts of 4EBP1 bound to eIF4E regardless of the presence or absence of SB216763 (Fig. 5B), suggesting that inhibition of GSK3 also does not impair the suppression of cap-dependent translation initiation by INK128. Interestingly, INK128 effectively decreased the levels of cyclin D1, an oncogenic protein known to be regulated by cap-dependent translation. Cotreatment of the cells with SB216763 and INK128 prevented cyclin D1 reduction by INK128 in all of the tested cell lines (Fig. 5D). These data clearly indicate that GSK3 is required for INK128 to decrease cyclin D1 levels.

INK128 under the tested conditions (100 nmol/L for 10 hours) reduced p-Akt (S473) levels in the tested three cell lines. When combined with SB216763, reduction of p-Akt levels was not altered (Fig. 5A). Thus, GSK3 inhibition does not interfere with the suppression of Akt phosphorylation (S473) by INK128. Interestingly, we noted that INK128 did not suppress GSK3α/β phosphorylation (S21/9) in either of the tested cell lines, despite its potent inhibition of Akt S473 phosphorylation (Fig. 5A). Hence, INK128 has no effect on activation of GSK3.

Figure 4. Detection of basal levels of p-GSK3 in human NSCLC cells (A) and tissues (D) and its correlation with cell sensitivity to INK128 (B and C). Whole-cell protein lysates were prepared from the listed cell lines and subjected to Western blotting for detection of the indicated proteins (A). The intensities of these proteins were quantified with NIH ImageJ software. The IC_{50} values of INK128 were determined from growth curves of the indicated cell lines exposed to different concentrations of INK128 for 3 days. The correlation between p-GSK3/GSK3 and IC_{50} values was calculated with GraphPad InStat software (B and C). p-GSK3 in human NSCLC tissues (D) was detected with IHC. SQCC, squamous cell carcinoma; ADC, adenocarcinoma.
INK128 and other TORKinibs decrease cyclin D1 levels through promoting its degradation

Because INK128 effectively decreased cyclin D1 levels, which could be prevented by GSK3 inhibition as demonstrated above, we conducted experiments to further explore the effect of INK128 on cyclin D1 expression. INK128-induced cyclin D1 reduction could be detected at 3 hours and was sustained up to 12 hours. At concentration ranges between 25 and 200 nmol/L, INK128 decreased the levels of cyclin D1 (Supplementary Fig. S3A and S3B). Thus, it is clear that INK128 rapidly and potently
decreases cyclin D1 levels. Several other TORKinibs, including AZD8055, Torin 1, and PP242, effectively reduced the levels of cyclin D1 as well (Supplementary Fig. S3C); these effects were also GSK3-dependent because inhibition of GSK3 with SB216763 abrogated the ability of these TORKinibs to decrease cyclin D1 (Fig. 5E). Hence, GSK3-dependent reduction of cyclin D1 is a general phenotype caused by TORKinibs. Here, we again observed that these TORKinibs decreased p-Akt (S473) levels without suppressing GSK3α/β phosphorylation (S21/9). Moreover, SB216763 did not affect the ability of the TORKinibs to inhibit Akt phosphorylation (Fig. 5E). We noted that SB216763 reduced p-GSK3β (S9) levels in the tested cell lines (Fig. 5A and E) although we do not know the underlying mechanism.

By examining the effects of INK128 or AZD8055 on cyclin D1 reduction in different NSCLC cell lines, we found that both INK128 and AZD8055 decreased cyclin D1 levels more effectively in three sensitive cell lines (H460, A549, and H1648) than in three relatively less sensitive cell lines (Calu-1, H23, and EKVX). Interestingly, the basal levels of cyclin D1 were higher in the less sensitive than in the sensitive cell lines. However, both agents effectively suppressed the phosphorylation of S6 and Akt in all of the tested cell lines regardless of the level of cell sensitivity (Supplementary Fig. S4). These data indicate that the inhibitory effects of TORKinibs on cyclin D1 are not associated with their abilities to inhibit either mTORC1 (e.g., p-S6) or mTORC2 (e.g., p-Akt) signaling.

Given that GSK3 inhibition blocks the reduction of cyclin D1 by INK128 without inhibiting mTORC1 signaling and cap-dependent translation initiation, we then examined the effects of INK128 on cyclin D1 stability. Compared with DMSO control, INK128 apparently shortened the half-life of cyclin D1 (Fig. 6A), indicating that INK128 decreases the stability of cyclin D1 protein. In the presence of the proteasome inhibitor MG132, INK128-induced cyclin D1 reduction was prevented in all of the tested cell lines, including H460, H157, and A549 (Fig. 6B), suggesting that INK128 facilitates cyclin D1 degradation. In agreement, INK128 also increased cyclin D1 ubiquitination (Fig. 6C), a required step for proteasome-dependent protein degradation. Collectively, we conclude that INK128 decreases cyclin D1 levels through promoting its degradation. Consistently, inhibition of the proteasome with MG132 rescued cyclin D1 reduction induced by other TORKinibs (Fig. 6D). Therefore, induction of cyclin D1 degradation is not specific to INK128, and is rather a general phenomenon caused by TORKinibs.

The ubiquitin E3 ligase FBX4 is involved in mediating TORKinib-induced cyclin D1 degradation

The F-Box ubiquitin E3 ligase FBX4 has been suggested to mediate GSK3-dependent cyclin D1 degradation (21, 34–36). Because cullin neddylation is essential for activating the SCF E3 ligase complex (consisting of Skp1, cullins, F-box proteins, and RING box protein/regulator of Cullins ring finger proteins), through which F-box E3 ligases function (37), we then tested whether inhibition of neddylation by the NEDD8 inhibitor MLN4924 prevented cyclin D1 reduction by INK128. Indeed, INK128 failed to decrease cyclin D1 levels in the presence of MLN4924 (Supplementary Fig. S5). Moreover, we used shRNA to stably knockdown FBX4 and then examined its impact on INK128-induced cyclin D1 reduction. Because of the lack of suitable antibodies to detect endogenous FBX4, we used reverse transcription PCR (RT-PCR) to detect FBX4 expression at the mRNA level to demonstrate its knockdown efficiency (Fig. 6E). We found that INK128 reduced cyclin D1 levels effectively in pLKO.1 cells, but not in two cell lines expressing FBX4 shRNA (shFBX4#1 and shFBX4#5). Similar results were generated when the cell lines were treated with AZD8055 (Fig. 6F). These results indicate that FBX4 is involved in mediating TORKinib-induced cyclin D1 degradation.

mTORC2, but not mTORC1, is responsible for positive regulation of cyclin D1 stability

To understand which mTORC is responsible for the regulation of cyclin D1 stability, we inhibited mTORC1 and mTORC2 by knocking down raptor or rictor, respectively, and then looked at their impact on cyclin D1 levels. We found that siRNA-mediated transient knockdown of both raptor and rictor decreased cyclin D1 levels in H157 cells. In A549 cells, transient knockdown of rictor, but not raptor, resulted in cyclin D1 reduction (Fig. 7A). Similarly, we also detected reduced cyclin D1 levels in H157 cells in which raptor or rictor expression was stably knocked down with shRNA (Fig. 7B). These results indicate that inhibition of both mTORC1 and mTORC2 reduces cyclin D1 levels.

To further demonstrate the role of mTORCs in the regulation of cyclin D1 levels, we then compared the effects of rictor and raptor knockdown on cyclin D1 stability. As presented in Fig. 7C, we detected a shorter half-life of cyclin D1 in H157-shRictor cells (about 15 minutes) than that in H157-scramble cells (about 30 minutes), meaning that cyclin D1 is degraded faster in H157-shRictor cells than in H157-scramble cells. Thus, it is clear that knockdown of rictor decreases cyclin D1 stability. In contrast, H157-scramble and H157-shRaptor cell lines had comparable cyclin D1 degradation rates, indicating that raptor knockdown does not alter cyclin D1 stability.

In our previous study, we have shown that enforced expression of ectopic rictor interacts with endogenous mTOR and increases Akt phosphorylation (38). To provide additional evidence in support of the role of the mTORC2 in regulation of cyclin D1 stability, we asked whether increasing mTORC2 activity by enforcing expression of ectopic rictor enhances cyclin D1 stability. Hence, we coexpressed myc-rictor with Flag-cyclin D1 or vector with Flag-cyclin D1 and then compared cyclin D1 stabilities. As shown in Fig. 7D, enforced expression of rictor substantially slowed down the cyclin D1 degradation rate in comparison with the vector control, indicating that rictor expression stabilizes cyclin D1 protein. In contrast, enforced expression of raptor did not affect cyclin D1 degradation rate. These results, thus, provide complementary evidence supporting the notion that mTORC2 indeed positively regulates cyclin D1 stability.

Moreover, we found that cyclin D1 reduction caused by rictor knockdown could be rescued by siRNA-mediated

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knockdown or inhibition of GSK3 (Fig. 7E) and by shRNA-mediated knockdown of FBX4 (Fig. 7F), demonstrating that rictor knockdown–mediated cyclin D1 is also GSK3- and FBX4-dependent. These data provide strong support for the notion that mTORC2 negatively regulates GSK3-dependent and FBX4-mediated cyclin D1 degradation.

Figure 6. INK128 (A–C) and other TORKinibs (D) decrease cyclin D1 levels through facilitating its degradation involving FBX4 (E and F). A, A549 cells were treated with DMSO or 100 nmol/L of INK128 for 6 hours. The cells were then washed with PBS three times and re-fed with fresh medium containing 10 μg/mL CHX. At the indicated times, the cells were harvested for preparation of whole-cell protein lysates and subsequent Western blot analysis. Protein levels were quantified with NIH ImageJ Software and were normalized to tubulin. B, the indicated cell lines were pretreated with 10 μmol/L MG132 for 30 minutes and then cotreated with 100 nmol/L INK128 for 6 hours. C, H1299 cells were cotransfected with HA-ubiquitin and Flag-cyclin D1 plasmids (top) or transfected with HA-ubiquitin plasmid alone (bottom) using Lipofectamine 2000 reagent. After 24 hours, the cells were then pretreated with 20 μmol/L MG132 for 30 minutes and then cotreated with 100 nmol/L INK128 for another 4 hours. Whole-cell protein lysates were then prepared for immunoprecipitation (IP) using anti-Flag or cyclin D1 antibody followed by Western blotting (WB) using the indicated antibodies. D, A549 cells were pretreated with 10 μmol/L MG132 for 60 minutes and then cotreated with 100 nmol/L AZD8055, 100 nmol/L Torin 1, or 1 μmol/L PP242 for an additional 4 hours. E, whole-cell protein lysates and total cellular total RNA were prepared from the indicated stable cell lines and used for detection of cyclin D1 and FBX4 expression by Western blot analysis and RT-PCR, respectively. F, the indicated cell lines were treated with 100 nmol/L INK128 for 8 hours. After the aforementioned treatments in B, D, and F, the cells were harvested for preparation of whole-cell protein lysates and subsequent Western blotting to detect the given proteins.
Figure 7. Genetic manipulation of rictor expression alters cyclin D1 stability (A–D) through a GSK3- and FBX4-dependent mechanism (E and F). A and B, whole-cell protein lysates were prepared from the indicated cell lines 48 hours after transfection with control (Ctrl), rictor or raptor siRNAs (A) or from the stable transfectants carrying scrambled, rictor or raptor shRNA. Western blot analysis was used to detect the indicated proteins. C, the indicated H157 stable transfectants were exposed to 10 μg/mL CHX. At the indicated times, the cells were harvested for preparation of whole-cell protein lysates and subsequent Western blot analysis. Protein levels were quantified with NIH ImageJ Software and were normalized to tubulin. D, HEK293T cells were cotransfected with cyclin D1 and rictor expression plasmids. After 48 hours, the cells were exposed to CHX as in C for the cyclin D1 stability assay. E and F, the indicated cell lines were transfected with the indicated siRNAs alone or their combination, and after 48 hours, were harvested for preparation of whole-cell protein lysates and subsequent Western blotting.
Discussion

This study provides the first preclinical evidence that GSK3 activity affects cell response or sensitivity to mTORkinibs based on the following findings: (i) the presence of the GSK3 inhibitor, SB216763, antagonizes the suppression of NSCLC cell growth by INK128 and other TORKinibs both in vitro and in vivo (Figs. 1 and 2); (ii) SB216763 abrogates the ability of INK128 to induce G1 arrest (Fig. 1B); (iii) knockdown or knockout of GSK3 (v) low basal GSK3 activity (i.e., high basal p-GSK3 levels) is significantly associated with low sensitivity of NSCLC cells to INK128 (Fig. 4). Thus, our findings warrant further study of the potential impact of GSK3 activity on the therapeutic outcomes of TORKinibs both preclinically and clinically.

It is well known that GSK3 is inactivated through phosphorylation by Akt, which is usually hyperactivated in many types of cancer cells (39). Previous studies have shown that Akt phosphorylation (or activation) is positive in 50% to 80% of NSCLC tumors (40–42). In our preliminary or pilot study, GSK3 phosphorylation was positive in approximately 80% of NSCLC tumors (Fig. 4). Because low basal GSK3 activity (i.e., high p-GSK3 levels) was significantly associated with low sensitivity of NSCLC cells to INK128 (Fig. 4), our prediction is that the subset of NSCLCs (approximately 20% based on our pilot study shown in Fig. 4) negative for p-GSK3 staining (i.e., having activated GSK3) may represent a population who may respond well to TORKinibs. Whether this implies that TORKinibs may not be very active in cancer types with hyperactivated Akt or whether GSK3 activity can be used as a predictive biomarker for TORKinib-based cancer therapy is worthy of further investigation.

Cyclin D1 is an oncogenic protein that promotes cell-cycle progression through the G1 phase by forming active hetero-dimers with cyclin-dependent kinase 4 and 6 (20). In our study, INK128 at the tested concentration ranges (e.g., 100 nmol/L) primarily induced G1 arrest with limited effect on induction of apoptosis. Moreover, INK128 and other TORKinibs effectively decreased cyclin D1 levels particularly in NSCLC cell lines sensitive to TORKinibs (Supplementary Figs. S3 and S4). Inhibition of GSK3 with SB216763 or siRNA-mediated knockdown prevented cyclin D1 reduction induced by INK128 (Fig. 5) and abrogated the ability of INK128 to induce G1 arrest (Fig. 1B). Consistently, other TORKinibs also induced GSK3-dependent cyclin D1 reduction (Fig. 5E). Hence, cyclin D1 reduction is likely to be a key reduction that mediates the growth-inhibitory effects of INK128 or TORKinibs. Accordingly, the blockage of cyclin D1 activity may be critical in mediating the antagocistic effect of GSK3 inhibition on the therapeutic efficacy of TORKinibs in cancer cells.

Cyclin D1 is known to be regulated by mTORC1 signaling through cap-dependent translation (3, 43). An intriguing and novel finding in this study is that TORKinibs reduce cyclin D1 levels through a GSK3-dependent mechanism independent of inhibition of mTORC1 signaling and cap-dependent translation based on the following results: (i) inhibition of GSK3 with SB216763 prevented cyclin D1 reduction by INK128 without interfering with the ability of INK128 to inhibit mTORC1 signaling (e.g., suppression of p-S6 and p-eIF4F) and eIF4 complex formation (Fig. 5B); and (ii) both INK128 and AZD8055 preferentially and potently decreased cyclin D1 levels in TORKinib-sensitive NSCLC cell lines (e.g., H460, A549, and H1648), but had comparable effects on inhibiting mTORC1 signaling (e.g., S6 phosphorylation), irrespective of cell sensitivities to TORKinibs (Supplementary Fig. S4).

Protein degradation is another important mechanism that regulates cyclin D1 levels. GSK3 has been suggested to be involved in this process (20, 44). In this study, we have demonstrated that INK128 and other TORKinibs reduce cyclin D levels through facilitating its proteasomal degradation. This conclusion is strongly supported by our findings that INK128 and other TORKinibs enhanced cyclin D1 ubiquitination, induced cyclin D1 proteasomal degradation, and decreased cyclin D1 stability as presented in Fig. 6. In addition, we have further demonstrated that the SCF E3 ligase FBX4 is involved in mediating cyclin D1 degradation induced by TORKinibs primarily because knockdown of FBX4 prevented cyclin D1 reduction by INK128 or AZD8055 (Fig. 6). Collectively, we conclude that TORKinibs such as INK128 induce GSK3-dependent, FBX4-mediated proteasomal degradation of cyclin D1, resulting in decreased cyclin D1 levels. Accordingly, we suggest a novel function that mTOR signaling positively regulates cyclin D1 levels through preventing its degradation.

Following this important finding, we have further shown that mTORC2 is in fact responsible for the positive regulation of cyclin D1 stability through preventing GSK3-dependent and FBX4-mediated cyclin D1 degradation. In our study, although knockdown of both raptor and rictor reduced cyclin D1 levels, knockdown of rictor, but not raptor, enhanced cyclin D1 degradation or decreased its stability (Fig. 7). Moreover, enforced expression of ectopic rictor, but not raptor, substantially stabilized cyclin D1 and rictor-induced cyclin D1 reduction could be prevented by knockdown of GSK3 or FBX4 (Fig. 7). Cyclin D1 reduction induced by rictor knockdown is likely to be the consequence of suppression of cap-dependent translation of cyclin D1 due to inhibition of the mTORC1. To the best of our knowledge, this is the first study revealing that the mTORC2, like the mTORC1, also positively regulates cyclin D1 levels, but through a different mechanism (i.e., stabilizing protein). Given that the mTORC2 has been implicated in tumorigenesis (45, 46), our findings warrant further study of the role of cyclin D1 in mTORC2-mediated oncogenesis.

Rictor has been shown to be involved in the regulation of protein degradation (e.g., SGK1 and cyclin E1) independent of mTOR (47, 48). Our findings differ from these in clearly showing an mTORC2-dependent regulation of cyclin D1 degradation. One significant implication of our findings is to suggest a new role for mTORC2 in the regulation of protein (e.g., cyclin D1) stability or degradation. Further study in this direction will shed light on the biology of the mTORC2. Although Akt may positively regulate cyclin D1 stability through inactivation of GSK3 (34), cyclin D1 reduction induced by TORKinibs is unlikely to be a consequence of Akt inhibition because our data clearly show
that inhibition of GSK3 with SB216763 rescued TOR kinase-induced cyclin D1 reduction without impairing the ability of TOR kinase inhibitors to suppress Akt phosphorylation in any of the tested cell lines (Fig. 5A and E). In addition, both INK128 and AZD8055 effectively inhibited Akt phosphorylation regardless of cell sensitivities to TOR kinase inhibitors, but potently reduced cyclin D1 levels preferentially in TOR kinase-sensitive cell lines (Supplementary Fig. S4). Moreover, none of the tested TOR kinase inhibitors could reduce p-GSK3β (S21/9) levels despite their potent suppression of Akt S473 phosphorylation (Fig. 5); this result is in line with a previous report showing that SIN1 knockout induced suppression of mTORC2, which abolished Akt S473 phosphorylation without affecting GSK3 phosphorylation (49). Our ongoing work is attempting to understand the mechanism by which mTORC2 positively regulates cyclin D1 stability.

In summary, this study has demonstrated the critical role of GSK3 activation in determining the response of cancer cells to TOR kinase inhibitors, and thus has high translational significance and impact in regard to cancer treatment with TOR kinase inhibitors. Our findings of the regulation of GSK3-dependent and FBX4-mediated cyclin D1 degradation by mTORC2 suggests a novel biologic function of the mTORC2 and increases our understanding of the involvement of the mTORC2 in the regulation of cancer development.

Disclosures of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biosististics, computational analysis): J. Koo, A.A. Gal, F.R. Khuri, S.-Y. Sun
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Maintaining Glycogen Synthase Kinase-3 Activity Is Critical for mTOR Kinase Inhibitors to Inhibit Cancer Cell Growth

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