Published in final edited form as:

Cell Signal. 2012 January ; 24(1): 316–324. doi:10.1016/j.cellsig.2011.09.017.

The p53-p21^{WAF1} checkpoint pathway plays a protective role in preventing DNA rereplication induced by abrogation of FOXF1 function

Pang-Kuo Lo^a, Ji Shin Lee^b, and Saraswati Sukumar^c

^aDepartment of Biological Sciences, University of South Carolina, Columbia, SC, USA

^bDepartment of Pathology, Chonnam National University Hwasun Hospital, Jeollanam-do, Republic of Korea

^cDepartment of Oncology, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

Abstract

We previously identified *FOXF1* as a potential tumor suppressor gene with an essential role in preventing DNA rereplication to maintain genomic stability, which is frequently inactivated in breast cancer through the epigenetic mechanism. Here we further addressed the role of the p53-p21^{WAF1} checkpoint pathway in DNA rereplication induced by silencing of FOXF1. Knockdown of FOXF1 by small interference RNA (siRNA) rendered colorectal p53-null and p21^{WAF1}-null HCT116 cancer cells more susceptible to rereplication and apoptosis than the wild-type parental cells. In parental HCT116 cells with a functional p53 checkpoint, the p53-p21^{WAF1} checkpoint pathway was activated upon FOXF1 knockdown, which was concurrent with suppression of the CDK2-Rb cascade and induction of G₁ arrest. In contrast, these events were not observed in FOXF1-depleted HCT116-p53^{-/-} and HCT116-p21^{-/-} cells, indicating the p53-dependent checkpoint function is vital for inhibiting CDK2 to induce G₁ arrest and protect cells from rereplication. The pharmacologic inhibitor (caffeine) of Ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3 related (ATR) protein kinases abolished activation of the p53-p21^{WAF1} pathway upon FOXF1 knockdown, suggesting that suppression of FOXF1 function triggered the ATM/ATR-mediated DNA damage response. Cosilencing of p53 by siRNA synergistically enhanced the effect of FOXF1 depletion on stimulation of DNA rereplication and apoptosis in wild-type HCT116. Finally, we show that FOXF1 expression is predominantly silenced in breast and colorectal cancer cell lines with inactive p53. Our study demonstrated that the p53-p21^{WAF1} checkpoint pathway is an intrinsically protective mechanism to prevent DNA rereplication induced by silencing of FOXF1.

© 2011 Elsevier Inc. All rights reserved.

Correspondence to: Pang-Kuo Lo, Ph.D., Research Assistant Professor, Department of Biological Sciences, University of South Carolina, 715 Sumter Street, PSC621, Columbia, SC 29208, TEL: 803-777-1855; FAX: 803-777-4002; LOP@mailbox.sc.edu; Saraswati Sukumar, Ph.D., Barbara B. Rubenstein Professor of Oncology, Breast Cancer Program, Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, 1650 Orleans Street, CRB 1, Rm 143, Baltimore, MD 21231-1000, TEL: 410-614-2479; FAX: 410-614-4073; saras@jhmi.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conflict of interest statement

None declared.

Keywords

FOXF1; tumor suppressor gene; DNA rereplication; p53; p21^{WAF1}

1. Introduction

Forkhead box (FOX) proteins are an evolutionarily conserved, ancient gene family that was discovered after the identification of the *Drosophila melanogaster* gene fork head (*fh*) [1-3]. FOX proteins function as transcription factors with the evolutionarily conserved DNA-binding domain termed forkhead box or winged helix domain [1-3]. Many members of the FOX gene family have been documented to play pivotal roles in embryonic development and also in the control of a variety of physiological processes, such as cell cycle progression, cell survival, cellular metabolism, life span, and immune responses [3-5]. Through the transcriptional regulation of genes involved in controlling the cell cycle machinery, several FOX gene members have been identified to play important roles in cell cycle regulation [6]. For example, FOXOs mediate stress responses to arrest cells in the G₁ phase; FOXM1 is implicated in controlling G₁-S and G₂-M cell cycle progression; FOXA1 collaborates with BRCA1 to synergistically enhance the *CDKN1B* gene expression and also interacts with estrogen receptors to regulate Cyclin D1 expression [6]. Consequently, deregulation of these FOX factors forces the development and progression of proliferative diseases, in particular cancer [3, 6]. Therefore, the identification and characterization of FOX gene members engaged in cell cycle regulation is of great importance for fundamental understanding of the molecular mechanisms underlying cell cycle regulation and the development of therapies for diseases caused by deregulation of the cell cycle machinery.

DNA replication is stringently controlled to occur only once in each cell cycle. This stringent control is essential for maintaining genome stability. The replication licensing system is responsible for this stringent control by permitting firing of replication origins only once in a single cell cycle [7, 8]. Licensing of DNA replication origins proceeds with the sequential loading of the origin recognition complex (ORC), replication licensing factors and the minichromosome maintenance (MCM) complex onto replication origin sites to form prereplication complexes (pre-RCs) [7, 9]. Chromosomal origins with the assembly of pre-RCs are licensed for replication and subsequently activated by Cdc7 and CDK2 kinases to initiate DNA replication when the cell cycle is committed to entering the S phase [7, 10]. Once licensed origins are initiated, pre-RCs are disassembled and the reloading of the MCM complex is forbidden until mitosis is complete. Deregulation of the replication licensing process disrupts the stringency of DNA replication and permits repeated firing of DNA replication origins, leading to DNA rereplication (also called over-replication) [8, 11]. DNA rereplication consequently gives rise to gene amplification and chromosomal alterations (e.g. translocation and deletion), which are known to promote cellular transformation and tumorigenesis [11]. In addition, DNA replication is regulated by S-phase checkpoint proteins, such as ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3 related (ATR), Fanconi anemia (FA) and Nijmegen breakage syndrome (NBS), which all play critical roles in DNA damage response. Defects in the S-phase checkpoints result in persistent DNA synthesis after DNA damage, in turn leading to genomic instability and mutagenesis [12]. Due to the crucial role of DNA replication regulation in tumorigenesis, it is important to identify and characterize molecules involved in the regulatory mechanisms underlying DNA replication control.

The human *FOXF1* gene, previously named as *Forkhead Related Activator (FREAC)-1* [13], encodes a homologue of the mouse forkhead box-F1 (Foxf1) transcription factor. Gene knockout studies have shown that the mouse Foxf1 takes an indispensable role of a regulator

in organ morphogenesis, including the lung, liver, gallbladder, esophagus, and trachea [14-16]. Although the roles of Foxf1 in mouse development have been extensively documented, the biochemical roles of its functions in mammalian cells are largely unknown. However, several studies have shown that mammalian forkhead box-F1 is the downstream mediator of the hedgehog (Hh) signaling pathway [14, 17, 18] and implicated in regulating migration of normal and cancer-associated fibroblasts as well as paracrine interactions between these fibroblasts and normal and cancer epithelial cells, which are involved in modulating developmental and tumorigenic progressions [17, 19, 20]. Dysregulation of FOXF1 expression has been identified in prostate and breast cancers [21, 22]. We recently identified human *FOXF1* as a potential tumor suppressor gene in mammary epithelial cells, which is frequently silenced in breast cancer through epigenetic mechanisms [22]. We have shown that ectopic overexpression of FOXF1 inhibits the CDK2-Rb-E2F cascade to block the G₁-S transition and inactivation of endogenous FOXF1 leads to genomic DNA rereplication [22]. Moreover, we also found that FOXF1 is engaged in negatively regulating expression of DNA replication initiation factor genes (e.g. *MCM3* and *CDC34*), suggesting that FOXF1 is involved in controlling DNA replication initiation [22].

In this study, we further extended our previous studies and addressed the role of the p53-p21^{WAF1} checkpoint pathway in regulating DNA rereplication induced by FOXF1 inhibition. Our study demonstrated that the abrogation of the *in vivo* FOXF1 function led to stimulation of the p53-p21^{WAF1} checkpoint pathway, which instigated G₁ arrest to prevent DNA rereplication and apoptosis. Our results also suggest that the ATM/ATR-mediated DNA damage response is implicated in activating this cell cycle checkpoint pathway. Furthermore, we found that FOXF1 expression is preferentially lost or underexpressed in breast and colorectal cancer cell lines with inactive p53. Therefore, our findings support the hypothesis that genetic inactivation of the p53 checkpoint function collaborates with aberrant silencing of FOXF1 to promote genomic instability and tumorigenesis.

2. Materials and methods

2.1. Cell lines

The human colorectal cancer cell line HCT116 and its derivative cell lines including HCT116-p53^{-/-} and HCT116-p21^{-/-}, kindly provided by Dr. B. Vogelstein, were cultured as described [23]. Immortalized, nontumorigenic human mammary epithelial cells (HBL100) and twenty breast cancer cell lines (obtained from the American Type Culture Collection, Manassas, VA) were cultured according to the ATCC online instructions.

2.2. Real-time quantitative reverse-transcription PCR (qRT-PCR) analysis

Total RNA isolated from cancer cell lines was reverse-transcribed and the SYBR green-based real-time quantitative RT-PCR reaction was carried out and then analyzed as previously described [24]. The primers for real-time quantitative RT-PCR analysis of FOXF1 and GAPDH mRNA expression are the same as previously described [22, 24]. The primers for analysis of p53 and PIG3 mRNA expression are: p53 forward primer, 5'-GGG AGC ACT AAG CGA GCA CTG-3'; p53 reverse primer, 5'-TTG GAC TTC AGG TGG CTG GA-3'; PIG3 forward primer, 5'-TCG ATG GGT TCT CTA TGG TCT GA-3'; PIG3 reverse primer, 5'-TCA CCA GCA TTT GCT TGT ACT TAT TG-3'.

2.3. siRNA transfection

siRNA transfections were performed as previously described [22]. The two siRNA sequences for targeting FOXF1 mRNA are: siFOXF1-1, 5'-GAA AGG AGU UUG UCU UCU C-3'; siFOXF1-2, 5'-UCA AGC CCA UGU ACA GCA U-3'. The FOXF1, p53 and non-targeting control siRNAs were purchased from Dharmacon (Boulder, CO). Transfected

cells were harvested at 48 h after siRNA transfection for qRT-PCR, BrdU incorporation and Western blot analyses.

For caffeine (Sigma-Aldrich, St. Louis, MO) and UCN-01 (Sigma-Aldrich) treatments after siRNA transfection, cells were treated with caffeine (5 mM) or UCN-01 (300 nM) at 24 h after siRNA transfection. Cells were harvested for Western blot analysis after drug treatment for 24 h.

2.4. Preparation of nuclear extracts

Nuclear extract was prepared according to the protocols described [25]. In brief, HCT116 cells with or without siRNA transfection were washed with ice-cold phosphate-buffered saline, and lysed in 500 μ l of ice-cold hypotonic solution (10 mM HEPES, 10 mM KCl, 1 mM Mg(OAc)₂, 1 mM dithiothreitol, 10% glycerol, and 1 mM phenylmethylsulfonyl fluoride). After centrifugation, the pellet was extracted with 100 μ l of hypertonic solution (hypotonic solution containing 0.5 M KCl) and then spun. The supernatant containing the nuclear extract was used for Western blot analysis of the FOXF1 protein.

2.5. BrdU incorporation and cell cycle analyses

BrdU incorporation assay was performed as described previously [22]. In brief, cells were incubated with 10 μ M BrdU (Sigma-Aldrich) for 30-min labeling. BrdU-labeled cells were fixed in 80% ethanol and denatured by 2N HCl with 0.5% Triton X-100, and then neutralized by 0.1 M sodium tetraborate (Na₂B₄O₇ *10H₂O, pH 8.5). BrdU was detected using the mouse monoclonal anti-BrdU antibody (Becton Dickinson, Sparks, MD) and Alexa Fluor® 488 goat anti-mouse IgG secondary antibody (Invitrogen). After washing and centrifugation, cells were resuspended in 1 ml of 1×PBS containing 20 μ g/ml of propidium iodide (Sigma-Aldrich). BrdU incorporation and DNA content were analyzed using the BD FACSCalibur flow cytometer. Apoptosis was determined by measuring the percent of cells with sub-G₁ DNA content (<2N). *P*-values for differences in apoptosis, cell cycle and rereplication were determined by two-tailed Student's *t*-tests.

2.6. Western blot analysis

Total or nuclear protein lysates from transfected cells were separated by SDS-PAGE and then blotted onto Hybond-C extra membranes (GE Healthcare, Buckinghamshire, UK) for Western blot analysis. Antibodies used for Western blot analysis are those purchased from Cell Signaling Technology (Beverly, MA), including phospho-Chk1 (Ser-345), phospho-Chk2 (Thr-68), phospho-CDK2 (Thr-160), phospho-Rb (Ser-795), Rb, phospho-CDC2 (Tyr-15) and CDC2, and other companies' antibodies, including FOXF1 (Human Protein Atlas antibody, Sigma-Aldrich) [20], CDK2 (Santa Cruz Biotechnology), α -tubulin (Invitrogen's Zymed), nucleolin (Sigma-Aldrich), p53 (DO-1, Invitrogen's Biosource), and p21^{WAF1} (Calbiochem, La Jolla, CA). Western blot data were quantified by densitometric analysis of autoradiograms, using a computerized densitometer (Typhoon System; Molecular Dynamics, Inc., Sunnyvale, CA).

2.7. In silico analysis of FOXF1 gene expression in colorectal cancer cell lines

The Oncomine's Cancer Microarray Database [26] (<http://www.oncomine.org>) was used to perform *in silico* analysis of *FOXF1* gene expression in 18 colorectal cancer cell lines. We fetched FOXF1 expression data from the microarray dataset by Wagner *et al.* [27]. The p53 status of 18 colorectal cancer cell lines was based on the information of studies by Liu *et al.* [28]. Mann-Whitney test was used to evaluate the statistical difference between p53 wild-type and p53 mutant datasets.

3. Results

3.1. Ablation of the p53-p21^{WAF1} checkpoint pathway promotes DNA rereplication in cells with FOXF1 knockdown

Given that our previous studies have shown that knockdown of FOXF1 led to genomic instability by triggering DNA rereplication, we investigated whether the endogenous p53-p21^{WAF1} checkpoint function plays a role in regulating this process due to its critical role in cell cycle checkpoints [29]. To decipher the effect of the p53-p21^{WAF1} checkpoint on rereplication after loss of FOXF1, we examined FOXF1 knockdown in the wild-type (HCT116-WT), p53-null (HCT116-p53^{-/-}) and p21^{WAF1}-null (HCT116-p21^{-/-}) HCT116 colon cancer cell lines. The knockdown effects of two FOXF1 siRNAs were examined first by Western blot analysis of nuclear protein extracts from siRNA-transfected HCT116-WT. As shown in Fig. 1A, both siFOXF1-1 and siFOXF1-2 siRNAs were able to deplete expression of endogenous nuclear FOXF1 protein in HCT116-WT cells; the siFOXF1-1 siRNA exhibited a stronger knockdown effect compared to siFOXF1-2. Also, as shown by real-time qRT-PCR analysis, the siFOXF1-1 and siFOXF1-2 siRNAs depleted over 90% and 60% of endogenous FOXF1 mRNA expression, respectively, in HCT116-WT cells as well as in derivative HCT116-p53^{-/-} and HCT116-p21^{-/-} cells (Fig. 1B).

The BrdU incorporation assay was performed to analyze the effect of FOXF1 knockdown on DNA replication, cell cycle progression and apoptosis in HCT116-WT, HCT116-p53^{-/-} and HCT116-p21^{-/-} cells. To selectively label cells in active DNA synthesis, cells were pulsed with BrdU for 30 min at 48 h after siRNA transfection. Flow cytometric analysis of BrdU-labeled, propidium iodide-stained cells revealed that FOXF1 knockdown resulted in an increase in the fraction of HCT116-WT, HCT116-p53^{-/-} and HCT116-p21^{-/-} cells with greater than 4N of BrdU-incorporated DNA compared to their corresponding control siRNA-transfected cells (Fig. 2 and Table 1). The appearance of BrdU-labeled polyploid cells indicates the occurrence of DNA rereplication. As mentioned in the “Introduction” Section, rereplication results from deregulated DNA replication, which occurs when initiation of DNA replication fires more than once in each cell cycle [8, 11, 12]. Noticeably, inactivation of the p53 or p21^{WAF1} function itself was able to cause a significant increase in the percentage of basal rereplicated cells in HCT116-p53^{-/-} (9.83±0.58%) or HCT116-p21^{-/-} (8.33±0.57%) cells compared to HCT116-WT cells (5.17±0.44%), indicating that the *in vivo* p53-p21^{WAF1} checkpoint function is required for maintaining the stringency of DNA replication in HCT116-WT cells. Knockdown of FOXF1, especially by the siFOXF1-1 siRNA, further augmented the fraction of rereplicated cells in HCT116-p53^{-/-} (12.89±0.65%) and HCT116-p21^{-/-} (14.55±0.53%) cell lines compared to HCT116-WT cells (6.68±0.51) (Table 1). Inactivation of either p53 or p21^{WAF1}, therefore, enhanced the effect of FOXF1 knockdown on promoting DNA rereplication. In addition, an increase in apoptotic cells induced by FOXF1 knockdown was more significant in HCT116-p53^{-/-} and HCT116-p21^{-/-} compared to HCT116-WT cells (Table 1). On the other hand, knockdown of FOXF1 in HCT116-WT cells led to a significant increment in G₁-phase cells (a 7.7% increase) and a parallel decrease in S-phase (a 6.2% decrease) and G₂/M-phase cells (a 3.3% decrease) (Table 1), whereas these events were not observed in HCT116-p53^{-/-} and HCT116-p21^{-/-} cells (Table 1). These results indicate that p53 and p21^{WAF1} are required for induction of G₁ arrest in cells with FOXF1 knockdown. Given the consistency between the data from both siFOXF1-1- and siFOXF1-2-knockdown experiments (despite a lower extent of the knockdown effect from siFOXF1-2), the results shown here were unlikely from an off-target effect of the siRNA. In addition, mRNA expression of *FOXF2*, the most similar *FOX* gene to *FOXF1*, was not affected by the FOXF1 siRNAs (data not shown), which further reduces the possibility of off-target effects. These findings, taken together, provide convincing evidence that the p53-p21^{WAF1} checkpoint function is required for inducing G₁ arrest in FOXF1-depleted cells to prevent DNA rereplication and apoptosis.

3.2. Inhibition of endogenous FOXF1 function results in activation of the p53-p21^{WAF1}-dependent DNA damage checkpoint pathway

The finding that inhibition of FOXF1 function induced G₁ arrest only in HCT116-WT cells, but not in p53^{-/-} or p21^{-/-} cells suggests that the p53-dependent DNA damage checkpoint mechanisms may have been activated. Previous studies have shown that an imbalance in molecular interactions during DNA replication initiation by overexpression of DNA replication initiation factors is able to induce activation of the ATM/ATR-Chk2-p53 DNA damage checkpoint pathways [11, 30]. We therefore postulated that inactivation of FOXF1 elicits the p53-dependent DNA damage checkpoint response in HCT116-WT cells. To address this possibility, protein extracts isolated from FOXF1 siRNA-transfected cells were subjected to Western blot analysis to examine the status of Chk1 and Chk2 kinase activities and expression levels of p53 and p21^{WAF1}. The phosphorylation sites Ser-345 of Chk1 and Thr-68 of Chk2 were examined in the study since phosphorylation of these two amino acid residues indicates activation of these two kinases. Due to the strong knockdown effect of the siFOXF1-1 siRNA, it was used throughout the following mechanism studies. In HCT116-WT cells, FOXF1 knockdown moderately increased phosphorylation at Thr-68 of Chk2, but not at Ser-345 of Chk1 (Fig. 3A). Therefore, upon FOXF1 knockdown, only Chk2 kinase activity was stimulated in HCT116-WT cells. Concurrently, protein levels of p53 and p21^{WAF1} were significantly elevated in HCT116-WT cells depleted of FOXF1 (Fig. 3A). Similar to the outcome of HCT116-WT cells, Chk2, but not Chk1, was modestly activated in HCT116-p53^{-/-} cells in response to abrogation of FOXF1 function. However, the elevation of p21^{WAF1} protein levels was completely abolished in HCT116-p53^{-/-} cells upon FOXF1 knockdown (Fig. 3A), indicating that induction of p21^{WAF1} levels following depletion of FOXF1 is p53-dependent. In addition to p21^{WAF1}, a two-fold induction of PIG3 mRNA levels (another p53 target gene) was also consistently observed in HCT116-WT cells, but not in HCT116-p53^{-/-} cells, as FOXF1 was inactivated (Fig. 3B). In HCT116-p21^{-/-} cells, both Chk1 and Chk2 were significantly activated by FOXF1 knockdown. Stimulation of both Chk1 and Chk2 kinase activities in FOXF1 siRNA-transfected HCT116-p21^{-/-} cells correlated with a dramatic increase in DNA rereplication and apoptosis (Fig. 2 and Table 1). Moreover, elevated protein levels of p53 were also consistently observed in HCT116-p21^{-/-} cells depleted of FOXF1 (Fig. 3A). To rule out the possibility that the results were attributable to the transcriptionally suppressive effect of FOXF1 on p53 mRNA expression, we performed quantitative RT-PCR analysis of p53 mRNA levels. As shown in Fig. 3C, mRNA levels of p53 were not significantly affected by knockdown of FOXF1 in HCT116-WT and HCT116-p21^{-/-} cells. This result suggests that p53, in all likelihood, is not the downstream target gene of FOXF1. Furthermore, activation of the Chk2-p53-p21^{WAF1} checkpoint cascade in FOXF1-depleted HCT116-WT cells correlated with inhibition of CDK2 activity (indicated by a reduction in phosphorylation of CDK2 at Thr-160) and dephosphorylation of the CDK2 downstream target Rb (Fig. 3A). These outcomes were concordant with the observed G₁ arrest in HCT116-WT cells depleted of FOXF1 (Table 1). Suppression of the CDK2-Rb cascade was not observed in HCT116-p53^{-/-} and HCT116-p21^{-/-} cells upon FOXF1 knockdown (Fig. 3A), demonstrating that this effect requires the intact p53-p21^{WAF1} checkpoint function. Intriguingly, total protein levels of CDK2 were increased upon FOXF1 knockdown (Fig. 3A). This change seems to be caused by regulation at the translational or posttranslational level since mRNA levels of CDK2 were not affected by knockdown of FOXF1 (data not shown). Due to activation of the p53-p21^{WAF1} pathway by FOXF1 knockdown to suppress CDK2 activity, the biological effects of upregulation of CDK2 protein in HCT116-WT cells were masked and unable to be detected in our studies.

To further elucidate whether the ATM/ATR-mediated DNA damage checkpoint pathways are responsible for stimulation of the p53-p21^{WAF1} pathway upon knockdown of FOXF1,

we treated siRNA-transfected HCT116-WT cells with DNA damage checkpoint inhibitors, including caffeine (an inhibitor of the ATM/ATR kinases) and UCN-01 (an inhibitor specific to Chk1). As shown in Fig. 3D, caffeine completely annihilated the elevation of p53 and p21^{WAF1} protein levels in HCT116-WT cells with FOXF1 knockdown. Consistent with the data shown in Fig. 3A that there was no activation of Chk1 activity in FOXF1-depleted HCT116-WT cells, UCN-01 had no suppressive effect on elevation of p53 and p21^{WAF1} protein levels (Fig. 3D). These data therefore suggest that abrogation of FOXF1 function activates the p53-p21^{WAF1} pathway through the ATM/ATR-dependent DNA damage checkpoint pathways.

3.3. Cosilencing of p53 synergistically enhances DNA rereplication and apoptosis induced by FOXF1 inhibition

To further validate the results from genetic knockout cell lines and unravel the effects of loss of both p53 and p21^{WAF1} functions in FOXF1-depleted cells, concurrent knockdown of p53 with siRNA was performed. Both wild-type and p21^{WAF1} cells were transfected with the FOXF1 siRNA or p53 siRNA alone, or with both siRNAs. After 48 h, siRNA-transfected cells were subjected to Western blot and BrdU incorporation analyses. As shown in Fig. 4A, the p53 siRNA almost completely depleted p53 protein expression and eradicated p21^{WAF1} induction upon FOXF1 knockdown, consistent with the data from the HCT116-p53^{-/-} cell line (Fig. 3A). For the CDK2-Rb cascade, suppression of CDK2 activity and Rb phosphorylation in HCT116-WT cells upon FOXF1 knockdown was abolished by cosilencing of p53 (Fig. 4A and 4B), agreeing with siRNA knockdown results from HCT116-p53^{-/-} cells (Fig. 3A). In HCT116-p21^{WAF1} cells, this G₁ checkpoint function was lost no matter whether p53 was silenced or not (Fig. 4A and 4B), indicating that p21^{WAF1} is required for the p53-mediated G₁ checkpoint function. In addition, we also investigated the status of CDC2, a critical kinase involved in regulating G₂-M progression. Although the total protein levels of CDC2 were downregulated in HCT116-WT cells upon FOXF1 knockdown, the kinase activity of CDC2 was increased, indicated by a decrease in inhibitory phosphorylation of CDC2 at Tyr-15 (Fig. 4A and 4B). This is consistent with the result that cell cycle of HCT116-WT cells was arrested in G₁, not in G₂/M, upon FOXF1 knockdown (Table 1). Given that activation of p53 has been reported to have an effect to downregulate CDC2 [31, 32] and Wee1 (a kinase to phosphorylate CDC2 at Tyr-14 and Tyr-15) protein levels [33], a decline in total CDC2 protein levels and their phosphorylation at Tyr-15 in HCT116-WT cells was likely to be caused by FOXF1-knockdown-induced activation of the p53 pathway. In contrast, single or concurrent knockdown of p53 resulted in a robust increase in phosphorylation of CDC2 at Tyr-15 (Fig. 4A and 4B), suggesting that CDC2 kinase activity was inhibited by activation of a p53-independent G₂/M checkpoint.

FACS analysis showed that HCT116-WT cells cotransfected with both FOXF1 and p53 siRNAs exhibited a synergistic induction in DNA rereplication and apoptosis compared to those transfected singly with each siRNA (Fig. 5A and 5B). Upon loss of p21^{WAF1} expression in HCT116-p21^{WAF1} cells, single knockdown of FOXF1 or p53 led to a maximal increase in DNA rereplication (Fig. 5A). Therefore, concurrent knockdown of both could not synergistically enhance this effect (Fig. 5A). However, cosilencing of FOXF1 and p53 was able to synergistically promote apoptosis compared to that in cells with single knockdown of FOXF1 or p53 (Fig. 5B). Furthermore, inactivation of p53 ablated G₁ arrest (Fig. 5C) and increased the fraction of S-phase cells (Fig. 5D) in HCT116-WT cells with FOXF1 knockdown, consistent with outcomes in FOXF1-depleted HCT116-p53^{-/-} cells (Table 1) and Western blot results (Fig. 4A and 4B). We also found that single knockdown of p53 led to induction of G₂/M arrest in both HCT116-WT and HCT116-p21^{WAF1} cells, which was persistent in cells with concurrent knockdown of p53 and FOXF1 (Fig. 5E). These events are consistent with inhibition of CDC2 kinase activity (Fig. 4A and 4B). Taken

together, these results again demonstrated that p53 and its downstream target p21^{WAF1} are vital for preventing cells from DNA rereplication and apoptosis after annihilation of FOXF1 function.

3.4. Abrogation of FOXF1 is highly associated with inactive p53 in breast and colorectal cancers

By using the HCT116 cell model system, we have demonstrated that silencing of FOXF1 function is able to activate the p53 checkpoint function. According to our findings, we speculated that aberrant silencing of FOXF1 would be preferentially found in cancers with inactive p53 since activation of the p53 checkpoint pathway by silencing of FOXF1 has a deleterious effect on growth and survival of cancer cells. To elucidate correlation between FOXF1 expression and the p53 status, we performed the quantitative RT-PCR assay to analyze the expression status of FOXF1 in 20 breast cancer cell lines with the clear information of p53 status [34]. As the summarized results shown in Table 2, 7 breast cancer cell lines expressing high, medium and low levels of FOXF1 possessed either active or inactive p53. For example, FOXF1-expressing cell lines BT20 and MDA-MB-175VII expressed the wild-type p53 protein; BT549 and HCC1187 expressed the mutant p53 protein; MDA-MB-361, MDA-MB-157 and HCC1937 lost expression of the p53 protein. Therefore, FOXF1-expressing breast cancer cell line exhibited no preference for the specific status of p53. In contrast, 11 out of 13 breast cancer cell lines with negative expression of FOXF1 harbored inactive p53 (either expression of the mutant p53 protein or loss of p53 expression) (Table 2). Overall the results suggest that loss of FOXF1 expression in breast cancer cell lines is associated with the inactive p53 status. In addition to analysis of breast cancer cell lines, we also performed *in silico* analysis of FOXF1 expression in 18 colorectal cancer cell lines with the known p53 status using Oncomine [26]. Consistent with the data from breast cancer cell lines (Table 2), FOXF1 was preferentially underexpressed in colorectal cancer cell lines with the mutated *p53* gene compared to those with the wild-type, functional *p53* gene (Fig. 6A). The association between FOXF1 silencing and inactive p53 is statistically significant ($p < 0.005$) (Fig. 6B). These data convincingly show that the occurrence of FOXF1 silencing is highly associated with inactivation of the p53 checkpoint function.

4. Discussion

This study demonstrated that inactivation of the p53-p21^{WAF1} checkpoint pathway enhanced DNA rereplication induced by ablation of FOXF1. In contrast, abrogation of FOXF1 in cells with the intact p53 checkpoint pathway induced G₁ arrest by activating the p53-p21^{WAF1} checkpoint cascade instead of substantial induction of DNA rereplication and apoptosis. Moreover, activation of the p53-p21^{WAF1} pathway could be completely abolished by pharmacological inhibition of ATM/ATR kinases (Fig. 3D), suggesting that the ATM/ATR-mediated DNA damage checkpoint pathways are the upstream signals to stimulate the p53-p21^{WAF1} pathway as the FOXF1 function is abrogated.

The mechanisms underlying activation of the ATM/ATR-p53-dependent DNA damage response by inactivation of FOXF1 is unclear. However, Vaziri *et al.* reported that disruption of the stringency of DNA replication initiation by overexpression of DNA replication initiation factors Cdt1 and Cdc6 triggers activation of the ATM/ATR-Chk2-p53-dependent DNA damage checkpoint response in p53^{+/+} cells to prevent DNA rereplication [11, 30]. In addition, Minella *et al.* reported similar findings that deregulated Cyclin E expression activated a p53-dependent response to prevent excess CDK2 activity by inducing expression of p21^{WAF1} [35]. Inactivation of either p53 or p21^{WAF1} renders excess Cyclin E catalytically active and leads to defects in S phase progression, increased ploidy, and genetic instability [35]. Owing to significant similarities between our and their findings, abrogation

of FOXF1 might employ the same surveillance mechanism responsible for sensing overexpression of Cdt1 plus Cdc6 and excess Cyclin E expression to activate the p53-p21^{WAF1} pathway [30, 36]. Furthermore, our results show that activation of the p53-p21^{WAF1} checkpoint pathway induced G₁ arrest to inhibit DNA rereplication, which indicates that the ATM/ATR-mediated DNA damage checkpoint pathways could be triggered in very early S phase or before cells entered into the S phase. This suggests that the DNA damage response we observed in the study of HCT116-WT cells might not be stimulated by DNA rereplication-generated DNA damage lesions proposed before [11, 37]. However, knockdown of FOXF1 in HCT116-p21^{-/-} cells induced a dramatic increase in DNA rereplication and activated both Chk1 and Chk2 kinase activities upon no induction of G₁ arrest, suggesting that severe rereplication might have caused DNA damage and in turn activated the ATM/ATR-dependent DNA damage response. The issue about how the ATM/ATR-mediated DNA damage checkpoint pathways are activated in cells with the functional p53-p21^{WAF1} pathway by impairment in the stringency of DNA replication licensing requires further work to clarify it.

Consistent with previous evidence that the p53-p21^{WAF1} checkpoint pathway is required for preventing DNA rereplication induced by overexpression of Cdt1 and Cdc6 or excess Cyclin E expression [30, 35], our studies demonstrated that p53 and its downstream target p21^{WAF1} are essential for maintaining genomic stability by inhibiting DNA rereplication induced by silencing of FOXF1. According to our (Fig. 3A and 4A) and other studies [38], the mechanism whereby activation of the p53 checkpoint pathway is able to impede DNA rereplication is very likely mediated by the inhibitory effect of p21^{WAF1} on CDK2, which is a key cyclin-dependent kinase associating with Cyclin A to promote rereplication [30]. Overall our findings suggest that the p53-dependent checkpoint pathway is the first layer of intrinsic surveillance mechanisms responsible for protecting cells from DNA rereplication induced by loss of FOXF1 function. In addition, we also found that loss of p53 function triggered a p53-independent G₂/M checkpoint response to induce G₂/M arrest in FOXF1-depleted cells. A recent report reveals that CDK2 is required for the p53-independent G₂/M checkpoint control [39]. Our studies also show the consistent results displaying characteristics of G₂/M checkpoint activation; CDK2 activity was dramatically activated by loss of p53 and CDC2 activity was concurrently inhibited. Therefore, the p53-independent G₂/M checkpoint is the second layer of surveillance mechanisms to protect cells from the deleterious consequences of replication deregulation when the p53-p21^{WAF1} checkpoint function is abrogated. However, its protective effect is not as efficient as the p53-dependent checkpoint control. Since frequent genetic alterations in the *p53* gene in various cancers have been widely documented [40], we propose that genetic abrogation of the p53-dependent checkpoint pathway may collaborate with aberrant inactivation of FOXF1 function to promote genomic instability and tumorigenesis (Fig. 7). Indeed, our studies of the correlation between FOXF1 silencing and the p53 status using a panel of breast and colorectal cancer cell lines further support this hypothesis.

In summary, these results highlight the importance of the p53-p21^{WAF1} checkpoint pathway as a first layer of protection in mammalian cells for preventing DNA rereplication and genomic instability stimulated by aberrant silencing of FOXF1.

Acknowledgments

We thank Dr. B. Vogelstein for kindly providing the colorectal cancer cell line HCT116 and its derivative cell lines p53^{-/-} and p21^{-/-}. This work was supported by the National Institutes of Health SPORE Grant P50-CA 88843 to S.S.

References

- [1]. Lai E, Clark KL, Burley SK, Darnell JE Jr. Proc. Natl. Acad. Sci. U.S.A. 1993; 90:10421–10423. [PubMed: 8248124]
- [2]. Katoh M, Katoh M. Int. J. Oncol. 2004; 25:1495–1500. [PubMed: 15492844]
- [3]. Hannenhalli S, Kaestner KH. Nat. Rev. Genet. 2009; 10:233–240. [PubMed: 19274050]
- [4]. Carlsson P, Mahlapuu M. Dev. Biol. 2002; 250:1–23. [PubMed: 12297093]
- [5]. van der Horst A, Burgering BM. Nat. Rev. Mol. Cell Biol. 2007; 8:440–450. [PubMed: 17522590]
- [6]. Myatt SS, Lam EW. Nat. Rev. Cancer. 2007; 7:847–859. [PubMed: 17943136]
- [7]. Nishitani H, Lygerou Z. Front. Biosci. 2004; 9:2115–2132. [PubMed: 15353274]
- [8]. Blow JJ, Dutta A. Nat. Rev. Mol. Cell Biol. 2005; 6:476–486. [PubMed: 15928711]
- [9]. Tsuyama T, Tada S, Watanabe S, Seki M, Enomoto T. Nucleic Acids Res. 2005; 33:765–775. [PubMed: 15687385]
- [10]. Kearsley SE, Cotterill S. Mol. Cell. 2003; 12:1067–1075. [PubMed: 14636567]
- [11]. Hook SS, Lin JJ, Dutta A. Curr. Opin. Cell Biol. 2007; 19:663–671. [PubMed: 18053699]
- [12]. Bartek J, Lukas C, Lukas J. Nat. Rev. Mol. Cell Biol. 2004; 5:792–804. [PubMed: 15459660]
- [13]. Hellqvist M, Mahlapuu M, Samuelsson L, Enerback S, Carlsson P. J. Biol. Chem. 1996; 271:4482–4490. [PubMed: 8626802]
- [14]. Mahlapuu M, Enerback S, Carlsson P. Development. 2001; 128:2397–2406. [PubMed: 11493558]
- [15]. Kalinichenko VV, Zhou Y, Bhattacharyya D, Kim W, Shin B, Bambal K, Costa RH. J. Biol. Chem. 2002; 277:12369–12374. [PubMed: 11809759]
- [16]. Kalinichenko VV, Bhattacharyya D, Zhou Y, Gusarova GA, Kim W, Shin B, Costa RH. Hepatology. 2003; 37:107–117. [PubMed: 12500195]
- [17]. Ormestad M, Astorga J, Landgren H, Wang T, Johansson BR, Miura N, Carlsson P. Development. 2006; 133:833–843. [PubMed: 16439479]
- [18]. Madison BB, McKenna LB, Dolson D, Epstein DJ, Kaestner KH. J. Biol. Chem. 2009; 284:5936–5944. [PubMed: 19049965]
- [19]. Malin D, Kim IM, Boetticher E, Kalin TV, Ramakrishna S, Meliton L, Ustiyani V, Zhu X, Kalinichenko VV. Mol. Cell. Biol. 2007; 27:2486–2498. [PubMed: 17261592]
- [20]. Saito RA, Micke P, Paulsson J, Augsten M, Pena C, Jonsson P, Botling J, Edlund K, Johansson L, Carlsson P, Jirstrom K, Miyazono K, Ostman A. Cancer Res. 2010; 70:2644–2654. [PubMed: 20233876]
- [21]. Watson JE, Doggett NA, Albertson DG, Andaya A, Chinnaiyan A, van Dekken H, Ginzinger D, Haqq C, James K, Kamkar S, Kowbel D, Pinkel D, Schmitt L, Simko JP, Volik S, Weinberg VK, Paris PL, Collins C. Oncogene. 2004; 23:3487–3494. [PubMed: 15007382]
- [22]. Lo PK, Lee JS, Liang X, Han L, Mori T, Fackler MJ, Sadik H, Argani P, Pandita TK, Sukumar S. Cancer Res. 2010; 70:6047–6058. [PubMed: 20587515]
- [23]. Bunz F, Dutriaux A, Lengauer C, Waldman T, Zhou S, Brown JP, Sedivy JM, Kinzler KW, Vogelstein B. Science. 1998; 282:1497–1501. [PubMed: 9822382]
- [24]. Lo PK, Mehrotra J, D'Costa A, Fackler MJ, Garrett-Mayer E, Argani P, Sukumar S. Cancer Biol. Ther. 2006; 5:281–286. [PubMed: 16410723]
- [25]. McLure KG, Lee PW. EMBO J. 1998; 17:3342–3350. [PubMed: 9628871]
- [26]. Rhodes DR, Yu J, Shanker K, Deshpande N, Varambally R, Ghosh D, Barrette T, Pandey A, Chinnaiyan AM. Neoplasia. 2004; 6:1–6. [PubMed: 15068665]
- [27]. Wagner KW, Punnoose EA, Januario T, Lawrence DA, Pitti RM, Lancaster K, Lee D, von Goetz M, Yee SF, Totpal K, Huw L, Katta V, Cavet G, Hymowitz SG, Amler L, Ashkenazi A. Nat. Med. 2007; 13:1070–1077. [PubMed: 17767167]
- [28]. Liu Y, Bodmer WF. Proc. Natl. Acad. Sci. U.S.A. 2006; 103:976–981. [PubMed: 16418264]
- [29]. Samuel T, Weber HO, Funk JO. Cell Cycle. 2002; 1:162–168. [PubMed: 12429926]
- [30]. Vaziri C, Saxena S, Jeon Y, Lee C, Murata K, Machida Y, Wagle N, Hwang DS, Dutta A. Mol. Cell. 2003; 11:997–1008. [PubMed: 12718885]

- [31]. Yun J, Chae HD, Choy HE, Chung J, Yoo HS, Han MH, Shin DY. *J. Biol. Chem.* 1999; 274:29677–29682. [PubMed: 10514438]
- [32]. Le Gac G, Esteve PO, Ferec C, Pradhan S. *J. Biol. Chem.* 2006; 281:24161–24170. [PubMed: 16807237]
- [33]. Leach SD, Scatena CD, Keefer CJ, Goodman HA, Song SY, Yang L, Pietenpol JA. *Cancer Res.* 1998; 58:3231–3236. [PubMed: 9699647]
- [34]. Neve RM, Chin K, Fridlyand J, Yeh J, Baehner FL, Fevr T, Clark L, Bayani N, Coppe JP, Tong F, Speed T, Spellman PT, DeVries S, Lapuk A, Wang NJ, Kuo WL, Stilwell JL, Pinkel D, Albertson DG, Waldman FM, McCormick F, Dickson RB, Johnson MD, Lippman M, Ethier S, Gazdar A, Gray JW. *Cancer Cell.* 2006; 10:515–527. [PubMed: 17157791]
- [35]. Minella AC, Swanger J, Bryant E, Welcker M, Hwang H, Clurman BE. *Curr. Biol.* 2002; 12:1817–1827. [PubMed: 12419181]
- [36]. Zhu W, Chen Y, Dutta A. *Mol. Cell. Biol.* 2004; 24:7140–7150. [PubMed: 15282313]
- [37]. Davidson IF, Li A, Blow JJ. *Mol. Cell.* 2006; 24:433–443. [PubMed: 17081992]
- [38]. Harper JW, Adami GR, Wei N, Keyomarsi K, Elledge SJ. *Cell.* 1993; 75:805–816. [PubMed: 8242751]
- [39]. Chung JH, Bunz F. *PLoS Genet.* 2010; 6:e1000863. [PubMed: 20195506]
- [40]. Vogelstein B, Lane D, Levine AJ. *Nature.* 2000; 408:307–310. [PubMed: 11099028]

Research Highlights

- ▶ FOXF1 knockdown renders p53-null and p21-null cells susceptible to DNA rereplication.
- ▶ FOXF1 knockdown activates the ATM/ATR-p53-p21 DNA damage checkpoint pathways.
- ▶ Activation of the p53-p21 pathway by FOXF1 knockdown inhibits the CDK2-Rb cascade.
- ▶ Inhibition of CDK2 by elevated p21 induces G₁ arrest to prevent DNA rereplication.
- ▶ FOXF1 is silenced in breast and colorectal cancer cell lines with inactive p53.

Fig. 1.

Analysis of the knockdown efficiency of FOXF1 siRNAs. (A) Analysis of the efficacy of FOXF1 siRNAs to knockdown endogenous FOXF1 protein expression. Western blot analysis was performed on nuclear protein lysates from HCT116 cells transfected with FOXF1 siRNAs (or the control siRNA) using antibodies specific to FOXF1 and nucleolin. Nucleolin was used as a loading control for nuclear proteins. (B) Analysis of the efficiency of siRNA-mediated knockdown of endogenous FOXF1 mRNA expression. The colon cancer cell line HCT116 (HCT116-WT) as well as its derived p53-null and p21^{WAF1}-null cell lines (HCT116-p53^{-/-} and HCT116-p21^{-/-}) were transfected with FOXF1 siRNA or control siRNA. 48 h later, total RNA was isolated from siRNA-transfected cells and subjected to real-time qRT-PCR for quantitative analysis of FOXF1 mRNA expression. For relative quantitation, the expression level of FOXF1 mRNA in siControl-transfected cells was set as 100%.

Fig. 2.

Disruption of the p53-p21^{WAF1} checkpoint function promotes DNA rereplication induced by silencing of FOXF1. FACS analysis was performed on HCT116-WT and its derived p53^{-/-} and p21^{-/-} cell lines transfected with the negative control siRNA (siControl) or FOXF1 siRNA (either siFOXF1-1 or siFOXF1-2) for 48 h. Before harvesting, the cells were pulsed with BrdU for 30 min and then fixed immediately. BrdU incorporation was detected by immunostaining with the anti-BrdU antibody and nuclear DNA was detected by staining with propidium iodide. The FACS analyses of BrdU incorporation and DNA content of siRNA-transfected cells are shown here as: y axis, fluorescent intensity of staining with the anti-BrdU antibody; x axis, propidium iodide fluorescence (DNA content). Cells with DNA content >4N are boxed in FACS dot plots. The percentage indicated inside the box is a measure of the number of cells displaying DNA rereplication.

Fig. 3.

Knockdown of endogenous FOXF1 triggers ATM/ATR-mediated activation of the p53-p21^{WAF1} checkpoint pathway. (A) Analysis of the functional status of Chk1, Chk2, CDK2 and Rb as well as expression levels of p53 and p21^{WAF1} in FOXF1-depleted cells. Western blot analysis was performed on protein extracts isolated from HCT116-WT, HCT116-p53^{-/-} and HCT116-p21^{-/-} cells transfected with control or FOXF1 siRNA using antibodies as indicated in the figure. On the right side of the Western blot gel data, p-Chk1 (S-345), p-Chk2 (T-68), p-CDK2 (T-160) and p-Rb (S-795) indicate their respective phosphorylated proteins. (B) Analysis of PIG3 mRNA expression in HCT116-WT and HCT116-p53^{-/-} cells transfected with control or FOXF1 siRNA. Triplicate siRNA transfection experiments were performed for analysis. The star symbol (*) indicates the statistically significant difference ($p < 0.05$) of PIG3 mRNA expression between siControl- and siFOXF1-1-transfected cells. (C) Analysis of p53 mRNA expression in HCT116-WT and HCT116-p21^{-/-} cells transfected with control or FOXF1 siRNA. Means and standard deviations were calculated from triplicate transfection experiments. (D) Activation of the p53-p21^{WAF1} checkpoint pathway by siRNA-mediated depletion of FOXF1 is abolished by the inhibitor of the ATM/ATR kinases. HCT116-WT cells were transfected with siControl or siFOXF1-1. At 24 h after siRNA transfection, cells were treated with caffeine (5 mM) or UCN-01 (300 nM) for another 24 h, and cells were then harvested for Western blot analysis of p53, p21^{WAF1} and α -tubulin protein expression.

Fig. 4.

Cosilencing of p53 ablates FOXF1 knockdown-induced suppression of the CDK2-Rb cascade and concurrently induces inhibition of CDC2 kinase activity. (A) Western blot analysis of p53, p21^{WAF1}, phospho-CDK2 (Thr-160), CDK2, phospho-Rb (Ser-795), Rb, phospho-CDC2 (Tyr-15) and CDC2 protein expression in HCT116-WT and HCT116-p21^{-/-} cells transfected with the control siRNA (siControl), FOXF1 siRNA (siFOXF1-1), p53 siRNA (si-p53), or the combination of both FOXF1 and p53 siRNAs for 48 h. Protein extracts isolated from siRNA-transfected cells were subjected to Western blot analysis using antibodies as indicated. (B) Quantitative analysis of Western blot results in (A) by computer-assisted densitometry. After normalization by α -tubulin, expression levels of phospho-CDK2 (Thr-160), CDK2, phospho-Rb (Ser-795), Rb, phospho-CDC2 (Tyr-15) and CDC2 are presented as folds relative to siControl-transfected controls (set as default 1).

Fig. 5.

Concurrent knockdown of both FOXF1 and p53 by siRNAs leads to a synergistic increase in rereplicated and apoptotic cells in HCT116-WT. Flow cytometric analysis was performed on BrdU-labeled, propidium iodide-stained HCT116-WT and HCT116-p21^{-/-} cells transfected with the same siRNA regimen as described in Fig. 4A. The percentage of cells showing rereplicated (A), apoptotic (B), G₁ (C), S (D) or G₂/M (E) nuclei is presented as mean $\pm$ SD of triplicate transfection experiments. Percentages of apoptosis and cell cycle phases were determined as described in “Materials and Methods”. DNA rereplication was scored by the same method as described in Fig. 2. A synergistic increase in apoptosis or DNA rereplication in HCT116-WT or HCT116-p21^{-/-} cells cotransfected with siFOXF1-1 and si-p53 is statistically significant (*, $p < 0.05$) compared to single siRNA-transfected cells.

Fig. 6.

Expression analysis of *FOXF1* gene in colorectal cancer cell lines. (A) *In silico* analysis of FOXF1 expression in 18 colorectal cancer cell lines. FOXF1 expression data were fetched from Wagner's microarray dataset [27] through Oncomine. For comparison, the FOXF1 expression value of HCT116 cells was set as 100% to normalize expression values of all the other colorectal cancer cell lines. The p53 states of these lines were indicated according to studies of Liu *et al.* [28]. (B) Statistical analysis of correlation between FOXF1 expression and the p53 status. The FOXF1 expression data in (A) were replotted into two groups based on the p53 status in the scatter plot and statistical analysis was performed according to the method described in "Materials and Methods".

Fig. 7.

A model to show collaboration between aberrant silencing of FOXF1 and genetic inactivation of p53 to promote DNA rereplication, which in turn leads to genomic instability and tumorigenesis.

Table 1

The impact of FOXF1 knockdown on cell cycle progression and DNA rereplication.

	HCT116-WT			HCT116-p53 ^{-/-}			HCT116-p21 ^{-/-}		
	siControl	siFOXF1-1	siFOXF1-2	siControl	siFOXF1-1	siFOXF1-2	siControl	siFOXF1-1	siFOXF1-2
Apoptosis	1.42±0.35	2.17±0.24 [*]	1.81±0.13	2.2±0.36	3.22±0.32 [*]	2.53±0.28	3.26±0.45	6.70±0.83 [*]	5.39±0.44 [*]
G ₁	61.21±1.14	68.93±1.53 [*]	65.63±1.19 [*]	47.56±1.40	42.14±1.21 [*]	44.59±1.23	52.31±0.56	42.80±0.54 [*]	46.35±1.34 [*]
S	20.66±1.80	14.42±1.05 [*]	17.14±0.59 [*]	24.12±0.67	26.56±0.72 [*]	25.16±1.06	23.81±0.58	21.89±0.53 [*]	22.96±0.50
G ₂ /M	12.48±1.10	9.18±0.61 [*]	11.23±0.48	16.90±0.46	15.09±0.47	15.86±0.38	12.41±0.28	14.48±0.66 [*]	13.35±0.54
Rereplication	5.17±0.44	6.68±0.51 [*]	5.97±0.24	9.83±0.58	12.89±0.65 [*]	12.19±0.53 [*]	8.33±0.57	14.55±0.53 [*]	11.82±0.61 [*]

The means and standard deviations resulted from triplicate experiments.

^{*} The star symbol indicates the data have statistically significant difference ($p < 0.05$) compared to those from siControl transfections.

Table 2

Correlation between the FOXF1 expression and the p53 status in breast cancer cell lines.

Cell Lines	FOXF1		p53 ^c	
	Expression fold ^a	Expression levels ^b	Expression levels	Genetic status ^d
BT20	51.16	++	++	WT
MDA-MB-361	45.43	++	—	WT
BT549	13.99	++	++	M
MDA-MB-157	6.07	+	—	ND
HCC1937	5.91	+	—	ND
HBL100	1.00	+	++	WT
MDA-MB-175VII	0.18	+/-	+/-	WT
HCC1187	0.11	+/-	++	M
Hs578T	0.039	—	+	M
T47D	0.037	—	++	M
MCF7	0.032	—	+/-	WT
HCC202	0.016	—	—	ND
MDA-MB-231	0.014	—	++	M
MDA-MB-435	0.011	—	+	M
MDA-MB-453	0.0103	—	—	WT
HCC38	0.0086	—	++	M
SUM159	0.0078	—	—	ND
HCC1143	0.0076	—	++	M
MDA-MB-134VI	0.0056	—	+/-	WT
ZR7530	0.00308	—	—	WT
ZR751	0.0025	—	—	ND

^aThe FOXF1 expression fold of each breast cancer cell line was calculated from the data of real-time quantitative RT-PCR analysis. The FOXF1 expression value of HBL100 cells (an immortalized human mammary epithelial cell line) was set as default 1 to normalize the data from all of the other cell lines.

^bThe FOXF1 expression levels of each cell line was indicated as: [++], the expression fold is between 10 and 100; [+], the expression fold is between 1 and 10; [+/-], the expression fold is between 0.1 and 1; [-], the expression fold is less than 0.1.

^cThe status of the p53 gene in each breast cancer cell line is based on the information reported by Neve et al.[26].

^dThe genetic status of the p53 gene in each cell line is dictated as: WT, wild-type; M, mutated; ND, not determined.