SCF^{FBXW7}/GSK3β-Mediated GFI1 Degradation Suppresses Proliferation of Gastric Cancer Cells

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Abstract

Gastric cancer is the third leading cause of cancer-related death worldwide. The regulatory mechanisms underlying gastric cancer cell proliferation are largely unclear. Here, we show that the transcription factor GFI1 is associated with advanced clinical gastric cancer progression and promoted gastric cancer cell proliferation partially through inhibition of gastrokine-2 (GKN2) transcription. GFI1 was a degrading substrate of FBXW7, whose loss was observed in gastric cancer. Mechanistically, GSK3β-mediated GFI1 S94/S98 phosphorylation triggered its interaction with FBXW7, resulting in SCFFBXW7-mediated ubiquitination and degradation. A nondegradable GFI1 S94A/S98A mutant was more potent in driving gastric cancer cell proliferation and tumorigenesis than wild-type GFI1. Overall, this study reveals the oncogenic role of GFI1 in gastric cancer and provides mechanistic insights into the tumor suppressor function of FBXW7.

Significance: These findings demonstrate the oncogenic role of the transcription factor GFI1 and the tumor suppressive function of FBXW7 in gastric cancer.

Introduction

As a global health threat (1), gastric cancer is the fourth most common cancer and the third leading cause of cancer-related deaths worldwide (2). In addition to the sustained high economic burden of gastric cancer in most countries, gastric cancer is one of the most serious public health problems in China because the incidence and mortality rate of gastric cancer is rapidly rising (3).

Although *Helicobacter pylori* infection is thought to be the main cause for sporadic distal-type gastric cancer (4), gastric cancer is distinct from other solid tumors and has many unique pathologic features. Therefore, a better understanding of the molecular mechanisms underlying the occurrence and progression of gastric cancer is urgently needed to improve clinical practice.

Growth factor independent 1 transcriptional repressor (GFI1) is located within chromosome 1p22 in the human genome (5). As a zinc finger protein, GFI1 mainly functions as a transcriptional repressor by direct or functional interaction with other cofactors, including protein lesion simulating disease 1 (LSD1; ref. 6). It has been shown that GFI1 plays an important role in hematopoietic stem cells. Together with Fosb, Runx1, and Spi1, GFI1 contributes to conversion of adult endothelium to immunocompetent hematopoietic stem cells (7). Subsequent studies have revealed that GFI1 restricts proliferation and preserves functional integrity in addition to regulating self-renewal and engraftment of hematopoietic stem cells (8). For the immune system, GFI1 has been reported to control Th17 cell immunosuppressive activity via regulation of ectonucleotidase expression (9), and functions in the specification of type 2 innate lymphocytes (10). Although a previous study indicated that GFI1 may promote medulloblastoma (11), the functional role of GFI1 in carcinogenesis, particularly in the alimentary tract, has not been fully investigated.

Recently, a few genetic alterations have been reported as emerging markers of gastric cancer, such as key modulators of kinase pathways (12–14). In addition, F-box and WD repeat domain-containing 7 (FBXW7), the substrate recognition component of the SCF^{FBXW7} ubiquitin ligase complex, was shown to be mutated in 9.2% to 18.5% of gastric tumors (15) and low FBXW7 expression was previously shown to be associated with poor prognosis in patients with gastric cancer (16) because FBXW7 controls apoptosis, proliferation, chemoresistance, and metastasis of gastric cancer (17, 18). Moreover, Fbxw7–/– mice are more prone to developing intestinal metaplasia, dysplasia, and
gastric cancer after carcinogen exposure (19). It is well-established that FBXW7 recognizes and degrades key factors, including C-MYC (20, 21), CCNE1 (22), C-JUN (23), and MCL1 (24), after phosphorylation by GSK3β (25), a kinase phosphorylated and inhibited by the activated RTK–PI3K–AKT pathway (26). With respect to functioning as a general tumor suppressor in many cancer types (27), little is known about how FBXW7 functions in gastric cancer cell proliferation and progression by modulating gastric tissue-specific factors. In contrast, gastrokine-2 (GKN2) belongs to a family of secreted proteins located on chromosome 2p13.3, the expression of which is restricted to normal gastric mucosal cells with a high prevalence in gastric juice (28, 29). GKN2 is downregulated in gastric cancer based on differential expression studies (30, 31), and GKN2 loss is correlated with poor survival in intestinal-type gastric cancer according to multivariate analysis (32). GKN2 mutations are rare (33), indicating that GKNs are putative stomach-specific tumor suppressor genes. In addition, Ghr2 knockout in the IL11/STAT3-dependent gp130F/F tumor suppressor gene model led to tumorigenesis of the proximal stomach (33); however, the mechanism underlying the low expression of GKN2 in gastric cancer has not been established.

Herein we characterized GFI1 as a potential oncoprotein in promoting gastric cancer cell proliferation via transcriptionally inhibiting GKN2, and we further revealed that GFI1 is subject to SCF FBXW7–E3 ligase/GSK3β-mediated degradation. Our results indicated that GFI1 is highly expressed in clinical gastric cancer samples and associated with poorer prognosis of patients. We further found that GFI1 promotes gastric cancer cell proliferation and anchorage-independent growth, which involves suppression of the gastric suppressor tumor suppressor, GKN2 (34). We continued to identify GFI1 as a FBXW7 binding protein in gastric cancer cells, the stability of which is governed by FBXW7. Moreover, a mechanistic study suggested that GSK3β controls GFI1 expression via phosphorylation of S94 and S98 sites, which leads to SCF FBXW7–mediated polyubiquitination and proteasome degradation. As a result, expression of a nondegradable form of the GFI1-S94A/S98A mutant dramatically increased xenograft tumorigenesis and anchorage-independent growth of gastric cancer cells. Taken together, our study elucidated the oncogenic function of GFI1 and implicated the mechanism by which GFI1 may be increased in gastric cancer tissues with deficient FBXW7/GSK3β.

Materials and Methods

Plasmids

The HA-tagged coding sequence of human GFI1 wild-type or the relevant GFI1-S94A, GFI1-S98A, and GFI1-S94A/S98A mutants were cloned into the lentiviral vector, pLex-MCS-CMV-puro (Addgene) to generate GFI1 expression plasmids. The cDNA of GKN2 was cloned into the pCDNA3.1 vector. The cDNA of TRCP, SKP2, FBXW7 (α, β, γ), GFI1, CUL11N, CUL11N2, CULLIN3, CULLIN4A, CULLIN4B, CUL5, and constitutively active GSK3β mutant were cloned into the pCMV-Flag vector. GST-GFI1 and relevant mutants were generated by subcloning corresponding cDNA into a pGEX-4T-1 vector.

Mass spectrometry analysis

To identify GFI1 binding protein and phosphorylation sites, 293T cells expressing HA-GFI1 were cultured in the presence of 15 μmol/L MG132 for 10 hours before harvest. Cells were pulse-synchronized in EBC buffer for 30 seconds, and cell lysates were collected to perform HA-IP. The sample was then resorbed on SDS–PAGE and stained with Gelcode Blue Safe protein staining reagents (Thermo Fisher Scientific). The corresponding bands were excised and digested using trypsin, and the resulting peptides were purified using C18 stage tips (3M Company). For LC/MS–MS, peptides were loaded and separated on a home-made micro-tip C18 column [ReproSil-Pur C18-AQ (1.9 μm, 75 μm × 300 mm)] on a nanoflow HPLC Easy-nLC 1200 system [buffer A (0.1% FA in H2O) and buffer B (0.1% FA in 80% ACN); Thermo Fisher Scientific]. The LC gradient was set as follows: 2% to 5% B for 1 minute; 5% to 33% B for 93 minutes; 33% to 45% B for 15 minutes; 45% to 100% B for 3 minutes; and 100% B for 8 minutes. MS data were collected using a data-dependent acquisition in the profile mode on a Q Exactive HF mass spectrometer. Mass spectrometric data were analyzed using MaxQuant 1.6.2.3 against the human UniProt database. The data were deposited (http://www.ebi.ac.uk/pride) with details as follows: project name, Description of GFI1 Function and Regulation in Gastric Cancer, and project accession, PXD013416.

Microarray data

The microarray data from gastric cancer cells with GFI1 overexpression is deposited as GSE115530 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE115530) with an access token: ijaxyqylvltusl.

Collection of human gastric cancer specimens

The clinical gastric tissue samples were histopathologically and clinically diagnosed in the Department of Gastroenterology of Nantong University Affiliated Hospital. The written informed consent was obtained from the participants. Tissue samples were collected after receiving written informed consent from participants and approval from the Institutional Research Ethics Committee. Tissue specimens were collected from 130 patients with gastric cancer who underwent gastrectomy; 13 patients had chronic gastritis, 28 patients had intestinal metaplasia, 16 patients had low-grade intraepithelial neoplasia, and 22 patients had high-grade intraepithelial neoplasia. All gastric cancer cases and adjacent nontumor tissues were diagnosed clinically and pathologically. Data on clinicopathologic features and prognoses of the patients were collected and analyzed retrospectively. The disease stage of each patient was classified or reclassified according to the 2009 AJCC staging system. A total of 130 patients were followed until the end of 2016.

Xenograft tumorigenesis assay

HS746T cells (1 × 107) wild type or overexpressed with GFI1, GFI1-S94A/S98A mutant, and GKN2/GFI1 double overexpression in PBS were subcutaneously inoculated into the flanks of nude mice (7 male mice/group; 4–6 weeks old) purchased from Shanghai Experimental Animal Center and maintained in pathogen-free conditions. Tumor sizes were measured every 3 days with a caliper and tumor volumes were determined using the following formula: V = L × W2 × 0.52, where L is the longest diameter and W is the shortest diameter. Eight weeks later, all mice were sacrificed and tumors were harvested, followed by photography and weighing of the specimens. All animals received humane care and all animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Shanghai Institutes for Biological Sciences.
Results
High GFI1 expression is associated with gastric tumorigenesis
To investigate the possible role of GFI1 in gastric disease, we first determined the level of GFI1 protein expression via IHC staining in samples from patients with chronic gastritis (n = 13), intestinal metaplasia (n = 28), and gastric cancer (n = 130). The samples were then grouped as low expression (score 0/1) and high expression (score 2/3) according to the intensity of GFI1 staining. The percentage of gastric cancer samples with high GFI1 staining (50%; Fig. 1A) was significantly higher than chronic gastritis (23.08%) and intestinal metaplasia (32.14%; Fig. 1B). Then, we further analyzed the association of high GFI1 expression with gastric cancer samples that were classified by 8 clinical factors. Although there was no correlation between GFI1 expression and several factors, including sex, age, and histologic type, the results suggested significant associations of high GFI1 expression with T grade (P = 0.0009), TNM stage (P = 0.002), and lymph node metastasis (P = 0.0109; Table 1). More importantly, Kaplan–Meier survival analysis from these clinical samples indicated that patients with high GFI1 expression (n = 65) had poorer overall survival than patients with low GFI1 expression (P < 0.001; Fig. 1C). Taken together, these results indicate that high GFI1 expression was associated with worse malignancy and poorer prognosis of gastric cancer.

GFI1 promotes gastric cancer cell proliferation and suppresses GKN2 expression
Although GFI1 has been shown to be an important transcriptional factor in hematopoietic stem cells, its role in oncogenesis of solid tumors was largely unknown. To investigate the function of GFI1 in gastric cancer, we ectopically expressed GFI1 in HS746T and BGC823 gastric cancer cells, and examined the proliferation and anchorage-independent colony formation of the resulting cells. Interestingly, GFI1 overexpressing cells proliferated faster and formed more colonies in both anchorage-dependent and -independent conditions (Fig. 2A; Supplementary Fig. S1A–S1B). In contrast, depletion of GFI1 in HS746T and HGC27 cells dramatically delayed cell growth and reduced the number of colonies (Fig. 2B and C; Supplementary Fig. S1C–S1D). Thus, GFI1 may exert a pro-proliferation function in gastric cancer cells, which potentially contributes to oncogenesis. Given that GFI1 usually functions as a transcription repressor, we examined the expression of several tumor suppressor genes in gastric cancer cells with ectopic GFI1 expression to identify possible target genes of GFI1.

Figure 1.
GFI1 expression is associated with gastric cancer progression. A, Representative IHC images of GFI1 staining in gastric cancer. GFI1 low, scored 0/1. GFI1 high, scored 2/3. Scale bar, 50 μm. B, Differential percentage of high GFI1 samples in various gastric disorders. GFI1 low, scored 0/1. GFI1 high, scored 2/3. Chi-square test was used for statistical analysis. ***, P < 0.001. C, Kaplan–Meier survival curves of gastric cancer patients based on GFI1 expression. Survival analysis was performed using log-rank test.
Table 1. Expression of GFI1 correlates with gastric cancer progression

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*P < 0.05.
*0.001 < P < 0.05.
*P < 0.001.

GFI1 via microarray analysis. Interestingly, the expression of GKN2 was significantly reduced upon ectopic GFI1 expression (Supplementary Fig. S1E). However, efficient knockdown of GFI1 caused a 10-fold increase in GKN2 transcripts and a 3-fold increase in secreted GKN2 protein in the supernatant, suggesting that GKN2 may be an important and sensitive downstream gene of GFI1 (Fig. 2D and E; Supplementary Fig. S1F). To further determine whether or not GKN2 is a direct target gene of GFI1, chromatin immunoprecipitation (ChIP) was performed to validate direct binding of GFI1 at the GKN2 promoter region (Fig. 2F). To validate whether or not GKN2 suppression contributes to GFI1-mediated gastric cancer cell proliferation, GKN2 was ectopically expressed in SGC7901 and HST746T cells with GFI1 overexpression. Indeed, GKN2 expression significantly reversed anchorage-dependent colony formation and S-phase entry that was promoted by GFI1 expression (Fig. 2G; Supplementary Fig. S1G–S1H). Importantly, additional GKN2 expression effectively blocked tumor growth driven by ectopic GFI1 expression in a mouse xenograft assay (Fig. 2H and I). Moreover, the inverse correlation between GFI1 and GKN2 levels was observed in clinical gastric cancer samples (Fig. 2J). Therefore, GFI1 is an oncogenic factor in gastric cancer cells and the function is at least partially through transcriptional inhibition of GKN2.

GFI1 interacts with and is degraded by FBXW7

To identify possible GFI1 modulators in gastric cancer cells, we generated SGC7901 cells expressing HA-GFI1 to perform immunoprecipitation (IP)–mass spectrometry analysis. In addition to the known GFI1 binding proteins, PRMT1 (35) and HDAC2 (36), FBXW7 was detected as a potential GFI1-interacting protein (Fig. 3A). The interaction between endogenous GFI1 and exogenous or endogenous FBXW7 (the α isoform) was further validated by IP (Fig. 3B–D; Supplementary Fig. S2A). Interestingly, minimal interaction was detected between GFI1 and other F-box proteins, including SKP2 and β-TRCP1 or CUL5-LIN 2-5, thus supporting a specific interaction between the SCF<sub>FBXW7</sub> complex and GFI1 (Fig. 3E and F). Reciprocally, ectopic expression of wild-type FBXW7α, but not SKP2 or β-TRCP1, caused a decrease in GFI1 and shortened the half-life (Fig. 3G and H). Moreover, the GFI1 degradation effect was only specific to wild-type FBXW7α, but not disease-derived R465C mutant (Fig. 3I; ref. 37), further suggesting that GFI1 is a possible physiologic substrate of FBXW7. Moreover, cotransfection of shRNAs targeting FBXW7 reduced GFI1 ubiquitination (Supplementary Fig. S2B). Because GFI1 suppressed GKN2 expression in gastric cancer cells and is governed by SCF<sub>FBXW7</sub>, we continued to investigate whether or not GKN2 expression is influenced by FBXW7 status. Importantly, depletion of endogenous FBXW7 in BGC823 GC cells caused a significant increase in endogenous GFI1 protein levels, which was very similar to the well-known FBXW7 substrate, C-MYC, and only had a minimal effect on C-MYC, and only had a minimal effect on FBXW7 mRNA abundance was observed in public data sets from gastric cancer cohorts (Fig. 3L and M; Supplementary Fig. S2C–S2E), indicating inhibition of FBXW7 may contribute to suppression of GKN2 in gastric cancer. Collectively, these results support the notion that GFI1 is a degradation substrate of the SCF<sub>FBXW7</sub> complex.

GSK3β destabilizes GFI1 in gastric cancer cells

In an attempt to screen for the specific upstream signaling that regulates GFI1 expression, we found that pazopanib, a potent multitarget receptor tyrosine kinase (RTK) inhibitor that has been approved for renal cell carcinoma and soft tissue sarcoma treatment by numerous agencies worldwide, effectively reduced ectopic GFI1 expression, which could be blocked by the proteasome inhibitor, MG132 (Supplementary Fig. S3A). Because pazopanib may alter different kinase pathways, including PI3K–Akt and MAPK–ERK, we examined the change in GFI1 expression upon treatment of chemicals inhibiting Akt, GSK3β, and MAPK. Interestingly, both exogenous and endogenous GFI1 levels were increased dramatically after treatment with the GSK3β inhibitors, CHIR99021 and LiCl (Fig. 4A; Supplementary Fig. S3B). Thus, we speculate that GSK3β may be the kinase that negatively regulates GFI1 stability. In keeping with this notion, the half-life of ectopically-expressed GFI1 was
Figure 2.
GFI1 promotes gastric cancer cell proliferation and suppresses GKN2 expression. A, Immunoblot (IB; left), cell growth curve analysis (middle), and soft agar assays (right) of GFI1 overexpression gastric cancer cell lines. B, Cell growth curve analysis of HGC27 cells with GFI1 knockdown (GFI1 KD) or empty vector (PLKO.1). C, Representative images and statistical analysis of colony formation assays of indicated cell line. Scale bar, 1 cm. D, qPCR analysis of GFI1 and GKN2 mRNA levels of indicated HS746T cells. E, ELISA analysis of GKN2 protein levels in SGC7901 control or GFI1 knockdown cells. F, ChIP-qPCR analysis of GKN2 promoter region precipitated with indicated antibodies in HS746T cells. G, Statistical analysis and representative images of colony formation assays of SGC7901 and HS746T cells with indicated gene expression. Scale bar, 1 cm. H and I, HS746T cells with ectopic GFI1 expression or GFI1/GKN2 double expression were used for xenograft tumorigenesis assay. Seven nude mice were used for each group and tumor growth curves were generated by measuring on the indicated days. Unpaired Student t test was used and data are shown as the mean ± SEM. J, GFI1 and GKN2 levels were inversely correlated in clinical GC samples. A chi-square test was used for statistical analysis. Scale bar, 50 μm. *, P < 0.05; **, 0.001 < P < 0.01; ***, P < 0.001, represents statistically significant.
Figure 3.
FBXW7 interacts with GFI1 and governs its stability. A, Coomassie blue staining of anti-HA-GFI1 IP. B, Immunoblot (IB) analysis of whole-cell lysates (WCL) and IP from SGC7901 cells transfected with Flag-FBXW7α or empty vector in the presence of MG132. C, Endogenous co-IP experiments in SGC7901 cells were performed with the indicated antibodies. D, Endogenous co-IP experiments in SGC7901 cells pretreated with FBXW7α siRNA or control siRNA (NC) were performed with the indicated antibodies. E, Immunoblot analysis of WCL and IP of 293T cells transfected with HA-GFI1 and different Cullin constructs and treated with MG132. F, Immunoblot analysis of whole-cell lysates and IP of 293T cells transfected with HA-GFI1 and different F-box and treated with MG132. G, Immunoblot analysis of whole-cell lysates of 293T cells transfected with HA-GFI1 and different F-box constructs. H, 293T cells were transfected with GFI1 and FBXW7α constructs as indicated and treated with 100 µg/mL of cycloheximide (CHX) before whole-cell lysates were collected at the indicated time points for immunoblot analysis. I, Immunoblot analysis of 293T cells transfected with Flag-GFI1 and wild-type FBXW7α (WT) or FBXW7α-R465C mutant. J and K, Immunoblot analysis or qPCR analysis of BGC823 cells treated with indicated siRNAs. L, The association (by Pearson) between FBXW7 and GKN2 transcripts was observed in public data sets. Unpaired Student t test was used. M, Model of FBXW7–GFI1–GKN2 axis. Data are shown as the mean ± SEM. *, 0.01 < P < 0.05; **, 0.001 < P < 0.01; ***, P < 0.001.
dramatically prolonged after CHIR99021 treatment (Fig. 4B), and cotransfection with GSK3β significantly decreased GF1 levels, which was effectively blocked by MG132 and GSK3 inhibitors (Fig. 4C and D). However, depletion of endogenous GSK3β in BGC823 gastric cancer cells caused marked upregulation of GF1 protein levels, but not GF1 transcripts, and resulted in suppressed GKN2 mRNA levels (Fig. 4E). Knockdown of GF1 restored GKN2 mRNA levels in GSK3β-depleted cells (Fig. 4F), further supporting SCF^{FBXW7}/GSK3β Degrades GF1 in Gastric Cancer

Figure 4.

GSK3β destabilizes GF1 and negatively regulates GF1 expression. A and B, Immunoblot (IB) analysis of whole-cell lysates from 293T cells transfected with HA–GF1 and treated with the indicated chemicals. GSK690693 (AKT inhibitor), 10 μmol/L; CHIR-99021 (GSK3β inhibitor), 10 μmol/L; U0126 (Erk1/2 inhibitor), 10 μmol/L. C, One hundred μg/mL of cycloheximide (CHX) in the presence or absence of 10 μmol/L CHIR-99021. D and E, Immunoblot analysis of whole-cell lysates from 293T cells transfected with indicated plasmids treated with MG132, CHIR-99021 (10 μmol/L), or LiCl (20 mmol/L). E, Immunoblot and qPCR analysis of BGC823 cells transfected with siRNAs targeting GSK3β or control siRNA. F, qPCR of the indicated gene mRNA levels in HST746T cells. Unpaired Student t test was used for E and F and data are shown as the mean ± SEM. Results are from n = 3 independent experiments. G, Significant inverse correlation (P = 0.019) between p-GSK3β (Y216) and GF1 levels was determined by a chi-square test in clinical gastric cancer samples. Scale bar, 50 μm. H, Immunoblot analysis of whole-cell lysates (WCL) and IP from 293T cells transfected with the indicated plasmids. I, Immunoblot analysis of whole-cell lysates and IP from 293T cells transfected with indicated plasmids treated with MG132 (10 μmol/L), CHIR-99021 (10 μmol/L), or LiCl (20 mmol/L). J–K, Immunoblot analysis of whole-cell lysates from 293T cells transfected with the indicated plasmids and treated with MG132 (10 μmol/L), CHIR-99021 (10 μmol/L), or LiCl (20 mmol/L). *P < 0.01; **P < 0.001; ***P < 0.0001, represents statistically significant.
a master regulatory role of GSK3β in modulating the GFI1–GKN2 axis. Next, we examined the association between GSK3β activity (represented by the pY216 signal) and GFI1 expression in 176 clinical gastric cancer tissues, and found that active GSK3β is significantly correlated with a low GFI1 level and better overall survival of patients with gastric cancer (Fig. 4G; Supplementary Fig. S3C). In support of an essential role of GSK3β in FBXW7-mediated GFI1 regulation, coexpression of GSK3β increased GFI1–FBXW7α interaction, whereas CHIR-99021 LiCl treatment drastically blocked such interaction and FBXW7α-coupled GFI1 reduction (Fig. 4H–K). Therefore, the results outlined above suggested that GFI1 protein stability is stringently regulated by FBXW7 in gastric cancer cells.

GSK3β-dependent S94/S98 phosphorylation mediates GFI1–FBXW7 interaction

SCF<sub>FBXW7</sub> has been shown to interact and degrade multiple key proteins, including C-JUN, C-MYC, MCL1, and others after phosphorylation by GSK3β at certain sites (namely phosphodegron; ref. 24). To gain a mechanistic insight of how GSK3β modulates GFI1, we analyzed the GFI1 sequence by mass spectrometry and identified S94/S98 as the putative GSK3β phosphorylation site matching the classic FBXW7 phosphodegron sequence (Fig. 5A; Supplementary Fig. S4A). We then generated GST-fused recombinant GFI1 proteins (wild-type and mutants) and incubated the proteins with GSK3β in the presence of [γ-32P]-ATP to perform an in vitro kinase assay. Indeed, mutation of either S94 or S98 to alanine (labeled as S94A and S98A) significantly reduced the incorporated [γ-32P]-ATP signal, and double mutation of both sites almost completely abolished the phosphorylation by GSK3β (Fig. 5B), suggesting that S94 and S98 are indeed the major GSK3β phosphorylation sites of GFI1. Mutation of S94 and S98 to alanine largely reduced the polyubiquitination of GFI1, and S94A and S98A mutants were resistant to ectopic GSK3β-mediated degradation (Fig. 5C and D). Furthermore, in support of an essential role for S94 and S98 in SCF<sub>FBXW7</sub>-mediated GFI1 degradation, S94A and S98A mutations abolished the FBXW7α–GFI1 interaction and ectopic FBXW7α-mediated degradation and resulted in a prolonged GFI1 half-life (Fig. 5E–G). To further strengthen a causal relationship between GSK3β-mediated GFI1 phosphorylation and the FBXW7α–GFI1 interaction, we first phosphorylated beads-bound recombinant GST-GFI1 (wild-type and S94A/S98A mutant) with GSK3β kinase in the presence of ATP, then incubated the resulting beads with Flag-FBXW7 (wild-type and R465C mutant, respectively) containing cell lysate to perform a GST-pulldown assay. As shown in Fig. 5H, only GSK3β pretreated wild-type GST-GFI1 protein, but not the S94A/S98A mutant, effectively pulled down wild-type Flag-FBXW7α, but not the Flag-FBXW7-R465C mutant. Collectively, these results indicated that phosphorylation of GFI1 at S94/S98 sites by GSK3β is both necessary and sufficient to trigger the GFI1–FBXW7 interaction and subsequent protein degradation.

Nondegradable GFI1 is more potent in promoting tumorigenesis of gastric cancer cells

As outlined above, GSK3β-mediated GFI1 S94/S98 phosphorylation was crucial for GFI1 stability control, and high GFI1 expression was correlated with a poorer clinical outcome of patients with gastric cancer. To gain an understanding of how the blockage of GFI1 degradation promotes the oncogenic potential of gastric cancer cells, we generated HS746T and BGC823 cells that stably express wild-type GFI1 and S94A/S98A mutants. As indicated in Fig. 6A, although mRNA levels of ectopic GFI1-WT and GFI1-S94A/S98A were quite similar, a much higher level of GFI1 protein product was observed in GFI1-S94A/S98A-expressing cells, which resulted in a lower level of endogenous GKN2 transcription and more colonies in the anchorage-independent soft agar assay in comparison with GFI1-WT expressing and parental control gastric cancer cells (Fig. 6B and C). We performed xenograft tumorigenesis experiments by inoculating HS746T gastric cancer cells expressing wild-type GFI1 (WT) and GFI1-S94A/S98A mutant in the flanks of nude mice and used parental HS746T gastric cancer cells as the control. Although all 3 groups of nude mice developed tumors, mice implanted cells expressing nondegradable GFI1-S94A/S98A mutant developed the largest tumors (Fig. 6D–F), implicating a potentially important functional role of deregulated GFI1 via inactivation of FBXW7 or GSK3β in gastric cancer (Fig. 6C). Taken together, these results suggest that blockade of GFI1 protein degradation was a tumor promoting factor of gastric cancer and efficient degradation of GFI1 by the FBXW7/GSK3β pathway may be crucial for suppressing gastric cancer progression.

Discussion

GFI1 has been shown to be a central regulator downstream of RUNX1 and P53, and by recruiting important epigenetic regulators, such as LSD1 and COREST, GFI1 plays a crucial role in the early development of the hematopoietic lineage (36). The GFI1 mutation in the human genome is associated with defective hematopoiesis. Although overexpression of GFI1 has been characterized in malignancies, including AML (38) and medullblastoma (39), the function and regulation of GFI1 in most solid tumors are largely unknown. In this study we first found a significant correlation between high GFI1 expression and advanced gastric cancer, which predicts a worse prognosis (Fig. 1). Furthermore, the expression of GFI1 promotes gastric cancer cell proliferation, a process that occurs at least in part via suppressing GKN2 transcription. Functionally, GKN2 has been reported as an antiproliferation factor, and results from a cell-based study and transgenic mice model suggested that GKN2 overexpression suppresses the oncogenic potential of gastric cancer cells and inhibits tumor growth in vivo. Importantly, although GKN2 protein was absent in 85% of diffuse and 54% of intestinal types of gastric cancer, and was associated with poorer survival; somatic mutation was rarely found in the coding region of the GKN2 allele (34). Therefore, GKN2 is mainly suppressed by a transcriptional/epigenetic mechanism in gastric cancer. In this study, we have characterized GFI1 as a major suppressor of GKN2, the depletion of which caused an approximate 10-fold upregulation of endogenous GKN2 transcription (Fig. 2D). More importantly, we further found that depletion of GSK3β by siRNA caused a dramatic decrease in GKN2 mRNA (Fig. 4E and F), which may be caused by upregulation of GFI1 protein. Therefore, our finding provided a potential link between decreased expression of the tumor suppressor, GKN2, and genetic lesions of the RTK–PI3K/PTEN pathway, which are often observed in gastric cancer and may cause inhibition of downstream GSK3β activity (13).

FBXW7 is among the most well-studied F-box proteins that form the SCF E3 ligase complex to degrade numerous substrates (27). By specifically recognizing the "phosphodegron" motif of substrates through the WD40 domain that forms a β propeller structure, FBXW7 only binds substrates after the substrates are properly phosphorylated at specific threonine or serine.
residues. First discovered as CDC4 in *Drosophila*, FBXW7 was characterized as the F-box protein responsible for cell-cycle regulation by degrading CCNE1, and subsequent studies revealed that FBXW7 governs the destruction of crucial oncogenic factors, including C-MYC (20, 21), C-JUN (23), MCL1 (24), and many others (22). Thus, FBXW7 is proposed to be a putative tumor suppressor.

Figure 5.

GSK3β phosphorylates S94/S98 sites of GFI1 to recruit FBXW7. A, Sequence alignment of GFI1 with the known phosphodegron sequences recognized by FBXW7. Ser94 and Ser98 sites were conserved in several species (bottom). B, Two μg recombinant GST-GFI1 or GST proteins were incubated with purified GSK3β for in vitro kinase assays in the presence of γ-32P-ATP. The reactions were resolved by SDS-PAGE and detected by autoradiography. C–F, Immunoblot (IB) analysis of whole-cell lysates (WCL) and IP from 293T cells transfected with the indicated plasmids. G, 293T cells were transfected with wild-type GFI1 or GFI1-S94A/S98A mutant and HA-FBXW7-Flag-GSK3β constructs. Cells were treated with 100 μg/mL of cycloheximide (CHX) before whole-cell lysates were collected at the indicated time points for immunoblot analysis. H, The flow chart for in vitro kinase pulldown assays (right). In vitro purified protein GST-GFI1 (WT and mutants) was phosphorylated by His-GSK3β first, then phosphorylated GST-GFI1 was added to pull down Flag-FBXW7α purified form 293T cells.
suppressor, and indeed genetic variations, including mutations and deletions, have been frequently observed in FBXW7 alleles. For example, in a comprehensive mutation analysis of FBXW7 genetic status in 534 human cancer samples, an overall approximate 6% mutation frequency was estimated with all cancer samples tested with the highest mutation rate (>30%) observed in cholangiocarcinomas and T-cell acute lymphocytic leukemia (40). Interestingly, 43% of all mutations occurred at the

Figure 6.
Nondegradable GFI1 mutant is more potent in driving tumorigenesis. A, qPCR (left) and immunoblot (IB) analysis (right) of gastric cancer cells with ectopic GFI1 expression. B, qPCR analysis of GKN2 transcription in HS746T cells with ectopic GFI1 expression. C, Representative images and quantification of soft agar assays of gastric cancer cells with ectopic GFI1 expression. Seven nude mice were used for each group and tumor growth curves were generated by measuring every 3 days (E). Scale bar, 1 cm. For D and E, unpaired Student t test was used and data are shown as the mean ± SEM. G, Model graph of how deregulated GFI1 may contribute to gastric tumorigenesis. GFI1 protein is stringently governed by SCFFBXW7/GSK3β so that GKN2 expresses and helps maintain homeostasis in healthy gastric tissues. When FBXW7 is mutated/downregulated or GSK3β is inhibited by elevated RTK signaling, GFI1 degradation is blocked and suppresses GKN2 transcription to promote gastric cancer cell proliferation and disease progression. *, P < 0.05; **, P < 0.01; ***, P < 0.001, represents statistically significant.
“hotspot” Arg residues responsible for binding the phosphodegron sequence of substrates. In another analysis of The Cancer Genome Atlas data, including 219 stomach cancer samples, the hotspot mutation rate was approximately 5% (41), whereas the overall mutation rate of FBXW7 was estimated to be 9.2% to 18.5% in gastric cancer (15). Therefore, FBXW7 is also an important tumor suppressor in gastric cancer. In addition to genetic mutations, the expression of FBXW7 is targeted by miRNAs, such as miR-223 (42) and miR-25 (43), and low FBXW7 expression is associated with poor prognosis and chemoresistance. Although BRG1 has been identified as a key FBXW7 substrate modulating gastric cancer metastasis recently (18), little is known about how low expression or mutation of FBXW7 contributes to disease-specific morphology and progressive features of gastric cancer. In this study, we have established GFI1 as a ubiquitination substrate of FBXW7, and our mechanistic study further connected FBXW7 loss to suppression of the GFI1 downstream target gene, GKN2, which is a gastric-specific tumor suppressor (34). Therefore, our work further supports the tumor suppressor function of FBXW7 in gastric cancer and provides new insight into how the loss of FBXW7 promotes gastric cancer progression via inhibition of the gastric tissue-specific tumor suppressor, GKN2.

As a very lethal type of cancer, gastric cancer is associated with bad outcomes and there are still very few therapeutic options for gastric cancer. Based on the result from a systematic study from the Asian Cancer Research Group with a large cohort analyzed, approximately 20% of gastric cancer cases were carrying amplifications or mutations in RTK genes, and about 18% cases were found with PIK3CA or PTEN mutations (44). Therefore, patients with gastric cancer with RTK mutations or impaired FBXW7 function may potentially suffer from subsequent accumulation of the GFI1 protein, which in turn would further contribute to the rapid proliferation of gastric cancer cells and promote disease progression. Because multiple RTK targeting drugs have been approved for gastric cancer treatment, such as bevacizumab and aptinib, it is possible that an elevated GFI1 protein level could serve as an additional indicative marker for usage of such therapeutic agents to treat patients with gastric cancer. Collectively, our study has characterized the oncogenic function and regulatory mechanism underlying high GFI1 expression in gastric cancer and may contribute to a better understanding and clinical treatment of gastric cancer.

Disclosure of Potential Conflicts of Interest
J. Li has ownership Interest (including stock, patents, etc.) for OrigInMed Inc. No potential conflicts of interest were disclosed by the other authors.

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