

Raf/MEK/MAPK signaling stimulates the nuclear translocation and transactivating activity of FOXM1c

Richard Y. M. Ma, Tommy H. K. Tong, Alice M. S. Cheung, Anthony C. C. Tsang, Wai Ying Leung and Kwok-Ming Yao*

Department of Biochemistry, Faculty of Medicine, The University of Hong Kong, 3/F Laboratory Block, The Faculty of Medicine Building, 21 Sassoon Road, Pokfulam, Hong Kong, China

*Author for correspondence (e-mail: kmyao@hkusua.hku.hk)

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Summary

The forkhead box (FOX) transcription factor FOXM1 is ubiquitously expressed in proliferating cells. FOXM1 expression peaks at the G2/M phase of the cell cycle and its functional deficiency in mice leads to defects in mitosis. To investigate the role of FOXM1 in the cell cycle, we used synchronized hTERT-BJ1 fibroblasts to examine the cell cycle-dependent regulation of FOXM1 function. We observed that FOXM1 is localized mainly in the cytoplasm in cells at late-G1 and S phases. Nuclear translocation occurs just before entry into the G2/M phase and is associated with phosphorylation of FOXM1. Consistent with the dependency of FOXM1 function on mitogenic signals, nuclear translocation of FOXM1 requires activity of the Raf/MEK/MAPK signaling pathway and is enhanced by the MAPK activator aurintricarboxylic acid. This activating effect was suppressed by the MEK1/2 inhibitor U0126. In transient reporter assays, constitutively active MEK1 enhances the transactivating effect of FOXM1c, but not FOXM1b, on the cyclin B1 promoter. RT-PCR analysis

confirmed that different cell lines and tissues predominantly express the FOXM1c transcript. Mutations of two ERK1/2 target sequences within FOXM1c completely abolish the MEK1 enhancing effect, suggesting a direct link between Raf/MEK/MAPK signaling and FOXM1 function. Importantly, inhibition of Raf/MEK/MAPK signaling by U0126 led to suppression of FOXM1 target gene expression and delayed progression through G2/M, verifying the functional relevance of FOXM1 activation by MEK1. In summary, we provide the first evidence that Raf/MEK/MAPK signaling exerts its G2/M regulatory effect via FOXM1c.

Supplementary material available online at
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Key words: FOXM1c, Raf/MEK/MAPK, Nucleocytoplasmic shuttling, Cyclin B1, G2/M

Introduction

FOX (Forkhead box) proteins, characterized by the possession of a 100 amino acid winged helix DNA binding domain, are chordate transcription factors that play important roles in the regulation of growth and development (Kaestner et al., 2000; Kaufmann and Knochel, 1996; Lehmann et al., 2003). Hitherto, 17 subclasses (A-Q) of FOX proteins have been defined based on phylogenetic analysis. FOXM1, in contrast to most FOX factors that are tissue- or cell type-specific, is expressed ubiquitously in all cells undergoing proliferation (Korver et al., 1997; Yao et al., 1997; Ye et al., 1997). Loss of FOXM1 function in knockout mice results in polyploidy, suggesting that FOXM1 function is required for normal coupling of DNA replication (at S phase) and chromosomal segregation (at M phase) during cell cycle progression (Korver et al., 1998).

FOXM1 expression peaks at G2/M and is believed to exert its S-M coupling role by promoting M phase entry and suppressing DNA re-replication (Korver et al., 1998; Leung et al., 2001). Using doxycycline-inducible FOXM1-expressing cell lines, we have previously demonstrated that overexpression of FOXM1 stimulates cyclin B1 expression and

facilitates progression through the G2/M phase (Leung et al., 2001). A similar growth stimulatory effect is also observed in transgenic mice overexpressing FOXM1 in regenerating hepatocytes (Ye et al., 1999). Furthermore, a liver-specific deletion of the mouse *Foxm1* gene clearly illustrates that FOXM1 function is essential for progression into mitosis (Wang et al., 2002).

Consistent with FOXM1 serving an M phase promoting function, expression of cyclin B1 and Cdc25B is significantly decreased in FOXM1 mutant mice (Wang et al., 2002). Cyclin B1 and Cdc25B are important regulators of cyclin-dependent kinase 1 (Cdk1) activity (Donzelli and Draetta, 2003; Doree and Hunt, 2002). Cyclin B1 allosterically binds and activates Cdk1 activity whereas Cdc25B, together with Cdc25C, removes the inhibitory phosphorylations on Cdk1 imposed by S-phase kinases Wee1 and Myt1. In transient reporter assays, FOXM1 overexpression stimulates the activity of both the cyclin B1 and Cdc25B promoters (Leung et al., 2001; Wang et al., 2002; Wang et al., 2001).

FOXM1 requires activation by mitogenic signals to exert its growth stimulatory effect. In transgenic mice overexpressing FOXM1 in the liver, growth stimulation of hepatocytes is only

evident upon partial hepatectomy when mitogenic signals are generated (Ye et al., 1999). In contrast, significant growth stimulation becomes detectable in cells overexpressing FOXM1 only upon serum deprivation (Leung et al., 2001). It has been proposed that requirement for FOXM1 function is not limiting in the presence of serum when mitogenic factors are abundant. Upon serum removal and subsequent decrease in mitogenic signals, FOXM1 function within cells becomes limiting and the cell cycle effect of FOXM1 overexpression manifested. Further understanding of the G2/M regulatory effect of FOXM1 requires identification of the signal transduction cascades that modulate FOXM1 activity.

The mitogen-activated protein (MAP) kinase cascade is activated by a variety of cellular stimuli to influence cell growth and proliferation (Wilkinson and Millar, 2000). In addition to its action on the G1/S phase, the Raf/MEK/MAPK pathway has a G2/M regulatory effect (Pouyssegur et al., 2002; Wilkinson and Millar, 2000). In synchronized NIH3T3 cells, ERK phosphorylation and hence activation of the Raf/MEK/MAPK pathway persists throughout the cell cycle (Roberts et al., 2002; Wright et al., 1999). When treated with the specific MEK1 inhibitor PD098059 or U0126, synchronized S-phase cells become arrested in the following G2 phase with a concomitant decrease in cyclin B-Cdk1 kinase activity (Roberts et al., 2002; Wright et al., 1999). Moreover, ectopic expression of dominant-negative forms of MEK1 (MAPKK1) and ERK2 retards progression through G2/M, suggesting that activation of the Raf/MEK/MAPK pathway is required for G2/M transition (Roberts et al., 2002; Wright et al., 1999). However, the molecular target(s) mediating this G2/M regulatory effect remain elusive.

We hypothesize that FOXM1 is modulated by the Raf/MEK/MAPK pathway and that it may mediate the G2/M regulatory effect of Raf/MEK/MAPK signaling. In this study, we use the human telomerase-immortalized cell line hTERT-BJ1 to investigate the cell cycle-dependent regulation of FOXM1. FOXM1 is found to be phosphorylated and translocated to the nucleus before entry into the G2/M phase. We present evidence that activity of the Raf/MEK/MAPK pathway is both necessary and sufficient for the nuclear translocation of FOXM1. Activation of the Raf/MEK/MAPK pathway by overexpression of constitutively active MEK1 also enhances the transactivating activity of FOXM1 on the cyclin B1 promoter and this activating effect is shown to be mediated through FOXM1c, the predominant isoform expressed in multiple cell types and tissues. Activated Raf/MEK/MAPK signaling promotes FOXM1 phosphorylation and mutations of two ERK1/2 target sequences within FOXM1c abolish the enhancing effect of Raf/MEK/MAPK signaling. Furthermore, inhibition of Raf/MEK/MAPK signaling leads to decreased expression of FOXM1 target genes, supporting the notion that FOXM1 is an effector of Raf/MEK/MAPK signaling in G2/M regulation. Our data provide important insights into the regulation of cell cycle progression through G2/M by FOXM1 and the Raf/MEK/MAPK pathway.

Materials and Methods

Cell culture and cell cycle synchronization

Human neonatal foreskin fibroblasts hTERT-BJ1 (BJ1, BD Biosciences) were cultured in Dulbecco's modified Eagle's

medium/medium 199 (4:1, v/v) and 10% (v/v) fetal bovine serum (FBS), supplemented with 4 mM L-glutamine and 1 mM sodium pyruvate. Mouse embryonic fibroblasts Swiss NIH 3T3 (American Type Culture Collection) were cultured in DMEM and 10% FBS. BJ1 cells were synchronized by serum deprivation/aphidicolin double block (Chen et al., 2001a). Briefly, $\sim 1.5 \times 10^5$ cells were seeded in 100 mm plates. After 36 hours, cells were washed three times with PBS and replenished with DMEM/M199 and 0.1% FBS for 48 hours. Afterwards, cells were treated with DMEM/M199 and 10% FBS containing 1 μ M aphidicolin for 20 hours. To release cells from arrest at the G1/S boundary, they were washed once with PBS and replenished with DMEM/M199 and 10% FBS. Subconfluent NIH 3T3 cells were synchronized at G1/S and G2 phases by incubation with 200 μ M L-mimosine and 500 ng/ml etoposide, respectively, for 48 hours.

Flow cytometry

Cells in 100 mm plates were trypsinized and resuspended in DMEM/M199 and 10% FBS. Cells were resuspended in 70% (v/v) ethanol for storage at -20°C before measurement. Prior to measurement, cells were resuspended in 1 ml serum-free medium and incubated with 16 μ g/ml RNase I for 3 minutes at room temperature. After RNA removal, cells were incubated with 250 μ l propidium iodide (PI) solution (250 μ g/ml PI in PBS with 10% TritonTM X-100) for 15 minutes at 4°C in the dark. Cells were then passed through an EPICS Elite ESP high performance cell sorter (Coulter Electronics), equipped with a red pass filter of 600 nm wavelength. Raw data were analyzed with EXPOTM for Windows 2.0 and ModfitTM LT version 2.0 (Verity software) to eliminate aggregated cells for determination of cell cycle distribution.

Western blotting

Cells were lysed with 10 mM Tris-HCl (pH 7.4) containing 1% SDS, 1 mM EGTA, 1 mM DTT and phosphatase inhibitors (0.1 mM sodium orthovanadate, 50 mM β -glycerophosphate). Phosphatase treatment of cell lysate was performed by incubation with 10 U calf intestinal phosphatase (New England Biolabs) at 37°C for 1 hour. Protein samples were separated by SDS-PAGE in 8% Laemmli gels and electroblotted onto Hybond C Super nitrocellulose membranes (Amersham Life Sciences). Blots were incubated with anti-FOXM1 antiserum (1:200, MPP2-C20 from Santa Cruz), anti- β -Tubulin antiserum (1:500, DM1B from Neomarker), anti-phosphoserine antiserum (1:250, Zymed), anti-ERK antiserum (1:200, C-14 from Santa Cruz), anti-phosphoERK antiserum [1:1000, p44/42 MAP Kinase (Thr202/Tyr204) from Cell Signaling Technology], anti-cyclin B1 antiserum (1:200, GNS1 from Santa Cruz) or anti-Cdc25B antiserum (1:200, H-85 from Santa Cruz). Antigen-antibody complexes were detected using secondary antibodies conjugated to horseradish peroxidase (1:2000, anti-rabbit or anti-mouse IgG purchased from BD Biosciences) and visualized using the SuperSignal ECL detection system (Pierce). Blots for reprobing were stripped in 0.1 M glycine buffer (pH 2.9).

Immunostaining and confocal microscopy

To study FOXM1 subcellular localization by immunostaining, 2.5×10^4 BJ1 cells were seeded on coverslips placed in 35 mm dishes. Cells were synchronized and harvested at different time intervals by fixing with methanol/acetone (1:1, v/v). Fixed cells were treated with anti-FOXM1 antibody (1:200, MPP2-C20 from Santa Cruz) and then the fluorochrome-tagged secondary antibody (1:200, Alexa fluor 488-conjugated goat anti-rabbit IgG from Molecular Probes). Following incubation in PBS with 12.5 μ g/ml propidium iodide and 0.5% Triton X-100, coverslips were mounted on slides with VectashieldTM (Vector Lab, Burlingame). To investigate whether U0126 could block FOXM1

nuclear translocation, synchronized cells were treated with 10 μ M U0126 (or U0124) for 1 hour at 7 hours after aphidicolin removal. Cells were harvested at 8 hours after release for immunostaining. To study the effect of activation of Raf/MEK/MAPK signaling on FOXM1 localization, asynchronized BJ1 cells were incubated with 200 μ M aurintricarboxylic acid (ATA) for 1 hour before their harvest for immunostaining. As controls, asynchronized cells were incubated with DMSO instead of ATA, or pretreated with 10 μ M U0126 (or U0124) for 1 hour before incubation with ATA.

Immunoprecipitation

FOXM1 was immunoprecipitated from BJ1 cells using the following procedure. Anti-FOXM1 antibody (4 μ g/reaction, MPP2-K19 from Santa Cruz) was allowed to bind to protein A/G plus agarose (20 μ g/reaction, Santa Cruz) by rotating overnight in 100 μ l PBS at 4°C. Antibody-bound agarose beads were washed twice with RIPA buffer (PBS, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, pH 7.2) and then once with PBS. BJ1 cells at 80-90% confluency were treated with 200 μ M ATA or 200nM TPA for 1 hour before harvest and lysis in RIPA buffer containing protease (Complete™ protease inhibitor cocktail, Roche) and phosphatase inhibitors. Cell lysates were subjected to one freeze-thaw cycle, passed through 21-gauge needles, briefly sonicated and then pre-cleared with 0.4 μ g normal rabbit serum and 20 μ g protein A/G plus agarose for 30 minutes at 4°C. Afterwards, lysates were transferred to tubes containing the antibody-bound beads and rotated for 4.5 hours at 4°C. Beads were then washed four times with RIPA buffer containing protease inhibitors and once with PBS containing protease inhibitors. Immunoprecipitates were resuspended in 50 μ l sample loading buffer, resolved by SDS-PAGE and immunoblotted with anti-FOXM1 (MPP2-C20) and anti-phosphoserine antibodies.

Chromatin immunoprecipitation (ChIP) assays were performed using the ChIP kit from Upstate Biotech with the following modifications. Phosphatase inhibitors were added to the SDS lysis and ChIP dilution buffers. BJ1 cells were lysed in 200 μ l PIPES buffer [5 mM (pH 8.0), 85 mM KCl, 0.5% NP-40] containing protease inhibitors for 10 minutes at 4°C. After resuspension in SDS lysis buffer, 2.5 μ g anti-FOXM1 (MPP2-K19) or anti-actin antibody was added. Cyclin B1 promoter sequence was amplified using AccuPrime SuperMix II PCR kit (Invitrogen) and two primers (5'-CCTCC-AACCCAGAGAGTTGTTGC-3' and 5'-AGCCAAGGACCTACAC-CCAGCA-3') for 35 cycles. PCR products were visualized by staining with SYBR Green I (1:10,000, Molecular Probes). As a positive control, 1/20 of the sonicated cell lysate was also PCR-amplified. Data were quantified using a Biorad imaging system and TotalLab™ software (Nonlinear Dynamics).

Transient reporter assays

The transactivating effect of FOXM1 on cyclin B1 promoter activity was assayed as previously described (Leung et al., 2001). Plasmids were transfected into NIH3T3 cells seeded in 24-well plates using Fugene 6 as transfection reagent (Roche). For a typical transfection, 100 ng cyclin B1 reporter (pGL3-CycBpro), 0.4 ng control reporter (pRL-SV40) and 100 ng expression plasmids were mixed with 0.6 μ g Fugene 6. The expression vectors pSR α -HA-MAPKK-SDSE (caMEK1) and pSR α -HA-MAPKK-SASA (dnMEK1) were kindly provided by E. Nishida (Fukuda et al., 1997). pcDNA3-FOXM1b was generated by deletion of exon Va from pcDNA3-FOXM1c using QuikChange™ site-directed mutagenesis kit (Stratagene) and two primers, 5'-GACATTGGACCAGGTGTTTAAAGCAGCAGAAAACG-ACCGAATCCAG-3' and 5'-CTGGATTCCGGTCGTTTCTGCTG-CTTAAACACTGGTCCAATGTC-3'. To generate pcDNA3-FOXM1c Δ Cter, coding sequence excluding the carboxyl 71 amino acids of FOXM1c was PCR-amplified using pcDNA3-FOXM1c as template and the primers 5'-GTTGTTGGTACCGAATCCACCAT

GGACTACAAGGACGACGATGACAAGAAAGCTAGCCCCCGTC-GG-3' and 5'-GTTGTTCTCGAGCTAGGGGACGTCTATATCTGA-GGG-3'. The resulting fragment was inserted into pcDNA3 using *EcoRI* and *XhoI* sites. Ser331 and Ser704 within the ERK1/2 target sequences were singly or doubly mutated to alanine in pcDNA3-FOXM1c using the QuikChange™ site-directed mutagenesis kit (Stratagene). pcDNA3 and pSR α were added to normalize the amount of vectors across all transfections. All experiments were conducted in triplicate using independent cultures and the results shown are the mean \pm s.e.m.

RNA extraction and RT-PCR

RNA from cells and tissues were extracted using the TRIzol™ reagent (GibcoBRL) according to the manufacturer's instruction. Tissue samples (10-20 ng) were homogenized with a Dounce homogenizer followed by brief sonication at 4°C. For expression analysis of FOXM1 isoforms, cDNAs were synthesized using 2 μ g total RNAs, SuperScript™ RT-PCR system (Invitrogen) and oligo(dT)₂₀ as the primer. cDNAs were subjected to PCR amplification using Platinum® Taq DNA polymerase kit (Invitrogen). FOXM1b and FOXM1c cDNAs (human), and Marathon-ready™ testis cDNA libraries (human and mouse, Clontech), were also PCR-amplified as controls. FOXM1 and GAPDH cDNAs were amplified using the following primers and cycle numbers: mouse FOXM1, 5'-ACCCAAGTGCCA-ATCGCCACTTG-3' and 5'-GAAGCGGGGCTATTCCTTCACTGG-3', 28 cycles; human FOXM1, 5'-CACCCAGTGCCAACCGCTA-CTTG-3' and 5'-AAAGAGGAGCTATCCCCTCCTCAG-3', 32 cycles; mouse GAPDH, 5'-GCAGGGGGAGCCAAAAGGG-3' and 5'-TGCCAGCCCCAGCGTCAAAG-3', 30 cycles. For semi-quantitative RT-PCR analysis to determine the effect of Raf/MEK/MAPK inhibition on cyclin B1 expression, synchronized hTERT-BJ1 cells were treated with 25 μ M U0126 at 5.5 hours after aphidicolin removal. Total RNAs were extracted at different time intervals and 0.5 μ g of each sample was subjected to reverse transcription using random hexamers to prime cDNA synthesis. cDNA amounts and cycle numbers were optimized to ensure that amplification was within the linear range for quantitative analysis. Cyclin B1 and GAPDH cDNAs were amplified using 25 and 24 cycles, respectively. PCR fragments were resolved in 1% (w/v) agarose gels and detected with SYBR Green I.

Results

FOXM1 is phosphorylated and translocated to the nucleus just before entry into the G2/M phase

FOXM1 expression is initiated before entry into S phase and its level persists during the S phase (Korver et al., 1998; Leung et al., 2001). However, dramatic transcriptional stimulation of the FOXM1 target gene cyclin B1 does not occur until the G2/M phase (Hwang et al., 1995; Hwang et al., 1998). This time lag could be due to a post-translational regulation of FOXM1 function and it has been previously reported that FOXM1 exhibits nuclear translocation in hepatocytes stimulated to proliferate (Ye et al., 1999), but it remains unclear how this nucleocytoplasmic shuttling of FOXM1 is regulated. As a first objective, we sought to investigate whether there is regulation of post-translational modification and nuclear translocation of FOXM1 in a cell cycle-dependent manner.

The human foreskin fibroblastic cell line hTERT-BJ1 (BJ1), which possesses a functional G1/S checkpoint for efficient synchronization at the G1/S boundary, was used for this analysis. BJ1 cells, synchronized using serum starvation/aphidicolin double block, were harvested for flow cytometric and immunoblot analyses at various time intervals after release

from cell cycle arrest. DNA analysis indicated that synchronized BJ1 cells gradually progressed through the cell cycle (Fig. 1A). More than 40% of cells entered G2/M phase at 9 hours after release and many cells re-entered the G1 phase of the next cycle at 12 hours. From 0.5 to 6 hours after release, western analysis revealed low levels of FOXM1 expression in late-G1 and S cells. FOXM1 level increased at 9 hours after release, when cells entered into the G2/M phase, and there was a concomitant mobility shift of the FOXM1 band (Fig. 1B, arrowhead). At 12 hours after release, a discrete FOXM1 band was discernible and a faint band of even slower mobility appeared. FOXM1 was previously shown to be phosphorylated at M phase (Korver et al., 1997; Westendorf et al., 1994). To test whether the early mobility shift at 9 hours after release also corresponded to phosphorylation of FOXM1, the cell lysate was treated with calf intestine phosphatase (CIP). This treatment abolished the FOXM1 mobility shift, suggesting that phosphorylation was the post-translational modification involved (Fig. 1C).

To determine whether FOXM1 phosphorylation was associated with any change in subcellular localization, BJ1 cells seeded on coverslips were similarly synchronized and harvested for immunostaining. Cells were immunostained with anti-FOXM1 antibody and counterstained with propidium iodide to label the nuclear DNA. Before removal of aphidicolin (0 hour), low levels of FOXM1 were detected in the cytoplasm (Fig. 1D a-c, arrow). At 3 hours and 6 hours after release, when most cells were in S phase, FOXM1 immunoreactivity was still predominately cytoplasmic (Fig. 1D d-i, arrows). Interestingly, there was a dramatic increase in nuclear-expressing cells at 9 hours after release, when cells at late S phase entered into the G2/M phase; FOXM1 remained nuclear-localized at 12 hours after release (Fig. 1D j-o, arrowheads). These findings suggest a tight regulation of FOXM1 subcellular localization. FOXM1 is mainly cytoplasmic in late-G1 and S phases. Late in S phase, FOXM1 becomes phosphorylated and there is an accompanying shuttling of the protein to the nucleus. These findings support the idea that FOXM1 plays a functional role at the G2/M phase when it activates expression of target genes like cyclin B1.

FOXM1 nuclear translocation requires Raf/MEK/MAPK signaling

Previous studies have shown that activation of the Raf/MEK/MAPK pathway is required for progression through the G2/M phase (Roberts et al., 2002; Wright et al., 1999) but little is known about the molecular mechanism underlying this G2/M regulatory effect. As FOXM1 exerts a modulatory effect on cell cycle progression through G2/M and its function requires activation by mitogenic signals (Leung et al., 2001; Ye et al., 1999), we speculate that FOXM1 may be one of the molecular targets of the Raf/MEK/MAPK pathway. To perturb activity of the Raf/MEK/MAPK pathway, we used the MEK1/2 inhibitor U0126 and aurintricarboxylic acid (ATA), an activator acting upstream of MEK1/2 in the Raf/MEK/MAPK pathway (Beery et al., 2001). Immunoblot analysis of ERK and phosphoERK expression indicated that U0126 at 10 μ M completely suppressed Raf/MEK/MAPK signaling (Fig. 2A). Pathway activity was stimulated by ATA at 200 μ M but this could be counteracted by pre-treatment with increasing concentrations of U0126.

To address whether the subcellular localization of FOXM1 is

modulated by Raf/MEK/MAPK signaling, synchronized BJ1 cells were treated with U0126 (10 μ M) at 7 hours after release, one hour before their harvest for immunostaining (Fig. 2B). Consistent with the results shown in Fig. 1, FOXM1 in cells without U0126 treatment entered the nucleus at 8 hours after release (Fig. 2C,D). This nuclear entry was abrogated in the presence of U0126 (Fig. 2E). U0124, an inactive analog of U0126, did not have any inhibitory effect on FOXM1 nuclear translocation (Fig. 2F). These results suggest that inhibition of the Raf/MEK/MAPK pathway prevents nuclear translocation of FOXM1.

If activity of the Raf/MEK/MAPK pathway is required for the nuclear translocation of FOXM1, we would expect an enhancement of FOXM1 nuclear import upon ATA treatment (Fig. 2G). Without synchronization and drug treatment, most BJ1 cells were at G1 or S phase with FOXM1 expressed in the cytoplasm (Fig. 2H). As expected, incubation of asynchronous BJ1 cells with ATA (200 μ M) for 1 hour increased dramatically the number of nuclear-stained cells (Fig. 2I), suggesting that ATA promoted nuclear translocation of FOXM1. To confirm that this ATA-induced nuclear translocation was mediated by Raf/MEK/MAPK signaling, we pre-treated BJ1 cells with U0126 or U0124 for 1 hour before ATA addition. ATA-induced nuclear translocation was abolished in the U0126-treated but not the U0124-treated cells (Fig. 2J,K). Taken together, we demonstrate that activation of the Raf/MEK/MAPK pathway is sufficient to induce the nuclear translocation of FOXM1.

Constitutively active MEK1 enhances the transactivating activity of FOXM1c, but not FOXM1b

FOXM1 has previously been shown to activate the cyclin B1 promoter in doxycycline-inducible cell lines and transient reporter assays (Leung et al., 2001; Wang et al., 2001). In this study, we could immunoprecipitate the cyclin B1 promoter sequence using an anti-FOXM1 antibody in a chromatin immunoprecipitation (ChIP) assay, suggesting that FOXM1 is a direct regulator of cyclin B1 transcription (Fig. 3A). To test whether the enhanced nuclear translocation of FOXM1 upon stimulation of Raf/MEK/MAPK signaling has any effect on the transactivating activity of FOXM1, a constitutively active form of MEK1 (caMEK1) (Fukuda et al., 1997) was coexpressed with FOXM1 and the cyclin B1 reporter in transient assays. Three isoforms of FOXM1, FOXM1a, FOXM1b and FOXM1c, have previously been reported (Kaestner et al., 2000) but only FOXM1b and FOXM1c exhibit transactivating activity in reporter assays (Leung et al., 2001; Ye et al., 1997). We have been doubtful about the physiological significance of FOXM1a, as this isoform is not conserved in mouse (Fig. S1 in supplementary material). Therefore, only the b and c isoforms (Fig. 3B) were individually tested in our assays. Similar to previous findings (Leung et al., 2001; Ye et al., 1997), FOXM1b and FOXM1c (60 ng) showed similar transactivating activity when tested in NIH 3T3 cells (Fig. 3C). However, only FOXM1c, but not FOXM1b, displayed significant enhancement of transactivating activity when caMEK1 (30 ng) was coexpressed (Fig. 3D). FOXM1c activity was enhanced 6.8-fold when amount of FOXM1 was increased from 0 to 60 ng.

Next, we tested whether both functional FOXM1c and MEK1 are required for the transcriptional enhancing effect. caMEK1 and FOXM1c, when individually tested, exhibited

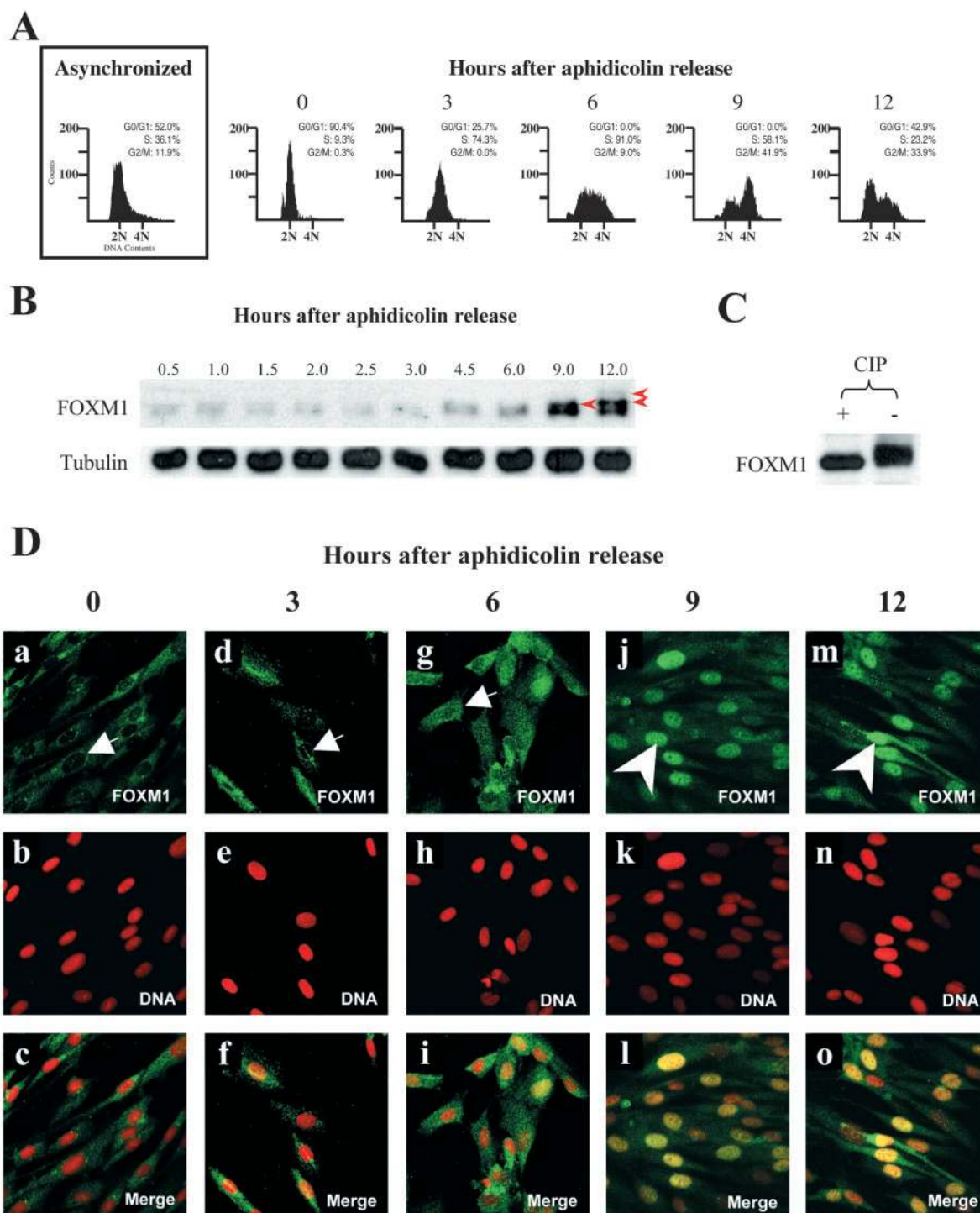


Fig. 1. FOXM1 is phosphorylated and translocated to the nucleus before entry into the G2/M phase. (A) Flow diagrams of asynchronized and synchronized BJ1 cells. BJ1 cells were synchronized at the G1/S boundary by serum starvation/aphidicolin double block. Cells were released from arrest by removal of aphidicolin. At different time intervals after release, cells were harvested and stained with propidium iodide for DNA analysis using a flow cytometer. Synchronized cells gradually progressed through the cell cycle and G2/M cells could be detected at both 9 hours and 12 hours after release. (B) Cells at different time intervals after aphidicolin release were harvested for immunoblot analysis using anti-FOXM1 and anti-tubulin antibodies. Progressive mobility shifts (denoted by arrowheads) of the FOXM1 band were observed at 9 hours and 12 hours after release. (C) The cell lysate at 9 hours after release was treated with calf intestine phosphatase (for 1 hour) before immunoblot analysis. Such treatment abolished the mobility up-shift of the FOXM1 band. (D) BJ1 cells grown on coverslips were synchronized by serum starvation/aphidicolin double block and fixed at various time points after removal of aphidicolin. Cells were immunostained with anti-FOXM1 antibody and counterstained with propidium iodide to detect nuclear DNA. Merged images of FOXM1 and nuclear staining are also shown. FOXM1 was predominantly cytoplasmic (arrows) at 0 hour, 3 hours and 6 hours after release; FOXM1 became mainly nuclear (arrowheads) at 9 hours and 12 hours after release.

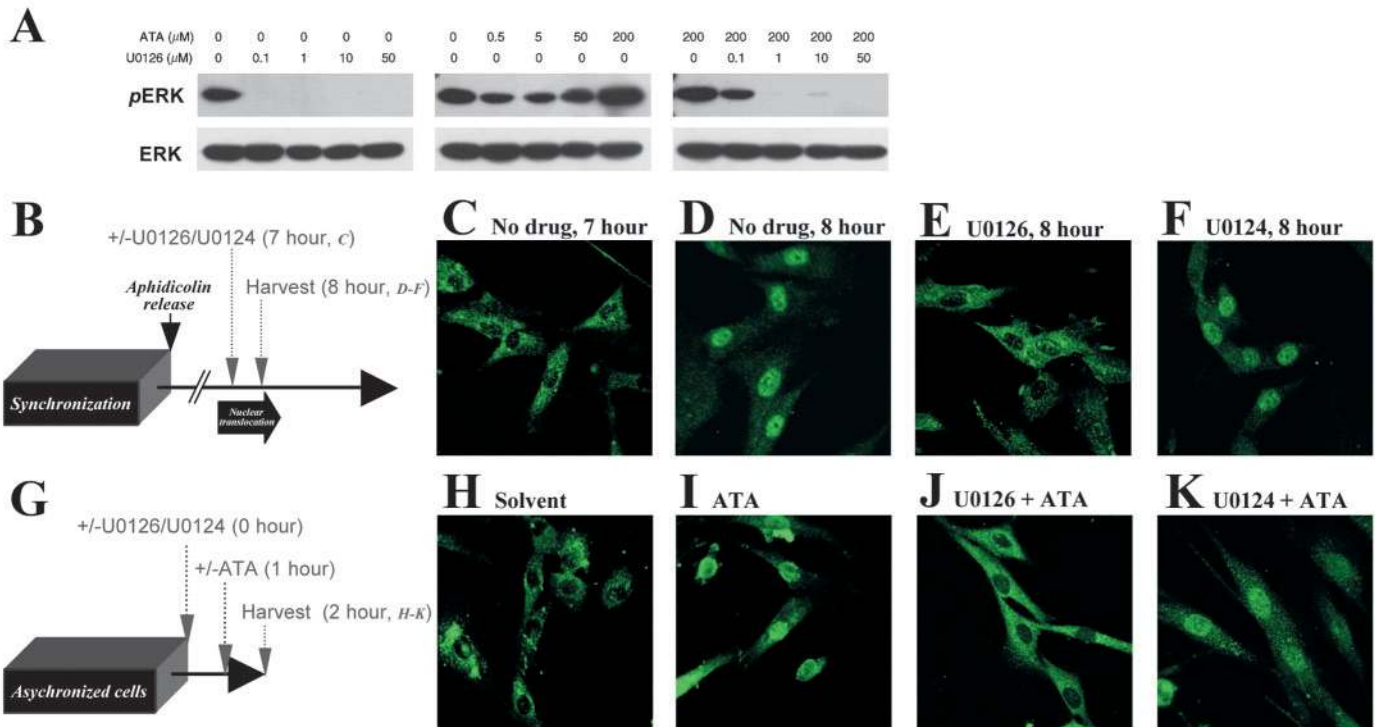


Fig. 2. Activity of the Raf/MEK/MAPK pathway is required for nuclear translocation of FOXM1. (A) Immunoblot analysis of total ERK and phosphoERK expression to monitor the effect of U0126 and ATA treatment on Raf/MEK/MAPK activity. (B) Scheme for drug treatment to inhibit MEK1/2. Synchronized BJ1 cells were incubated with U0126 (10 μM) or the inactive analog U0124 (10 μM) from 7 hours to 8 hours after aphidicolin release. Cells were harvested 1 hour later (i.e. at 8 hours after release) and immunostained for FOXM1. (C) Without drug treatment, FOXM1 was predominantly cytoplasmic at 7 hours after release. (D) 1 hour later, FOXM1 became mainly nuclear. (E) Treatment with U0126 abolished FOXM1 nuclear translocation at 8 hours after release. (F) FOXM1 nuclear translocation was not affected by U0124 treatment. (G-K) ATA promotes nuclear translocation of FOXM1. (G) Scheme for ATA treatment and U0126/U0124 pre-treatment. (H) In cells treated with solvent (DMSO), FOXM1 was predominantly expressed in the cytoplasm. (I) After incubation with 200 μM ATA for 1 hour, FOXM1 became localized mainly to the nucleus. (J) ATA stimulation of FOXM1 nuclear translocation was abrogated by pre-incubation with 10 μM U0126 for 1 hour. (K) U0124 pre-treatment could not counteract the stimulatory effect of ATA.

only a twofold stimulatory effect owing to the presence of endogenous FOXM1 and MEK1 activity (Fig. 3E). The effect was increased to 15.5-fold when caMEK1 and FOXM1 were coexpressed. To verify that FOXM1 is downstream of MEK1 to mediate the activating effect, a FOXM1 mutant missing the carboxyl 71 amino acids (FOXM1 Δ Cter; Fig. 3B) was tested. Based on previous structure-function analysis, this deleted region is within the transactivation domain and the mutant protein synthesized is transcriptionally inactive (R.Y.M.M. and K.-M.Y., unpublished). FOXM1 Δ Cter, when coexpressed with caMEK1, displayed a mildly suppressive effect (Fig. 3E). The enhancing effect was also lost when a dominant negative form of MEK1 (dnMEK1) (Fukuda et al., 1997) was coexpressed. In transiently transfected cells, overexpression of caMEK1 and dnMEK1 did show the expected effects on Raf/MEK/MAPK activity, monitored by immunoblot analysis of phosphoERK expression (Fig. 3F). These data strongly support the fact that caMEK1 enhancement of FOXM1 activity requires the presence of functional FOXM1 protein.

FOXM1c is the major isoform expressed in cells and tissues

The responsiveness of FOXM1 to Raf/MEK/MAPK stimulation

in BJ1 cells points to FOXM1c being the predominantly expressed isoform. To test this notion, we subjected total RNA isolated from BJ1 cells to RT-PCR analysis using primers that flank the coding sequence of exon Va, which is missing in the FOXM1b isoform (Fig. 4A). The primers should generate PCR products of 323 bp and 368 bp if FOXM1b and FOXM1c transcripts, respectively, are expressed. As the testis has previously been found to express both transcripts (Yao et al., 1997), testis cDNA libraries were similarly amplified to generate the FOXM1b- and FOXM1c-specific fragments as size controls. In BJ1 cells synchronized at different cell cycle phases, only the FOXM1c fragment was detectable (Fig. 4B), supporting a predominant expression of FOXM1c. The NIH3T3 cell line used as host in our transient reporter assays, and a few neonate and adult tissues that are rich in mitotically active progenitor-like cells and hence FOXM1 expression, were also analyzed. Again, NIH3T3 cells, synchronized or not, and the various tissues mainly showed amplification of the FOXM1c fragment (Fig. 4C,D).

Identification of ERK1/2 target sites within FOXM1c and their requirement for caMEK1 enhancement

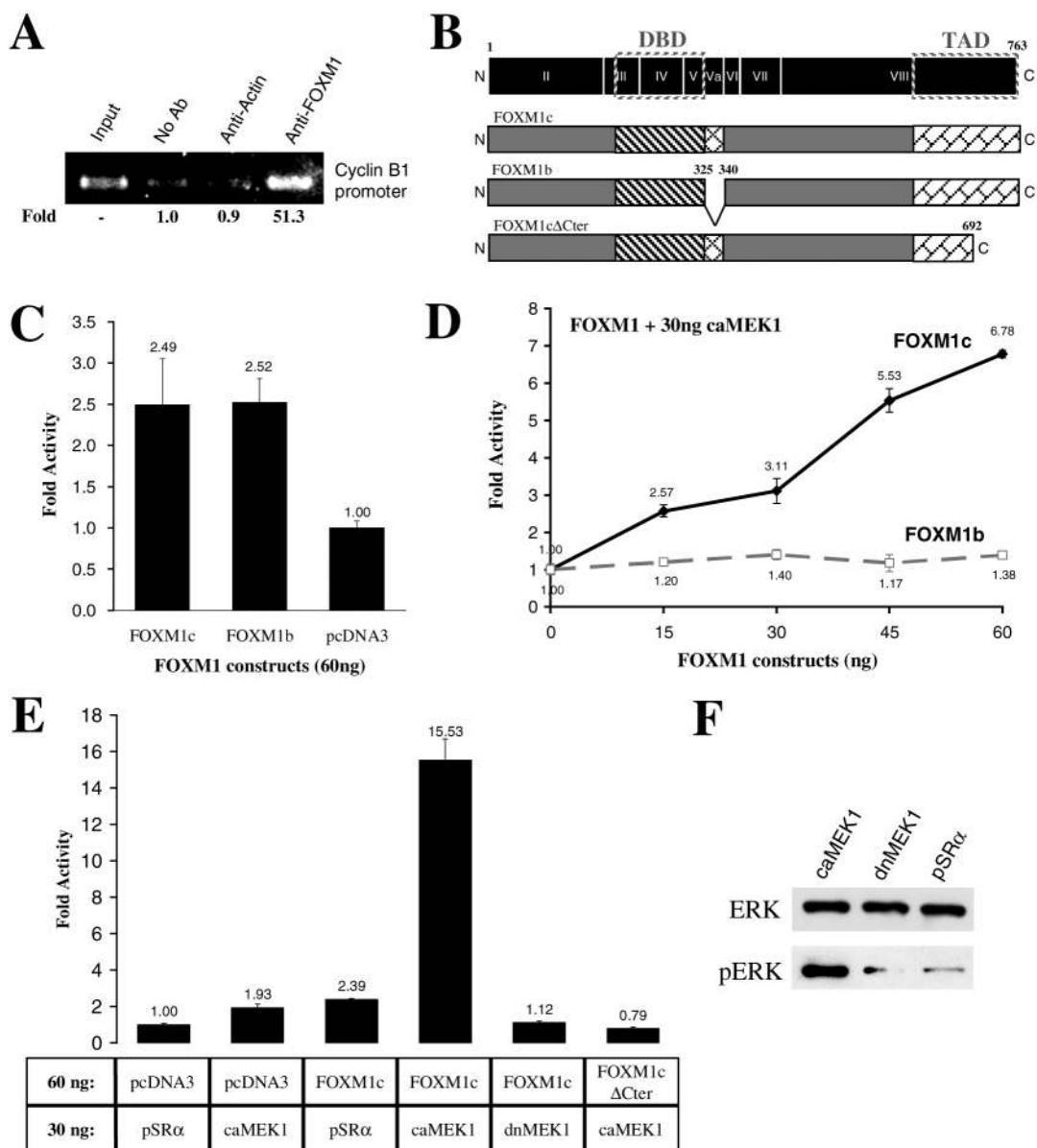
ERK1 and ERK2 are the effector serine/threonine kinases

believed to mediate the regulatory effect of the Raf/MEK/MAPK pathway by directly phosphorylating target proteins (Wilkinson and Millar, 2000). They recognize Pro-X-Ser/Thr-Pro (where X represents any amino acid) as the substrate consensus sequence (Thomas, 1992). Analysis of the FOXM1c coding sequence identifies three motifs (PGS331P, PRT620P and PGS704P; Fig. 5A) that resemble the ERK1/2 target sequence. Of particular interest are the 331 and 704 motifs, which are conserved among the human, rat and mouse FOXM1 sequences (Fig. 5B).

Aurintricarboxylic acid (ATA) and TPA, which are activators of IGF-1 receptor and protein kinase C signaling, respectively, have been previously used to stimulate the Raf/MEK/MAPK pathway (Beery et al., 2001; Chen et al., 2001b). To provide

more evidence that FOXM1 is a direct target of Raf/MEK/MAPK signaling, we tested whether ATA and TPA are able to induce phosphorylation of FOXM1. FOXM1 was immunoprecipitated from activator-treated and non-treated BJ1 cells using anti-FOXM1 antibody. The immunoprecipitates were subjected to immunoblot analysis using both anti-FOXM1 and anti-phosphoserine antibodies (Fig. 5C). Treatment with ATA and to a lesser extent TPA, enhanced the intensity of the phosphorylated FOXM1 (up-shifted) band. Notably, anti-phosphoserine antibody mainly detected the upper band of the FOXM1 doublet, indicating that FOXM1 was highly phosphorylated at serines with ATA treatment. Phosphorylation was weaker with the TPA-stimulated or non-treated sample.

Fig. 3. Constitutively active MEK1 enhances the transactivating activity of FOXM1c. (A) Chromatin immunoprecipitation assays of asynchronous BJ1 cells were carried out as described in Methods section to detect FOXM1 binding to the cyclin B1 promoter. Cyclin B1 DNA was enriched more than 50-fold by the anti-FOXM1 antiserum. (B) Schematic diagrams of FOXM1c, FOXM1b and FOXM1cΔCter. Positions of the DNA binding domain (DBD), exon Va and transactivation domain (TAD) are shown. FOXM1b lacks exon Va. FOXM1cΔCter was generated by deletion of the last 71 amino acids of FOXM1. (C-E) Transient reporter assays. NIH3T3 cells were co-transfected with the various expression plasmids and cyclin B1 luciferase reporter. 48 hours after transfection, cells were harvested for luciferase assay. (C) 60 ng of FOXM1c, FOXM1b or the control vector pcDNA3 was co-transfected with the cyclin B1 reporter. Both FOXM1c and FOXM1b showed ~2.5-fold stimulation of the cyclin B1 promoter when compared with the vector control. (D) caMEK1 enhances the transactivating activity of FOXM1c. Co-transfection of caMEK1 (30 ng) with an increasing amount of FOXM1 strongly enhanced the transactivating activity of FOXM1c, but not FOXM1b. (E) The caMEK1 enhancing effect requires the presence of functional FOXM1 protein. Various amounts of FOXM1 and MEK1 expression plasmids, and empty vectors (pcDNA3 and pSRα), were co-transfected as indicated. Both caMEK1 and functional FOXM1c are required for the synergistic activation of cyclin B1 promoter. (F) Western blot to demonstrate the activating and inhibitory effect of caMEK1 and dnMEK1, respectively, on Raf/MEK/MAPK signaling.



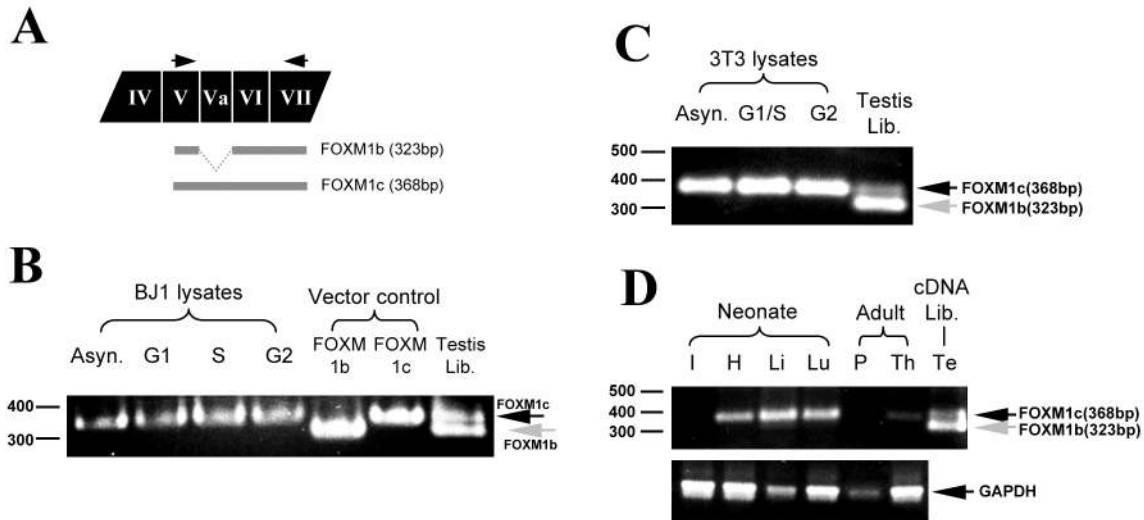


Fig. 4. FOXM1c is the predominant isoform expressed in BJ1, NIH 3T3 and various mouse tissues. (A) RT-PCR assays were designed using human and mouse primers, which flank exon Va, to assess the expression of FOXM1b and FOXM1c transcripts. FOXM1b and FOXM1c transcripts generate PCR fragments of 323 and 368 base pairs, respectively. (B-D) RT-PCR analysis. As positive controls, cDNAs encoding human FOXM1b and FOXM1c, and human and mouse testis cDNA libraries, were PCR-amplified to generate the FOXM1b and FOXM1c bands. GAPDH transcripts were also PCR-amplified in the mouse tissue samples to control for possible loading differences. mRNA of asynchronous and synchronized BJ1 cells [0 hour (G1), 4.5 hours (S), 9 hours (G2) after aphidicolin release] (B) and asynchronous, L-mimosine-arrested (G1/S) and etoposide-arrested (G2) NIH 3T3 cells (C) were extracted for RT-PCR analysis. Intestine (I), heart (H), liver (Li) and lung (Lu) of day 1 neonates, and pancreas (P) and thymus (Th) of adult mice, were harvested for RT-PCR analysis and compared to that of testis (Te) cDNA library (D).

To test directly the regulatory importance of the 331 and 704 motifs, serine residues of these motifs were singly or doubly substituted with the unphosphorylatable amino acid alanine and the mutant constructs assessed for caMEK1 activation using the cyclin B1 reporter in transient assays (Fig. 5D). Mutation of the 331 motif led to a 48% suppression of caMEK1 enhancement. A 60% loss of caMEK1 enhancement was detected when the 704 motif was similarly mutated. caMEK1 enhancement was totally lost when both motifs were substituted in the SASA double mutant. All the mutant constructs were HA-tagged and western analysis confirmed expression of the different mutant FOXM1 proteins (Fig. 5D). These results demonstrate that both the 331 and 704 motifs are required for mediating the enhancing effect of the Raf/MEK/MAPK pathway.

Inhibition of the Raf/MEK/MAPK pathway slows down transition at G2/M and reduces expression of FOXM1 target genes

The data presented above are supportive of our hypothesis that Raf/MEK/MAPK signaling promotes cell cycle progression through G2/M via activation of FOXM1 function. If FOXM1 is indeed a G2/M effector of the Raf/MEK/MAPK pathway, we would expect G2/M delay and suppression of FOXM1 target gene expression upon inhibition of the Raf/MEK/MAPK pathway. To test this argument, we inhibited Raf/MEK/MAPK signaling in BJ1 cells at S and G2/M phases and followed the effect on cell cycle progression and gene expression. BJ1 cells were again synchronized at the G1/S boundary by serum deprivation/aphidicolin double block. Cells were allowed to re-enter S phase following the removal of aphidicolin. The MEK1 inhibitor U0126 was added at 5.5 hours after release to inhibit the Raf/MEK/MAPK pathway. Afterwards, cells were harvested

at various time points for expression and DNA analyses. Rate of progression through G2/M was deduced based on the kinetics of re-entry into the subsequent G1 phase.

Western blots of total ERK revealed that ERK levels were rather constant throughout the cell cycle (Fig. 6C). Without drug treatment, ERK was highly phosphorylated from 1.5 hours to 10 hours after release, suggesting activation of the Raf/MEK/MAPK pathway. When U0126 was added at 5.5 hours, phosphorylation of ERK was abolished henceforth. Both non-treated and U0126-treated BJ1 cells progressed into the G2/M phase and a G2/M peak became discernible at 9 hours after release (Fig. 6A). Without drug treatment, the percentage of G1 cells increased at 11 hours after release and thereafter, indicating G1 re-entry of cells previously in the G2/M phase (Fig. 6A). In contrast, G1 re-entry of U0126-treated cells was delayed, as indicated by the lower percentage of G1 cells at 12.5-15.5 hours after release. Therefore, inhibition of Raf/MEK/MAPK signaling slows down cell cycle progression through G2/M.

Cyclin B1 is a direct transcriptional target of FOXM1. To investigate the effect of U0126 treatment on cyclin B1 expression, RNA isolated from the samples at different time points were subjected to semi-quantitative RT-PCR analysis. GAPDH levels were also analyzed in parallel as loading controls. Cyclin B1 levels were found to increase 8 hours after release, just before cells entered the G2/M phase (Fig. 6B). This increase was greatly attenuated with U0126 treatment, suggesting an inhibition of FOXM1 function. The inhibitory effect of U0126 on the expression of cyclin B1 and another putative FOXM1 target Cdc25B was also analyzed by immunoblotting (Fig. 6C). In control cells, the protein levels of cyclin B1 increased at around 9 hours after release and peaked at 11 hours. In U0126-treated cells, the increase was strongly suppressed and cyclin B1 levels became barely detectable

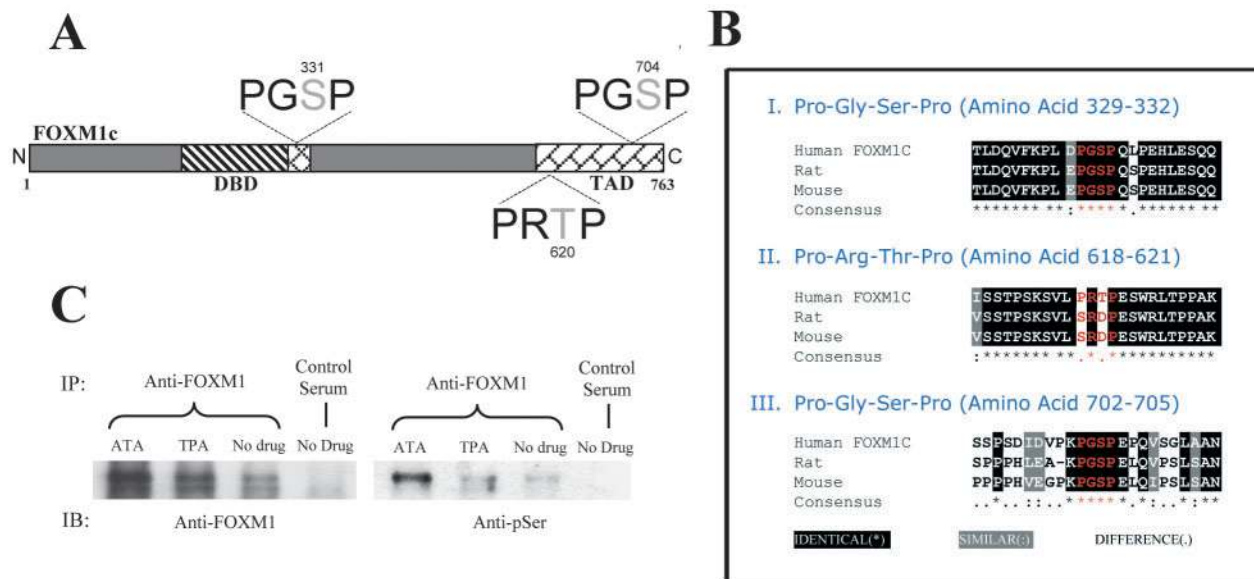


Fig. 5. Identification and functional analysis of the ERK1/2 target sites within FOXM1c. (A) Sequence analysis of FOXM1c identifies three putative ERK1/2 phosphorylation sites (PXS/TP) at amino acids 329-332, 618-621 and 702-705. DBD, DNA binding domain; TAD, transactivating domain. (B) The 331 and 704 motifs are conserved in the FOXM1c coding sequences from multiple species. (C) Activated Raf/MEK/MAPK signaling stimulates FOXM1 phosphorylation. Asynchronous BJ1 cells were incubated with 200 μ M ATA or 200 nM TPA for 1 hour. Cells with or without drug treatment were lysed and endogenous FOXM1 immunoprecipitated with anti-FOXM1 antibody or control rabbit antiserum. The immunoprecipitates were immunoblotted with anti-FOXM1 and anti-phosphoserine antibodies. ATA and to a lesser extent TPA, enhanced the phosphorylation of FOXM1. Note that anti-phosphoserine antibody selectively detected the upper band of the FOXM1 doublet. (D) Both the 331 and 704 motifs are important for mediating the caMEK1 enhancing effect. The wild type (WT) and various substitutive mutants (S331A, S704A, SASA) were co-transfected with caMEK1 in transient reporter assays as described in Fig. 3. caMEK1 enhancement is shown as percentage of activation compared to the wild-type control (100%). Immunoblot analysis revealed expression of the various HA-tagged, mutant FOXM1c proteins.

between 11 and 14 hours after release. A milder inhibitory effect was also observed for Cdc25B expression. Thus, inhibition of Raf/MEK/MAPK signaling leads to suppression of G2/M transition and FOXM1 function, consistent with the idea that Raf/MEK/MAPK signaling mediates its G2/M effect via regulation of FOXM1 function.

Discussion

The loss of S-M coupling in mice deficient in FOXM1 function first implicates FOXM1 as a critical regulator of cell proliferation (Korver et al., 1998). The polyploidy phenotype suggests a requirement of FOXM1 function for entry into mitosis. This M-phase-promoting function of FOXM1 is corroborated by a recent demonstration (Wang et al., 2002) that

FOXM1 function is essential for hepatocytes to enter into mitosis. One salient feature of the M-phase-promoting function of FOXM1 is its dependency on mitogenic stimulation (Leung et al., 2001; Ye et al., 1999). Our demonstration that Raf/MEK/MAPK signaling stimulates FOXM1 nuclear translocation and transactivating activity offers an explanation to this mitogenic dependency, and provides an important insight into how the Raf/MEK/MAPK pathway modulates cell cycle progression through the G2/M phase.

Using synchronized BJ1 cells, we show that FOXM1 is phosphorylated just before G2 entry and there is an associated nuclear translocation of FOXM1. The phosphorylation of FOXM1 observed in this study appears earlier than the M-phase phosphorylation of FOXM1 detected in Rat-1 cells (Korver et al., 1997). Increased up-shifts of the FOXM1 band from 9 to 12

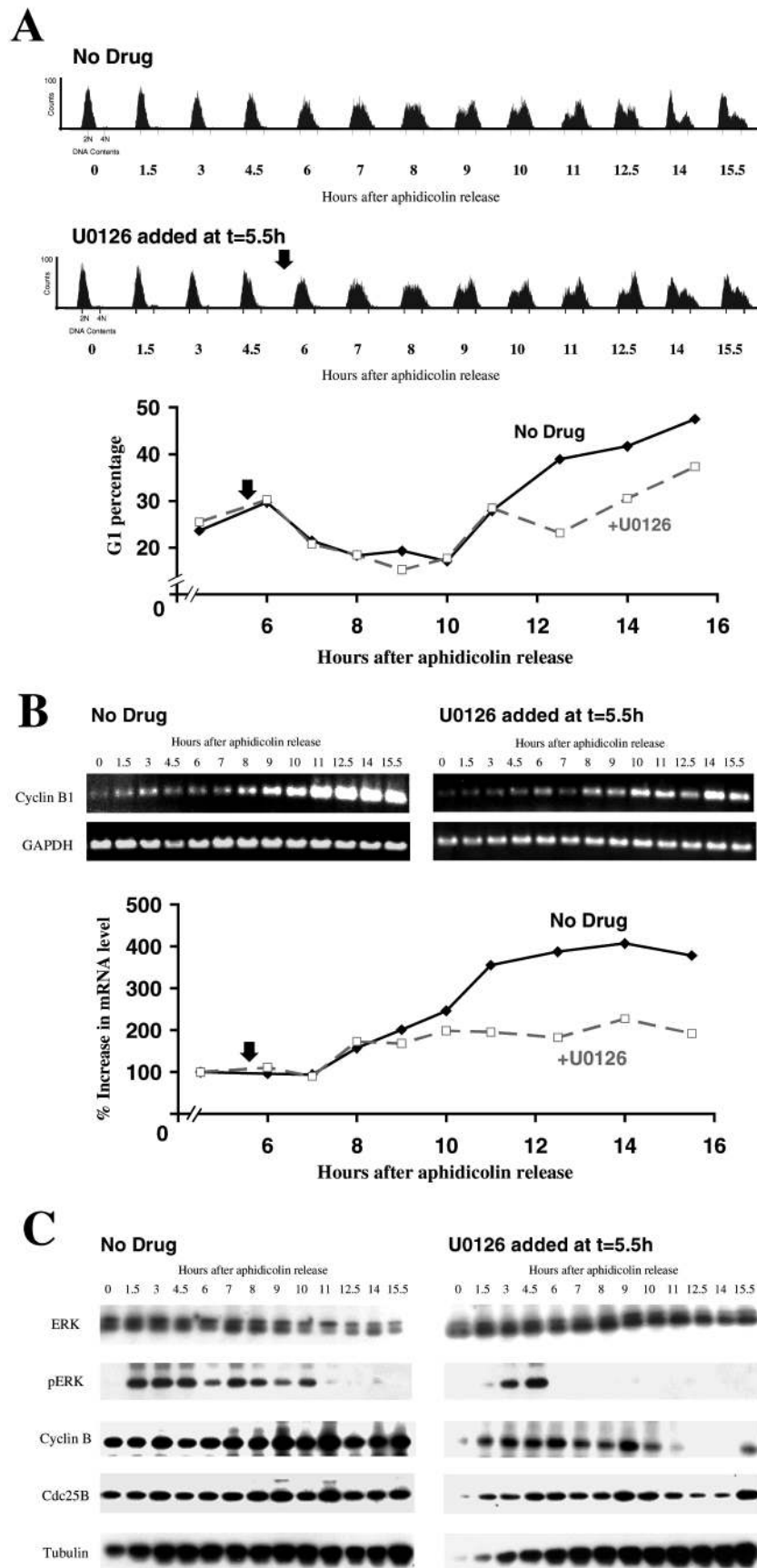


Fig. 6. Inhibition of Raf/MEK/MAPK signaling leads to G2/M delay and downregulation of FOXM1 target genes. U0126 (25 μ M) was added to synchronized BJ1 cells at 5.5 hours after aphidicolin release (black arrows). Cells were harvested for flow cytometric (A), RT-PCR (B) and western blot (C) analyses. Data in A and B were quantified using Modfit and TotalLab software, respectively.

(A) After U0126 treatment, slower progression through G2/M was revealed by the delayed re-entry of cells into the subsequent G1 phase. (B) RT-PCR analysis indicated that U0126 treatment attenuated the increase in cyclin B1 mRNA levels associated with cell cycle progression through G2/M. GAPDH mRNA levels were analyzed in parallel as loading control. (C) Cell lysates were immunoblotted with anti-ERK, anti-phosphoERK, anti-cyclin B1, anti-Cdc25B and anti-tubulin antibodies. U0126 suppressed the phosphorylation of ERK, and the expression of cyclin B1 and Cdc25B, but had no effect on the expression of ERK and tubulin.

hours after release (Fig. 1B) are suggestive of multiple FOXM1 phosphorylations catalyzed by multiple kinases. Our findings are consistent with phosphorylation of FOXM1 by the Raf/MEK/MAPK pathway at late-S phase, later followed by FOXM1 phosphorylation by activated Cdk1 at M phase.

Activity of the Raf/MEK/MAPK pathway is both necessary and sufficient for stimulating nuclear translocation of FOXM1. FOXM1 nuclear entry is abolished by incubation with the MEK1/2 inhibitor U0126 at S phase. Activating the Raf/MEK/MAPK pathway with ATA stimulates FOXM1 nuclear entry. This activating effect is abrogated by U0126, affirming that the effect is mediated via MEK1/2. The stimulatory effect on FOXM1 by the Raf/MEK/MAPK pathway is an interesting contrast to the inhibitory effect on FOXOs by the PI3K-PKB pathway (Burgering and Kops, 2002; Van Der Heide et al., 2004). The PI3K-PKB pathway exerts a negative effect on FOXO activity via PKB-mediated phosphorylation and nuclear export of FOXO proteins. It is worth noting that similar treatment of BJ1 cells with the PI3K inhibitors Wortmannin and LY294002 did not have any significant effect on FOXM1 subcellular localization (R.Y.M.M. and K.-M.Y., unpublished). Both FOXM1 and FOXO factors have previously been reported to regulate cyclin B1 expression (Alvarez et al., 2001; Leung et al., 2001). The different FOX factors may be integrating signals from multiple signaling pathways to effect changes in cell cycle progression.

Besides nuclear translocation, activation of the Raf/MEK/MAPK pathway strongly enhances the ability of FOXM1 to transactivate cyclin B1 promoter in transient reporter assays. ChIP assays affirm binding of cyclin B1 promoter by

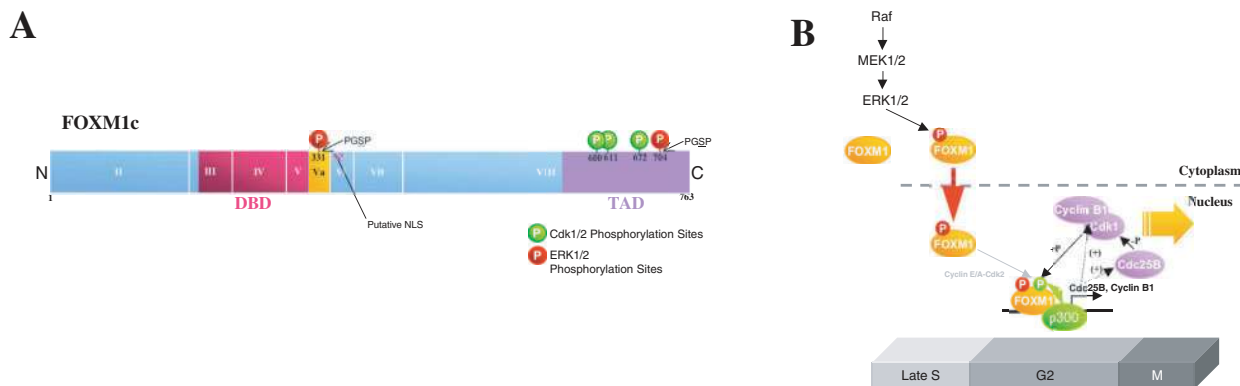


Fig. 7. A model to illustrate the cell cycle-dependent regulation of FOXM1c function. (A) Location of the PGS331P and PGS704P ERK1/2 phosphorylation sites (shown in red circles). The 331 motif within exon Va (yellow) is close to the DNA binding domain (DBD, red) and a putative NLS (sequence of the NLS is provided in Fig. S2 of the supplementary material). The 704 motif is in close proximity to the Cdk phosphorylation sites (green circles) identified within the transactivating domain (TAD, violet). (B) During late S phase, the Raf/MEK/MAPK pathway phosphorylates FOXM1 and stimulates its nuclear translocation and transactivating activity. Active cyclin-Cdk2 (Cyclin E/A-Cdk2) phosphorylates FOXM1 at the Cdk1/2 phosphorylation sites within the TAD. This promotes recruitment of p300/CBP and transcription of FOXM1 target genes like cyclin B1 and Cdc25B. Cdk1 becomes activated by binding of cyclin B1 and dephosphorylation by Cdc25B. Active cyclin B1-Cdk1 phosphorylates FOXM1 and further promotes transcription of cyclin B1 and Cdc25B. This positive-feedback loop produces a burst of cyclin B1-Cdk1 activity to drive the cell into mitosis.

FOXM1 *in vivo*. The enhancement of cyclin B1 promoter activity by caMEK1 requires coexpression of functional FOXM1, supporting the idea that the enhancing effect is mediated through FOXM1. Unexpectedly, caMEK1 exerts an enhancing effect on FOXM1c but not FOXM1b. It has previously been demonstrated using semi-quantitative RT-PCR, that FOXM1b is the major isoform expressed in skin whereas FOXM1c is abundantly expressed in a variety of primary and secondary cultured cell lines (Teh et al., 2002). Similar RT-PCR analysis conducted in this study reveals FOXM1c as the predominant isoform expressed in BJ1, NIH3T3 and several tissues rich in mitotically active cells. High levels of the FOXM1b transcript are only detectable in testis.

Sequence analysis identifies two ERK1/2 target sites (PGS331P and PGS704P; Fig. 7A) that are conserved among the human, mouse and rat FOXM1 proteins. Site-directed mutagenesis and transient reporter assays demonstrate that both the 331 and 704 motifs are of regulatory significance. Increased phosphorylation of FOXM1 at serine residues upon ATA treatment is also consistent with FOXM1 being a direct target of Raf/MEK/MAPK signaling. The 331 motif is located within exon Va. The difference in responsiveness of the two FOXM1 isoforms to caMEK1 enhancement pinpoints exon Va (containing 15 amino acids), which is missing in FOXM1b, as the regulatory site. The close proximity of exon Va to a putative bipartite nuclear localization signal (NLS; Fig. S2 in supplementary material) identified within exon VI suggests that phosphorylation at exon Va may be relevant to regulation of FOXM1 nuclear entry. It is interesting to note that the 704 motif, localized within the transactivation domain, is close to the cyclin-Cdk recruitment and Cdk1/2 phosphorylation sites recently shown to be important for recruitment of p300/CBP coactivators (Fig. 7A) (Major et al., 2004).

Consistent with our hypothesis that the Raf/MEK/MAPK pathway mediates its G2/M regulatory effect via activation of FOXM1 function, inhibition of Raf/MEK/MAPK signaling at S and G2/M phases suppresses the G2/M-stimulated expression of

cyclin B1 at both RNA and protein levels. The dramatic reduction in protein levels associated with the milder decrease in transcript levels suggests active regulation of cyclin B1 by protein degradation. Interestingly, decreased expression of another putative FOXM1 target Cdc25B also precedes the G2/M delay revealed by monitoring of G1 re-entry.

Based on these findings, we propose a model to depict regulation of FOXM1 function by the Raf/MEK/MAPK pathway and Cdk1/2 (Major et al., 2004) and hence cell cycle progression through G2/M (Fig. 7B). FOXM1c is mainly localized in the cytoplasm in S phase. Activation of the Raf/MEK/MAPK pathway in late S phase leads to phosphorylation of FOXM1c at the ERK1/2 sites. This phosphorylation stimulates the nuclear import and transactivating activity of FOXM1c. In the presence of active cyclin-Cdk2 complexes (cyclin E/A-Cdk2), FOXM1c becomes phosphorylated at the C-terminal Cdk1/2 sites and recruits p300/CBP for transcriptional activation of target genes like cyclin B1 and Cdc25B. Elevated cyclin B1 levels generate more cyclin B1-Cdk1 complexes. These complexes become activated by Cdc25B, which also accumulates and activates Cdk1 by dephosphorylation. The heightened Cdk1 activity will further phosphorylate and activate FOXM1c in a positive-feedback loop, resulting in a burst of synthesis of active cyclin B1-Cdk1 to drive the cell into mitosis. This mode of regulation has a switch-like property similar to other inputs previously shown to regulate Cdk1 activity (O'Farrell, 2001) and is a very interesting parallel to the transcriptional regulation of the 'CLB2' cluster of genes (also peaking at G2/M) by the forkhead transcription factors Fkh1 and Fkh2 in yeast (Kumar et al., 2000; Pic et al., 2000; Zhu et al., 2000).

In summary, we demonstrate that nucleocytoplasmic shuttling of FOXM1 is regulated in a cell cycle-dependent manner; FOXM1 is translocated to the nucleus just before G2 phase. Activation of the Raf/MEK/MAPK pathway is required for promoting the nuclear entry and transactivating activity of FOXM1. Expression of FOXM1 target genes and G2/M

progression is suppressed when Raf/MEK/MAPK signaling is inhibited. Our data provide the first evidence to support a functional link between Raf/MEK/MAPK signaling and FOXM1 in the regulation of cell cycle progression through G2/M.

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