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GSK3 β phosphorylation of the KLF6 tumor suppressor promotes its transactivation of p21

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Abstract

KLF6, a ubiquitously expressed Krüppel-like transcription factor, is frequently inactivated in human cancer and has significant roles in cellular proliferation, apoptosis, differentiation and development. A key mechanism of KLF6-mediated growth suppression is through p53-independent transactivation of p21. Several cancer-derived KLF6 mutants lead to the loss of p21-mediated growth suppression through an unknown mechanism. Because several colorectal cancer and hepatocellular carcinoma-derived KLF6 mutations affect a glycogen synthase kinase 3 β (GSK3 β) phosphorylation consensus site, we investigated the role of GSK3 β in the regulation of KLF6 function. Based on transient transfection, GSK3 β augments the transactivation of a p21 promoter luciferase by KLF6. Reciprocal co-immunoprecipitation of hemagglutinin (HA)-GSK3 β and Flag-KLF6 validated the interaction between these two proteins. KLF6 phosphorylation is augmented in the presence of GSK3 β based on *in vitro* and *in vivo* ³²P incorporation assays. Site-directed mutagenesis of the candidate phosphorylation sites to alanines ('KLF6-4A' phosphomutant) eliminated a higher molecular weight phosphorylated isoform of KLF6 based on western blot. GSK3 β augmented the transactivation by wild-type KLF6, but not KLF6-4A, towards the p21 promoter, and increased p21 protein. Functionally, GSK3 β enhanced KLF6-mediated growth suppression, which was abrogated by the KLF6-4A phosphomutant. These data establish that GSK3 β directly phosphorylates KLF6, which augments its induction of p21 and resultant growth suppression. This interaction may account for the growth-promoting effects of cancer-derived KLF6 mutants that lack tumor suppressor activity.

Keywords

KLF6; GSK3 β ; p21; phosphorylation

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the seventh most common cancer and the third leading cause of cancer death worldwide, with few therapeutic options.¹ Major pathways implicated in HCC pathogenesis include defects in the RAF/MEK/ERK pathway, phosphatidylinositol-3 kinase/AKT/mammalian target of rapamycin pathway, p53, insulin-like growth factor pathway, hepatocyte growth factor/c-MET pathway, growth factor-regulated angiogenic signaling (vascular endothelial growth factor receptor, fibroblast growth factor) and the WNT/ β -catenin pathway.²

A key mediator of the WNT/ β -catenin pathway is glycogen synthesis kinase 3 β (GSK3 β), which suppresses tumor progression by downregulating the Wnt survival pathway through phosphorylation and degradation of β -catenin. The importance of GSK3 β to the maintenance of β -catenin degradation is highlighted by the frequency of mutations in HCC affecting the GSK3 β phosphorylation sites within β -catenin.^{3,4} The kinase activity of GSK3 β can also be suppressed in HCC, illustrating another mechanism by which GSK3 β targets protein dysregulation. This decrease in GSK3 β kinase activity results in part through downregulation of miR-122, which is also frequently decreased in HCC and associated with poorer survival.⁵

Krüppel-like factor 6 (KLF6) is a zinc-finger transcription factor with tumor suppressor properties that can both activate and suppress gene expression.^{6–10} Mutations within the *klf6* gene, decreased expression and/or loss of heterozygosity are associated with the development of several human cancers, including HCC.^{11,12} Most studies describing reduced KLF6 expression in cancer have been limited to the analysis of mRNA levels; however, in one study, KLF6 protein was reduced in 39% of HCC tissues compared with matched surrounding livers.¹³ Thus, reduced KLF6 protein expression may also contribute to its inactivation in cancer; however, the mechanism of decreased expression has not been explored. Indeed, knowledge about post-translational regulation of KLF6 protein stability is limited. A prostate cancer-derived mutation of a lysine to arginine change, K209R, has been previously identified as an acetylation site on KLF6 that is required for p21 transactivation.¹⁴ In addition, phosphorylation of KLF6 at serine and tyrosine residues may be important for binding to the inducible nitric oxide synthase promoter in response to hypoxia,¹⁵ hinting at the possibility that KLF6 phosphorylation regulates its activity. However, kinases responsible for KLF6 phosphorylation have not been identified.

In this study, we have examined the post-translational regulation of KLF6 by GSK3 β based on the presence of cancer-derived mutations in KLF6 that disrupt a predicted consensus phosphorylation site for GSK3 β .^{16,17} We hypothesized that these mutations could disrupt the ability of GSK3 β to phosphorylate KLF6, thereby affecting its transcriptional activity towards p21, a key cdk/cyclin inhibitor and known transcriptional target of KLF6.¹⁸

RESULTS

GSK3 β alters levels of KLF6 isoforms

Because KLF6 contains a highly conserved GSK3 β consensus site (Table 1), we hypothesized that GSK3 β may post-translationally regulate KLF6 through phosphorylation. Wild-type KLF6 (KLF6-WT) typically appears on western blot as a double band, but the explanation for two isoforms has not been established. To examine the effect of GSK3 β on either isoform of endogenous KLF6, GSK3 β was overexpressed in HepG2 cells for 24 h. The level of KLF6 protein increased in the presence of increased GSK3 β , in particular the upper band of KLF6 (Figure 1a). To support the hypothesis that the upper band is hyperphosphorylated, we used calf intestinal phosphatase (CIP) on cellular lysates, and

found that the upper band partially collapsed (Figure 1b). Taken together, these data suggested that GSK3 β was preferentially increasing the protein levels of phosphorylated form of KLF6-WT.

We next determined the impact of mutating the GSK3 β consensus site on this putative phosphoisoform of KLF6. Therefore, three serines and one tyrosine from amino acids 143 to 155 in human KLF6-WT were mutated either to alanine or aspartic acid at residues 143, 147, 151 and 155 by site-directed mutagenesis. Alanine amino acids are unable to become phosphorylated, whereas aspartic acid amino acids mimic phosphorylation. Accordingly, the phosphomutant was designated 'KLF6-4A' and the phosphomimetic as 'KLF6-4D' (Figure 2a).

The KLF6-WT, KLF6-4A or KLF6-4D constructs were each overexpressed in HepG2 or 293T cells, with or without GSK3 β , to analyze the impact on KLF6 protein. There was a clear difference in apparent molecular size of the KLF6-4A and KLF6-4D mutants when compared with KLF6-WT. Interestingly, KLF6-4A migrated similar to the hypophosphorylated band of KLF6-WT and KLF6-4D migrated to the same size as the hyperphosphorylated KLF6-WT band. All three isoforms of KLF6 responded to GSK3 β with an increase in KLF6 protein (Figure 2b).

Previous studies have reported that GSK3 β targets KLF5 for proteosomal degradation,¹⁹ which is in concordance with GSK3 β altering the stability of many of its substrates. Moreover, previous studies have indicated that KLF6 is ubiquitinated and targeted for proteasomal degradation.²⁰ Therefore, we assessed the impact of proteasomal inhibition on protein levels of KLF6-WT, KLF6-4A and KLF6-4D. The 293T cells were transfected with each of these plasmids and subjected to MG132 (a proteasome inhibitor) for 12 h, after 24 h of transfection. As shown in Figure 2c, the hyperphosphorylated band of KLF6-WT accumulated with proteasomal inhibition, which suggests that the hyperphosphorylated band is normally targeted for degradation. Interestingly, the hypophosphorylated band of KLF6-WT disappeared after treatment with inhibitor, which may reflect the accumulation of another protease that degrades the hypophosphorylated KLF6 band. After preliminary experiments with KLF6-4D showed an inability to recapitulate KLF6-WT function as a growth suppressor in cell culture (Supplementary Figure S1), we focused further experiments only on the KLF6-4A phosphomutant.

We next sought to link GSK3 β activity to increased KLF6-WT and KLF6-4A protein levels. Preliminary experiments had suggested that GSK3 β increased both KLF6-WT and KLF6-4A protein levels (Figure 3a), but the increase was only significant for the KLF6-WT protein ($P = 0.002$), with only a trend of increase of KLF6-4A ($P = 0.12$) (Figure 3b). Moreover, the amount of protein was increasing proportionally more in the hyperphosphorylated band of KLF6-WT than the hypophosphorylated band ($P = 0.03$) (Figure 3c).

We next examined whether the two bands of KLF6-WT result from post-translational modifications. The effect of CIP on endogenous KLF6 suggested that the upper band was phosphorylated, leading to a higher molecular weight. Detection of the two bands expressed as Flag-fusion cDNAs by anti-Flag antibody indicated that they were not alternative splice forms. Loss of the higher molecular weight isoform following mutation of the GSK3 β consensus site in KLF6 was consistent with loss of phosphorylation. We addressed this prospect experimentally by treating cell lysates from cells overexpressing KLF6-WT or KLF6-4A, with or without GSK3 β , with CIP. The CIP treatment led to a reduced size of the higher molecular weight isoform of KLF6, but had no impact on the KLF6 phosphomutant (Figure 3d). The lack of complete shift of the upper band could represent other post-translational modifications not affected by CIP, whereas dephosphorylation by CIP

treatment is less likely, as a distinct band, not a smear was apparent on western blot. These data also suggest that the four residues mutated in the KLF6-4A protein are likely important for phosphorylation of KLF6, as the upper phosphorylated form of KLF6 is completely lost with KLF6-4A.

Inhibition of GSK3 β abrogates KLF6 protein increase

To confirm that the effect of GSK3 β on KLF6 was reversible, we assessed the impact of both a dominant negative and a constitutively active GSK3 β on KLF6 protein levels. Two GSK3 β plasmids: hemagglutinin (HA)-GSK3 β -S9A (constitutively active) and HA-GSK3 β -DN (dominant negative) were transiently transfected into 293T cells for 24 h before collection. Western blot demonstrated an increase in endogenous KLF6 hyperphosphorylated band intensity following both HA-GSK3 β and HA-GSK3 β -S9A transfection, whereas the dominant-negative GSK3 β was unable to increase KLF6 protein levels (Figure 4a). These findings suggested that the kinase activity of GSK3 β was necessary to increase KLF6 protein levels.

To complement use of the dominant-negative plasmid, HepG2 cells were incubated with SB-216763 (10 μ M), a potent and selective ATP-competitive inhibitor of GSK3 α and GSK3 β isozymes. In the presence of GSK3 β overexpression in HepG2 cells, this inhibitor abrogated the increase in endogenous KLF6 protein, particularly the hyperphosphorylated band (Figure 4b). We also assessed the inhibitor's impact on HepG2 cells following transient transfection with Flag-KLF6-WT or Flag-KLF6-4A, with or without HA-GSK3 β . The addition of SB-216763 abrogated the increase in KLF6 protein in response to GSK3 β for both KLF6-WT and KLF6-4A (Figure 4c). Taken together, these results are consistent with the hypothesis that GSK3 β increases total KLF6 protein.

GSK3 β interacts with and phosphorylates KLF6 *in vitro* and *in vivo*

We next determined whether GSK3 β had a direct effect on KLF6 or was altered indirectly through an upstream pathway involving other GSK3 β substrates. Direct interaction between GSK3 β with KLF6 was established by transfecting HA-GSK3 β and Flag-KLF6 into HepG2 cells and reciprocally immunoprecipitating with either anti-HA or anti-Flag antibodies (Figure 5a).

We next determined if KLF6 was a direct substrate for GSK3 β kinase activity. Previous studies have shown that KLF6 can be phosphorylated at serine and threonine residues,²¹ but the specific kinases responsible for this regulation have not been identified. To examine whether KLF6 is phosphorylated by GSK3 β , GST-tagged KLF6 was expressed in bacteria, and then purified by glutathione affinity chromatography. HA-GSK3 β was transfected into 293T cells and purified by immunoprecipitation (IP) with an anti-HA antibody. Both proteins were then incubated with ATP in an *in vitro* kinase reaction, separated by electrophoresis, and the phosphorylated protein was identified using a Multiplexed Proteomics Phosphoprotein Gel Stain Kit. This analysis demonstrated a band of approximately 45 kDa, which likely represents the GST-KLF6 phosphoprotein (Figure 5b). This finding was validated using *in vivo* kinase assays with the addition of [³²P]orthophosphate in cell culture to confirm phosphorylation of KLF6 by GSK3 β . HeLa cells were transfected with KLF6, with or without GSK3 β , and grown in the presence of [³²P]orthophosphate. IP of KLF6 followed by western blot and autoradiography demonstrated KLF6 phosphorylation by GSK3 β (Figure 5c). Lastly, we demonstrated that KLF6 is phosphorylated on serine residues by incubating Flag-KLF6 with GSK3 β and using a serine-specific antibody on western blot (Figure 5d). These results are consistent with a direct interaction between GSK3 β and KLF6 that leads to KLF6 phosphorylation.

GSK3 β does not affect KLF6 protein stability

We next examined whether the increase in KLF6 protein following interaction with GSK3 β resulted from altered protein stability. Previous studies of KLF6 half-life using GFP-conjugated KLF6 cDNA in HeLa cells reported the half-life of KLF6 as ~18 min.²² Flag-KLF6, with or without GSK3 β , was transfected into 293T cells and *de novo* protein synthesis inhibited with cycloheximide for 0, 30, 60 and 120 min. This indicated a half life of 50–70 min.²² There was no significant impact of GSK3 β on KLF6 protein stability (Supplementary Figure S2).

GSK3 β increases KLF6-mediated transactivation of p21 and growth suppression

We next assessed the functional impact of GSK3 β on KLF6. Transactivation of p21 is a well-characterized pathway of KLF6-mediated growth suppression.^{6,12,23} As the cancer-derived mutations found within GSK3 β phosphorylation sites of KLF6 fail to decrease cellular proliferation,¹⁷ we examined whether GSK3 β -mediated phosphorylation of WT KLF6 could contribute to its growth-suppressive activity by assessing the ability of GSK3 β to augment KLF6 transactivation of the p21 promoter. KLF6-WT or KLF6-4A was co-transfected with a p21 promoter luciferase construct in 293T cells. The addition of GSK3 β to KLF6-WT significantly increased p21-luciferase activity compared with either alone. This transactivation was partially abrogated with the KLF6-4A mutant, although a significant increase was still evident with the addition of GSK3 β , possibly through the impact of GSK3 β on other proteins that affect p21 transactivation (Figure 6a). To confirm an effect of KLF6 on endogenous p21, 293T cells were transfected with KLF6-WT or KLF6-4A plasmids, with or without GSK3 β , and p21 was quantified by western blot (Figure 6b). The addition of GSK3 β increased protein levels of both wild-type and phosphomutant KLF6, yet only KLF6-WT increased p21 protein levels ($P < 0.05$), while KLF6-4A had a minimal effect ($P < 0.5$) (Figure 6c).

To correlate transactivation of p21 with cellular growth, HepG2 cells were transiently transfected with KLF6-WT or KLF6-4A, with or without GSK3 β , and DNA synthesis was assessed by [³H]thymidine incorporation. GSK3 β enhanced the growth-suppressive effect of KLF6-WT but not that of the KLF6-4A phosphomutant (Figure 6d). This result does not directly link the enhanced growth suppression to an increase in p21 as there could be other downstream changes mediated through enhanced KLF6 that were not addressed in this study. Taken together, however, these results indicate that GSK3 β enhances the growth-suppressive activity of KLF6 at least in part through phosphorylation, thereby enhancing KLF6-mediated transactivation of p21.

KLF6 cancer-derived mutants are not stabilized by GSK3 β

Finally, we sought to functionally link the loss of GSK3 β -mediated phosphorylation in KLF6 cancer-derived mutants with reduced KLF6 protein, the P149S (HCC) and P148L (HCC and colon cancer) mutations that fall within the GSK3 β consensus site of KLF6 provoke a significant increase rather than inhibition of cellular proliferation.^{16,17} To do so, HepG2 cells were transiently transfected with Flag-KLF6-WT, Flag-KLF6-P149S or Flag-KLF6-P148L in the presence or absence of HA-GSK3 β . There was no effect of GSK3 β on the cancer-derived KLF6 mutants (Figure 7), suggesting that the cancer-derived mutants may lose their growth-suppressive function in part through a blunted response to GSK3 β .

DISCUSSION

Although most studies exploring KLF6's tumor suppressor activity have focused on the regulation of mRNA expression, very few have examined the control of KLF6 protein stability and activity. Here we report that GSK3 β regulates KLF6 protein activity through

phosphorylation at amino acids 143, 147, 151 and 155, leading to the accumulation of KLF6 protein and augmented KLF6-mediated growth suppression by p21. Based on the loss of activity of cancer-derived mutations affecting these residues, the findings suggest that loss of GSK3 β -mediated KLF6 phosphorylation impairs its growth-suppressive activity.

Cell cycle control is an essential component of development and growth, and when dysregulated promotes neoplasia.²⁴ KLF6 regulates cell cycle through several pathways, including transactivation of p21, by sequestering cyclin D1, and by antagonizing the c-Jun proto-oncogene.²¹ Sequestration of cyclin D1 prevents its interaction with cdk4, leading to decreased Rb phosphorylation at Ser⁷⁹⁵.^{6,25}

Our findings indicate that KLF6 is a novel GSK3 β substrate, which enhances KLF6-mediated transactivation of the p21 promoter. Moreover, GSK3 β primarily increases the phosphorylated isoform of KLF6, and mutation of the conserved GSK3 β phosphorylation site within KLF6 leads to selective loss of the higher molecular weight, hyperphosphorylated isoform of KLF6. We further validate this effect by inhibiting GSK3 β , which reduces KLF6 protein levels. The finding that GSK3 β stabilizes rather than suppresses KLF6 protein is in contrast to the recently described role of GSK3 β in targeting KLF5 for ubiquitin-mediated protein degradation.¹⁹ In both cases, GSK3 β leads to growth inhibition; however, in the case of KLF5, which is proliferative, it does so by targeting KLF5 for degradation, whereas it increases the protein of the growth-suppressive KLF6. The divergent regulation of KLF5 and KLF6 by GSK3 β to achieve the same cellular effect is novel among KLFs, and it is unknown whether KLF5 and KLF6 also interact directly.

We found that GSK3 β was able to increase all forms of KLF6 to some extent, although the mechanism of increase of KLF6-4A is not completely clear. One possible explanation is that in the absence of native KLF6, GSK3 β is made available for interaction with lower affinity substrate residues elsewhere on KLF6-4A protein. The hypophosphorylated band of KLF6 acts in a manner opposite to the hyperphosphorylated band in response to MG132, which suggests that they are indeed regulated differently in response to proteasomal inhibition. In contrast to the effect of GSK3 β , the impact of MG132 is more straightforward, in that the wild-type KLF6 is stabilized, allowing more phosphorylation of the protein, leading to a shift from the hypo- to the hyperphosphorylated form, whereas the phospho-deficient mutant KLF6-4A cannot be stabilized through phosphorylation and thus is not increased in amount or molecular size.

This study partially clarifies the post-translational regulation of KLF6 by phosphorylation, but further studies are required to fully characterize GSK3 β -mediated KLF6 regulation, specifically the requirement for kinase priming to maximize phosphorylation. Based on sequence analysis by the online phosphorylation prediction program, KinasePhos,²⁶ there is a strongly predicted casein kinase 1 phosphorylation site at residue 159, which is the kinase that primes β -catenin for GSK3 β phosphorylation.²⁷ As casein kinase 1 also requires pre-priming for efficient phosphorylation, an additional layer of complexity is likely to activate casein kinase 1.

In evaluating how KLF6 regulation by GSK3 β fits into upstream pathways, it is important to consider that GSK3 β also regulates β -catenin in cell cycle control. The axin/GSK3 β /APC complex normally promotes growth suppression by the proteolytic degradation of β -catenin, through its phosphorylation by GSK3 β . In hepatocytespecific APC knockout mice, β -catenin is no longer phosphorylated by GSK3 β , leading to increased levels of β -catenin protein and subsequent hepatocyte proliferation.²⁸ The Wnt pathway that regulates GSK3 β kinase activity could be involved in regulating the phosphorylation state of KLF6 in parallel with

its regulation of β -catenin. Both these pathways lead to a decrease in cellular proliferation, and may have GSK3 β as a common regulatory kinase.

In summary, loss of GSK3 β regulation either by downregulation of its kinase activity, mutation of KLF6 phosphorylation sites or disrupted interaction between the two could contribute to decreased KLF6 function as a tumor suppressor. Future studies should address GSK3 β regulation of KLF6 in the context of disease progression in HCC. Coupling the biochemistry of KLF6 with its biology in the setting of HCC and other cancers will help clarify its contribution to human cancer.

MATERIALS AND METHODS

Antibodies and reagents

Flagged KLF6 PCIneo wild-type plasmid was used to generate flagged KLF6-4A phosphomutant with QuikChange XL Site-Directed Mutagenesis Kit (Catalog no. 200516) from Stratagene (Santa Clara, CA, USA). Anti-Flag antibody produced in rabbit (Catalog no. F7425) from Sigma (St Louis, MO, USA) and HA tag antibody (Catalog no. ab18181) mouse monoclonal from Abcam (Cambridge, MA, USA) were used in IP and western blotting reactions. Alkaline phosphatase, CIP (Catalog no. M0290S) from BioLabs (Ipswich, MA, USA) was used to de-phosphorylate KLF6. Antibody to p21 (Catalog no. sc-397) from Santa Cruz Technology (Santa Cruz, CA, USA) and anti-phosphoserine antibody (Catalog no. ab9332) from Abcam was used in western blotting. Lithium chloride (20 mM) was used to inhibit GSK3 β .

Cell culture and transfection

HepG2 and 293T cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (GIBCO-BRL, Grand Island, NY, USA), 2 mM L-glutamine (GIBCO-BRL), 50 U/ml penicillin (GIBCO-BRL) and 50 μ g/ml streptomycin (GIBCO-BRL). Cells were grown in a humidified incubator at 37°C and 5% CO₂ atmosphere. Lipofectamine 2000 Transfection Reagent (Catalog no. 11668500) from Invitrogen was used to transfect transiently PCIneo plasmids encoding Flag-KLF6-WT, Flag-KLF6-4A, Flag-KLF6-4D, HA-GSK3 β and p21-luciferase.

Co-IP and western blotting

All protein extracts were collected in cold IP buffer supplemented with protease and phosphatase inhibitors (10 mM Tris-HCl (pH 7.5), 10 mM NaCl, 10 mM EDTA, 0.5% Triton X-100, 1 mM phenylmethanesulfonylfluoride, 1 μ M aprotinin, 1 μ M leupeptin). After centrifuging the extracts, 500 μ g of the supernatant and 2 μ g of corresponding antibody was used for IP with overnight rotation at 4°C. The next day, protein A/G sepharose beads were washed with lysis buffer and 25 μ l were added per tube and incubated for 1 h at 4°C. Beads were then washed three times with co-IP buffer. Proteins were subsequently boiled off the beads in 2 $\times$ sodium dodecyl sulfate (SDS) protein loading buffer and separated on SDS-polyacrylamide gel electrophoresis gels for western analysis.

Cell extracts for western blotting were harvested in lysis buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.1% NP40, 10% glycerol, 20 mM β -glycerophosphate, 0.1 mM sodium vanadate, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride and 1 protease inhibitor tablet per 10 ml buffer) according to standard protocols using 30–60 μ g protein per lane.

Non-radioactive phosphodetection staining

Recombinant GST-tagged KLF6 was produced in bacteria and column-purified on Glutathione-Superflow Resin (Clontech, Mountain View, CA, USA). HA-GSK3 β was

transfected into 293T cells and IP purified using HA antibody. *In vitro* kinase reactions were carried out with 3 μ g of GST-KLF6, IP-purified HA-GSK3 β and 150 μ M ATP (Catalog no. PV3227; Invitrogen) for 30 min at room temperature, followed by 30°C for 15 min. Reactions were run on a 10% gel and phosphorylated KLF6 was identified using Multiplexed Proteomics Phosphoprotein Gel Stain Kit (Catalog no. MPM33306) and Pro-Q Diamond phosphoprotein gel destaining solution (Catalog no. P33310) from Invitrogen.

***In vivo* [³²P]orthophosphate cell labeling**

HeLa cells were transfected with Flag-KLF6 together, with or without GSK3 β , and labeled with [³²P]orthophosphate (0.5 mCi/ml) in minimum Eagle's medium without phosphate for 4 h. KLF6 was immunoprecipitated with anti-Flag antibody. The immunoprecipitates were separated on SDS-PAGE and transferred to membranes. Phosphorylated KLF6 was detected by autoradiography.

Luciferase assay

HepG2 cells cultured in 12-well plates (Corning Glass, Tewksbury, MA, USA) were transfected with 1 μ g of PCIneo empty vector, PCIneo-Flag-KLF6, PCIneo-Flag-KLF6-4A, with or without GSK3 β , with p21 promotor construct as described previously. At 24 h after transfection, luciferase activity was assayed in 10 μ l of lysate using the Dual-Luciferase reporter assay system (Catalog no. E1910) from Promega (Madison, WI, USA) and a Dynex (Sunnyvale, CA, USA) luminometer. Transfection efficiency was normalized to *Renilla* luciferase activity.

Cellular proliferation assay

Proliferation was determined by assaying [³H]thymidine incorporation into DNA. HepG2 cells were seeded in 12-well plates (50 000 cells per ml) and transfected the following day. [³H]thymidine (1 μ Ci/ml) was added 4 h before collection, which was performed 24 h post-transfection. Cells were washed three times with ice-cold phosphate-buffered saline and solubilized in 0.25% sodium hydroxide/0.25% SDS and neutralized with hydrochloric acid (1 N). Disintegrations per minute were evaluated in a liquid scintillation counter.

Statistical analyses

Results are obtained using Microsoft Excel software from at least three independent calculations and expressed as mean $\pm$ s.e. *P*-values (Student's two-tailed, unpaired *t*-test). Statistical significance is considered for data with *P* < 0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

GSK3 β increases KLF6 protein, possibly through phosphorylation. **(a)** HepG2 cells were transfected with HA-GSK3 β or PCIneo control plasmids for 24 h before collecting the protein. HA-GSK3 β overexpression migrates as a band slightly higher than the endogenous protein, and hence the double band seen on western blot. **(b)** HepG2 cell lysates with or without GSK3 β transfection were treated with CIP enzyme for 1 h at 37°C and representative western blot is shown.

Figure 2.

KLF6 phosphomutant, KLF6-4A, and KLF6 phosphomimetic, KLF6-4D, lead to changes in upper versus lower band accumulation. **(a)** KLF6-WT plasmid was used to produce the KLF6 phosphomutant and phosphomimetic with site-directed mutagenesis. The consensus site for GSK3 β phosphorylation, amino acids 143, 147 and 151, as well as a possible priming site amino acid 155 were all mutated to either alanine or aspartic acid and designated KLF6-4A or KLF6-4D. **(b)** KLF6-WT, KLF6-4A and KLF6-4D transiently overexpressed in 293T cells, with or without GSK3 β , were separated by western blotting. **(c)** The 293T cells were transfected with Flag-KLF6-WT, Flag-KLF6-4A or Flag-KLF6-4D for 24 h before the addition of 10 μ M of MG132 for an additional 12 h. Total cell lysates were run separated by western blot and probed with a Flag antibody. Glyceraldehyde 3-phosphate dehydrogenase (Gapdh) was used as a loading control.

Figure 3.

GSK3 β causes a significant increase in total KLF6-WT but not KLF6-4A protein abundance. (a) KLF6-WT and KLF6-4A overexpression in HepG2 cells, with or without GSK3 β , collected after 24 h of transient transfection. (b) Western blot of the total protein was quantified using ImageJ (NIH, Bethesda, MD, USA) of nine independent experiments and the mean intensity was graphed (** $P < 0.005$). The significance was calculated using a two-tailed Student's *t*-test. The error bars are representative of the standard error. (c) The hyperphosphorylated band of KLF6-WT was quantified as a percentage of the total amount. The results are graphed as a fold change of KLF6-WT upper band % of total protein with GSK3 β over KLF6-WT upper band % of total protein without GSK3 β (* $P < 0.05$). Graph represents quantification of nine independent experiments and significance was assessed by two-tailed Student's *t*-test. (d) Flag-KLF6-WT or Flag-KLF6-4A plasmids were transfected into HepG2 cells, with or without GSK3 β , for 24 h before collecting proteins in lysis buffer $\pm$ phosphatase inhibitors. A measure of 5 μ g of crude cell lysates were treated with CIP for 1 h at 37°C and separated on a 10% SDS-PAGE gel. Representative western blot from at least three independent experiments is shown. NS, not significant.

Figure 4.

GSK3β inhibition or overexpression of a GSK3β dominant-negative protein abrogates KLF6 protein increase. **(a)** The 293T cells were transfected with GSK3β-WT, GSK3β-S9A (constitutively active) or GSK3β-DN (dominant-negative) plasmids. Endogenous KLF6 protein is shown by western blot. **(b)** HepG2 cells were incubated with either dimethylsulfoxide (DMSO) (control) or 10 μM SB-216763 (GSK3 inhibitor) for 16 h before collection. Western blot for KLF6 protein is shown. **(c)** HepG2 cells were transfected with KLF6-WT or KLF6-4A for 24 h, followed by incubation for 16 h with either DMSO or SB-216763. Western blot for KLF6 protein is shown. These western blots are representative of at least three experiments.

Figure 5.

GSK3β interacts with and phosphorylates KLF6 *in vitro* and *in vivo*. (a) Flag-KLF6 and HA-GSK3β were transiently transfected into 293T cells for 24 h before collection. Reciprocal co-IP was performed with either flag or HA antibodies. Western blot shown was probed with either GSK3β or KLF6 antibodies. (b) HA-GSK3β was transfected into HeLa cells and purified with an antibody to the HA-tag and A/G sepharose beads. Recombinant GST-KLF6 was purified from bacteria and then incubated with HA-GSK3β with non-radioactive ATP. The phosphorylated protein was detected by Phosphoprotein Gel Stain kit. The band marked by the arrow is the phosphorylated protein. (c) GSK3β and Flag-KLF6 were transfected into HeLa cells, which contained [³²P]orthophosphate, in the media for 4 h before collecting of the protein lysates. Flag-KLF6 was immunoprecipitated, and radiograph and western blot of KLF6 are shown. (d) GSK3β and Flag-KLF6 were transiently transfected into 293T cells for 24 h, followed by IP of Flag-KLF6 and western blotted for phosphorylated serine residues. IB, immunoblotting. A full colour version of this figure is available at the *Oncogene* journal online.

Figure 6.

KLF6-WT and KLF6-4A have differential capacities to upregulate p21 and augment growth suppression. **(a)** KLF6 functional response to GSK3 β was measured by using a p21 luciferase promoter construct. Flag-KLF6-WT or Flag-KLF6-4A plasmids with or without HA-GSK3 β and p21 promoter luciferase plasmid with *Renilla* (internal control) were overexpressed in 293T cells for 24 h. Luciferase luminescence was graphed as a ratio relative to PCIneo, negative control. Data represent three independent experiments. A two-tailed Student's *t*-test was performed and (*) denotes $P < 0.05$. **(b)** Flag-KLF6-WT or Flag-KLF6-4A plasmids were transiently overexpressed in 293T cells for 24 h, with or without HA-GSK3 β , and western blot performed for KLF6 downstream target gene, *p21*. Representative western blot from nine independent experiments is shown. **(c)** Quantification of blots (from **b**) was carried out by ImageJ and represented as a fold change of GSK3 β + KLF6-WT or GSK3 β + KLF6-4A over KLF6-WT or KLF6-4A alone. A two-tailed Student's *t*-test was performed and (*) denotes $P < 0.05$. **(d)** HepG2 cells were transfected with HA-GSK3 β , Flag-KLF6-WT and Flag-KLF6-4A, alone or in combination for 24 h. [3 H]Thymidine was added 4 h before collection, non-incorporated [3 H]thymidine was washed away and cell lysates were read on a scintillation counter. Results are graphed as a fold change to control cells. Data represent three independent experiments and (*) denotes $P < 0.05$. Representative western blots from three independent experiments are shown.

Figure 7.

GSK3 β effects on cancer-derived KLF6 mutants. **(a)** HepG2 cells were transiently transfected with KLF6 cancer-derived mutants (Flag-P149S, P148L), in the presence or absence of GSK3 β , for 24 h before collection. Flag-KLF6-WT was added for reference. In all, 30 mg of crude cell lysates were separated on a 10% SDS gel and analyzed with indicated antibodies. Both western blots, right and left, were developed at the same time with the same exposure. **(b)** Western blot quantification using ImageJ from three independent experiments. Statistics were carried out with a Student's *t*-test and (*) denotes *P* < 0.05.

Table 1GSK3 β consensus sequence within KLF6.

KLF6 sequence	Species
141 SSSVTSTPPSSPELSREPSQ 160	Human
141 SSSVISTPPSSPEVNRESSQ 160	Mouse
137 SSSVTSTPPSSPEQSKDPSC 156	Xenopus
137 SSSIISTPPSSPEMPQEPGV 156	Zebrafish

Abbreviations: GSK3 β , glycogen synthase kinase 3 β ; KLF6, Krüppel-like factor 6. Consensus sequence: Ser/Thr-X-X-Ser/Thr-P.