Tumor and Stem Cell Biology

Cancer Research

The Fbw7 Tumor Suppressor Targets KLF5 for Ubiquitin-Mediated Degradation and Suppresses Breast Cell Proliferation

Dong Zhao, Han-Qiu Zheng, Zhongmei Zhou, and Ceshi Chen

Abstract

Fbw7 is a tumor suppressor frequently inactivated in cancers. The KLF5 transcription factor promotes breast cell proliferation and tumorigenesis through upregulating *FGF-BP*. The KLF5 protein degrades rapidly through the ubiquitin proteasome pathway. Here, we show that the Skp1-CUL1-Fbw7 E3 ubiquitin ligase complex (SCF^{Fbw7}) targets KLF5 for ubiquitin-mediated degradation in a GSK3β-mediated KLF5 phosphorylation–dependent manner. Mutation of the critical S303 residue in the KLF5 Cdc4 phospho-degrons motif ([303]**S**PPSS) abolishes the protein interaction, ubiquitination, and degradation by Fbw7. Inactivation of endogenous Fbw7 remarkably increases the endogenous KLF5 protein abundances. Endogenous Fbw7 suppresses the *FGF-BP* gene expression and breast cell proliferation through targeting KLF5 for degradation. These findings suggest that Fbw7 inhibits breast cell proliferation at least partially through targeting KLF5 for proteolysis. This new regulatory mechanism of KLF5 degradation may result in useful diagnostic and therapeutic targets for breast cancer and other cancers. *Cancer Res; 70(11); 4728–38. ©2010 AACR.*

Introduction

The F-box and WD40 repeat domain–containing 7 (Fbw7/Cdc4) protein is a bona fide tumor suppressor inhibiting cell division and growth (1). Fbw7 is inactivated in numerous human malignances, including breast cancer by gene mutation (2–4) and expression downregulation (5, 6). Fbw7 is an F-box protein that recruits substrates for the SCF^{Fbw7} (a complex of *S*kp1, *C*UL1, and *F*-box proteins) E3 ubiquitin ligase. SCF^{Fbw7} degrades several well-known oncoproteins, including MYC (7, 8), Cyclin E (4, 9), Notch (10), c-Jun (11), and mammalian target of rapamycin (12, 13). All Fbw7 substrates contain at least one conserved Cdc4 phospho-degrons (CPD) sequence (**T/S**)PXX(S/T/E) in which the T/S residue can be phosphorylated by GSK3 (1).

The KLF5 transcription factor has been shown to play important roles in cancer (14). Accumulated evidence suggests that KLF5 promotes fibroblast, colon, bladder, and breast cell proliferation (15–17). KLF5 is highly expressed in estrogen receptor (ER)α–negative basal-type breast cancer and is an unfavorable prognostic biomarker correlated with shorter survival for breast cancer patients (18, 19). Our previous studies suggest that KLF5 promotes breast cell proliferation

through directly upregulating the *FGF-BP* gene transcription (17). More recently, inhibition of KLF5 by small interfering RNA (siRNA) using nanoparticles has been shown to efficiently inhibit tumor growth *in vivo* (20). These findings define KLF5 as an oncogenic transcription factor and a potential therapeutic target for invasive breast cancer and other cancers.

KLF5 is an unstable protein with a short half-life (21). KLF5 can be degraded through the ubiquitin-dependent and ubiquitin-independent mechanisms (21, 22). Previously, we showed that the major KLF5 TAD contains destruction motifs (degrons) that recruit E3 ligases for KLF5 ubiquitination and degradation (21). Besides the PY ([325]PPSY) motif that recruits WWP1 (23), we noticed that the KLF5 TAD also contains two putative evolution-conserved CPD motifs ([303]**S**PPSS and [323]**T**PPPS) that could recruit Fbw7 containing E3 ligase complex SCF^{Fbw7}. Given the significant roles of Fbw7 and KLF5 in human cancers, it is important to know whether Fbw7 promotes KLF5 degradation.

In this article, we show that Fbw7 targets KLF5 for ubiquitin-mediated proteasomal degradation. We show that the GSK3β kinase is involved in the KLF5 S303 phosphorylation that is required for Fbw7-mediated KLF5 degradation. Importantly, we found that Fbw7 suppresses breast cell proliferation at least partially through promoting KLF5 proteolysis. These findings help us understand the regulatory mechanism of KLF5 in human cancer.

Materials and Methods

Antibodies and reagents

The rabbit polyclonal anti-KLF5 and anti-WWP1 antibodies (Ab) are kindly provided by Dr. J.T. Dong (Emory University,

Authors' Affiliation: The Center for Cell Biology and Cancer Research, Albany Medical College, Albany, New York

Corresponding Author: Ceshi Chen, Albany Medical College, Albany Medical College, 47 New Scotland Avenue, Albany, NY 12208. Phone: 518-262-2936; Fax: 518-262-3065; E-mail: chenc@mail.amc.edu.

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Atlanta, GA). The anti–β-actin, anti-FLAG, and anti–glutathione *S*-transferase Abs are from Sigma. The anti–glyceraldehyde-3-phosphate dehydrogenase Ab is from Cell Signaling. The anti–MYC 9E10, anti-HA, anti-Ub, and anti-ERα Abs are from Santa Cruz Biotechnology. The anti–FGF-BP and goat anti-KLF5 Abs are from R&D Systems. The rabbit polyclonal anti-KLF5 pS303 Ab was generated using the keyhole limpet hemocyanin–conjugated peptide "*FLPQQATYFPPS(p303)PPS*" (Panora Biotech). The sera were collected and affinity purified. The Ab was diluted with 1:10,000 in 3% bovine serum albumin (BSA) for Western blotting. Calf intestinal alkaline phosphatase (CIP; 20 U/μL) is from Promega.

Cell culture and transfection

Wild-type (WT) and Fbw7 null DLD1 cells (kindly provided by Drs. B. Vogelstein and K.W. Kinzler, Johns Hopkins University, Baltimore, MD) were cultured in McCOY'S 5A supplemented with 10% fetal bovine serum (FBS). SUM149 was cultured in Ham's F-12 supplemented with 5% FBS, 5 μg/mL insulin, and 1 μg/mL hydrocortisone. All transient transfections for plasmids and siRNAs were performed using Lipofectamine 2000 (Invitrogen). All chemically synthesized siRNAs were purchased from Ambion and transfected at 10 nmol/L final concentration. The siRNA target sequences are provided in Supplementary Table S1.

Expression plasmids

The pcDNA3 plasmids expressing WT KLF5, Δ323-248, Δ299-348, and Δ321-328 have been described in our previous study (21). The KLF5-S303A, KLF5-S307A, KLF5-T323A, and KLF5-T323A/T327A were generated using the PCR-directed mutagenesis method. Three FLAG tags were added to the COOH-terminus of KLF5 and its mutants. All FLAG- and MYC-tagged WT and mutant Fbw7 plasmids are kindly provided by Dr. B.E. Clurman (Fred Hutchinson Cancer Research Center, Seattle, WA). The *Fbw7-γ* and *Fbw7-γ-F* genes were amplified and subcloned into the pLenti6 vector.

Quantitative reverse transcription-PCR assays

Total RNA was isolated from cells using the Trizol reagent (Invitrogen) and subjected to reverse transcription with random hexanucleotide primers using the iScript cDNA Synthesis kit (Bio-Rad). Quantitative real-time PCR was performed on the ABI-7300 system, using Roche FastStart SYBR Green Master containing Rox (Roche Diagnostics). The primer sequences used in this study are listed in Supplementary Table S2.

Immunofluorescence staining

HEK293FT cells (5×10^4) were plated on a gelatin-coated glass slide. The cells were transfected with KLF5 and FLAG-tagged Fbw7 isoforms, respectively. Two days after transfection, the cells were treated with 10 μmol/L MG132 for 4 hours and then were fixed using 4% paraformaldehyde at 4°C overnight. The slides were washed with PBS and permealized by 0.2% Triton X-100 in PBS for 5 minutes. The cells were then quenched with 50 mmol/L NH_4Cl for 5 minutes

and blocked with 10% goat serum for 1 hour at room temperature. The anti-KLF5 Ab (1:100) and anti-FLAG M2 mouse monoclonal Ab (1:300) were diluted with 0.1% BSA to stain the cells at 4°C overnight. The slides were washed and incubated with secondary Abs [rhodamine goat anti-rabbit Ab from Jackson ImmunoResearch (1:150) and Alexa-488 goat anti-mouse from Molecular Probes (1:150)] in 5% goat serum for 1 hour at room temperature. Fluorescent images were captured using an Olympus BX-61 microscopy at total ×400 magnification.

Protein ubiquitination assays

The *in vitro* ubiquitination assay is performed using an ubiquitination kit from Enzo Life Science. The 3×FLAG-tagged KLF5 and KLF5-S303A substrate proteins were purified from HEK293FT cells using immunoprecipitation (IP) with FLAG M2 beads. The KLF5 proteins were eluted by using 3× Flag peptide (100 μg/mL; Sigma). Similarly, the SCF^{Fbw7} E3 complexes were purified from HEK293FT cells using IP. Myc-CUL1, Rbx1, Skp1, and FLAG-Fbw7γ/-F/R338L were cotransfected. The reaction was performed with 0.75 μL E1, 1.5 μL E2 (UbcH5c), 0.75 μL Mg-ATP buffer, 1.5 μL 10× ubiquitination buffer, 0.75 μL Ub, 0.75 μL ubiquitin aldehyde, 2 μL KLF5, 2 μL E3 (SCF^{Fbw7} or its mutants), and H_2O in a 15 μL volume at 30°C for 1 hour. The ubiquitinated KLF5 proteins were detected by Western blotting. The KLF5 ubiquitination assay in cells has been described in our previous study (23).

In vitro kinase assays

FLAG-tagged KLF5 and its mutants (S303A and S307A) were purified by IP. The KLF5 proteins (5 μL) were incubated with and without 0.4 μL active GSK3β enzyme (New England Biolabs), 2 μL 10× reaction buffer, 0.4 μL 10 mmol/L ATP, 0.2 μL γ-^{32}P-ATP (25Ci/mmol, MP Biomedicals), and H_2O up to 20 μL. The mixtures were incubated for 1 hour at 30°C and subjected to SDS-PAGE and autoradiography.

Results

Fbw7 interacts with KLF5

To test if all Fbw7 isoforms (α, β, and γ) interact with KLF5, we cotransfected FLAG-Fbw7 and FLAG-Fbw7-F (without the intact F-box that interacts with Skp1; ref. 7) with KLF5 into HEK293FT cells. The cells were treated with the MG132 proteasome inhibitor to protect the KLF5 protein from degradation by Fbw7. We performed IP with the anti-FLAG Ab and found that all (α, β, and γ) WT and -F Fbw7 proteins interact with the KLF5 protein (Fig. 1A). These results indicate that all Fbw7 isoforms interact with KLF5 in an F-box–independent manner.

To further test if the Fbw7 WD40 repeats are responsible for KLF5 binding, we cotransfected FLAG-Fbw7-WD (8 WD40 repeats only; ref. 7) with KLF5 into HEK293FT cells and performed IP. We found that the WD40 repeats are sufficient for KLF5 binding (Fig. 1A). When the key R338 residue is mutated into L in Fbw7γ, the protein-protein interaction is dramatically reduced (Fig. 1B). Thus, the WD40 repeats are sufficient and necessary for KLF5 binding. To test if Fbw7
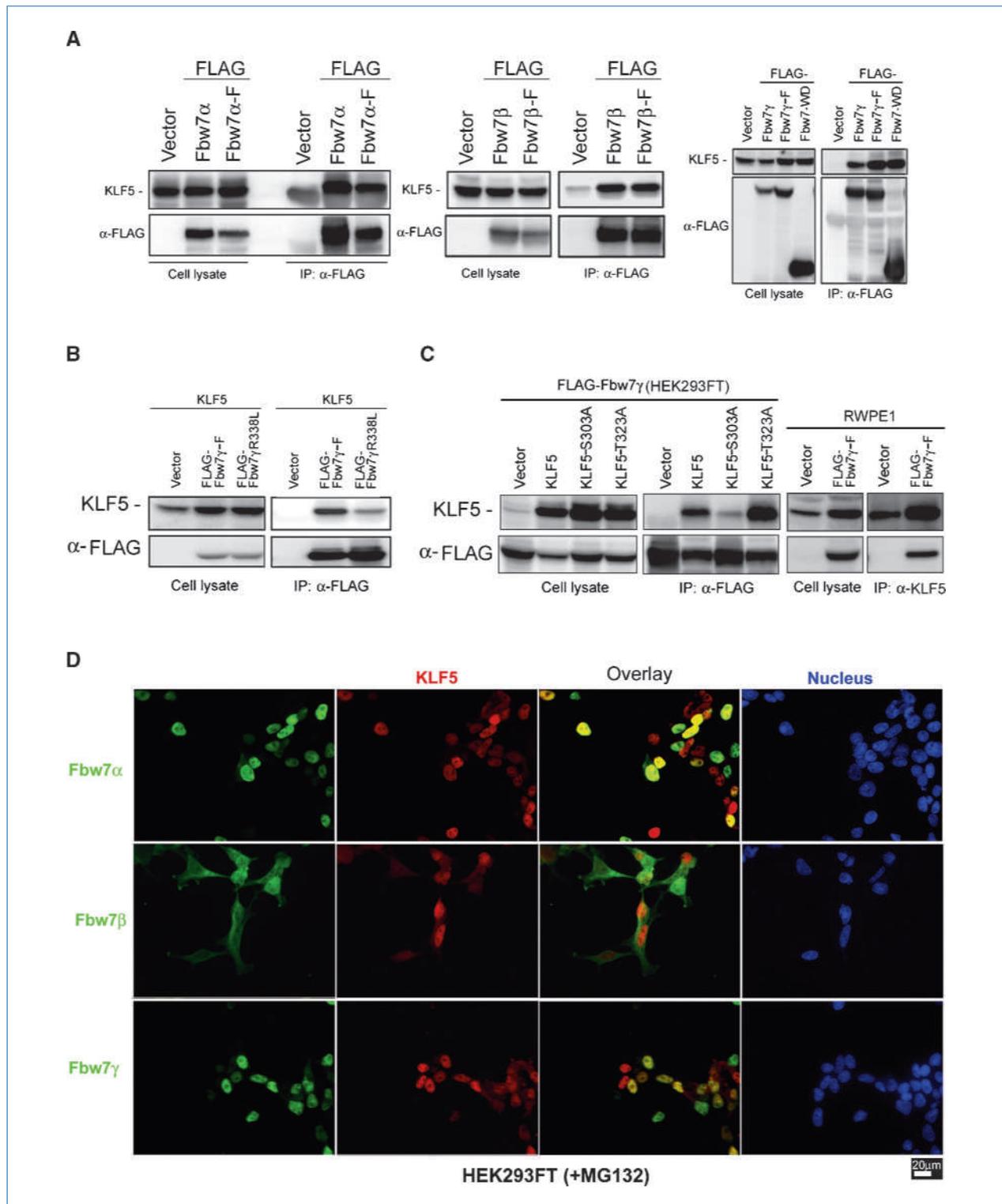
Figure 1. The Fbw7 protein interacts with KLF5 through the WD40 repeats/CPD motif. A, KLF5 is coprecipitated with FLAG-Fbw7 (α, β, and γ; WT and Fbw7γ-F mutant). HEK293FT cells were cotransfected with different combinations of expression plasmids. IP was performed using the FLAG-M2 affinity gel. B, FLAG-Fbw7γ-R338L does not efficiently interact with KLF5 compared with WT FLAG-Fbw7γ. C, KLF5-S303A dramatically loses interaction with FLAG-Fbw7γ compared with WT KLF5 and KLF5-T323A. The endogenous KLF5 and FLAG-Fbw7γ-F interact in RWPE1. FLAG-Fbw7γ-F increases the KLF5 protein level because it may function as a dominant-negative mutant of Fbw7. D, the subcellular localization of KLF5 and FLAG-Fbw7 (α, β, and γ) in HEK293FT cells, as determined by immunofluorescence. The cells were treated with MG132 to prevent KLF5 from degradation by Fbw7.

binds to KLF5 CPD motifs, we mapped the KLF5 CPD motifs that are responsible for Fbw7 binding. We found that the S303A mutation in the first CPD motif dramatically decreases the protein-protein interaction whereas the T323A mutation in the second CPD motif does not (Fig. 1C). These results indicate that the KLF5 CPD motif (^{303}SPPSS) is responsible for Fbw7 binding. Additionally, we showed that the endogenous KLF5 interacts with FLAG-Fbw7γ-F in the RWPE1 cells (Fig. 1C).

It has been documented that Fbw7 α and γ are in the nucleoplasm and nucleolus, respectively, in U2OS cells whereas Fbw7β is in the cytoplasm (1). To determine whether Fbw7 isoforms are colocalized with KLF5, we cotransfected FLAG-Fbw7 isoforms and KLF5 into HEK293FT cells and found that Fbw7 α and γ are localized in the nucleus whereas Fbw7β is in the cytoplasm by immunofluorescence staining (Fig. 1D). As expected, KLF5 is predominately localized in the nucleus, although it can also be detected in the cytoplasm (Fig. 1D). The colocalization of KLF5 and Fbw7 α and γ is obvious.

Fbw7 overexpression promotes the KLF5 protein proteasomal degradation

Next, we asked whether Fbw7 overexpression decreases the KLF5 protein levels. To test this, we first cotransfected Fbw7γ and Fbw7γ-F with KLF5 into HEK293FT cells and found that WT Fbw7γ dramatically reduces the KLF5 steady level compared with the empty vector and Fbw7γ-F (Fig. 2A). MG132 can increase the KLF5 steady level in the presence of Fbw7γ (Fig. 2A). Similar results were observed for Fbw7 α and β (Supplementary Fig. S1A and C).

To further evaluate whether Fbw7 promotes KLF5 degradation, we overexpressed WT Fbw7γ and KLF5 into HEK293FT cells and measured the KLF5 protein half-lives by cycloheximide chase assays. We found that Fbw7γ dramatically reduces the KLF5 half-life compared with the empty vector, Fbw7γ-F, and Fbw7γ-R338L (Fig. 2B). The Fbw7γ-mediated KLF5 protein half-life decrease is completely blocked by MG132 (Fig. 2B). Similarly, Fbw7α also significantly decreases the KLF5 protein half-life in HEK293FT cells (Supplementary Fig. S1B).

Because the KLF5-S303A loses the protein interaction with Fbw7γ (Fig. 1C), we tested the KLF5-S303A protein degradation by Fbw7. Consequently, the KLF5-S303A protein half-life cannot be decreased by WT Fbw7γ compared with the Fbw7γ-F mutant in HEK293FT cells (Fig. 2C). In contrast, mutation and deletion of the other CPD motif is still sensitive to Fbw7γ-mediated degradation (Supplementary Fig. S1E). These results clearly suggest that the CPD (^{303}SPPSS) motif is responsible for Fbw7γ-mediated KLF5 degradation.

## The KLF5 S303 is phosphorylated by GSK3β

KLF5-S303A cannot be recognized by Fbw7 (Fig. 1C) and cannot be degraded by Fbw7 (Fig. 2D). To further investigate whether the phosphorylation occurs at S303, we generated a KLF5-S303 phosphorylation–specific Ab using a synthesized phosphorylated peptide. This anti-KLF5 pS303 Ab works well for Western blotting as it specifically detects the phosphorylated KLF5 band from WT KLF5 and KLF5-S307A, but not KLF5-S303A (Fig. 3A). To further test whether the KLF5

phosphorylation is required for Fbw7 binding, we treated the FLAG-Fbw7γ and KLF5-transfected HEK293FT cell lysate with different dosages of CIP and performed IP. We confirmed that the CIP treatment almost completely eliminated the KLF5 S303 phosphorylation (Fig. 3A). Importantly, the binding between FLAG-Fbw7γ and KLF5 is significantly reduced after the CIP treatment (Fig. 3A).

It is well known that GSK3β is the kinase for the first Ser/Thr in the CPD motifs of several Fbw7 substrates, such as MYC (1). To test if the KLF5 degradation is regulated by GSK3β in cultured cells, we treated HeLa cells with the GSK3 inhibitor LiCl and the negative control KCl. As expected,
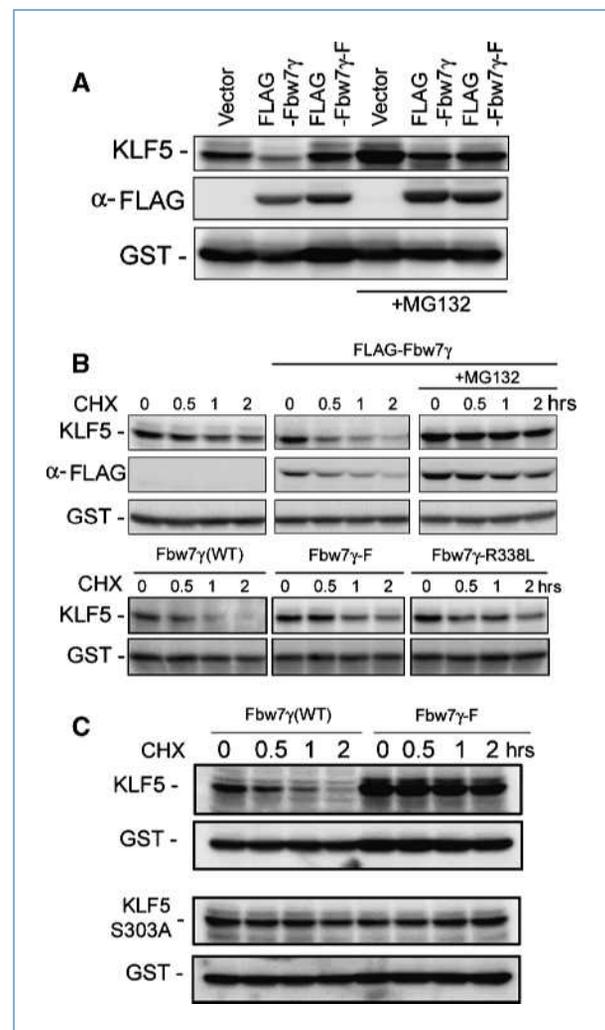
Figure 2. Fbw7 promotes proteasomal degradation of the KLF5 protein. A, FLAG-Fbw7γ decreases the steady levels of KLF5 in HEK293FT cells, as determined by Western blotting. An empty vector and FLAG-Fbw7γ-F were used as controls. B, FLAG-Fbw7γ, but not FLAG-Fbw7γ-F and FLAG-Fbw7γ-R338L, decreases the KLF5 protein half-life in HEK293FT cells. The protein half-lives were measured by cycloheximide (CHX; 50 μg/mL) chase assays and Western blotting. Glutathione S-transferase was used as a transfection control. The exposure times have been adjusted for each panel to compare protein degradation. C, KLF5-S303A is resistant to FLAG-Fbw7γ–mediated degradation.
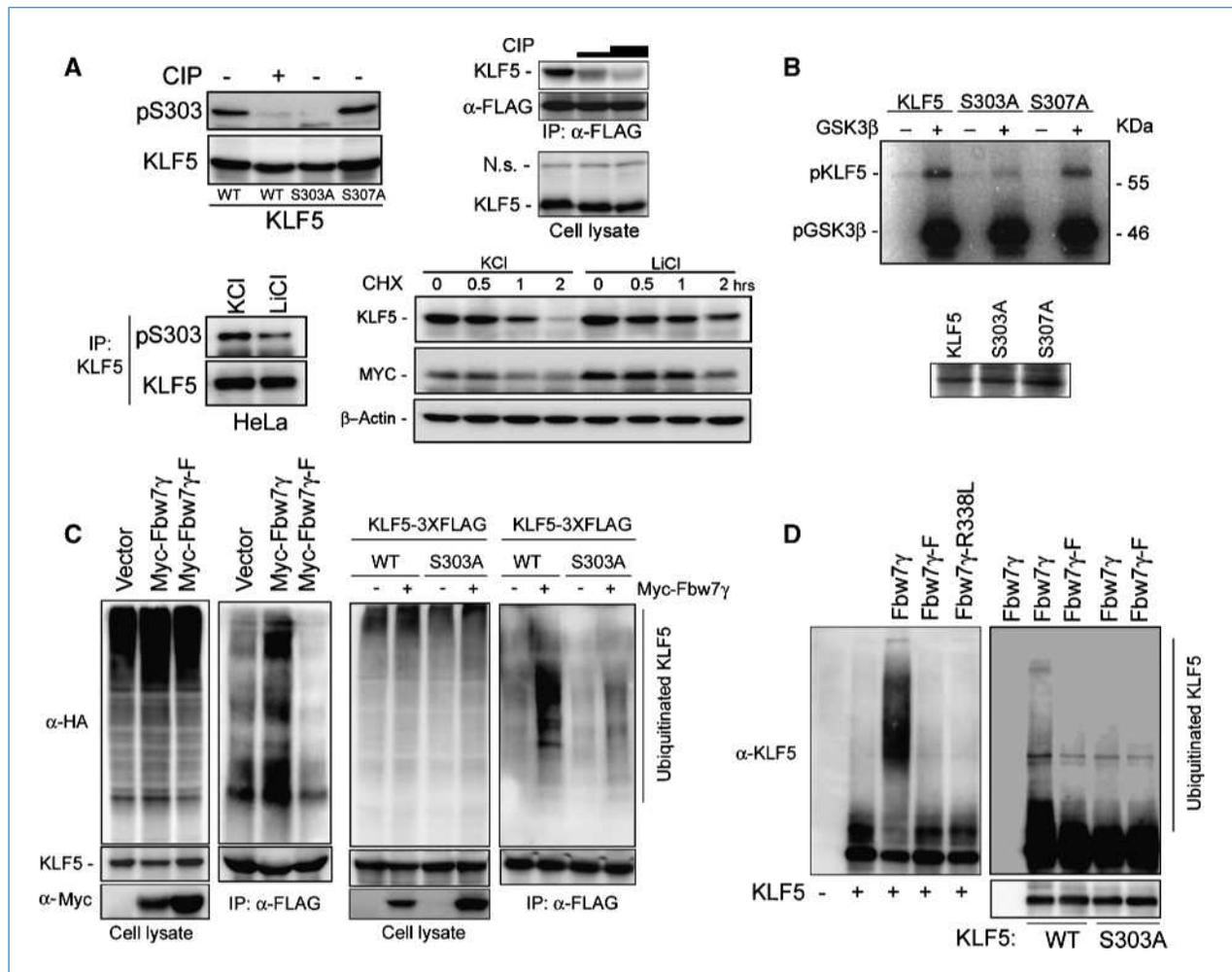
Figure 3. GSK3β-mediated KLF5 phosphorylation at S303 regulates the KLF5-Fbw7 protein interaction and the KLF5 protein ubiquitination and degradation. A, KLF5 phosphorylation at S303 regulates the KLF5-Fbw7 protein interaction and the KLF5 protein degradation. CIP (100 units) decreases the S303 phosphorylation, as determined by Western blotting using the KLF5 pS303–specific Ab. The Fbw7-KLF5 protein interaction was disrupted by CIP. The cell lysate (200 μL) was treated with different dosages of CIP (0, 100 U, and 200 units) for 1 h at 30°C. The GSK3 inhibitor LiCl (20 mmol/L) decreases the endogenous KLF5 phosphorylation at S303, as determined by IP-Western blotting, and extends the KLF5 and MYC protein half-lives in HeLa, as determined by the cycloheximide chase assay. KCl was used as the negative control. N.s., a nonspecific band. B, the GSK3β kinase efficiently phosphorylates WT KLF5 and KLF5-S307A, as determined by the *in vitro* kinase assay using γ-^{32}P-ATP. The loading of the KLF5, KLF5-S303A, and KLF5-S307A proteins is shown by silver staining. C, Fbw7γ increases the ubiquitination of KLF5 but not KLF5-S303A in HEK293FT cells. Expressing plasmids for HA-Ub, Myc-Fbw7γ, Myc-Fbw7γ-F, and KLF5-3 × FLAG were transfected. The cells were treated with MG132 to accumulate the ubiquitinated KLF5. The IP was performed with the anti-FLAG M2 beads under denaturing conditions. An empty vector and FLAG-Fbw7γ-F were used as controls. D, Fbw7γ ubiquitinates KLF5 but not KLF5-S303A *in vitro* using its E3 ligase activity. The intact SCF[Fbw7] E3 ligase complex was specifically purified by IP using the anti-FLAG M2 beads. FLAG-Fbw7γ-F and FLAG-Fbw7γ-R338L cannot ubiquitinate KLF5 under the same conditions.

LiCl decreases the KLF5 pS303 levels (Fig. 3A) and extends both the KLF5 and MYC protein half-lives in HeLa (Fig. 3A). These results suggest that GSK3β could be the kinase for KLF5 S303 phosphorylation.

To directly test whether GSK3β phosphorylates KLF5 at S303, we performed the *in vitro* kinase assay using the purified recombinant GSK3β kinase and the purified recombinant KLF5/KLF5-S303A/KLF5-S307A proteins in the presence of γ-^{32}P-ATP. We found that GSK3β can efficiently phosphorylate WT KLF5 and KLF5-S307A but not KLF5-S303A (Fig. 3B). These results indicate that the KLF5

protein phosphorylation at S303 can be mediated by GSK3β *in vitro*.

Fbw7 ubiquitinates KLF5

To test whether Fbw7 ubiquitinates KLF5 in cultured cells, we performed the KLF5 ubiquitination assay in HEK293FT cells as described in our previous study (23). We found that WT Fbw7γ but not the Fbw7γ-F mutant increases the KLF5 ubiquitination (Fig. 3C). Under the same condition, KLF5-S303 cannot be efficiently ubiquitinated by Fbw7γ (Fig. 3C). Additionally, we examined the endogenous KLF5

ubiquitination in the Fbw7 WT and knockout DLD1 colon cancer cell lines (4) and found that the endogenous KLF5 ubiquitination is decreased in Fbw7 knockout DLD1 cells (Supplementary Fig. S2A).

To test whether Fbw7 directly ubiquitinates KLF5 *in vitro*, we purified KLF5 and the SCF^{Fbw7} E3 ligase complex from HEK293FT cells. The purified FLAG-Fbw7γ/Fbw7γ-R338L, but not Fbw7γ-F, complexes contain the cooverexpressed Myc-CUL1 protein (Supplementary Fig. S2B), suggesting that the intact SCF^{Fbw7} E3 complex was purified by IP Fbw7γ, Fbw7γ-R338L, but not Fbw7γ-F. In the presence of the KLF5 substrate, Ub, E1, E2 (UbcH5a), and ATP, the ubiquitinated KLF5 is dramatically increased by WT Fbw7γ, but not by Fbw7γ-F or Fbw7γ-R338L (Fig. 3D). Finally, we showed that Fbw7γ cannot ubiquitinate KLF5-S303A efficiently compared with WT KLF5 *in vitro* (Fig. 3D). These results suggest that the SCF^{Fbw7} E3 ligase specifically ubiquitinates KLF5 *in vitro*.

Endogenous SCF^{Fbw7} promotes KLF5 degradation

To test if endogenous KLF5 is the substrate of endogenous Fbw7, we examined the KLF5 protein levels in the Fbw7 knockout DLD1 cells and found that KLF5 is upregulated,

like another Fbw7 substrate MYC (Fig. 4A). In the presence of MG132, there is no difference for the KLF5 protein levels between the WT and Fbw7 null cells, suggesting that WT Fbw7 targets KLF5 for proteasomal degradation. To further test whether Fbw7 promotes the KLF5 protein degradation, we compared the KLF5 protein half-lives. As shown in Fig. 4A, both the KLF5 and MYC protein half-lives are dramatically extended in the Fbw7-deficient cells compared with the WT DLD1 cells.

To further test if endogenous Fbw7 suppresses the KLF5 protein expression in other cells, we knocked down Fbw7 by two different siRNAs in HeLa, MCF10A, and BT20 cells. The Fbw7 knockdown efficiencies are about 60% to 80% in these cell lines (Fig. 4B). We could not detect the endogenous Fbw7 proteins in these cell lines (data not shown) because there are no effective anti-Fbw7 Abs for Western blotting to date (24). We found that the endogenous KLF5 and MYC protein levels are significantly elevated in all these cell lines (Fig. 4B). As expected, the *KLF5* mRNA levels are not increased by Fbw7 siRNAs (Supplementary Fig. S2C). Furthermore, knockdown of Fbw7 by two different siRNAs clearly extends both the KLF5 and MYC protein half-lives in HeLa (Fig. 4B). Consistently, the KLF5 and MYC protein half-lives
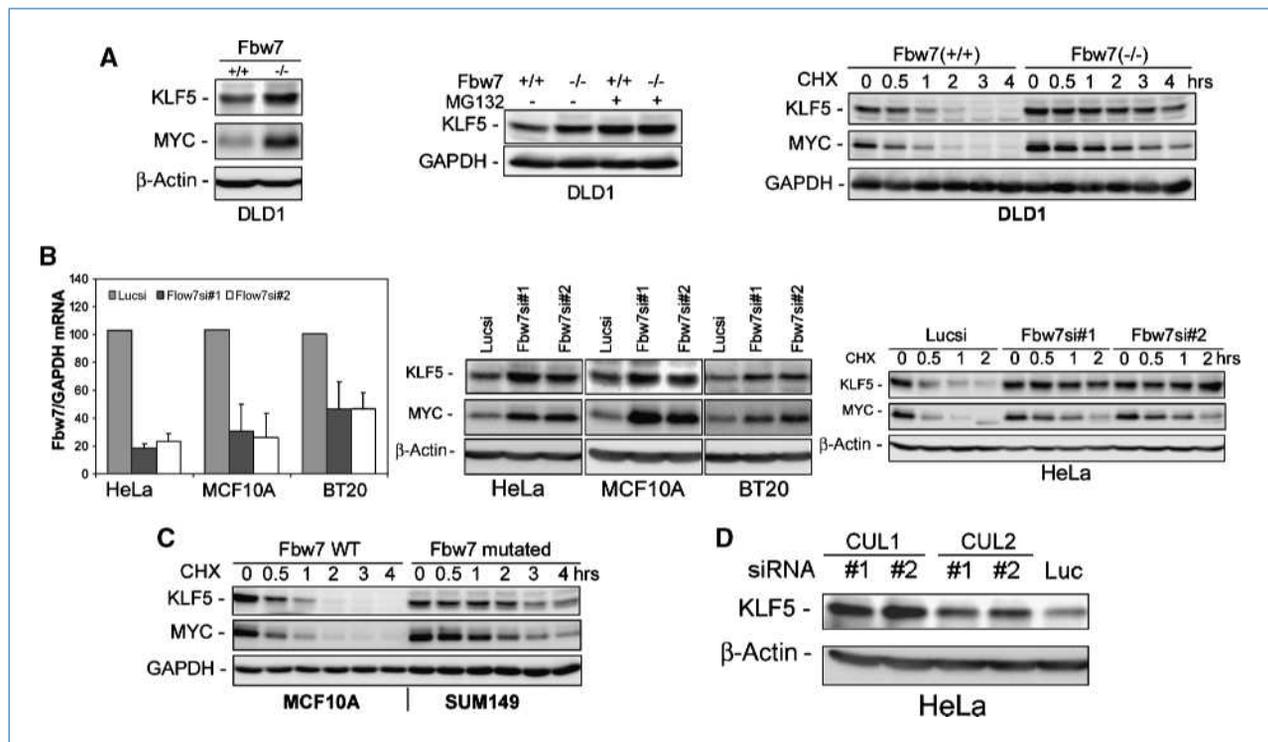
Figure 4. Inactivation of SCF^{Fbw7} increases the endogenous KLF5 protein levels through preventing KLF5 from degradation. A, endogenous KLF5 and MYC protein levels and half-lives are increased in the *Fbw7* knockout cells compared with the WT DLD1 cells, as determined by the cycloheximide chase assay and Western blotting. The Fbw7-mediated endogenous KLF5 protein degradation can be blocked by MG132. B, knockdown of *Fbw7* increases the endogenous protein levels of KLF5 and MYC in HeLa, MCF10A, and BT20, as determined by Western blotting. *Fbw7* is knocked down by two different siRNAs, as determined by qRT-PCR. Lucsi was used as a negative control. The *KLF5* mRNA levels are not upregulated by *Fbw7* siRNAs in these cells (Supplementary Fig. S2C). The protein half-lives of endogenous KLF5 and MYC are dramatically extended in *Fbw7* knockdown HeLa cells, as determined by the cycloheximide chase assay. C, the protein half-lives of endogenous KLF5 and MYC are dramatically extended in Fbw7 mutated SUM149 breast cancer cells, as determined by the cycloheximide chase assay. D, depletion of *CUL1*, but not *CUL2*, by two different siRNAs increases the endogenous KLF5 protein levels in HeLa.

in the Fbw7-mutated SUM149 breast cancer cell line are much longer than those in MCF10A that has WT Fbw7 (Fig. 4C). These results strongly suggest that inactivation of endogenous Fbw7 by gene knockout, knockdown, or mutation increases the KLF5 protein stability.

Because Fbw7 functions as an adaptor for SCF^{Fbw7}, we asked whether knockdown of other SCF components, such as CUL1 (25), also increases the KLF5 protein expression. We knocked down CUL1 and CUL2 in HeLa, respectively, and found the KLF5 protein levels are specifically upregulated by knocking down CUL1 but not CUL2 (Fig. 4D). The knockdown efficiencies of CUL1 and CUL2 are ~90% as monitored by quantitative reverse transcriptase PCR (qRT-PCR; Supplementary Fig. S2D). Importantly, the *KLF5* mRNA levels are not upregulated by knocking down CUL1, suggesting that the upregulation of KLF5 occurs at the post-transcriptional level. Similar to the knockdown of Fbw7, knockdown of Rbx1 by siRNA also upregulates the KLF5 protein level in HeLa (data not shown). These findings suggest that the SCF^{Fbw7} E3 complex suppresses the KLF5 protein expression.

The expression of Fbw7 and KLF5 in breast cancer

Because the degradation of MYC by Fbw7 is isoform and cell line specific (24, 26), we asked whether the endogenous Fbw7 isoforms (α, β, and γ) also promote KLF5 degradation in a cell line–dependent manner. To test this, we knocked down Fbw7 using the isoform-specific siRNAs (27) in HeLa, MCF10A, and 184B5 cell lines. We found that knockdown of any Fbw7 isoforms (α, β, and γ) upregulates the KLF5 protein levels in HeLa with a similar extent (Fig. 5A). In the MCF10A breast cell line, knockdown of Fbw7α and β isoforms upregulates KLF5 with a similar efficiency. However, knockdown of Fbw7γ does not show any significant changes for KLF5 (Fig. 5A). In the 184B5 breast cell line, only knockdown of Fbw7α clearly upregulates KLF5 (Fig. 5A). These results suggest that the degradation of KLF5 by Fbw7 is also isoform and cell line specific. Consistent with a previous report that Fbw7α is the predominant isoform expressed in breast cancer cell lines (3), endogenous Fbw7α appears as the major active isoform for KLF5.

Previously, we showed that KLF5 is expressed in ERα-negative breast cell lines and downregulated in ERα-positive
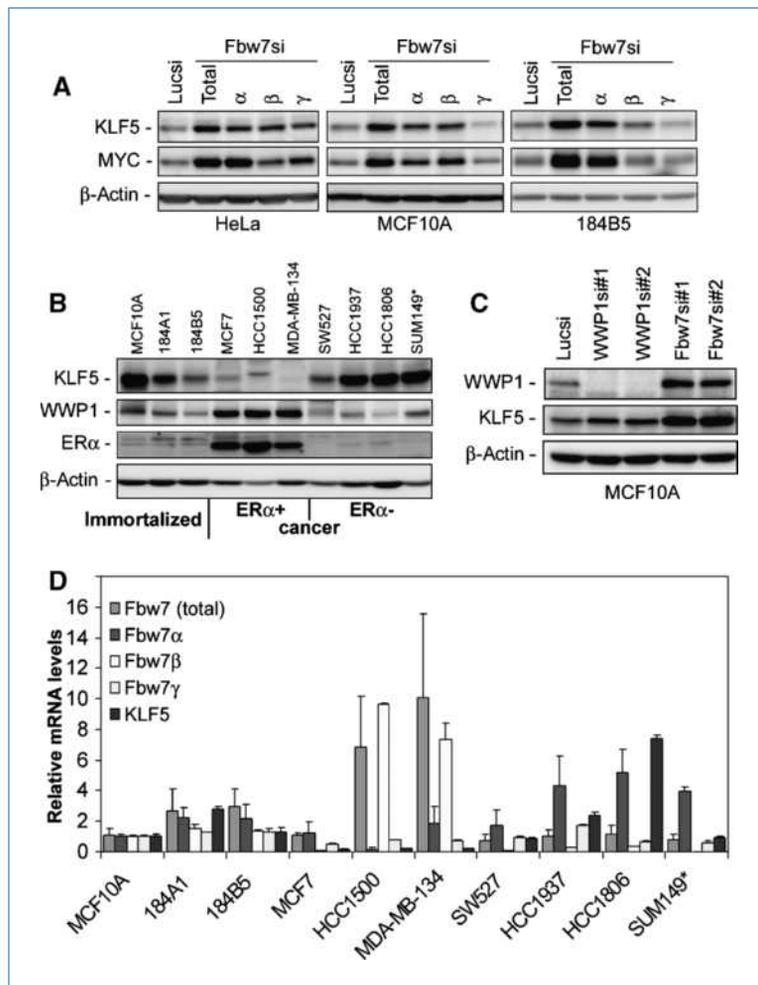
Figure 5. The expression of Fbw7 isoforms and KLF5 in breast cell lines. A, knockdown of Fbw7 by isoform-specific siRNAs differently increases the endogenous KLF5 and MYC protein levels in HeLa, MCF10A, and 184B5. B, the negative protein expression correlation between KLF5 and WWP1 in a panel of 10 breast cell lines. *, the *Fbw7* gene is inactively mutated in SUM149. C, knockdown of WWP1 and Fbw7 by two different siRNAs upregulates the endogenous KLF5 protein levels in MCF10A cells. D, the mRNA levels of *Fbw7* isoforms and *KLF5* in breast cell lines, as determined by qRT-PCR.

breast cell lines (17). The KLF5 E3 ligase WWP1 is overexpressed in ERα-positive breast cancer (28). As shown in Fig. 5B, there is a negative correlation between the KLF5 and WWP1 protein expression in a panel of 10 breast cell lines. To test whether both KLF5 E3 ligases function at the same time, we knocked down WWP1 and Fbw7 by two different siRNAs, respectively, in MCF10A and found that knockdown of Fbw7 increases the KLF5 protein levels to higher levels compared with knockdown of WWP1 (Fig. 5C). These results suggest that Fbw7 is the major E3 ligase for KLF5 in MCF10A, although both endogenous Fbw7 and WWP1 target KLF5 for degradation. Interestingly, Fbw7 and WWP1 appear to compensate each other because knockdown of either Fbw7 or WWP1 causes the expression upregulation of the other KLF5 E3 ligase (Supplementary Fig. S2E; Fig. 5C).

Finally, we measured the relative mRNA levels of *KLF5*, total *Fbw7*, and the individual *Fbw7* isoforms (α, β, and γ) in 10 breast cell lines by qRT-PCR (Fig. 5D). Among three immortalized cell lines (MCF10A, 184A1, and 184B5), the mRNA levels of *Fbw7α* and total *Fbw7* negatively correlate with the KLF5 protein levels (Fig. 5B and D). Consistent with our previous reports (17, 29), the low levels of KLF5 protein expression in ERα-positive breast cancer cells seem to be driven by the loss of *KLF5* mRNA expression. In ERα-negative cancer cell lines, the downregulation of Fbw7 is obvious compared with 184A1 and 184B5. Interestingly, inactivation of Fbw7 in SUM149 leads to the accumulation of a high level of KLF5 protein without upregulating the *KLF5* mRNA level (Fig. 5B and D).

Fbw7 suppresses the *FGF-BP* expression and breast cell proliferation through promoting KLF5 degradation

Recently, we showed that KLF5 promotes breast cell proliferation through upregulating the *FGF-BP* expression (17). To test whether Fbw7 suppresses the KLF5 transactivation function, we knocked down *Fbw7* by siRNA in MCF10A, 184B5, and MCF7 breast cells (Supplementary Fig. S3A) and found that the KLF5 protein levels are upregulated (Fig. 6A). In agreement with the fact that *FGF-BP* is one of the KLF5 transcriptional targets (17), we found that the *FGF-BP* expression upregulation occurs at the mRNA and/or protein levels (Fig. 6A). Knockdown of Fbw7 by two different short hairpin RNAs in 184B5 shows similar results (Supplementary Fig. S3B). Importantly, the depletion of KLF5 can significantly rescue the Fbw7 siRNA–induced *FGF-BP* upregulation in all three cell lines (Fig. 6A). These observations suggest that endogenous Fbw7 suppresses the KLF5 function of inducing the *FGF-BP* gene transcription in breast cells.

Because KLF5 promotes breast cell proliferation (17) and Fbw7 also targets several other oncoproteins for degradation, it is important to elucidate whether Fbw7 suppresses breast cell proliferation through KLF5. As shown in Fig. 6B, knockdown of Fbw7 significantly increases DNA synthesis in MCF10A, 184B5, and MCF7. Knockdown of Fbw7 by two different short hairpin RNAs in 184B5 also increases DNA synthesis and cell proliferation (Supplementary Fig. S3C). In agreement with our earlier report (17), depletion of KLF5 can almost completely block the Fbw7 siRNA–induced

DNA synthesis increase in all three cell lines. Additionally, depletion of KLF5 in MCF7 can rescue the Fbw7 siRNA–induced colony formation increase in soft-agar (Supplementary Fig. S3D). These results strongly argue that endogenous Fbw7 suppresses breast cell proliferation through targeting the endogenous KLF5 for degradation.

Finally, we tested whether restoring the Fbw7 expression in the SUM149 breast cancer cell line, in which the endogenous Fbw7 loses its activity by gene mutation (3), inhibits *FGF-BP* expression and DNA synthesis. FLAG-Fbw7γ, FLAG-Fbw7γ-F, and LacZ were overexpressed in SUM149 by lentiviruses. As expected, the KLF5 protein level, but not its mRNA level, is specifically downregulated by the WT Fbw7γ compared with LacZ and Fbw7γ-F (Fig. 6C). Furthermore, restoring WT Fbw7γ in SUM149 significantly decreases the *FGF-BP* mRNA level (Fig. 6C) and DNA synthesis (Fig. 6D) compared with LacZ and Fbw7γ-F.

Discussion

This is the first study to report that the KLF5 protein degradation is targeted by the SCF^Fbw7 E3 ligase. We provide several lines of evidence to support that Fbw7 targets the KLF5 protein for ubiquitin-mediated proteasomal degradation and suppresses breast cell proliferation. First, Fbw7 binds to the KLF5 protein through the WD40/CPD motif interaction. Second, Fbw7 overexpression decreases the KLF5 protein level and half-life. Third, the phosphorylation of KLF5 at S303 by GSK3β is indispensable for Fbw7 to target KLF5 for ubiquitination and degradation. Fourth, Fbw7 ubiquitinates KLF5 through its E3 ligase activity. Additionally, the inactivation of Fbw7 increases the endogenous KLF5 protein level and half-life. Finally, Fbw7 suppresses the KLF5 functions of promoting the *FGF-BP* gene expression and breast cell proliferation.

The KLF5 protein is an unstable protein with a short half-life. Previously, we reported that KLF5 degrades rapidly through the ubiquitin proteasome pathway (21). The degrons overlap with its TAD between 299 and 348. We first identified a PY motif from this region that recruits WWP1 (23). However, Fbw7 binds exclusively to the KLF5 CPD motif in a S303 phosphorylation–dependent manner (Supplementary Fig. S4). Thus, KLF5 TAD contains two different degrons that recruit Fbw7 and WWP1, respectively (Supplementary Fig. S4). Depletion of either WWP1 or Fbw7 increases the KLF5 protein levels in MCF10A; however, Fbw7 is the major E3 ligase for KLF5. Interestingly, when Fbw7 is knocked down in MCF10A, the WWP1 expression level is upregulated and vice versa (Supplementary Fig. S2E; Fig. 5C). Thus, Fbw7 and WWP1 are coordinately activated to target KLF5 for degradation.

The KLF5 protein is phosphorylated by PKC (30) and extracellular signal-regulated kinase (31). For the fist time, we found that the phosphorylation of KLF5 at S303 by GSK3β promotes the protein ubiquitination by Fbw7. Additionally, GSK3β-mediated phosphorylation usually needs priming phosphorylation at +4 site (S307 in KLF5; ref. 1). However, KLF5-S307A can still be efficiently phosphorylated
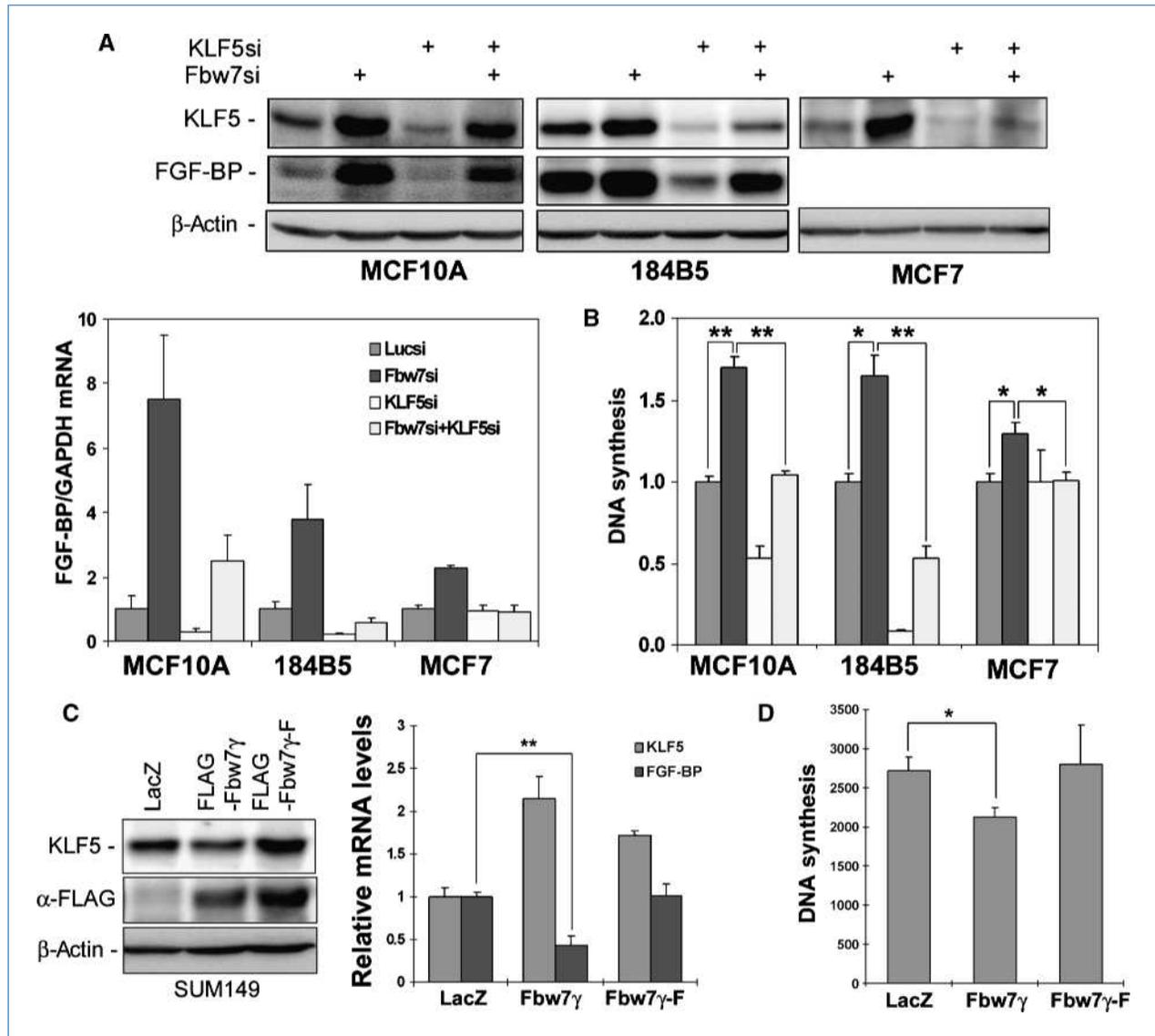
Figure 6. Fbw7 suppresses the FGF-BP expression and breast cell proliferation through promoting KLF5 degradation. A, knockdown of *Fbw7* by siRNA increases the FGF-BP protein and/or mRNA levels in a KLF5-dependent manner in MCF10A, 184B5, and MCF7 (the FGF-BP protein cannot be detected in MCF7 by Western blotting). The *Fbw7* and *FGF-BP* mRNA levels were determined by qRT-PCR. Knockdown of Fbw7 by siRNA increases the KLF5 protein levels. Knockdown of KLF5 by siRNA rescues the depletion of Fbw7-induced FGF-BP protein and/or mRNA expression upregulation. B, knockdown of *Fbw7* by siRNA significantly increases the DNA synthesis in MCF10A, 184B5, and MCF7, as determined by the ^{3}H-thymidine incorporation. Knockdown of KLF5 by siRNA significantly rescues the depletion of *Fbw7*-induced DNA synthesis increase. C, the overexpression of FLAG-Fbw7γ decreases the endogenous KLF5 protein level, but not *KLF5* mRNA level, compared with LacZ and FLAG-Fbw7γ-F in SUM149, as determined by Western blotting and qRT-PCR. The overexpression of FLAG-Fbw7γ significantly decreases the *FGF-BP* mRNA level. D, the overexpression of FLAG-Fbw7γ significantly decreases the DNA synthesis compared with LacZ and FLAG-Fbw7γ-F in SUM149. *, $P < 0.05$; **, $P < 0.01$ (*t* test).

by GSK3β *in vitro* and in cultured cells (Fig. 3). In addition, we found that KLF5-S307A can still efficiently interact with Fbw7γ and be ubiquitinated by Fbw7γ (data not shown). These results suggest that the priming phosphorylation could be from other sites.

Three Fbw7 isoforms (α, β, and γ) show different subcellular localization in U2OS (1) and HEK293FT cells (Fig. 1D). KLF5 is predominately localized in the nucleus of HEK293FT cells (Fig. 1D). However, a small fraction of KLF5 has been shown to localize in the cytoplasm (32). Nevertheless, all Fbw7 isoforms interact with KLF5 in our IP experiments after the disruption of the intact cell structures (Fig. 1A). Overexpression of any Fbw7 isoforms decrease the KLF5 protein levels (Supplementary Fig. S1A and C; Fig. 2A). Importantly, knockdown of any endogenous Fbw7 isoforms in HeLa increases the endogenous KLF5 protein levels (Fig. 5A). In breast cells, Fbw7α seems to be the major functional endogenous isoform for KLF5.

Fbw7 has been documented to be inactivated by somatic gene mutation in a small subset (~1%) of breast cancers based on the Catalogue of Somatic Mutations in Cancer Database. Interestingly, polymorphism of the *Fbw7* gene was found to be associated with high-stage and ERα-negative breast cancers (33). *Fbw7* has been reported to be induced by the p53 tumor suppressor (6) that is frequently mutated in ERα-negative breast tumors. Indeed, the total *Fbw7* mRNA levels in ERα-negative breast cancer cell lines are generally lower than that in ERα-positive breast cancer cell lines (Fig. 5D). In addition to breast cancer, Fbw7 is more frequently mutated in tumors from the endometrium (15%), large intestine (9%), thyroid (8%), hematopoietic and lymphoid tissue (8%), pancreas (3%), and others. Consistently, the conditional knockout of *Fbw7* in the T-cell lineage of mice shows thymic hyperplasia and eventually the mice develops thymic lymphoma (34). The *Fbw7* heterozygous knockout mice increase susceptibility to radiation-induced tumorigenesis (6). It is well documented that KLF5 plays oncogenic roles in breast cancer (17), colon cancer (35), leukemia (36), and pancreatic cancer (37). These findings suggest that genetic inactivation of Fbw7 in a variety of cancers could promote cancer progression through accumulating KLF5.

Fbw7 has been suggested to be a tumor suppressor controlling the level of the key cell cycle regulatory protein Cyclin E (1). In this study, we show that Fbw7 inhibits KLF5. Importantly, KLF5 seems to be a critical substrate for Fbw7 to suppress breast cell proliferation because depletion of KLF5 can rescue the inactivation of Fbw7-induced DNA synthesis increase (Fig. 6A and B). KLF5 has been shown to promote G_1-S and G_2-M cell cycle transition through upregulating the *FGF-BP*, *Cyclin D1*, and *Cyclin B1* protein levels (15, 16). Thus, Fbw7 suppresses cell cycle progression by directly and indirectly controlling multiple cell cycle regulatory proteins.

In summary, we show that Fbw7 targets KLF5 proteins for ubiquitin-mediated proteasomal degradation in a S303 phosphorylation–dependent manner. We show that Fbw7 suppresses KLF5's functions of promoting gene transcription and breast cell proliferation. Given the frequent inactivation of Fbw7 in breast and other cancers, these findings may help us further understand the roles of Fbw7 and KLF5 in cancer development.

Disclosure of Potential Conflicts of Interest

Acknowledgments

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The Fbw7 Tumor Suppressor Targets KLF5 for Ubiquitin-Mediated Degradation and Suppresses Breast Cell Proliferation

Dong Zhao, Han-Qiu Zheng, Zhongmei Zhou, et al.

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