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Over-expression of FoxM1 stimulates cyclin B1 expression

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Abstract FoxM1 (previously named WIN, HFH-11 or Trident) is a Forkhead box (Fox) transcription factor widely expressed in proliferating cells. Various findings, including a recent analysis of FoxM1 knockout mice, suggest that FoxM1 is required for normal S–M coupling during cell cycle progression. To study the regulatory role of FoxM1 and its downstream regulatory targets, three stably transfected HeLa lines that display doxycycline (dox)-inducible FoxM1 expression were established. Over-expression of FoxM1 by dox induction facilitates growth recovery from serum starvation. Quantitation of cyclin B1 and D1 levels using flow cytometric, Western and Northern analyses reveals that elevated FoxM1 levels lead to stimulation of cyclin B1 but not cyclin D1 expression. Transient reporter assays in the dox-inducible lines and upon co-transfection with a constitutive FoxM1 expression plasmid suggest that FoxM1 can activate the cyclin B1 promoter. **© 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Forkhead box transcription factor; Doxycycline-inducible gene expression; Cell cycle; Cyclin B1; Promoter

1. Introduction

The cell cycle is a highly regulated event with periods of DNA replication (S phase) alternating with periods of chromosomal segregation and cell division (M phase). S and M phases are separated by preparative phases (G1 and G2) but are tightly coupled so that chromosomal DNA is replicated precisely once per cell cycle. Failure of this S–M coupling results in aneuploidy (gain or loss of DNA) leading to abnormality in cell growth and function [1].

Regulation of S–M coupling is best understood in yeast [2]. To maintain stable DNA ploidy in dividing cells, each origin of replication fires only once per cycle and the pre-replication complex at each origin is only formed after exit from M phase. Pre-replication complex formation and hence DNA re-replication is prevented during S, G2 and early M phase by the inhibitory action of Clb1-4-Cdk1 kinases (yeast equivalents of mammalian cyclin A-Cdk1 and cyclin B1-Cdk1), which are also required for triggering M phase. Proteolysis of Clbs at anaphase terminates Clb1-4-Cdk1 action. In the presence of Cdc6, pre-replication complexes can be re-formed upon re-entry into the cell cycle. Therefore, Clb1-4-Cdk1 kinases play a major role in S–M coupling by suppressing DNA re-replication and promoting M phase entry.

Cyclin-Cdk activities are partly regulated by periodic changes in cyclin transcription. Transcriptional regulation of cyclin synthesis has been extensively studied in yeast *Saccharomyces cerevisiae* due to its powerful genetics. In *S. cerevisiae*, the activation of G1 cyclins Cln1 and -2 and expression of S cyclins Clb5 and -6 at START is coordinated by transcriptional complexes SBF (Swi4 and Swi6) and MBF (Swi6 and Mbp1) reviewed in [3,4]. Another transcriptional complex, Mcm1/Sff (Swi five factor), controls the cell cycle-dependent expression of the major mitotic cyclin Clb2 [5]. Most recently, the forkhead/winged helix protein Fkh2 was demonstrated to be a component of Sff and deletion of *FKH2* was shown to dramatically reduce *CLB2* mRNA levels [6–8]. Much less is known about the transcriptional regulation of mitotic cyclins in higher eukaryotes although a recent molecular dissection of the human cyclin B1 promoter has identified regions important for constitutive and cell cycle-dependent expression of cyclin B1 [9,10].

In higher eukaryotes, E2Fs have been proposed to be the mammalian functional homologs of the yeast Mbp1/Swi4/Swi6 family of proteins because of their critical role in regulating the G1/S checkpoint. Recent structural analyses of the E2F and Mbp1 DNA binding domains support this relatedness as both domains display a folding pattern similar to forkhead/winged helix domains [11,12].

Forkhead box (Fox)M1, previously known as HFH-11, Trident and FKL16, is a Fox transcription factor expressed ubiquitously in many if not all cell types undergoing proliferation [13–16]. Several different lines of evidence suggest that FoxM1 is required for proper cell cycle function. First, in Rat-1 cells synchronized by serum starvation, FoxM1 levels increase at the start of DNA replication and persist until the end of mitosis [16]. Second, Western blots reveal phosphorylation and degradation of FoxM1 upon entry into M phase [16]. This M phase phosphorylation explains why a 3′ partial FoxM1 cDNA (named MPP2: M Phase Phosphoprotein 2) was previously isolated in an expression screen for clones encoding substrates of M phase kinases [17]. The presence of multiple destruction box sequences (RXXL, X = any amino acid) at the amino-terminus of FoxM1 suggests that FoxM1, like mitotic cyclins, is degraded before M phase exit. Third,

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FoxM1/Trident knockout mice generated by Korver et al. [18] exhibit cell division defects. Homozygous mutant mice die in the perinatal period and examination of mutant embryos reveals DNA polyploidy in cardiomyocytes and hepatocytes. This finding suggests that FoxM1 is required for normal S–M coupling of the cell cycle as loss of suppression of DNA re-replication and/or defects in mitosis are known to lead to polyploidy. Fourth, transgenic over-expression of FoxM1/HFH-11 in mice was recently demonstrated to accelerate hepatocyte regeneration upon partial hepatectomy [19].

The apparent cell cycle regulatory role in animal models warrants testing of FoxM1 in cultured cells more amenable to cell cycle analysis. To investigate the cell cycle regulatory role of FoxM1 and to identify FoxM1-regulated genes, we have established several doxycycline (dox)-inducible FoxM1-expressing HeLa lines. Using these lines, we demonstrate that over-expression of FoxM1 alters cell cycle kinetics by facilitating progression through G2/M. This facilitatory effect is consistent with maximum levels of FoxM1 detected in cycling G2/M cells by flow cytometry. Further study of cyclin expression using flow cytometry, Western and Northern blot analyses indicates that FoxM1 activates the expression of cyclin B1 but not of cyclin D1 which explains why FoxM1 over-expression in HeLa cells accelerates progression through G2/M. Finally, we show that FoxM1 activates the cyclin B1 promoter in luciferase reporter gene assays. Our data provide evidence that FoxM1 exerts its S–M coupling function by regulating the expression of cyclin B1.

2. Materials and methods

2.1. Generation of FoxM1-inducible HeLa cells

Human FoxM1 cDNA (GenBank accession number U83113) was PCR-amplified using a High-Fidelity polymerase (Clontech) for subcloning into the Tet-On™ mammalian expression plasmid pTRE (Clontech) to generate pTRE-FoxM1. Nucleotide sequence of the construct was confirmed by DNA sequencing. HeLa Tet-On™ cells (Clontech) were maintained in Dulbecco's modified Eagle medium (Sigma) supplemented with 10% Tet-On™ approved fetal bovine serum (FBS) (Clontech), streptomycin, penicillin and 100 mg/l Geneticin® (Gibco-BRL) at 37°C in a humidified atmosphere containing 5% (v/v) CO_2. 40 μg pTRE-FoxM1 and 2 μg of the selection plasmid, pTK-Hyg, were delivered into HeLa Tet-On™ cells by electroporation (at 950 μF and 0.22 kV/cm). Stably transfected clones were selected and maintained in the presence of 50 mg/l hygromycin B (Gibco-BRL) as described by the manufacturer.

2.2. Preparation of cell extracts and analysis by Western blotting

Cells were lysed in denaturing lysis buffer (10 mM Tris–HCl, pH 7.4, 1 mM sodium orthovanadate, 1% SDS). Aliquots of cell lysates were subjected to SDS–PAGE in a 12% Laemmli gel and blotted onto HyBond™-C Super nitrocellulose membrane (Amersham Pharmacia Biotech). Anti-MPP2 antibodies (K19 and C20), anti-cyclin B1 (H-433) and anti-cyclin D1 (R-124) were purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA. Anti-MPP2 antibodies recognize the FoxM1 protein from human, rat and mouse. Antigen–antibody complexes were detected using a secondary antibody conjugated to horseradish peroxidase and visualized using the SuperSignal® enhanced chemiluminescence detection system (Pierce Chemical).

2.3. Cell proliferation assays, analysis of cell cycle distribution and cell synchronization

A fixed number of cells was seeded onto 24-well culture plates. After serum starvation overnight, cells were replenished with FBS-containing culture medium with or without dox (2 μg/ml). At daily intervals, cells were trypsinized and those that excluded trypan blue were counted as viable cells under light microscopy. Cell proliferation was also assayed using the Cell Proliferation kit (MTT) from Boehringer Mannheim. Analysis of cell cycle distribution was performed by flow cytometry as follows. Ethanol-fixed cells were treated with DNase-free RNase followed by propidium iodide (PI) staining (250 μg/ml in PBS with 1% Triton™ X-100). The PI-stained cells were detected for far-red fluorescence intensity using the EPICS Elite *ESP* high performance cell sorter from Coulter Electronic. Data were analyzed by the cell cycle analysis software, ModFit LT version 2.0 (Verity software House), to tabulate percentages of cells at G0/G1, S and G2/M phases. Cell growth was arrested at S phase and at the G1/S junction by treatment with L-mimosine (0.5 mM) for 16 h in serum-containing culture medium [20].

2.4. Bivariate antigen-DNA flow analysis

Cells were fixed in ethanol as described above and washed three times with PBS supplemented with 1% FCS. Fixed cells were immunostained with primary antibodies (against MPP2, cyclin B1 or cyclin D1) or isotype control IgG followed by the corresponding secondary antibodies (anti-rabbit IgG for anti-MPP2 and anti-cyclin B1; anti-mouse IgG for anti-cyclin D1) conjugated with fluorescein isothiocyanate (FITC). After antibody binding, cellular DNA was stained with PI as mentioned above. Cells were detected by FITC and PI fluorescence using a 514-nm bandpass filter and 600-nm red pass filter, respectively. Data were interpreted using EXPO2™ flow cytometer analysis software (Coulter Electronic).

2.5. Transient reporter assays and Northern analysis

The cyclin B1 promoter (−831 to +54; [9,10]) was PCR-amplified from HeLa genomic DNA while the cyclin D1 promoter (−1179 to +162; [21]) was subcloned as a 1.34-kb *Cla*I–*Nco*I fragment from the plasmid pRc/ND1-A, which carries a PRAD1 gene fragment. Both promoter fragments were cloned upstream of the firefly luciferase reporter gene in pGL3-Basic (Promega). PCR-based mutagenesis of the cyclin B1 promoter was performed using the Advantage-HF2 PCR kit from Clontech according to Harlow et al. [22]. All constructs were checked by DNA sequencing. Superfect (Qiagen) and FuGENE 6 (Boehringer) were used as transfection reagents for HeLa and NIH3T3 cells, respectively. As an internal control for transfection efficiency, each sample was co-transfected with 2 ng of the *Renilla* luciferase vector pRL-SV40. The Promega dual-luciferase reporter assay system was used to sequentially quantitate firefly and *Renilla* luciferases in cell lysates to measure the activity of the tested promoters normalized against the internal control. The CMV promoter-driven expression plasmid, pcDNA3-FoxM1, was constructed by subcloning the 3.3-kb *Eco*RI–*Xho*I FoxM1 fragment excised from pTRE-FoxM1 into pcDNA3 (Invitrogen). All experiments were conducted in triplicate using independent cultures. For Northern analysis, total RNAs were isolated using the Trizol reagent (Gibco-BRL) and separated on 1% agarose–formaldehyde gels, blotted onto HyBond™-N nylon membrane (Amersham Pharmacia Biotech) and probed with ^{32}P-labelled FoxM1 and cyclin B1 cDNA fragments. Blots were stripped and reprobed with GAPDH to check for loading difference.

3. Results

3.1. Cell cycle-dependent expression of FoxM1

To examine more closely the cell cycle-dependent expression of FoxM1 at the single-cell level, expression of FoxM1 protein was quantitated in dividing HeLa cells using bivariate FoxM1/FITC and DNA/PI flow cytometric analysis (Fig. 1A,B). This single-cell analysis indicates that FoxM1 expression is first initiated in a sub-population of G1 cells, increases slightly during S phase and reaches peak levels in a subset of G2/M cells with doubled DNA content (Fig. 1A). The expression profile is FoxM1-specific as FITC signal could be competed away by pre-incubation with FoxM1 blocking peptide (Fig. 1B). This strong expression in G2/M supports the S–M coupling role predicted based on phenotypic analysis of FoxM1/Trident knockout mice [18].

3.2. Establishment of FoxM1-inducible HeLa cell lines

Disruption of the *FoxM1/Trident* gene function in mouse

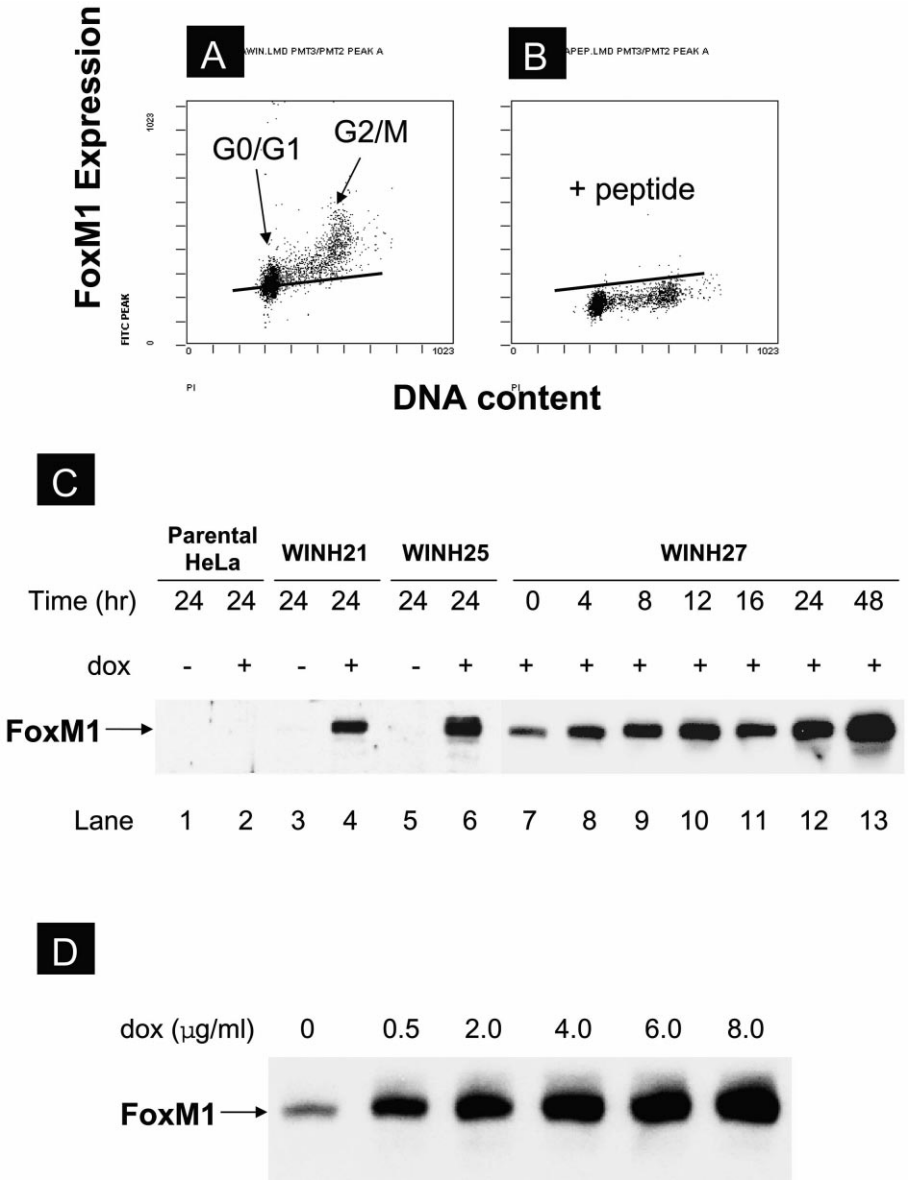

Fig. 1. Single-cell analysis of FoxM1 expression and characterization of FoxM1-inducible HeLa lines. A,B: Bivariate flow cytometric analysis of FoxM1 expression and cellular DNA content in HeLa cells. FoxM1 was detected in cycling cells by immunostaining with anti-MPP2 antibody and FITC-conjugated secondary antibody. Cellular DNA content was measured by PI staining. The doubly stained cells were analyzed by flow cytometry as mentioned in Section 2. HeLa cells were immunostained in the absence (A) and presence (B) of a specific blocking peptide which competes for primary antibody binding. Each spot represents an individual cell with a specific FoxM1 level (*y*-axis) and DNA content (*x*-axis). Identical reference lines were drawn in corresponding panels to assist comparison of expression levels. C,D: Western analysis was conducted to follow dox-inducible FoxM1 over-expression in three stably transfected HeLa lines. C: Time-dependent over-expression of FoxM1. The HeLa lines, WINH21, WINH25 and WINH27, were treated with dox (2 μg/ml) and harvested at the indicated time points after dox induction. Parental HeLa line was used as a negative control. After longer exposure, background level of FoxM1 expression was also detectable in lanes 1, 2, 3 and 5. D: Dosage-dependent over-expression of FoxM1 in WINH27. WINH27 cells were treated for 24 h with dox at the indicated concentrations (from 0 to 8 μg/ml). 10 μg of whole cell lysate was analyzed in each sample as described in Section 2.

leads to polyploidy in hepatocytes and cardiomyocytes [18]. This phenotype can be explained by a cell cycle defect in M phase entry and/or uncontrolled DNA re-replication. However, Korver et al. [16] could not detect any effect on cell cycle kinetics by ectopic over-expression of FoxM1/Trident in transfected cells. To further investigate the cell cycle regulatory role of FoxM1 in cultured cells, we set out to conditionally over-express FoxM1 in HeLa cells using the Tet-On™-inducible gene expression systems (Clontech). Multiple human FoxM1 isoforms have been reported [14,15]; the FoxM1 cDNA expressed in our cell lines encodes the major ubiquitous isoform (FoxM1c encoded by Class b transcripts as described in [14]).

After screening 60 stably transfected HeLa cell lines by Western blot analysis, we selected three lines (WINH21, WINH25 and WINH27) displaying FoxM1 expression inducible by dox (a tetracycline derivative) (Fig. 1C, 2 μg/ml for 24 h). In comparison, the parental HeLa cell line shows no detectable induction of FoxM1 expression (Fig. 1C, lanes 1 and 2). In WINH27, which has the highest basal FoxM1 level (compare lanes 3, 5 and 7), expression doubles within 4 h of induction and these high levels persist even 48 h after induc-

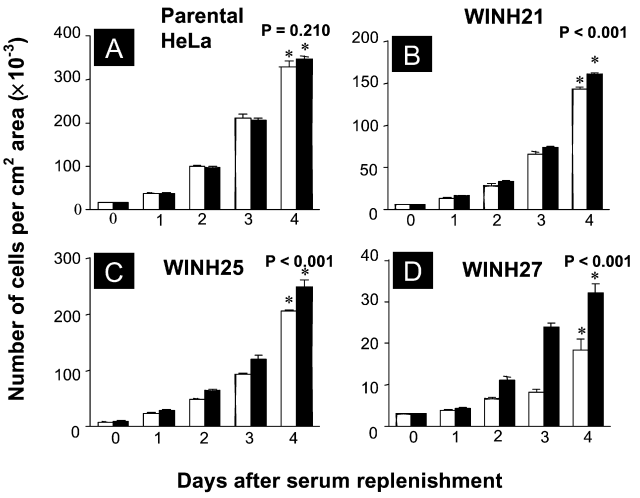

Fig. 2. Dox-inducible FoxM1 over-expression facilitates growth recovery from serum deprivation. The indicated cell clones were serum-starved overnight prior to serum replenishment in the presence (black bar) or absence (white bar) of dox stimulation (2 μg/ml) for an experimental period of four days. The number of viable cells was counted daily as described in Section 2 and expressed as number of cells per square centimeter. Results shown are the means ± S.E.M. of four countings. For each of the three FoxM1-inducible cell lines, the *P*-value (calculated by GraphPad Prizm® software version 2.01) indicates a significant difference between the day-4 samples with and without dox treatment (asterisks).

tion (Fig. 1C). Background levels of FoxM1 were also detectable in lanes 1, 2, 3 and 5 after longer exposure (data not shown). FoxM1 induction is dependent on dox concentration; increase in dox concentration from 0 to 8 μg/ml gradually enhances FoxM1 expression in WINH27 (Fig. 1D). WINH21 shows similar time and dosage dependency in their dox-mediated FoxM1 over-expression while WINH25 displays more rapid induction kinetics (data not shown).

3.3. Over-expression of FoxM1 facilitates growth recovery after serum starvation

To investigate the effect of FoxM1 induction on cell growth, dox-inducible cell lines were seeded at ~10 000 cells/cm^2 and serum-starved overnight to slow down initial cell growth. The number of viable cells per square centimeter was counted daily for 4 days, using the trypan blue dye exclusion method, in the presence or absence of dox in serum-containing medium (Fig. 2). A growth facilitatory effect was observed in both WINH21 and WINH25 after dox induction (Fig. 2B,C). The greatest effect was detected in WINH27, which could be seeded at a lower cell density (~3000 cells/cm^2), and dox induction led to a much faster growth recovery (Fig. 2D). The growth effect in WINH27 became more comparable to WINH21 and WINH25 when seeded at ~10 000 cells/cm^2. The ability of WINH27 but not WINH21 and WINH25 to survive at lower cell densities may be related to its higher basal level of FoxM1 expression (Fig. 1). In contrast, the control parental HeLa line did not show significant growth difference (Fig. 2A) and no significant cell death was detectable in any cell line. The effect of dox induction on cell proliferation could not be observed if the initial serum starvation step was omitted. The growth effect was also observed in a cell proliferation assay (MTT-based) for viable cells (data not shown).

3.4. FoxM1 induction in synchronized cells facilitates transition through the G2/M phase

L-Mimosine is a potent inhibitor of DNA replication that prevents formation of replication forks [20]. To determine the underlying change in cell cycle kinetics that contributes to faster growth recovery after serum deprivation, we followed the growth kinetics of WINH27 cells synchronized at the G1/S boundary and S phase by L-mimosine treatment. Typically, after 16 h of drug treatment, ~75% of cells became arrested at the G1/S boundary with around 25% of cells in S phase (Fig. 3, zero time points). After L-mimosine treatment, WINH27 cells were placed in serum-free medium with or without dox and the change in cell cycle distribution was determined by DNA/PI analysis over a period of 12 h (Fig. 3). The major G1/S population progressed through S phase with comparable kinetics in both dox-induced and non-induced cells. Interestingly, a new G1 peak (indicated by arrows) corresponding to cells previously in S phase appeared first in the dox-treated cells after 4 h. However, the corresponding peak in the non-induced cells appeared later and grew much more slowly. This finding suggests that FoxM1

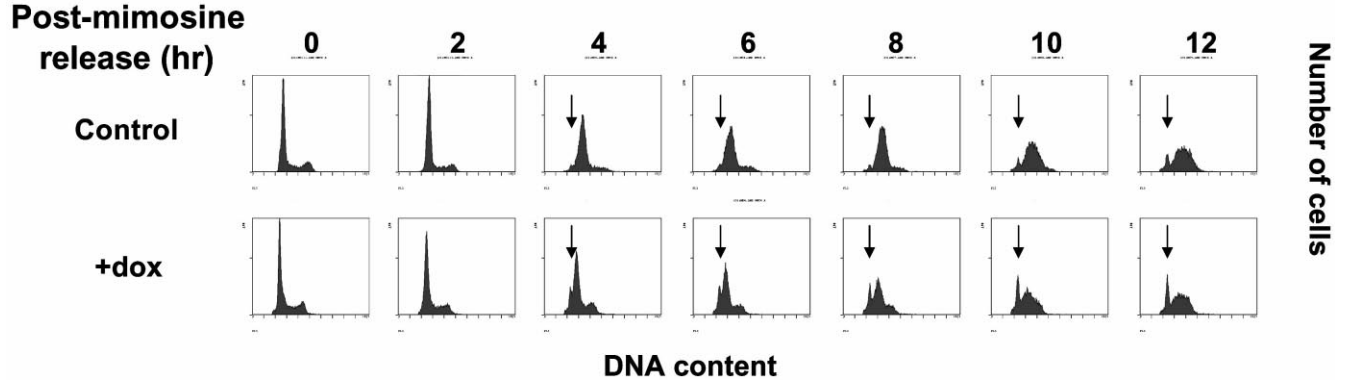

Fig. 3. FoxM1 over-expression accelerates transition through G2/M. WINH27 cells were synchronized in S phase and at the G1/S junction by overnight (16 h) administration of L-mimosine (0.5 mM). Thereafter, cells were released from L-mimosine arrest by replenishment with serum-free medium with (lower panels) or without (upper panels) dox (2 μg/ml). At indicated time intervals, cells were fixed in cold 70% ethanol and prepared for DNA analysis as described in Section 2. Histograms indicate number of cells (*y*-axis) plotted against corresponding cellular DNA content (*x*-axis). Arrows denote gradual appearance of the new G1 peaks.

over-expression facilitates progression of cells through G2/M. Accelerated G2/M progression was also observed in WINH21 and WINH25, but not in the parental HeLa line, similarly analyzed following cell synchronization (data not shown).

3.5. Over-expression of FoxM1 induces cyclin B1 but not cyclin D1 expression

To study the molecular basis of FoxM1 action, we analyzed the effect of FoxM1 over-expression on cyclin expression in our dox-inducible cell lines. Since endogenous FoxM1 and cyclin expression is cell cycle-dependent, perturbation of cell cycle kinetics following FoxM1 over-expression will change the absolute level of expression of the different proteins. To prevent the cell cycle effect of FoxM1 over-expression from interfering with expression analysis, all cells in subsequent expression analysis were grown in serum-containing media. Without serum deprivation, FoxM1 over-expression by dox induction or transient transfection (see below) did not have any significant effect on cell cycle distribution as determined by flow cytometry (data not shown).

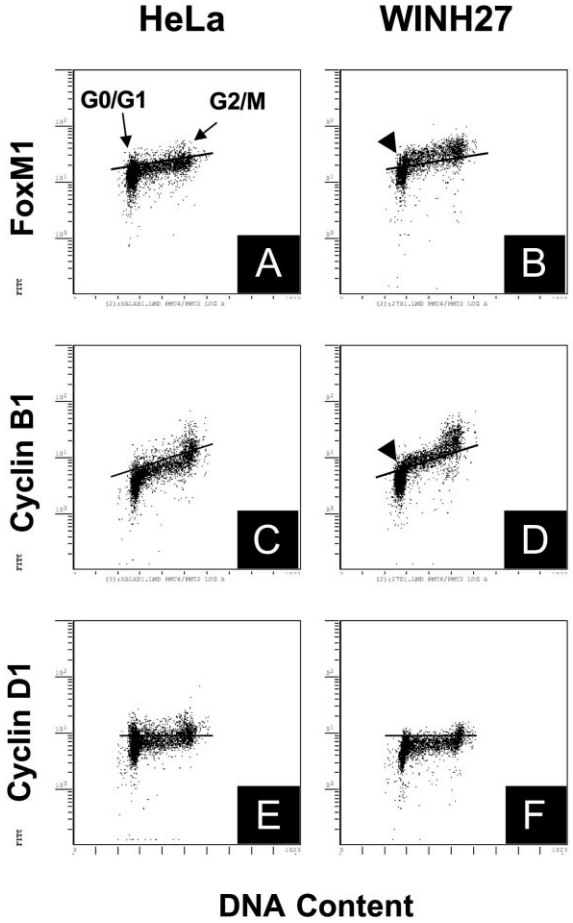

Fig. 4. Comparison of FoxM1 and cyclin expression in WINH27 and parental HeLa cells by bivariate flow analysis. Dividing parental HeLa (A,C,E) or WINH27 (B,D,F) cells were fixed and immunostained with different primary antibodies (anti-MPP2 (A,B), anti-cyclin B1 (C,D) or anti-cyclin D1 (E,F)), followed by the corresponding FITC-conjugated secondary antibodies. After PI staining to quantitate cellular DNA content, the doubly stained cells were subjected to flow analysis as described in Section 2. Expression of different proteins was displayed in log scale to reduce scattering at the y-axis and as a result the FoxM1/DNA profile shown differs from that of Fig. 1. Identical reference lines were drawn in corresponding panels to aid comparison. Arrowheads (B,D) indicate the G1/S junctions that show a smooth curvature in WINH27 as a result of expression increases in S and G2/M cells.

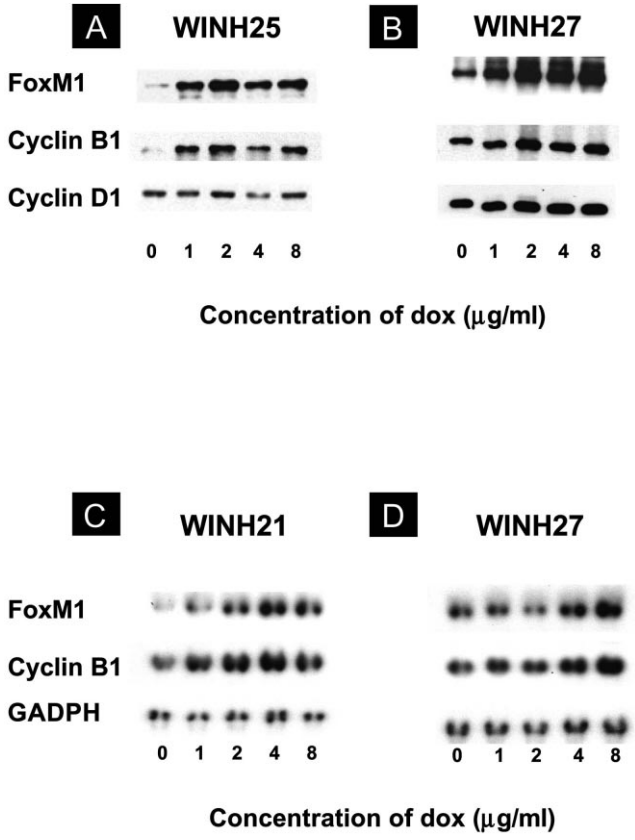

Fig. 5. Dox-induced over-expression of FoxM1 is associated with increased expression of cyclin B1. A,B: Western analysis. WINH25 (A) and WINH27 (B) cells were treated with different doses of dox (0–8 μg/ml) for 9 and 24 h, respectively. Cell lysates were prepared for Western analysis as described in Section 2. 10 μg of whole cell lysate was analyzed in each lane. C,D: Northern analysis. WINH21 (C) and WINH27 (D) cells were subjected to dox treatment of increasing doses to stimulate FoxM1 over-expression. After 24 h, cells were harvested for Northern analysis and levels of FoxM1 and Cyclin B1 transcripts were detected. 20 μg of total RNA was loaded per lane. Blots were stripped and reprobed with GAPDH to control for possible loading difference.

Because FoxM1 accelerates transition through G2/M, cyclin B1, a mitotic cyclin, was analyzed with cyclin D1 serving as a control. Notable differences were observed when we compared FoxM1 and cyclin protein expression in WINH27 to the parental HeLa line using bivariate flow analysis (Fig. 4). The WINH27 cell line was chosen for this experiment due to its high basal level of FoxM1 expression relative to the parental cell line, especially evident in the S and G2/M phases (compare Fig. 4A and B). Concomitant with the elevated FoxM1 levels, there is increased expression of cyclin B1 in the WINH27 cells relative to the parental HeLa cells, again especially evident at the S and G2/M phases (Fig. 4C,D) so that both the FoxM1 and cyclin B1 profiles show a smooth curvature at the G1/S junction (see corresponding arrowheads in Fig. 4B,D). This suggests that FoxM1 may induce cyclin B1 expression. In contrast, cyclin D1 levels appear the same in WINH27 and the parental cell line, showing that the FoxM1 effect on cyclin B1 is cyclin-specific (Fig. 4E,F). Isotype controls conducted using non-specific antibodies did not reveal

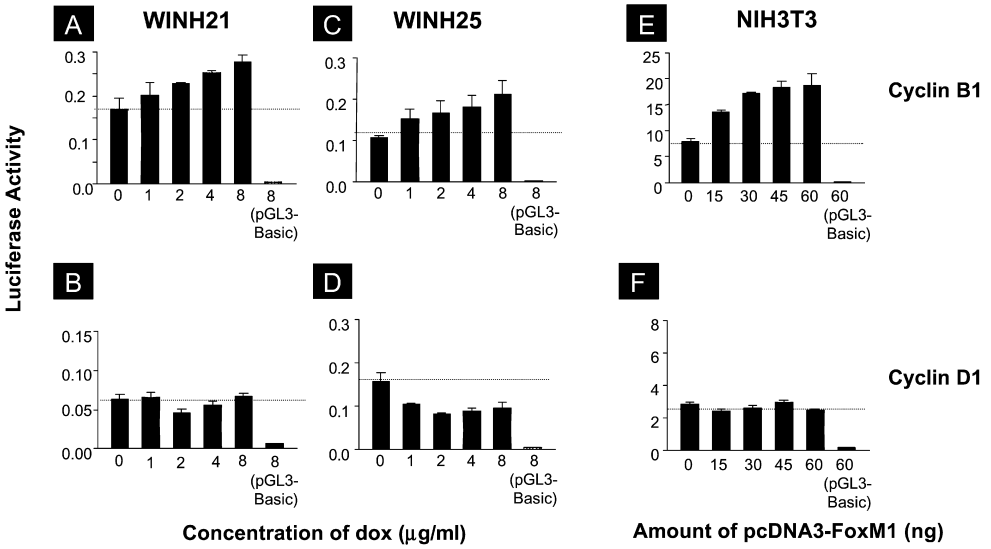

Fig. 6. FoxM1 over-expression stimulates cyclin B1 promoter activity. A–D: Over-expression by dox induction. WINH21 (A,B) and WINH25 (C,D) cells were transfected with luciferase reporters driven by cyclin B1 (A,C) or cyclin D1 (B,D) promoters. 16 h after transfection, cells were treated with dox at different concentrations (0–8 μg/ml). Luciferase assay was performed as described in Section 2 24 h (A,B) or 9 h (C,D) after dox treatment. Reporter gene activity was expressed as a ratio of firefly luciferase activity to control *Renilla* luciferase activity. E,F: Over-expression by transient co-transfection. NIH3T3 cells were transfected with different amounts of pcDNA3-FoxM1 plus either the cyclin B1 (E) or cyclin D1 (F) luciferase reporter. pcDNA3 was added to normalize the amount of pcDNA3 vectors across all transfections. Luciferase assay was performed 48 h after transfection as described in Section 2. Results shown are the means ± S.E.M. of triplicate cultures.

any difference in background signals between WINH27 and the parental HeLa line (data not shown).

The inducing effect of FoxM1 on cyclin B1 expression was also observed in Western blot analysis (Fig. 5). In both WINH25 and WINH27 cells, induction of FoxM1 expression resulting from increasing doses of dox was associated with increased cyclin B1 levels (Fig. 5A,B). Cyclin B1 levels closely followed the variations in FoxM1 expression while cyclin D1 levels stayed relatively constant. A similar correlation of FoxM1 and cyclin B1 expression at the RNA level was also detected in Northern blots (Fig. 5C,D). These data suggest that FoxM1 as a transcription factor induces cyclin B1 expression.

3.6. *FoxM1 activates the cyclin B1 but not the cyclin D1 promoter*

If FoxM1 is a Fox transcription factor that induces cyclin B1 expression, it would be expected to activate the cyclin B1 promoter. Both the cyclin B1 and D1 promoters have been cloned and sequenced, and the transcriptional start sites defined [9,10,21]. To study the effect of FoxM1 over-expression on the activity of the cyclin B1 and cyclin D1 promoters, we PCR-amplified both promoters for subcloning into pGL3-Basic (Promega) upstream of the firefly luciferase-coding sequences.

Each of the cyclin promoter-luciferase reporters plus pRL-SV40 (internal control) was transfected into WINH21 and WINH25, the dox-inducible lines with lower FoxM1 basal levels (see Fig. 1). After transfection, cells were treated with dox to stimulate FoxM1 over-expression and the effect on promoter activity was monitored using luciferase assays. When dox concentration was increased from 0 to 8 μg/ml, a gradual increase in cyclin B1 promoter activity was detected (Fig. 6A,C). At 8 μg/ml of dox, there was a 1.65-fold and 1.96-fold increase in cyclin B1 promoter activity in WINH21 and WINH25, respectively, when compared with the no dox controls. The cyclin D1 reporter did not show any increase in promoter activity when subjected to similar analysis (Fig. 6B,D); the difference in cyclin D1 response between the two lines may be due to clonal variation.

To further assess the activating effect of FoxM1 on cyclin B1 promoter activity, a CMV-driven FoxM1 expression plasmid, pcDNA3-FoxM1, was also co-transfected with the individual cyclin reporters into NIH3T3 cells. A dramatic stimulation of cyclin B1 reporter activity was observed with increasing amounts of the FoxM1 expression plasmid (Fig. 6E). The increase leveled off with 45 ng pcDNA3-FoxM1 and at greater than 45 ng a 2-fold stimulation of cyclin B1 promoter activity is similar in magnitude to the activating effect of β-catenin on cyclin D1 promoter activity [23]. When similarly assayed, activity of the cyclin D1 reporter did not show any FoxM1 dependency (Fig. 6F). Taken together, these findings show that FoxM1 over-expression enhances cyclin B1 expression by stimulating the cyclin B1 promoter.

3.7. *FoxM1-dependent activation of the cyclin B1 promoter does not require intact CDE and CHR elements*

Transcription of human cyclin B1 is regulated by two promoters, which direct constitutive and G2/M-stimulated expression of the gene [9,10]. Hwang et al. [10] identified a 65-bp region between the constitutive and G2/M-activated transcriptional start sites that is important for directing G2/M-stimulated cyclin B1 transcription. Within this 65-bp sequence, different *cis*-acting elements including the CCAAT box, CDE and CHR elements and GGCT repeats were shown to be important for cyclin B1 promoter activity (Fig. 7A) [10,24,25]. The CDE and CHR elements mediate the repressive effect of a p53-dependent, DNA damage-activated pathway on cyclin B1 transcription [25] and are also present in the promoters of *cyclin A*, *cdc25C* and *cdc2* genes [26].

To determine whether FoxM1 activation of the cyclin B1

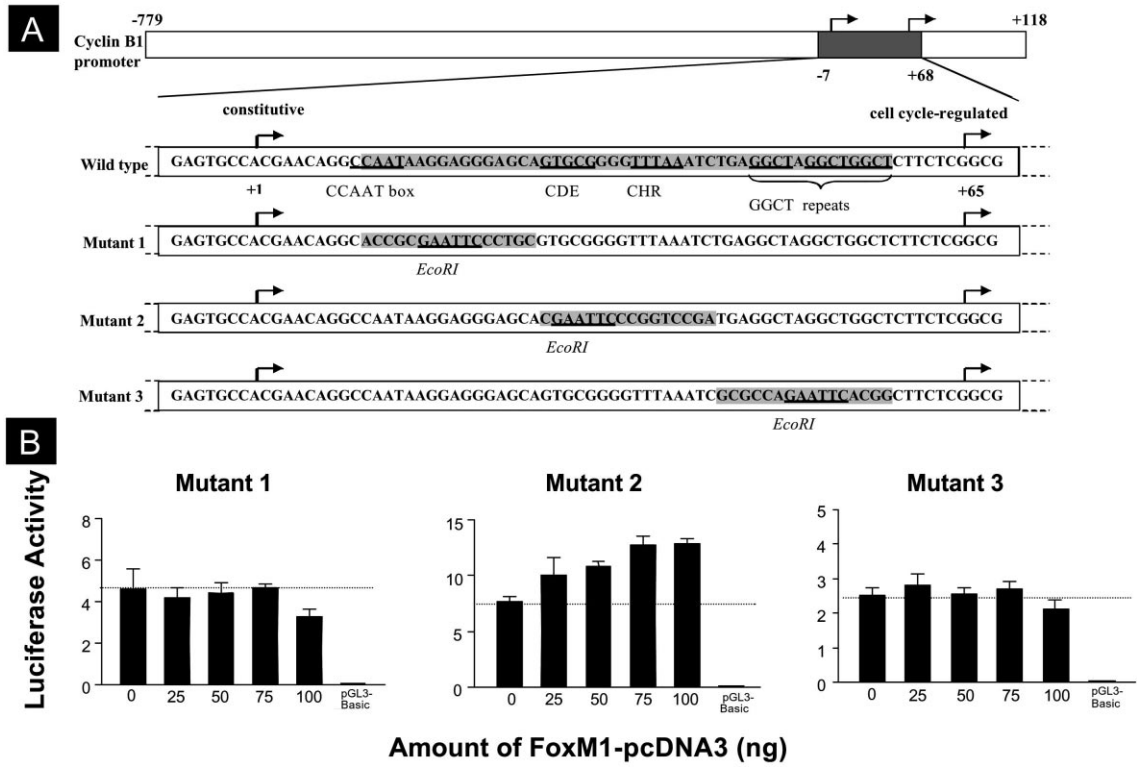

Fig. 7. FoxM1-dependent stimulation of cyclin B1 promoter activity does not require the CDE and CHR elements. A: Structure of the wild-type [10] and mutant cyclin B1 promoters. Consecutive 16-bp sequences (+11 to +26, +27 to +42, +43 to +58) were scrambled in Mutants 1, 2 and 3 to destroy the CCAAT box, CDE and CHR elements, and GGCT repeats, respectively. *Eco*RI sites were introduced into the mutant sequences to assist identification of mutant clones. B: Luciferase assay of mutant reporters. HeLa cells were transfected with luciferase reporters driven by the different mutant cyclin B1 promoters and increasing amounts of pcDNA3-FoxM1. pcDNA3 was added to normalize the amount of pcDNA3 vectors across all transfections. Luciferase assay was performed 48 h after transfection as described in Section 2. Reporter gene activity was expressed as a ratio of firefly luciferase activity to control *Renilla* luciferase activity. Results shown are the means ± S.E.M. of triplicate cultures.

promoter is mediated through the CDE and CHR elements, we scrambled the CDE/CHR sites and flanking sequences by PCR-based site-directed mutagenesis to generate promoter Mutants 1, 2 and 3 (Fig. 7A). Consecutive 16-bp sequences were substituted in these three mutants, with Mutant 2 covering the CDE/CHR sites. These mutant reporter constructs were individually co-transfected with pRL-SV40 (internal control) and different amounts of pcDNA3-FoxM1 into HeLa cells to test for their responsiveness to FoxM1 stimulation. Interestingly, the FoxM1-dependent activating effect could still be observed when CDE/CHR sites were substituted in Mutant 2 (Fig. 7B), strongly suggesting that promoter activation is not mediated by de-repression through the CDE and CHR elements. However, the dose-dependent activating effect of FoxM1 was abolished in both Mutants 1 and 3 (Fig. 7B). This implies that sequences substituted in Mutants 1 and 3, including the CCAAT box and GGCT repeats, are necessary for FoxM1 to exert its activating effect. Transfection into NIH3T3 cells gave similar results (data not shown).

4. Discussion

Mutation of the FoxM1 gene in mice revealed a cell cycle defect only in heart and liver cells although FoxM1 is expressed broadly in all cycling cells. To study whether FoxM1 plays a wider role in cell cycle regulation, an inducible gain-of-function approach was undertaken to investigate FoxM1 function in HeLa cells. Using multiple dox-inducible HeLa lines, we showed that FoxM1 over-expression facilitates growth recovery after serum deprivation and shortens transition through the G2/M phase of the cell cycle. Based on further bivariate flow analysis and Western and Northern blot analyses, FoxM1 over-expression was found to be associated with increased cyclin B1 expression. Further, we have shown that FoxM1 as a transcription factor activates the cyclin B1 promoter in transient reporter assays and this activating effect does not require the CDE/CHR sites within the human cyclin B1 promoter. The stimulatory effect of FoxM1 on cyclin B1 promoter activity is in support of FoxM1 playing a G2/M regulatory role.

The growth stimulatory effect of FoxM1 over-expression is reminiscent of the accelerated regeneration of hepatocytes in transgenic mice over-expressing FoxM1b/HFH-11B in liver [19]. In this animal model, growth stimulation of hepatocytes was only evident upon partial hepatectomy when mitogenic signals were generated. In contrast, significant growth stimulation became detectable in our cell-based system only upon serum deprivation. We argue 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 over-expression manifested. Our observation corroborates the notion that FoxM1 requires activation by mitogenic signals to

function. In the transgenic analysis, it was argued that heightened FoxM1b expression led to earlier S and M phase entry. Our demonstration that FoxM1 over-expression can activate cyclin B1 expression and shorten G2/M transition strongly supports earlier M phase entry upon FoxM1 induction.

In our study, FoxM1 over-expression by both dox induction of integrated transgenes and transient co-transfection led to increase in cyclin B1 promoter activity. The fact that FoxM1 levels peak in cells during G2/M and the observation that high cyclin B1 levels are always associated with high FoxM1 levels in cells examined by immunocytochemical methods (Yao et al., unpublished data) are consistent with FoxM1 being a regulator of cyclin B1 level in vivo. It is interesting to note that FoxM1 activation of the cyclin B1 promoter does not require the CDE/CHR sites, which were shown to mediate a negative effect on cyclin B1 promoter activity. Our current focus is to determine how FoxM1 acts on the cyclin B1 promoter by further mutagenesis and DNA binding analysis, and to test whether FoxM1 stimulation of cyclin B1 promoter activity is cell cycle-dependent. Further promoter analysis will also reveal the functional relationship between FoxM1 and other pathway(s) in regulating cyclin B1 promoter activity.

Transcriptional regulation of cyclins has been intensively studied in yeast. Yeast Fkh2, a forkhead transcription factor, was recently demonstrated to activate transcription of the major mitotic cyclin Clb2 and many other co-regulated genes within the 'CLB2' cluster [7,8]. Transcription of genes within the 'CLB2' cluster is cell cycle-regulated and peaks at G2/M. We speculate that FoxM1, as a forkhead transcription factor that shows peak expressions at G2/M and activates mitotic cyclin expression, may be the mammalian equivalent of yeast Fkh2 and thus may play a wider role in coordinating cell cycle-dependent transcriptional periodicities. Recently, genome wide analysis of gene expression using cDNA microarrays has revealed that, in addition to cyclins, many other genes are periodically transcribed. From 6 to 12% of all genes show cell cycle-dependent periodicity in yeast [27,28] while 6% of 8600 human transcripts analyzed display cell cycle-dependent variation in gene expression following serum stimulation of human fibroblasts [29]. These findings reflect the molecular complexity of cell cycle regulation at the transcriptional level and illustrate the challenge of understanding how transcription factors coordinate cell cycle-dependent periodicities. Expression profiling using the FoxM1-inducible cell lines will reveal how extensive a role FoxM1 plays in the regulation of cell cycle periodicities.

Based on phylogenetic analysis, the M, N and O classes of Fox factors are much more closely related to one another than to the A–K classes [13]. It is interesting to note that over-expression of members of the O subclass of Fox factors (AFX, FKHR and FKHR-L1) was recently shown to mediate G1 arrest by activating $p27^{kip1}$ expression [30]. Another member of the N class, CHES1, was demonstrated to complement a G2 checkpoint defect in yeast [31]. It will be worth examining whether FoxM1, which is required for proper cell cycle function, is actually involved in the regulation of the G2 checkpoint.

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