A Colon Cancer-derived Mutant of Krüppel-like Factor 5 (KLF5) Is Resistant to Degradation by Glycogen Synthase Kinase 3β (GSK3β) and the E3 Ubiquitin Ligase F-box and WD Repeat Domain-containing 7α (FBW7α)*

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Background: Stability of KLF5 is regulated by GSK3β- and FBW7α-mediated proteasomal degradation.

Results: Colon cancer-derived P301S KLF5 mutant is degradation-resistant due to the inability to interact with FBW7α.

Conclusion: Residue 301 of KLF5 is critical for the proper recognition by FBW7α that is involved in the degradation of KLF5.

Significance: Our results suggest that the P301S KLF5 mutant is potentially oncogenic in colon cancer.

Krüppel-like factor 5 (KLF5) is a zinc finger transcription factor that is highly expressed in the crypt epithelial cells of the intestine and plays a critical role in regulating proliferation of both normal intestinal epithelial cells and colorectal cancer cells. Stability of the KLF5 is mediated by proteasomal degradation via phosphorylation by glycogen synthase kinase 3β (GSK3β) and recognition by F-box and WD repeat domain-containing 7 (FBW7) of a phosphodegron sequence surrounding serine 303 in KLF5. A genomic analysis of colorectal cancer tissues identified a somatic mutation (P301S) in KLF5 within the phosphodegron sequence. We hypothesized that due to its close proximity to the phosphodegron sequence, the P301S mutation may affect signaling that is involved in proper KLF5 degradation. We demonstrated that the P301S KLF5 mutant has a longer half-life than wild type (WT) KLF5. Furthermore, P301S KLF5 has a higher transcriptional activity than WT KLF5 as demonstrated by luciferase assays using cyclin D1 and CDC2 promoter constructs. In contrast to WT KLF5, P301S KLF5 does not physically interact with FBW7α. Concomitantly, the P301S KLF5 mutant displays reduced levels of phosphorylation at serine 303 in comparison with WT KLF5. These results of our study indicate that amino acid residue 301 of KLF5 is critical for proper recognition of the phosphodegron sequence by FBW7α and that the P301S mutation inhibits this recognition, leading to a degradation-resistant protein with elevated levels and enhanced transcriptional activity. These findings raise a potentially oncogenic role for the P301S KLF5 mutant in colorectal cancer.

Homeostasis of the intestinal epithelium is ensured by appropriate mechanisms governing expression, activity, and degradation of key transcriptional components of the cells.

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‡ The abbreviations used are: KLF5, Krüppel-like factor 5; GSK3, glycogen synthase kinase 3β; FBW7, F-box and WD repeat domain-containing 7; APC, adenomatous polyposis coli; CDC, cell division cycle; DMSO, dimethyl sulfoxide.
Here, we demonstrate that the P301S mutation interferes with GSK3β phosphorylation of serine 303 in KLF5 that is required for FBW7α-mediated degradation of KLF5. We show that the P301S KLF5 mutant has decreased levels of phosphorylation at serine 303 and reduced interaction with FBW7α. We also find that P301S KLF5 mutant is characterized by a prolonged half-life and increased transcriptional activity. Collectively, our results provide evidence that proline residue in amino acid position 301 in KLF5 is necessary for the recognition of human KLF5 phosphodegron sequence by GSK3β and FBW7α.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—The human embryonic kidney (HEK) cell line and DLD-1 and HCT116 colorectal cancer cell lines were purchased from the American Type Culture Collection (ATCC). HEK cells were maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. DLD-1 cells were maintained in RPMI1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin, and HCT116 cells were maintained in McCoy’s medium supplemented with 10% FBS and 1% penicillin/streptomycin.

**Reagents**—Cell culture media, FBS, penicillin/streptomycin, Lipofectamine 2000, and Lipofectamine RNAiMAX were purchased from Life Technologies. Cycloheximide and MG132 were purchased from Calbiochem. Rabbit polyclonal antibodies generated against amino acid positions 95–111 of the KLF5 protein was manufactured by SDIX/OriGene. Rabbit antibodies against FBW7α were purchased from Bethyl Laboratories, and GSK3β was from Cell Signaling. Rabbit antibodies against phospho-Ser-303 KLF5 were purchased from Panora Biotech. Mouse monoclonal antibodies against HA and β-actin were purchased from Covance and Sigma-Aldrich, respectively.

**Plasmid Constructs**—WT Human KLF5 cDNA with an N-terminal HA tag was inserted into pEFGP-N1 after excision of gene encoding EGFP (WT KLF5). S303A and P301S KLF5 constructs were provided by the Custom Cloning Core Facility of Emory University, Atlanta, GA. P301A and P301E KLF5 constructs were provided by the Molecular Cloning Core Facility of Stony Brook University, Stony Brook, NY. All KLF5 constructs have an N-terminal HA tag. Plasmid encoding FBW7α was a gift from Dr. Michelle Pagano (Howard Hughes Medical Institute, HHMI, New York University), and GSK3β was a gift from Dr. Peter Klein (University of Pennsylvania).

**Cycloheximide Chase Experiment**—Cells were transfected with WT, S303A, and P301S human KLF5 constructs alone or in combination with plasmid encoding FBW7α. At 24 h after transfection, cycloheximide was added to the cells to a final concentration of 10 μM. Protein samples were collected at 0, 1, 2, and 3 h after the addition of cycloheximide and subjected to Western blot analysis.

**Transfection Experiment**—HEK, DLD-1, and HCT116 cells at 80–90% confluence were transfected with WT, S303A, or P301S human KLF5 constructs or in combination with GSK3β and FBW7α plasmids, using Lipofectamine 2000. Protein extracts were collected 24 h after transfection and subjected to Western blot analysis.

**Ubiquitin Assay**—HEK cells at 80–90% confluence were transfected with WT, S303A, or P301S human KLF5 constructs, or in combination, using Lipofectamine 2000. Cells were additionally transfected with firefly luciferase reporter constructs bearing cyclin D1 or CDC2 promoters, and a Renilla luciferase reporter (Promega) for transfection efficiencies. The firefly luciferase activity was normalized to protein concentrations. Luciferase activities were determined at 24 h after transfection using the Dual-Luciferase assay system kit (Promega).

**siRNA Experiment**—For small interfering RNA (siRNA) experiments, validated Stealth Select siRNAs for human FBW7α were obtained from Life Technologies. HEK and HCT116 cells were transfected using Lipofectamine RNAiMAX with either nonspecific siRNA or siRNAs specific for FBW7α with a combination of WT, S303A, or P301S KLF5 constructs. Protein extracts were collected 24 h after transfection and subjected to Western blot analysis.

**Cycloheximide Experiment**—To detect interaction between KLF5 and FBW7α, HEK cells were transfected with the indicated plasmids. At 24 h after transfection, cells were treated with 10 μM MG132 or DMSO (vehicle) for 6 h followed by co-immunoprecipitation. Briefly, cells were washed twice with phosphate-buffered saline (PBS)/phosphatase inhibitor solution and collected in homemade immunoprecipitation buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% Nonidet P-40, and protease inhibitor cocktail (purchased from Calbiochem). The solutions were spun for 5 min at 1,100 rpm at 4 °C; cells were suspended in immunoprecipitation buffer and sonicated twice for 10 s. The lysates were spun for 10 min at 10,000 × g at 4 °C, and supernatants containing protein extracts were collected. Supernatants were immunoprecipitated with the appropriate antibodies overnight at 4 °C followed by incubation with protein G magnetic beads (Cell Signaling) for 10 min at room temperature. The immune complexes were washed six times with immunoprecipitation buffer and subjected to Western blot analysis.

**Statistical Analysis**—Statistical analysis was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA).

**RESULTS**

**Human P301S KLF5 Mutant Is More Stable than WT KLF5**

**Protein**—A genomic analysis of colorectal cancer tissues identified a somatic mutation, P301S, in KLF5 (21). Because the identified mutation is localized close to or within the phosphodegron sequence in KLF5, we examined the impact of the P301S mutation on KLF5 protein stability. Previously, it has been shown that S303A mutation within human KLF5 protein renders it insensitive to GSK3β-dependent degradation facilitated by FBW7α (19). We therefore employed a S303A KLF5 construct throughout our studies as a positive control. First, to determine the stability of the P301S KLF5 mutant, we per-
formed cycloheximide chase experiments in HEK cells transfected with WT, P301S, or S303A KLF5 constructs. Following the addition of cycloheximide, protein samples were collected hourly for 3 h. As shown in Fig. 1A, WT KLF5 levels decreased over the time course with a half-life of ~1.6 h (Fig. 1B, Table 1). In contrast, P301S and S303A KLF5 levels were relatively stable over the 3 h of treatment and had significantly longer half-lives than WT KLF5 protein (Fig. 1, A and B, and Table 1). We also performed cycloheximide chase experiments in human colorectal cancer cell lines HCT116 and DLD-1 (Fig. 2). As shown, P301S and S303A KLF5 have significantly increased protein half-lives in comparison with WT KLF5 in HCT116 and DLD-1 cell lines (Fig. 2 and Table 1). Noted also is that the rate of degradation of all KLF5 proteins in DLD-1 and HCT116 cells was slightly higher than in HEK cells.

To test whether the increased stability of the tested mutants results in augmented activities, we examined the transcriptional activity of WT, P301S, and S303A KLF5 constructs using luciferase assays. As it has previously been shown that KLF5 positively regulates expression of cyclin D1 and CDC2 (5, 6), we co-transfected the KLF5 plasmids with luciferase reporter constructs linked to the cyclin D1 or CDC2 promoters. As shown in Fig. 1C, both P301S and S303A KLF5 activated the cyclin D1 and CDC2 to a significantly higher extent than WT KLF5.

The P301S KLF5 Protein Is More Resistant to FBW7α-mediated Degradation than WT KLF5—To test the sensitivity of the P301S KLF5 mutant to FBW7α-mediated degradation, we overexpressed WT, P301S, or S303A KLF5 constructs with increasing levels of FBW7α in HEK cells and measured the rate of KLF5 degradation. As shown in Fig. 3, A and B, P301S and S303A KLF5 mutants were significantly more resistant to FBW7α-mediated degradation than WT KLF5. Additionally, we performed cycloheximide chase assays after co-expressing FBW7α with WT, P301S, or S303A KLF5 constructs in HEK, HCT116, and DLD-1 cell lines. FBW7α overexpression in HEK cells reduced the half-life of WT KLF5 protein significantly more than those of P301S and S303A KLF5 proteins (Fig. 3, C and D, and Table 2). Similar results were obtained when the same experiments were performed in HCT116 and DLD-1 cells (Fig. 4 and Table 2). These results indicate that P301S KLF5 is less sensitive to FBW7α-mediated degradation than WT KLF5 in both colorectal cancer-derived and non-colorectal cancer-derived cells.

Human P301S KLF5 Does Not Interact with FBW7α—To examine the effects of decreased levels of FBW7α on P301S

![FIGURE 1. Human P301S KLF5 protein has increased stability and higher transcriptional activity as compared with WT KLF5 as tested in HEK cells. HEK cells transfected with WT, S303A, and P301S KLF5 were subjected to cycloheximide (CHX) chase assays for 3 h, and protein lysates were collected at each time point. The expression levels of proteins in whole cell lysates were detected by Western blotting using antibodies against HA (KLF5) and β-actin. A, a representative example of Western blot analysis. B, data represent mean ± S.D. (n = 3), ● depicts WT KLF5, ■ depicts P301S KLF5, and ▲ depicts S303A KLF5, * p < 0.005, ** p < 0.0005 as compared with the WT. C, luciferase assays were performed in HEK cells transfected with expression constructs as indicated and measured for activity of cyclin D1 and CDC2 promoters 24 h after transfection. Data represent mean ± S.D. (n = 3), *, p < 0.05, **, p < 0.005, *** p < 0.001 as compared with the WT.]

![FIGURE 2. Human P301S KLF5 protein has increased stability as compared with WT KLF5 as tested in HCT116 and DLD-1 colorectal cancer cell lines. HCT116 and DLD-1 cells transfected with WT, S303A, and P301S KLF5 were subjected to cycloheximide (CHX) chase assays for 3 h, and protein lysates were collected at each time point. The expression levels of proteins in whole cell lysates were detected by Western blotting using antibodies against HA (KLF5) and β-actin. A and C, representative examples of Western blot analysis in HCT116 and DLD-1 cell lines, respectively. B and D, data represent mean ± S.D. (n = 3) in HCT116 and DLD-1 cell lines, respectively. ● depicts WT KLF5, ■ depicts P301S KLF5, and ▲ depicts S303A KLF5, * p < 0.01, ** p < 0.05 as compared with the WT.]

| TABLE 1 |
| --- |
| Estimated half-lives of WT, P301S, and S303A KLF5 proteins in HEK, HCT116 and DLD-1 cell lines from cycloheximide chase experiments based on overexpression alone |
| | Cell line | Protein |
| | | WT | S303A | P301S |
| | HEK | 1.6 | 33.9 | 15.8 |
| | HCT116 | 1.6 | 4.9 | 2.4 |
| | DLD-1 | 1.8 | 3.5 | 4.9 |
KLF5 mutant, we transfected HEK and HCT116 cells with WT, P301S, or S303A KLF5 constructs and control siRNA or siRNA against FBW7/H9251, collected the lysates 24 h after transfection, and performed Western blot analysis. As shown in Fig. 5, A and B, the relative protein levels at 0 h were set as 1, and time points 1, 2, and 3 h are shown as relative to those at 0 h. Data represent mean ± S.D. (n = 3), ● depicts WT KLF5, ■ depicts P301S KLF5, and ▲ depicts S303A KLF5. *, p < 0.01, **, p < 0.005 as compared with the WT. C, a representative example of Western blot analysis using antibodies against FBW7α, HA (KLF5), and β-actin. Cycloheximide (CHX) chase experiments were performed with WT, P301S, or S303A KLF5 constructs co-expressed with FBW7α in HEK cells. D, the relative protein levels at 0 h were set as 1, and time points 1, 2 and 3 h are shown as relative to those at 0 h. Data represent mean ± S.D. (n = 3), ● depicts WT KLF5, ■ depicts P301S KLF5, and ▲ depicts S303A KLF5. *, p < 0.01, ***, p < 0.0001 as compared with the WT.

### TABLE 2

| Cell line | Protein | WT | S303A | P301S |
|-----------|---------|----|-------|-------|
| HEK       |         | 0.7| 3.1   | 4.7   |
| HCT116    |         | 0.9| 9.6   | 4.4   |
| DLD-1     |         | 0.9| 3.4   | 4.2   |

**P301S KLF5 Mutant Is Resistant to FBW7α-mediated Degradation**

MGI32 (an inhibitor of proteasomal degradation) or vehicle. Inhibition of proteasomal degradation increased the levels of WT KLF5 protein in HEK and HCT116 cells (Fig. 5, C and D, respectively). In contrast, P301S and S303A KLF5 levels were not affected by suppression of proteasomal activity in either cell type (Fig. 5, C and D). Furthermore, to examine whether the decrease in FBW7α-mediated degradation of P301S KLF5 was the result of a loss of interaction between FBW7α and the KLF5 mutants, we performed co-immunoprecipitation experiments. We transfected HEK cells with WT, P301S, or S303A KLF5 and FBW7α constructs and treated the cells with MGI32 or vehicle. The supernatants were immunoprecipitated with HA antibodies, and protein complexes were subjected to Western blot analysis with antibodies against KLF5 and FBW7α. As shown in Fig. 5E, whereas WT KLF5 interacted with FBW7α, there was no detected interaction between P301S or S303A and FBW7α.

The P301S Mutation Prevents the Phosphorylation of Serine Residue 303 in KLF5 by GSK3β—FBW7α facilitates proteasomal degradation of WT KLF5 protein via phosphorylation of serine 303 and recognition of a phosphodegron sequence surrounding serine 303, which are mediated by GSK3β and FBW7α, respectively (19, 20). As shown earlier, the P301S mutation reduces FBW7α-mediated degradation of KLF5. We
investigated whether overexpression of GSK3β influences the recognition and binding of P301S KLF5 by FBW7α. Expression of FBW7α alone or in combination with GSK3β or GSK3β constructs caused effective degradation of WT KLF5 in HEK and HCT116 cells (Fig. 6, A and B, respectively). However, co-expression of GSK3β and FBW7α did not change the level of either P301S or S303A KLF5 mutant in HEK and had only meager effect on P301S and S303A KLF5 mutants in HCT116 cells (Fig. 6, A and B, respectively).

Thereafter, we examined the phosphorylation levels of serine 303 in WT, P301S, and S303A KLF5 constructs. We co-expressed GSK3β construct with WT, P301S, and S303A KLF5 constructs in HEK cells and treated cells with MG132 to stabilize KLF5 protein levels. As shown in Fig. 6C, overexpression of GSK3β with a combination of MG132 treatment increased the levels of both phosphorylated serine 303 and total protein of WT KLF5. In contrast, the P301S and S303A KLF5 mutants had much less serine 303 phosphorylation as compared with WT

FIGURE 4. Human P301S KLF5 protein is more resistant to FBW7α-mediated degradation than WT KLF5 in HCT116 and DLD-1 colorectal cancer cell lines. A–D, cycloheximide (CHX) chase experiments with WT, P301S, or S303A KLF5 constructs co-expressed with FBW7α in HCT116 (A and B) and DLD-1 (C and D) cells. A and C, representative examples of Western blot analysis using antibodies against HA (KLF5) and β-actin in HCT116 and DLD-1 cells, respectively. B and D, the relative protein levels at 0 h were set as 1, and time points 1, 2, and 3 h are shown as relative to those at 0 h. Data represent mean ± S.D. (n = 3), ● depicts WT KLF5, ■ depicts P301S KLF5, and ▲ depicts S303A KLF5, *, p < 0.0001, **, p < 0.05, ***, p < 0.005 as compared with the WT.

FIGURE 5. P301S KLF5 does not interact with FBW7α. A and B, HEK (A) and HCT116 (B) cells were transfected with WT, P301S, or S303A KLF5 constructs and control siRNA or siRNA against FBW7α; lysates were collected 24 h after transfection, and Western blotting was performed using antibodies against HA (KLF5), and β-actin. C and D, HEK (C) and HCT116 (D) cells were transfected with WT, P301S, or S303A KLF5 and FBW7α constructs and treated with MG132 or vehicle. Lysates were collected, and Western blot analysis of the samples was performed using antibodies against HA (KLF5), phospho-Ser-303 (pSer303), KLF5, GSK3β, and β-actin. D, HEK cells were transfected with WT, P301S, or S303A KLF5 in combination with ubiquitin (Ubi) and FBW7α expression plasmids and treated with MG132. Lysates were subjected to Western blot analysis with antibodies against HA (KLF5) and β-actin.
Furthermore, we examined the ubiquitination status of WT, P301S, and S303A KLF5 alongside overexpression with ubiquitin and FBW7. As shown in Fig. 6D, WT KLF5 has higher level of ubiquitination than P301S and S303A KLF5. Taken together, these data suggest that reduced phosphorylation of serine 303 is responsible for the decreased degradation of P301S KLF5 mutant.

The Proline Residue in the 301 Position Is Necessary for FBW7-mediated Degradation of KLF5—To assess the importance of proline residue in the 301 position of KLF5, we tested FBW7-mediated degradation of P301A (resistant to phosphorylation) and P301E (mimics phosphorylation) KLF5 mutants. We overexpressed WT, S303A, P301S, P301A, or P301E KLF5 constructs with FBW7 and treated with MG132 or vehicle. A, example of Western blot analysis using antibodies against FBW7, HA (KLF5), and β-actin. A, a representative example of Western blot analysis using protein lysates from HEK cells. 8, the relative protein levels of WT KLF5 were set as 1 (lane 1) and the other (lanes 2–10) are shown as relative to this one. Data represent mean ± S.D. (n = 3). *, p < 0.05.

P301S, P301A, and S303A KLF5 mutants in HEK cells. As shown in Fig. 7B, there is a significant difference in the protein levels of WT KLF5 as compared with S303A, P301S, P301A, and P301E KLF5 mutants upon FBW7 overexpression. Complementary experiment performed in HCT116 cells (Fig. 7C) showed reduction in WT KLF5 levels upon FBW7 overexpression and less so on the KLF5 mutants. Overexpression of FBW7 and subsequent inhibition of proteasomal degradation with MG132 in HEK cells increased levels of WT KLF5 protein (Fig. 8, A and B). However, the same treatment did not significantly modify levels of P301S, P301A, P301E, and S303A KLF5 mutants (Fig. 8, A and B). Taken together, modification of proline 301 into serine, alanine, or glutamate resulted in a decrease of degradation for each of these mutants. These results indicate that the 301 proline residue is critical for the proper recognition of KLF5 by GSK3β and FBW7α.

DISCUSSION
Development and progression of cancer is caused by a myriad of mechanisms. One of the most studied mechanisms is mutations in genes that function as tumor suppressors and oncogenes. Mutations in tumor suppressors cause their inactivation and lead to higher activity of downstream effectors. Mutations in oncogenes usually lead to increased expression, prolonged stability, and increased activity of the proteins that they encode for. In colorectal cancer, there are many examples of mutations in both tumor suppressors and oncogenes that occur within the same pathway (e.g. Wnt and PI3K signaling.

**FIGURE 7.** Effects of FBW7 on P301A and P301E KLF5 mutants. A-C, HEK (A and B) and HCT116 (C) cells were transfected with WT, S303A, P301S, P301A, or P301E KLF5 alone or in combination with FBW7α, and lysates were subjected to Western blotting with antibodies against FBW7α, HA (KLF5), and β-actin. A, a representative example of Western blot analysis using protein lysates from HEK cells. 8, the relative protein levels of WT KLF5 were set as 1 (lane 1) and the other (lanes 2–10) are shown as relative to this one. Data represent mean ± S.D. (n = 3). *, p < 0.05.

**FIGURE 8.** Decrease of FBW7α-mediated proteasomal degradation of P301A and P301E KLF5 mutants. HEK cells were transfected with WT, S303A, P301S, P301A, or P301E KLF5 constructs with FBW7α and treated with MG132 or vehicle. A, example of Western blot analysis using antibodies against FBW7α, HA (KLF5), and β-actin. B, the relative protein levels of WT KLF5 were set as 1 (lane 1), and the others (lanes 2–10) are shown as relative to this one. Data represent mean ± S.D. (n = 3). *, p < 0.05.
303 of WT KLF5 is phosphorylated by GSK3 and phosphorylation by GSK3 does not permit binding by FBW7 and inhibits efficacious degradation. Our results demonstrated that the P301S KLF5 mutant is resistant to FBW7-α-mediated proteasomal degradation of KLF5. A, serine 303 of WT KLF5 is phosphorylated by GSK3β, and FBW7 α recognizes and binds to phosphorylated serine 303 and marks WT KLF5 for proteasomal degradation. αα, amino acid; B, mutated P301S, P301A, and P301E (P301S(A/E)) residues in KLF5 protein interfere with phosphorylation of serine 303 by GSK3β. Inefficient phosphorylation by GSK3β does not permit binding by FBW7α and inhibits efficacious degradation.

FIGURE 9. A model of the interference of the P301S, P301A, and P301E mutations with FBW7α-mediated proteasomal degradation of KLF5. A, serine 303 of WT KLF5 is phosphorylated by GSK3β, and FBW7α recognizes and binds to phosphorylated serine 303 and marks WT KLF5 for proteasomal degradation. αα, amino acid; B, mutated P301S, P301A, and P301E (P301S(A/E)) residues in KLF5 protein interfere with phosphorylation of serine 303 by GSK3β. Inefficient phosphorylation by GSK3β does not permit binding by FBW7α and inhibits efficacious degradation.

It has been postulated that KLF5 can function as a tumor suppressor or oncogene and that its feature depends on the type of tissue (11). Our previous study showed that, in the context of colorectal cancer, KLF5 participates in promoting the β-catenin activity at the tumor-initiating steps (3). Here, we provide the first analysis of the properties of a naturally derived KLF5 mutant, P301S, identified in colorectal cancer (21). Previous evidence demonstrating that KLF5 levels are regulated by proteasomal degradation was provided by Zhang and Teng (17). They determined that KLF5 fragments with high transcriptional activity are unstable and undergo rapid degradation, and this process can be prevented by inhibition of proteasome activity with MG132. Subsequently, Chen et al. (10) showed that KLF5 protein levels are in part regulated by ubiquitin-mediated proteasomal degradation. They confirmed that the degradation signal of 56 amino acids (amino acids 293–348) is localized within a transactivation domain (TAD) of KLF5, and its deletion results in decreased ubiquitination and delayed degradation of KLF5. Inevitably, the S303A mutant of human KLF5 is resistant to GSK3β phosphorylation and subsequent steps of degradation. Our data indicate that proline residue 301 of KLF5 is critical for the proper recognition of the phosphodegron sequence in KLF5. Based on the studies from Liu et al. (20) and Zhao et al. (19), this mutation is localized near the phosphodegron sequence that is involved in signaling that leads to KLF5 degradation. Our results demonstrated that the P301S KLF5 mutant is more stable than WT protein, is not affected by FBW7α-mediated degradation, and is characterized by increased transcriptional activity. This suggests that the P301S KLF5 mutant may have oncogenic potential. Additionally, in this study we showed that P301A and P301E KLF5 mutants act in a similar manner to the P301S KLF5 mutant. Based on our results, we propose a model by which P301S mutation hinders KLF5 protein degradation (Fig. 9). Under normal conditions (Fig. 9A), GSK3β recognizes phosphodegron sequence in WT KLF5 and phosphorylates serine 303. This phosphorylation is in turn recognized by FBW7α, which binds to it and facilitates KLF5 proteasomal degradation. Mutations P301S, P301A, and P301E (Fig. 9B) inhibit phosphorylation of serine 303 by GSK3β and consequently impair efficient binding of FBW7α and degradation of KLF5. It is worth mentioning that since the first publication of the endogenous KLF5 mutant, significant progress has been made in this field of study. The Sanger Institute Catalogue of Somatic Mutations in Cancer performs large scale genomic sequencing of carcinoma from multiple tissues (32). They identified mutations in the KLF5 gene in tissues that originated from various carcinomas including large intestine, lung, ovary, esophagus, pancreas, prostate, urinary tract, endometrium, upper aerodigestive tract, and breast and from lymphoid neoplasm. The largest number of KLF5 mutations has been identified in samples from large intestine and lung cancers. It is noteworthy that there are several other identified mutations in large intestine carcinomas that are localized adjacent to or within the degradation sequence of KLF5, for example, P301H, P301L, S307A, S311delS, and S311L. It is of interest that P301S has been identified in large intestine and lung carcinomas and that P301H and P301L until now have been identified only in large intestine carcinomas, suggesting the importance of this residue in the context of colorectal cancer. Another potentially important residue, serine 307, is localized in the priming +4 position with regard to serine 303. However, this site has not been identified as a substrate for GSK3β phosphorylation (19). Based on computational prediction using GPS 3.0 (Group-based Prediction System, version 3.0), it may however be phosphorylated by different protein kinases from families of cell division cycle (CDC), cyclin-dependent kinases (CDK), and mitogen-activated protein kinases (MAPK). Furthermore, it has been recently reported that an R482Q mutation in mouse Fbxw7 (also known as FBW7, hCDC4) causes increased Klf5 protein levels and promotes early tumor formation in ApcMinR482Q+/− mice (33). This finding is very interesting as residue 482 of mouse Fbxw7 corresponds to the human residue 479 frequently mutated in colorectal cancer as reported by the Catalogue of Somatic Mutations in Cancer (COSMIC) (32, 34, 35). This suggests an
additional mechanism of impairing KLF5 degradation and enhancing its activity that may lead to cancer development.

In summary, we showed that residue 301 of human KLF5 protein is critical for proper recognition of the phosphodegron sequence and that P301S KLF5 potentially has oncogenic properties. Future studies to correlate the status of KLF5 and FBW7 mutations with the respective protein levels in colorectal cancer tissues need to be performed.

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