FoxM1 Regulates Growth Factor-induced Expression of Kinase-interacting Stathmin (KIS) to Promote Cell Cycle Progression*

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The Forkhead box M1 (FoxM1) transcription factor is essential for cell cycle progression and mitosis. FoxM1 regulates expression of Skp2 and Cks1, subunits of the SCF ubiquitin ligase complex, which ubiquitinitates p27Kip1 and targets it for degradation. Kinase-interacting stathmin (KIS) is a growth factor-dependent nuclear kinase that regulates cell cycle progression by phosphorylating p27Kip1 to promote its nuclear export. Here we present an additional mechanism of FoxM1-mediated regulation of p27Kip1 and provide evidence that FoxM1 regulates growth factor-induced expression of KIS. In cells harboring FoxM1 deletion or expressing FoxM1-short interfering RNA, the expression of KIS is impaired, leading to an accumulation of p27Kip1 in the nucleus. Furthermore, we show that KIS is a direct transcriptional target of FoxM1. Thus FoxM1 promotes cell cycle progression by down-regulating p27Kip1 through multiple mechanisms.

The mammalian Forkhead box family includes more than 50 proteins that operate as transcription factors. Their unique characteristic is shared homology in a winged helix DNA binding domain (1). The FoxM1 (Trident, HFH-11, WIN) transcription factor is a well studied member of that family (1–5 and reviewed in Ref. 6). Expression of FoxM1 is induced during the G1 phase of the cell cycle, and its expression is maintained during S phase and mitosis (2). In mice, it is expressed throughout embryonic development and in all proliferating adult cells (3). Upon terminal differentiation or exit from the cell cycle, expression of FoxM1 is completely extinguished (3) or exported out of the nucleus (2). In contrast, tumor cells constitutively express FoxM1 (7). The constitutive expression of FoxM1 is important for maintenance of the tumor cell phenotype because deletion of the FoxM1 gene or inhibition of the FoxM1 activity by a peptide inhibitor derived from the tumor suppressor ARF leads to regression of hepatocellular carcinomas in mice (8). Inhibition of FoxM1 by thiazole compounds was also found to correlate with apoptosis of human tumor cells (9).

FoxM1 is important for transcriptional activation of several genes that regulate G1/S phase progression and the G2/M transition (6, 10). FoxM1 target genes include Cdc25B and Plk1, which are important for the activation of Cdk1 for mitosis (11), and CENP-F that regulates the mitotic spindle checkpoint (12). In FoxM1−/− mice, liver regeneration after partial hepatectomy is impaired partly because of deficiency in S phase (5). It has been suggested that defects in S phase progression in FoxM1 null hepatocytes are because of accumulation of the Cdk inhibitor p27Kip1 (13). Aged mice do not express FoxM1 and have higher levels of p27Kip1 in the liver, leading to inefficient liver regeneration following partial hepatectomy (4). Interestingly, restoration of FoxM1 expression reduced the level of p27Kip1 and reversed the deficiency in liver regeneration in old mice (4).

The level of p27Kip1 protein is primarily regulated by the ubiquitin-proteasome pathway (13). Skp2 and Cks1, specificity subunits of ubiquitin-protein isopeptidase ligase complexes, play important roles in targeting p27Kip1 for proteolysis by the ubiquitin-proteasome pathway (14, 15). Previously we showed that FoxM1 directly binds to regulatory elements in promoters of the human Skp2 and Cks1 genes, thereby stimulating their transcription (11).

In quiescent cells, p27Kip1 is expressed at a relatively high level, but levels decrease rapidly upon entry into the G1 phase. Growth factor stimulation of resting cells results in degradation of p27Kip1 protein in cytoplasm (16). It has been suggested that the growth factor-induced proteolysis of p27Kip1 involves a distinct pathway that is independent of Skp2. Nakayama and coworkers (17) identified a cytoplasmic ubiquitin-protein isopeptidase ligase (KPC ligase) consisting of KPC1 and KPC2 subunits that polyubiquitinates p27Kip1 causing degradation of p27Kip1 in the cytoplasm. The KPC pathway of proteolysis of p27Kip1 is not dependent on Thr-187 phosphorylation, which is a prerequisite for Skp2/Cks1-mediated ubiquitination (18).

Kinase-interacting stathmin (KIS)⁴ was originally identified based on its interaction and phosphorylation of the ubiquitous

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4 The abbreviations used are: KIS, kinase-interacting stathmin; siRNA, short interfering RNA; MEF, mouse embryonic fibroblast; BSA, bovine serum albumin; PBS, phosphate-buffered saline; DAPI, 4′,6-diamidino-2-phenylindole; CMV, cytomegalovirus; ChIP, chromatin immunoprecipitation; RT, reverse transcription; WT, wild type; DEN, diethylnitrosamine; PB, phenobarbital; S, sense; AS, antisense; Ta, annealing temperature; HCC, hepatocellular carcinoma.
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cytosolic protein, stathmin (19). KIS is a nuclear protein kinase that possesses an RNA recognition motif, which suggests that it is involved in the control of trafficking and/or splicing of RNAs probably through phosphorylation of associated factors (20). Interestingly, it was shown that KIS could bind to the C-terminal region of p27Kip1 and phosphorylate it at Ser-10. Phosphorylation of p27Kip1 at Ser-10 is essential for its nuclear export and accumulation in the cytoplasm (21), where it is targeted for proteolysis by the KPC ligase (17). KIS is expressed during growth factor-induced entry into the cell cycle. It has been suggested that KIS promotes cell cycle re-entry by inactivating p27Kip1 following mitogen stimulation (21). Here we show that FoxM1 regulates KIS expression following growth factor stimulation. FoxM1, by stimulating expression of KIS, enhances phosphorylation of p27Kip1 at Ser-10 leading to increased proteolysis of p27Kip1. Our results provide further evidence that FoxM1 is important for growth factor-induced proteolysis of p27Kip1.

MATERIALS AND METHODS

Cell Culture and Cell Fractions—U2OS cells were obtained from the ATCC (HTB-96) and maintained as a monolayer using the conditions described previously (11). To generate FoxM1 null MEFS, we bred FoxM1+/- mice (5) to produce 13.5-day embryos with FoxM1+/- (wild type), FoxM1+/- (heterozygous), or FoxM1-/- (knock-out) genotypes. MEFS were isolated from the embryos using standard procedures described by Hogan et al. (22). The MEFS were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 0.1 mM minimum Eagle’s medium nonessential amino acids, and 5 mM 2-mercaptoethanol in a humidified 9% CO2 incubator under conditions described by Zindy et al. (23). Cytoplasmic and nuclear protein extracts were made using a nuclear/cytosol fractionation kit (K266–100; BioVision Inc., Mountain View, CA) following protocols provided by the manufacturer.

siRNA Transfection—Two 21-nucleotide siRNA duplexes specific for human FoxM1 and KIS mRNA, named siFoxM1 (GGACCACUUUUCCACUUU) (11), and siKIS RNA duplex (AAGCAGUUCCCACCGCCAGGA) (21) were synthesized by Dharmacon Research (Lafayette, CO). These siRNA duplexes were transfected into U2OS cells using Lipofectamine 2000 reagent (Invitrogen) in serum-free tissue culture medium following the manufacturer’s protocol. U2OS cells were harvested 48 h after FoxM1 siRNA transfection for total RNA or 72 h after FoxM1 siRNA transfection to prepare protein extracts for Western blot analysis or immunofluorescent staining.

Antibodies and Immunoblotting—Protein preparation and immunoblotting was performed as described previously (24, 25). The signals from the primary antibody were amplified by horseradish peroxidase-conjugated anti-mouse immunoglobulin G (IgG; Bio-Rad) and detected with enhanced chemiluminescence (ECL Plus; Amersham Biosciences). The following commercially available antibodies and dilutions were used for immunoblotting: mouse anti-β-actin (AC-15; 1:5000) (Sigma), mouse anti-p27Kip1 (1:3000) (BD Transduction Laboratories), and rabbit anti-KIS (AP8067a 1:1000) (Abgent, San Diego). The mouse anti-phospho-Ser-10 p27Kip1 antibody (1:1000) was a gift from Dr. Elizabeth G. Nabel, Cardiovascular Branch, NHLBI, National Institutes of Health. Rabbit antisera specific for the human C-terminal FoxM1 protein region (1:5000) was obtained as described previously (11).

Immunofluorescent Staining of U2OS Cells—U2OS cells were fixed with 10% buffered formalin (Fisher) for 20 min at room temperature, rinsed with PBS, and permeabilized with PBS supplemented with 1% bovine serum albumin (BSA; Sigma) and 0.2% Triton X-100 (Fisher). After washing in PBS with 1% BSA, proteins of interest were visualized by staining cells with specific antibodies in PBS containing 0.5% BSA at 25 °C for 16 h. Mouse anti-p27Kip1 (1:200) (BD Transduction Laboratories) was used for immunofluorescent staining. After being washed with PBS, cells were incubated with fluorescein isothiocyanate-conjugated polyclonal anti-mouse immunoglobulins (1:100; DakoCytomation, Denmark) in PBS containing 0.5% BSA at 25 °C for 30 min. The slides were washed with PBS, and cover glasses were mounted with Vectashield mounting medium with 4’,6’-diamidino-2-phenylindole (DAPI; H-1200; Vector Laboratories, Burlingame, CA). Immunofluorescence was performed with primary antibodies followed by secondary antibodies conjugated to fluorescein isothiocyanate.

Primers Used for Real Time Reverse Transcriptase-PCR (RT-PCR) to Determine mRNA Expression Levels—RNA was isolated from U2OS cells or MEFS using RNA-STAT-60 (Tel-Test B Inc., Friendswood, TX). Following RNase-free DNase I (New England Biolabs, Boston) digestion of total RNA to remove contaminating genomic DNA, we used the Bio-Rad cDNA synthesis kit containing both oligo(dT) and random hexamer primers to synthesize cDNA from 10 μg of total RNA. The following reaction mixture was used for all PCR samples: 1X IQ SybrGreen supermix (Bio-Rad), 100–200 nM of each primer, and 2.5 μl of cDNA in a 25-μl total volume. Reactions were amplified and analyzed in triplicate using a MyiQ single-color real time PCR detection system (Bio-Rad).

The following sense (S) and antisense (AS) primer sequences and annealing temperatures (Ta) were used to amplify and measure the amount of human mRNA by real time RT-PCR: FoxM1-S, 5’-GGA GGA AAT GCC ACA ATG GAC G-3’; and FoxM1-AS, 5’-TAG GAC TCT TGG GGT CCT GGG GTG-3’ (Ta, 55.7 °C); KIS-S, 5’-CCA TCA CGC TGT CTG TTG CTT G-3’, and KIS-AS, 5’-GGG CAC AAT GCT GTA TCA TCC AC-3’ (Ta, 61.3 °C). These real time RT-PCR RNA levels were normalized to human cyclophilin mRNA levels, and these primers are as follows: cyclophilin-S, 5’-GCA GAC AAG GTC CCA AAG ACA G-3’, and cyclophilin-AS, 5’-CCT GAC CCT GAC ACA TAA ACC CTG G-3’ (Ta, 55.7 °C).

The following sense and antisense primer sequences and annealing temperatures were used to amplify and measure the amount of mouse mRNA by real time RT-PCR: FoxM1-S, 5’-CAG TGG TAT TGA GGA CCA CCTT C-3’, and FoxM1-AS, 5’-GTC GTT TCT GCT GTG ATG CC-3’ (Ta, 55.7 °C); KIS-S, 5’-AGT ATG GTT GCC GCA AAG AGA GG-3’, and KIS-AS, 5’-ATG GCA CAT TGG GAG AGA AGT G-3’ (Ta, 52.0 °C); cyclinD1-S, 5’-CCT GAC ACG AAC CAA CTC TCC AAC G-3’, and cyclin D1-AS 5’-TCT CGC TAC TGT TGC TCC TCA C-3’ (Ta, 62.0 °C); and cyclin E1-S 5’-GAA AGA...
AGA AGG TGG CTC CGA C-3’, and cyclin E1-AS 5’-GTT AGG GGT GGG GAT GAA AGA G-3’ (Ta, 62.0 °C). These real time RT-PCR RNA levels were normalized to mouse cyclophilin mRNA levels, and these primers were as follows: cyclophilin-S, 5’-GGG AAA TGG TGC TGG ACC AAA CAC-3’, and cyclophilin-AS, 5’-TTT CTG GAC CCA AAA CGC TC-3’ (Ta, 57.5 °C).

Preparation of KIS Promoter/Binding Sites; Luciferase Constructs and Dual Luciferase Assays —We used PCR to amplify a 1.1-kb region of the human KIS promoter from genomic DNA. This PCR-amplified promoter region was cloned in the correct orientation in the pGL3-basic luciferase reporter plasmid (Promega, Madison, WI). Additional DNA duplexes representing putative FoxM1 23-kb binding site and its mutant were cloned in front of the -1.1-kb promoter region. Oligonucleotides used for creating DNA duplexes were: CGA TTT TGT TGG TTT GTT TTT GC and TCG AGC AAA AAC AAA CAA ACA AAA TCG GTA C for the intact region and CAA TTT TAT TTA TTT ATT TTT AC and TCG AGG AAA AAG AAA GAA AGA AAA TGG GTA C for the mutated site. The following PCR primers were used to amplify the KIS promoter region: forward, 5’-AAA TAA AAA TTT GGA ACA GAT C-3; reverse, 5’-CAG CCA TGA CAC CGG AAG ACG TTT G-3. The KIS kinase promoter region was confirmed by DNA sequencing.

We used FuGENE 6 reagent (Roche Applied Science) to transfected U2OS cells with 200 ng of either cytomegalovirus (CMV) FoxM1 expression construct or CMV empty vector with 1.5 µg of -1.1-kb KIS promoter or -1.1-kb promoter fused with either intact or mutated binding site duplex luciferase reporter with 10 ng of CMV- Renilla luciferase, which served as an internal control. Luciferase assays were performed as described (25). Expression was represented as the fold induction of transcriptional activity by the FoxM1 expression vector ± the S.D., where binding site fused promoter activity resulting from transfection with CMV empty vector was set at 1. Experiments were performed in triplicate, and statistical analysis was performed with Microsoft Excel tools.

ChIP Assay —FoxM1-depleted or untreated U2OS cells were processed for ChIP assays 3 days after siRNA transfection using published methods (26). For the immunoprecipitation step, specific amounts of antibody as indicated were added to the preclarified and clarified sample, which was incubated at 4 °C for 2 h and washed according to ChIP assay protocol (Upstate). The following antibodies were used in the indicated amounts: 10, 25, or 50 µl of rabbit antiserum specific for FoxM1 protein (amino acids 365–748) and 2 µg of rabbit serum (Vector Laboratories, Burlingame, CA). We used 2.5 µl of ChIP DNA sample in the subsequent 25-µl real time PCR mixtures. The total input sample was diluted 1:10, and 2.5 µl was used for real time PCR (10% total input).

PCR Primers and Reaction Conditions for ChIP Assay —The primers used to amplify the following human gene fragments are annotated with the binding position relevant to the transcription start site, annealing temperature, and whether in the sense or antisense orientation: KIS 1.3S, 5’-CAA TGG CGA GAT CAC AGT TCA CTC-3’, and KIS 1.3AS, 5’-ATT AGC CAG GCT TGG TGC TAC G-3’ (Ta, 62 °C); KIS 23S, 5’-GAG CAC ATA CAG TGT CGT GAT CTT G-3, and KIS 23AS, 5’-CAA AAT CTA ACT GGG CGT GAT AGC-3’ (Ta, 62 °C); and KIS 33S, 5’-TCT GTA TTC CTA GCA CCT AGC CCC-3, and KIS 33AS, 5’-CCA ACT GGA TGC CAG ATT CTA TG-3’ (Ta, 62 °C). The following reaction mixture was used for all PCR samples: 1 X IQ SybrGreen Supermix (Bio-Rad), 100 nM of each primer, and 2.5 µl of each purified ChIP extract in a 25-µl total volume. Reactions were amplified and analyzed in triplicate using a MyiQ single color real time PCR detection system (Bio-Rad). Normalization was carried out using the Ct method as described previously (11).

Electrophoretic Mobility Shift Assays —Electrophoretic mobility shift assay experiments were performed with the U2OS cell nuclear extract isolated as described above. We used 3.5 pmol of the double-stranded FoxM1-binding site oligonucleotide for radioactive labeling with [32P]ATP isotope (ICN, Irvine, CA) and T4 polynucleotide kinase (Promega, Madison, WI). Formation of the FoxM1 protein-DNA complex was performed by incubating ~0.2 pmol of [32P]-labeled FoxM1-binding site oligonucleotide, 2 µg of U2OS cell nuclear protein extract, and 1X gel shift buffer (Promega, Madison, WI) for 20 min at room temperature. The FoxM1 protein-DNA complexes were resolved by electrophoresis on a nondenaturing 5% polyacrylamide gel run at 4 °C and detected by autoradiography as described previously (27). We also performed DNA competition experiments that included in the binding reaction mixture a 100-fold molar excess of unlabeled FoxM1-binding site oligonucleotide from a mouse CDX2 promoter as described previously (control) (28).

Statistical Analysis —Experiments were repeated three times, and results were combined and represented graphically as the mean values ± S.D. We used the Microsoft Excel program to calculate the S.D. and statistically significant differences between samples using the Student’s t test. The asterisks in each graph indicate statistically significant changes, with p values calculated by the Student t test. p values of <0.05 were considered statistically significant.

RESULTS

FoxM1-depleted Cells Fail to Regulate p27Kip1 Protein Expression in Response to Serum Stimulation —When quiescent mouse embryonic fibroblasts (MEFs) were stimulated by serum addition, they entered the G1 phase of the cell cycle, and levels of endogenous p27Kip decreased. To examine regulation of p27Kip1 in FoxM1-depleted MEFs, we used a Cre Lox system to delete the FoxM1 alleles by delivering Cre recombinase via an adenoviral vector into MEFs containing two LoxP sites surrounding essential FoxM1 exons 4–7. These MEFs were kept in low serum conditions (0.1%) for 60 h to render them quiescent, after which medium containing 10% fetal bovine serum was added. Nuclear cell protein extracts were prepared from these synchronized cells at 0, 12, 18, 24, and 36 h following serum addition and used for immunoblot analysis with p27Kip1-specific monoclonal antibody (Fig. 1A). In the control AdLacZ-infected MEFs, dramatic down-regulation of p27Kip1 protein levels was observed after serum stimulation. However, p27Kip1 levels did not decrease in AdCre-infected, FoxM1-deficient MEFs. Similar results were also obtained with FoxM1-depleted MEFs from FoxM1−/− mouse embryos (data not shown). Even

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—FoxM1-depleted or untreated U2OS cells were processed for ChIP assays 3 days after siRNA transfection using published methods (26). For the immunoprecipitation step, specific amounts of antibody as indicated were added to the preclarified and clarified sample, which was incubated at 4 °C with rotation for 12–16 h and washed according to ChIP assay protocol (Upstate). The following antibodies were used in the indicated amounts: 10, 25, or 50 µl of rabbit antiserum specific for FoxM1 protein (amino acids 365–748) and 2 µg of rabbit serum (Vector Laboratories, Burlingame, CA). We used 2.5 µl of ChIP DNA sample in the subsequent 25-µl real time PCR mixtures. The total input sample was diluted 1:10, and 2.5 µl was used for real time PCR (10% total input).

PCR Primers and Reaction Conditions for ChIP Assay —The primers used to amplify the following human gene fragments are annotated with the binding position relevant to the transcription start site, annealing temperature, and whether in the sense or antisense orientation: KIS 1.3S, 5’-CAA TGG CGA GAT CAC AGT TCA CTC-3’, and KIS 1.3AS, 5’-ATT AGC CAG GCT TGG TGC TAC G-3’ (Ta, 62 °C); KIS 23S, 5’-GAG CAC ATA CAG TGT CGT GAT CTT G-3, and KIS 23AS, 5’-CAA AAT CTA ACT GGG CGT GAT AGC-3’ (Ta, 62 °C); and KIS 33S, 5’-TCT GTA TTC CTA GCA CCT AGC CCC-3, and KIS 33AS, 5’-CCA ACT GGA TGC CAG ATT CTA TG-3’ (Ta, 62 °C). The following reaction mixture was used for all PCR samples: 1 X IQ SybrGreen Supermix (Bio-Rad), 100 nM of each primer, and 2.5 µl of each purified ChIP extract in a 25-µl total volume. Reactions were amplified and analyzed in triplicate using a MyiQ single color real time PCR detection system (Bio-Rad). Normalization was carried out using the Ct method as described previously (11).

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Statistical Analysis —Experiments were repeated three times, and results were combined and represented graphically as the mean values ± S.D. We used the Microsoft Excel program to calculate the S.D. and statistically significant differences between samples using the Student’s t test. The asterisks in each graph indicate statistically significant changes, with p values calculated by the Student t test. p values of <0.05 were considered statistically significant.
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**Figure 1.** FoxM1−/− MEFS fail to regulate p27^Kip1^ protein expression in response to serum stimulation. A, double-floxed FoxM1 MEFS were infected with either AdCre or AdLacZ (control) viruses, followed by a 60-h period of serum starvation (0.1%), after which the cells were replenished with medium containing 10% fetal bovine serum, and nuclear lysates were prepared at the indicated time points following serum addition. Immunoblot analysis was performed with p27^Kip1^ monoclonal antibody. B, infection of double floxed FoxM1 MEFS effectively mediates the loss of FoxM1−/− allele as shown by a 20-fold decrease in FoxM1 mRNA expression 48 h following adenoviral infection. C, normalized values for p27^Kip1^ protein expression shown in A were calculated and represented by a histogram. Each bar represents the mean values for three experiments ± S.D.

though the FoxM1-depleted quiescent cells were stimulated by high serum concentration, they failed to down-regulate nuclear and total p27^Kip1^ protein expression as compared with their FoxM1-expressing counterpart cells. This suggests that FoxM1 is involved in regulation of p27^Kip1^ metabolism and nuclear export in the early G1 phase of the cell cycle. Interestingly, we observed lower p27^Kip1^ levels in FoxM1-depleted MEFS at the 0 h timepoint when compared with control cells, which may be due to some sort of compensatory effect allowing these cells to adapt for survival under *in vitro* conditions with marginal levels of FoxM1 expression.

**FoxM1 Is Essential for Expression of KIS Gene**—KIS is an important regulator of p27^Kip1^ in the G1 phase of the cell cycle. KIS phosphorylates p27^Kip1^ on Ser-10, promoting its nuclear export and degradation in the cytoplasm (21). Because FoxM1-depleted cells fail to down-regulate p27^Kip1^ expression upon serum stimulation, we set out to test whether FoxM1 is involved in the regulation of KIS expression.

We rendered either FoxM1−/− MEFS (Fig. 2A) or WT MEFS (Fig. 2B) quiescent as described above, and isolated total RNA from these cells at the indicated time points following serum addition. These RNA extracts were used for real time RT-PCR analysis with primers specific for mouse FoxM1 and KIS mRNA (Fig. 2). FoxM1−/− MEFS showed no significant change in KIS mRNA expression even 24 h following serum stimulation, whereas WT MEFS displayed rapid onset of FoxM1 and KIS mRNA induction after the serum was added. We also analyzed the expression of cyclin D1 and cyclin E1 mRNA in FoxM1−/− cells and demonstrated changes in expression of both genes, suggesting that cells were able to progress through the cell cycle, indicating that lack of KIS up-regulation was not because of cell cycle arrest but rather FoxM1 deficiency.

Next we compared levels of KIS mRNA in asynchronous cells. WT and FoxM1−/− MEFS were subjected to real time RT-PCR analysis that shows significantly diminished expression of KIS mRNA in FoxM1−/− MEFS as compared with WT cells (Fig. 3A).

To investigate whether KIS mRNA expression is regulated by FoxM1 in human cells, we transfected U2OS cells with 100 nm siFoxM1 or control si duplexes and determined the relative mRNA expression as described above. FoxM1-depleted U2OS cells show substantial down-regulation of KIS mRNA expression as compared with untransfected or control siRNA-transfected cells (Fig. 3B). Furthermore, we found that overexpression of FoxM1 leads to an increase in KIS protein expression. U2OS cells were transfected with either the empty CMV vector as control or a CMV promoter-FoxM1 expression construct. Total cell protein extracts were isolated 72 h following transfection and subjected to immunoblot analysis with polyclonal antibodies specific for FoxM1 and KIS (Fig. 3C). The U2OS cells ectopically expressing FoxM1 displayed elevated expression of KIS protein when compared with control cells. Together these results suggest that FoxM1 is essential for regulating the expression of KIS kinase, which is critical for phosphorylation and inactivation of p27^Kip1^ protein during the G1 phase of the cell cycle.
FoxM1 Is Required for KIS-mediated Phosphorylation of p27Kip1 on Ser-10 and Nuclear Export—Previous studies indicated that KIS kinase is necessary for phosphorylation of p27Kip1 on Ser-10 (21). To determine whether FoxM1 expression affects phosphorylation of Ser-10 on p27Kip1 protein, we transfected U2OS cells with either siFoxM1 or a CMV-FoxM1 expression construct and isolated total cell protein extracts. Immunoprecipitations were performed with monoclonal p27Kip1 antibody. Immunoblotting performed with Ser-10 phosphospecific antibody revealed lower levels of Ser-10 phosphorylation in FoxM1-depleted cells and higher levels in cells ectopically expressing FoxM1 as compared with untreated cells (Fig. 4A). The increase in phosphorylation is dramatic because p27Kip1 is down-regulated in cells ectopically expressing FoxM1. These data further indicate that FoxM1 is essential for KIS expression by demonstrating that FoxM1 is required for KIS functions and phosphorylation of p27Kip1.

To further explore the consequences of FoxM1 regulation of KIS, we transfected U2OS cells with either the CMV-FoxM1 expression construct, KIS siRNA, or both, and we isolated total cell protein extracts. Immunoprecipitations performed with p27Kip1 antibody, followed by immunoblotting with Ser-10 phosphospecific antibody, revealed that the increase in phosphorylation caused by FoxM1 ectopic expression was significantly reduced upon co-transfection with KIS-specific siRNA (Fig. 5A). Real time RT-PCR analysis of total RNA extracts isolated from these cells showed diminished expression of KIS mRNA upon KIS-specific siRNA transfection, and also an increase in relative KIS mRNA levels upon ectopic expression of FoxM1 (Fig. 5B). This supports the idea that KIS kinase is the main mediator of the effect of FoxM1 on Ser-10 phosphorylation of p27Kip1 protein.

We detected reduced levels of total p27Kip1 protein in cells ectopically expressing FoxM1 as compared with untransfected cells, which is probably caused by FoxM1-induced up-regulation of Skp2 and Cks1 (11). These FoxM1 targets regulate ubiquitination and degradation of p27Kip1 protein. Thus, FoxM1 can down-regulate p27Kip1 levels by multiple mechanisms.

KIS-mediated phosphorylation of p27Kip1 on Ser-10 leads to rapid nuclear export and subsequent degradation of p27Kip1 protein (21). To test whether FoxM1 depletion affects the nuclear accumulation of p27Kip1, we transfected U2OS cells with siRNAs targeting FoxM1, KIS, or control siRNA. Cells were fixed 72 h following transfection and incubated with the
p27^{kip1}-specific monoclonal antibody. Cells were then counterstained with DAPI and analyzed by immunofluorescent microscopy (Fig. 6). We found that U2OS cells depleted of FoxM1 as well as cells depleted of KIS accumulate significantly higher levels of p27^{kip1} in the nuclei as compared with cells transfected with control siRNA. This supports the hypothesis that FoxM1 is required for the KIS-induced nuclear export of p27^{kip1} protein.

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**Figure 5.** KIS kinase is required for FoxM1 induced Ser-10 phosphorylation of p27^{kip1}. A, U2OS cells were transfected with either 100 nM of siKIS, the CMV-FoxM1 expression construct, or both. Total cell protein extracts were isolated, and immunoprecipitations were performed with monoclonal p27^{kip1} antibody and analyzed by immunoblot with phospho-Ser-10 p27^{kip1} phosphospecific antibody. 1:10 of the lysates used for co-immunoprecipitation were loaded on an SDS-polyacrylamide gel and blotted with FoxM1- and p27^{kip1}-specific antibodies. FoxM1-induced phosphorylation of p27^{kip1} is diminished by KIS siRNA. B, total RNA was extracted from U2OS cells treated as described above and used in a real time RT-PCR analysis revealing the relative levels of KIS mRNA in the samples. C, graphical representation of relative Ser-10 phosphorylated p27^{kip1} protein levels normalized to total levels of p27^{kip1}. Bars represent the mean values from three experiments ± S.D. UNTR, untransfected.

**Figure 6.** Depletion of FoxM1 and KIS causes nuclear accumulation of p27^{kip1} protein. U2OS cells were transfected with 100 nM of siRNAs targeting FoxM1 or KIS or a control siRNA. Localization of p27^{kip1} was examined at 72 h post-transfection. The cells were fixed and stained with p27^{kip1} antibody (fluorescein isothiocyanate, green) and DAPI (blue nuclei) and examined by immunofluorescence.

**Figure 7.** FoxM1 binds to a regulatory region of a human KIS gene. A, schematic presentation of FoxM1 protein putative binding sites within the KIS gene as determined by the computer analysis. 3′-UTR, 3′-untranslated (UNTR) region. B, FoxM1-depleted or untreated U2OS cells were processed by quantitative ChIP assay. The cross-linked and sonicated human chromatin was immunoprecipitated with antibody specific for FoxM1 or rabbit serum (control), and the amount of promoter DNA associated with the IP was quantified by real time PCR with primers specific for the indicated regions predicted to contain FoxM1-binding sites. C, luciferase reporter constructs containing 1.1-kb proximal human KIS promoter alone or with addition of fused 23-kb putative binding sequence and its mutant (mut) were used to co-transfect U2OS cells along with a plasmid expressing GFP-FoxM1 fusion protein. Protein extracts were prepared and analyzed for dual luciferase activity. D, electrophoretic mobility shift assays were performed using U2OS cell nuclear extracts. The extracts were incubated with ^32P-labeled oligonucleotides, alone or in the presence of 100-fold excess of unlabeled Cdx2 oligonucleotide (oligo) (specific cold competitor, cc). The FoxM1 protein-DNA complexes were resolved by electrophoresis on a nondenaturing PAGE and detected by autoradiography. The asterisk denotes nonspecific binding.

FoxM1 Binds to an Internal Regulatory Region of the KIS Gene and Activates Its Transcription—The human KIS gene sequence was analyzed using MacVector 8.0 software, and the analysis revealed several clusters of potential FoxM1-binding sites based on sequence homology. These putative binding sites are positioned within the introns of KIS gene at 1.3, 23, and 33-kb distance from the transcriptional start site and are represented with dark boxes in a schematic diagram (Fig. 7A).

To determine whether FoxM1 binds to any of these putative binding sites within the KIS gene, FoxM1-depleted or untreated U2OS cells were subjected to quantitative ChIP
analysis. The cross-linked and sonicated human chromatin was immunoprecipitated with antibodies specific for FoxM1 or rabbit serum (control), and the amount of human KIS gene DNA associated with the IP protein was quantified by real time PCR. The primers for PCR amplification were designed to amplify the segments considered as putative FoxM1-binding sites. These ChIP assays demonstrated that FoxM1 protein binds to the putative element at 23-kb endogenous KIS gene region, whereas the other two predicted regions showed no association (Fig. 7B). This suggests that endogenous FoxM1 protein binds specifically to the 23-kb site within the KIS gene.

To obtain further evidence for the binding of endogenous FoxM1 to the 23-kb element, electrophoretic mobility shift assays were performed with U2OS nuclear extract. The 23-kb region FoxM1-binding site oligonucleotide and a specific mutant containing four A introduced in place of G were synthesized and annealed as well as the specific FoxM1-binding Cdx2 promoter region (28) as a binding competitor. These oligonucleotide DNA duplexes were 32P-labeled and incubated with the nuclear extracts either alone or in the presence of 100-fold excess unlabeled CDX2 oligonucleotide. The FoxM1 protein-DNA complexes were resolved by electrophoresis on a nondenaturing PAGE and detected by autoradiography (Fig. 7D). The 23-kb region oligonucleotide showed formation of a specific FoxM1-DNA complex that was significantly reduced in the presence of competing CDX2 oligonucleotide.

To investigate functionality of the 23-kb element, the DNA element was synthesized as a pair of two 23-bp oligonucleotides, annealed to form a DNA duplex, and cloned in front of 1.1 kb of the proximal human KIS promoter in the vector pGII3 to generate a luciferase reporter construct. As a control, another set of oligonucleotides representing a mutated 23-kb region was also annealed and cloned upstream of the KIS promoter in the same vector. We performed co-transfection assays with the CMV GFP-FoxM1 expression vector and the 23-kb region 1.1 KIS promoter luciferase reporter plasmid, prepared protein extracts from U2OS cells at 24 h following transfection, and used them to measure dual luciferase enzyme activity. Co-transfection of the FoxM1 vector caused a nearly 3-fold increase in transcriptional activity of constructs containing the 23-kb region site when compared with plasmids harboring the promoter alone or the mutated 23-kb region, demonstrating that FoxM1 protein can transcriptionally activate the KIS gene (Fig. 7C). These results suggest that the element within the 23-kb region is a functional FoxM1-binding site.

DISCUSSION

FoxM1 is a transcription factor that regulates proliferation and cell cycle progression. It is expressed in a number of tumor-derived cell lines (3, 28, 29) and was identified as a differentially expressed gene in a number of solid tumors (7, 30, 31). FoxM1 is up-regulated in human carcinomas originating from different tissues, such as prostate, breast, lung, ovary, colon, pancreas, stomach, bladder, liver, and kidney (7). The human FOXM1 gene is located on the chromosomal band 12p13 (32), which is commonly amplified in advanced stage cervical squamous carcinomas (33), breast adenocarcinomas (34), nasopharyngeal carcinomas (35), head and neck squamous cell carcinomas (36), and also in peripheral cytotoxic T cell lymphomas not otherwise specified (PTCLNOS) (37).

Previously Costa and co-workers (38) demonstrated FoxM1 plays an essential role in the development of HCC. It was demonstrated that liver-specific ablation of FoxM1 made mice resistant to developing HCC tumors in response to diethylnitrosamine (DEN)/phenobarbital (PB) exposure, an established method for induction of HCC tumors in mouse liver. Hepatocytes in the FoxM1 null liver failed to undergo extensive proliferation following DEN/PB exposure that is required for liver tumor progression, while accumulating p27Kip1 protein in the nuclei (24).

p27Kip1 inhibits Cdk2-mediated phosphorylation of the retinoblastoma protein that is required for activation of E2F transcription factors (39, 40) leading to diminished proliferation and cell cycle arrest. A recent study showed that p27Kip1 expression is reduced at relatively early stages of HCC, and its reduction correlates with a higher recurrence rate after surgical resection, whereas patients with intense p27Kip1 staining in more than 50% of the cells had a lower recurrence rate (41). In a related study, p27Kip1 protein expression was significantly lower in advanced stages of HCC and in multiple tumor nodules. Evaluation of p27Kip1 expression in hepatocellular carcinoma may have prognostic significance and could guide decisions of usefulness of various therapeutic regimes in the clinic (42).

It was shown previously that FoxM1 is essential for transcription of the Skp2 and Cks1 genes, which are a part of the SCF ubiquitin ligase complex. Following its phosphorylation by the Cdk2-CyclinE complex, p27Kip1 binds to Skp2 and Cks1 and is targeted for ubiquitin-mediated proteasome degradation (11). Here we present an additional mechanism of FoxM1-mediated regulation of p27Kip1 protein. By up-regulating KIS expression, FoxM1 stimulates phosphorylation of p27Kip1 on Ser-10, leading to its nuclear export and degradation. Accumulating data support a role for FoxM1 in promoting cell proliferation and tumor development and mark it as a potential therapeutic target (reviewed in Refs. 6, 43, 44). Previously it was shown that disruption of the FoxM1 gene, or inhibition of FoxM1 by ARF peptide, can reduce development of HCC using the DEN/PB protocol in mouse models (8, 24). A recently identified chemical inhibitor also shows promise for targeting tumor cells with high FoxM1 expression (9). Thus, it will be important to further evaluate the efficacy of inhibiting FoxM1 activity by chemical inhibitors, cell-penetrating ARF peptide, or siRNA as a therapeutic approaches for the treatment of HCC.

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