Live-cell monitoring of periodic gene expression in synchronous human cells identifies Forkhead genes involved in cell cycle control

Gavin D. Grant, Joshua Gamsby, Viktor Martyanov, Lionel Brooks 3rd, Lacy K. George, J. Matthew Mahoney, Jennifer J. Loros, Jay C. Dunlap, and Michael L. Whitfield

ABSTRACT We developed a system to monitor periodic luciferase activity from cell cycle-regulated promoters in synchronous cells. Reporters were driven by a minimal human E2F1 promoter with peak expression in G1/S or a basal promoter with six Forkhead DNA-binding sites with peak expression at G2/M. After cell cycle synchronization, luciferase activity was measured in live cells at 10-min intervals across three to four synchronous cell cycles, allowing unprecedented resolution of cell cycle-regulated gene expression. We used this assay to screen Forkhead transcription factors for control of periodic gene expression. We confirmed a role for FOXM1 and identified two novel cell cycle regulators, FOXJ3 and FOXK1. Knockdown of FOXJ3 and FOXK1 eliminated cell cycle-dependent oscillations and resulted in decreased cell proliferation rates. Analysis of genes regulated by FOXJ3 and FOXK1 showed that FOXJ3 may regulate a network of zinc finger proteins and that FOXK1 binds to the promoter and regulates DHFR, TYMS, GSDMD, and the E2F binding partner TFDP1. Chromatin immunoprecipitation followed by high-throughput sequencing analysis identified 4329 genomic loci bound by FOXK1, 83% of which contained a FOXK1-binding motif. We verified that a subset of these loci are activated by wild-type FOXK1 but not by a FOXK1 (H355A) DNA-binding mutant.

INTRODUCTION

The cell cycle, which governs the timing and progression of DNA replication (S phase) and mitosis (M phase), is one of the most tightly regulated cellular processes. Because misregulation can lead to catastrophic cellular events such as programmed cell death or cancer, determining the key regulators and pathways controlling periodic, cell cycle-dependent gene expression is critical. Cell cycle-regulated gene expression has been studied extensively by DNA microarray (Cho et al., 2001; Whitfield et al., 2002; Bar-Joseph et al., 2008), luciferase reporter assays (Hwang et al., 2004), and cell division in yeast (Welsh et al., 2004) and cell cycle–regulated gene expression has been studied extensively by DNA microarray (Cho et al., 2001; Whitfield et al., 2002; Bar-Joseph et al., 2008), luciferase reporter assays (Hwang et al., 2004), and cell division in yeast (Welsh et al., 2004) and cell division in yeast (Welsh et al., 2004). Although these assays can provide extraordinary levels of detail with regard to the number of genes measured, they are too costly and time-consuming to use as a screening strategy for genes that affect the regulation of periodic gene expression. To study genetic interactions between regulators and periodic gene expression, we developed a system to measure cell cycle–driven luciferase expression in living cells over multiple synchronous cell cycles in real time. We then used this system in conjunction with RNA interference (RNAi) to identify novel regulators of cell cycle progression.

The ability to follow promoter-regulated luciferase activity in live cells has been used successfully to probe the molecular nature of both circadian rhythms (Welsh et al., 2004) and cell division in yeast (Robertson et al., 2008). Recently a similar system has been used to...
show that progression through mitosis is independent of the circadian rhythm in Rat-1 fibroblasts (Yeom et al., 2010). In the case of mammalian circadian rhythms, cells transfected with a circadian-regulated promoter driving luciferase are synchronized, and luciferase activity is followed over time in a luminometer (Gamsby et al., 2009). In addition, circadian rhythms can be followed with this method in cells or tissue isolated from transgenic mice containing clock genes fused to luciferase at their endogenous loci (Yoo et al., 2004). Furthermore, RNAi has been used with this system to uncover novel components of the mammalian molecular clock (Zhang et al., 2009).

Measurement of luciferase activity in real time allows for simpler data collection and for greater accuracy, as the sampling intervals are more frequent than with other methods. Furthermore, since the measurements are nondestructive, the readings are from the same cells throughout the experiment, thus removing the variability of sample-to-sample comparison and greatly reducing the number of replicates needed. Because the cells are viable during data collection, drug treatments with agents such as DNA-damage inducers are possible throughout the experiment, allowing for great flexibility in experimental design (Gamsby et al., 2009). In addition, since luciferase remains bound to olyxiferin after the light-emitting reaction (Lemasters and Hackenbrock, 1977) and the media contains an excess of luciferin, the readings obtained report the activity of newly synthesized luciferase, giving a clearer snapshot of reporter activity.

Forkhead Box (FOX) genes form a family of transcription factors found in species from yeast to human and are involved in a number of different cellular processes, including cell cycle progression, organel development, and oxidative stress responses (Huang and Tindall, 2007). The FOX family of transcription factors comprises >30 genes in humans, all of which contain a monomeric DNA-binding domain (Kaestner et al., 2000).

The transcription factor FOXM1 is known to regulate gene transcription during the transition from G2 to M phase of the cell cycle and to be overexpressed in cancer cells (Wierstra and Alves, 2007). RNAi-mediated knockdown in U2OS or BT-20 human cells results in decreased mitotic indexes and a concomitant increase in the number of cells in G2 (Laoukili et al., 2005; Wonsey and Follettie, 2005). In G2, FOXM1 up-regulates the expression of Cdc25B, cyclin B1, Aurora B kinase, Plk1, CENPA, CENPB, and survivin. In addition, FOXM1 is progressively phosphorylated by a number of kinases, including Plk1 and cyclin B/cdk1 complexes (Fu et al., 2008), as well as by cyclin A/cdk2 or cyclin E/cdk2 complexes (Wierstra and Alves, 2007), which then leads to its degradation by the APC/C after targeting by CDH1 at the end of mitosis (Laoukili et al., 2008). These phosphorylation events restrict the period of FOXM1 DNA-binding activity to the G2 and M phases of the cell cycle. Given this, a reporter driven by a synthetic promoter that is responsive to FOXM1 activity will accurately report the G2/M-specific activity of FOXM1.

E2F1 is a member of the E2F family of transcription factors, which control the expression of genes involved in apoptosis, differentiation, DNA synthesis, and mitosis (Shibutani et al., 2008). E2F1 is specifically involved in the transition from G1 to S phase of the cell cycle (Johnson et al., 1994). Of the many regulatory mechanisms that control E2F1 activity during the cell cycle, two of the best characterized are the binding of E2Fs to the retinoblastoma family of proteins and cell cycle phase–specific degradation (Marti et al., 1999). In addition, cell cycle regulation of the E2F1 promoter restricts expression to the correct cell cycle phase. The E2F1 promoter is autoregulated by active E2F1 during G1/S (Johnson et al., 1994), driving a rapid increase of active E2F1. After the period of E2F1 activity is complete, E2F7 and E2F8 bind to and repress the E2F1 promoter (Li et al., 2008; Zalmas et al., 2008). The combination of E2F1 promoter autoregulation and E2F7 and E2F8 repression restricts the promoter activity to G1/S.

To exploit the well-characterized, cell cycle–controlled oscillatory behavior of E2F1 at G1/S and FOXM1 at G2/M, we used constructs with either a minimal E2F1 promoter or FOX DNA consensus binding sites (Furuyama et al., 2000) driving luciferase expression. Using cell cycle synchronization protocols combined with selective small interfering RNA (siRNA) knockdowns, we followed the G1/S or G2/M reporters and identified two genes necessary for proper oscillation of cell cycle–regulated gene expression in human cells, FOXJ3 and FOXK1 (myocyte nuclear factor). We identified target genes regulated by FOXJ3 and FOXK1. We also identified the genomic loci bound by FOXJ1 with chromatin immunoprecipitation followed by short read sequencing (ChIP-seq), which showed that FOXK1 might have a direct role in regulating the transcription of key cell cycle effectors. We identified a FOXK1 DNA motif present in 81.6% of the intersecting ChIP-seq peaks identified by MACS, and we show that select ChIP-seq peaks are responsive to FOXK1 in the live-cell luciferase reporter assay. These results show that FOXK1 regulates an expansive network of genes, including many that are critical for cell cycle progression, most notably DHFR, TYMS, GSDMD, and TFDP1.

RESULTS
We designed experiments to identify Forkhead transcription factors that contribute to the periodic oscillation of cell cycle–regulated genes. We optimized thymidine-thymidine (thy-thy) and thymidine-nocodazole (thy-noc) cell cycle synchronizations (Whitfield et al., 2002) in U2OS cells, a human osteosarcoma line widely used for cell cycle studies (Fu et al., 2008; Laoukili et al., 2008). To easily and inexpensively monitor cell cycle progression in living cells across multiple, synchronous cell cycles, we developed stable cell lines that expressed cell cycle–regulated luciferase reporters with peak activity in either G1/S or G2/M.

Development of real-time cell cycle reporter assay for periodic gene expression
Previous studies reported that FOXM1 activity oscillates in a cell cycle–dependent manner (Laoukili et al., 2005, 2008; Fu et al., 2008). To report FOXM1 activity, we used a construct that has six Forkhead DNA-binding domains (6xDB) that drive expression of luciferase. Because estrogen receptors (ERs) localize to the nucleus upon ligand binding and this property can be exploited to artificially shuttle proteins to the nucleus (Chandrian et al., 2009), we designed a construct that expresses a fusion of FOXM1 and ER (FOXM1-ER). We then transiently transfected U2OS cells with both the 6xDB and FOXM1-ER constructs and confirmed by chromatin immunoprecipitation that FOXM1 is bound to the FOX-specific consensus sequences after addition of 4-hydroxytamoxifen (4OHT; Supplemental Figure S1D, inset). Addition of ER ligand 4OHT induced nuclear translocation of the FOXM1-ER fusion protein, which elicited an increase of luciferase activity beginning between 7.5 and 9 h, with peak luciferase expression around 34 h (Supplemental Figure S1D). Luciferase activity increased approximately eightfold in the 4OHT-treated cells compared with the vehicle-only negative control cells. Thus FOXM1 binds directly to FOX-specific DNA-binding sites and activates the 6xDB construct in our U2OS system.

After synchronization of U2OS cells stably expressing the 6xDB reporter at G1/S by thy-thy synchronization, FOXM1 displays peak levels and increased phosphorylation in late G2 (Figure 1A, top), and luciferase activity peaks concomitantly (Figure 1B). As expected, FOXM1 phosphorylation peaks during G2 before FOXM1 is
Numerous Forkhead genes are involved in proper cell cycle regulation

The ability to monitor simultaneously and with precision the different cell cycle stages suggested to us that this system would be useful in screening potential cell cycle regulators. As a proof of principle, we treated 6xDB and hE2F1 cells with siRNAs specific to FOXM1 and then synchronized the treated cells in G1/S with a thy-thy block (Supplemental Figure S1). Knowing that cells treated with siRNAs targeting FOXM1 have aberrant mitosis and undergo mitotic catastrophe (Wierstra and Alves, 2007), we anticipated dampened or lost oscillations in both markers, assuming the degree of knockdown was sufficient; this was observed (Figure 3, A and B). Empowered by these positive controls and the lack of cell cycle effects in negative controls (see Supplemental Figure S2, A and B), we screened siRNAs against five FOX genes. Four were implicated in cell cycle regulation (see Supplemental Figure S2, A and B), and one showed periodic mRNA expression during the cell cycle in one of three genome-wide mRNA profiling experiments for cell cycle-regulated genes (FOXP1; G.D.G., L.K.G., and M.L.W., unpublished data).
FIGURE 2: Luciferase activity from hE2F1-Luc and 6xDB-Luc reporters are antiphasic after thy-thy and thy-noc synchronizations. (A) Baseline-subtracted luciferase activity over 54 h after thy-thy synchronization in cells expressing 6xDB-Luc and hE2F1-Luc. Raw data are shown as an inset at the upper right. (B) Baseline-subtracted luciferase activity in cells expressing hE2F1-Luc and 6xDB-Luc over 78 h after a thy-noc synchronization. Mean values of quadruplicate samples are shown. Raw data are shown as an inset at the upper right.

Overlap between the different RNAi screens and mRNA expression data was minimal, resulting in each gene being identified in only a single screen. FOXM1, a known regulator of cell cycle gene expression, was only identified in Whitfield et al. (2002).

In independent experiments, knockdown of FOXJ3 and FOXK1 greatly decreased the cell cycle–dependent oscillations in both 6xDB and HE2F1 cell lines (Figure 3, E–L). FOXL2 was found not to be expressed in U2OS cells and, consistent with this finding, had no cell cycle phenotype (unpublished data). Quantitative PCR demonstrated knockdown efficiencies of 90 and 60% of wild-type gene expression (Figure 3M). Knockdown of the remaining Forkhead proteins gave intermediate phenotypes; detailed characterization will be reported elsewhere.

We then used a human PLK1 promoter luciferase reporter construct to show that loss of G2/M oscillations also occurred with an endogenous human G2/M promoter. U2OS cells stably expressing this reporter faithfully reproduced the same periodic, G2/M-specific oscillation and phasing of luciferase activity as the synthetic 6xDB Forkhead Box promoter construct (Figure 4A). RNAi knockdown with the two independent siRNAs against FOXJ3 (Figure 4, B and C) and FOXK1 (Figure 4, D and E) shows a loss of cell cycle phase–specific luciferase activity. This suggests that loss of G2/M oscillations is due to cell cycle arrest and not to a direct interaction between the reporter and FOXK1 proteins, since FOXK1 does not appear to target PLK1 directly (see ChIP-seq data presented later).

Consistent with this, all siRNAs against FOXJ3, FOXK1, or FOXM1 resulted in significantly reduced proliferation rates compared with controls (Figure 5). A cell viability assay revealed no noticeable increases in dead cells between the control and the siRNA-treated cells, indicating that the decrease in cell numbers reflects a decrease in proliferation rate. Disruption of cell cycle progression by these FOX-specific siRNAs was further confirmed by FACS analysis of propidium iodide (PI)–stained cells, which revealed an increase in the number of cells in G1 (Figure 5A). These data indicate that in addition to FOXM1, the Forkhead Box transcription factors FOXJ3 and FOXK1 have important roles in regulating cell proliferation. This also suggests that an extensive network of Forkhead transcription factors regulates cell cycle progression.

Transcriptional targets of FOXJ3 and FOXK1
To identify potential gene targets of FOXJ3 and FOXK1, we performed three independent siRNA knockdowns for these two genes in asynchronous U2OS cells. As a negative control, we used an siRNA that does not target any known cellular mRNAs (siGENOME Non-Targeting siRNA #2). We collected time points before siRNA treatment (0 h) and at 12, 24, 48, and 72 h after knockdown. We prepared RNA from each time point and analyzed it using DNA microarray hybridization. We selected 1651 genes that changed at least 2.14-fold from the 0 time point in at least three samples and clustered them hierarchically (Figure 6A).

Multiple clusters of coordinately regulated genes were evident after siRNA knockdown. The genes showing the most consistent decrease in expression were those associated with cell division. As expected, these genes showed decreased expression in both FOXJ3 and FOXK1 treatments, and there is an enrichment of well-characterized cell cycle–regulated genes (Figure 6E). These include genes known to cause cell cycle defects after knockdown (Mukherji et al., 2006; Root et al., 2006; Kittler et al., 2007; Rines et al., 2008), as well as genes expressed in S (RRM2) and G2/M phases (CENPE, PLK1, and STK6; Whitfield et al., 2002). Of the 52 genes with decreased expression in this cluster, 35 (69%) were cell cycle regulated in HeLa cells (Whitfield et al., 2002). Analysis of the 52 genes in this cluster by the Database for Annotation, Visualization and Integrated Discovery (DAVID; Dennis et al., 2003; Huang da et al., 2009) showed enrichment for genes associated with cell cycle–specific processes (Supplemental Table S1). A second general group of genes included the immediate early genes that are increased by serum stimulation (Iyer et al., 1999; Figure 6C) and decrease with time after treatment in both the siRNA knockdowns and the mock treatment.

To identify genes that might be specifically regulated by either FOXJ3 or FOXK1, we examined the data for genes whose expression levels changed due to the knockdown of one Forkhead but not the other. As expected, FOXJ3 mRNA levels were decreased by the siRNA specific for FOXJ3 but not by the siRNA for FOXK1 or the control siRNA. Examination of genes that cluster with FOXJ3 shows that 9 of 32 genes are uncharacterized zinc finger proteins (Figure 6D), whereas others include a ring finger protein (RNF138) and a putative RNA-binding protein (RBM25). This suggests that FOXJ3 might have a role in regulating this subset of zinc finger DNA and/or RNA-binding proteins that might be involved in cell cycle progression (Supplemental Table S1). We also uncovered a group of genes (Figure 6B) that showed increased expression after FOXJ3 knockdown and decreased expression in the FOXK1 knockdown,
indicating that these genes might be repressed by FOXJ3 activity and activated by FOXK1. This set of genes could represent indirect FOXK1 targets, although many of these genes do not have a clear role in cell cycle regulation. However, there is a significant enrichment for proteins involved in cell signaling ($p = 1.02 \times 10^{-4}$; Supplemental Table S1) as well as secreted, extracellular matrix proteins ($p = 0.001$).

**FOKK1 binds to the promoter and regulates key cell cycle regulators**

To understand how knockdown of FOXK1 results in cell cycle arrest, we sought to identify direct targets of FOXK1 by analyzing the DNA regions bound by endogenous FOXK1 by ChIP-seq using two independent antibodies against FOXK1 (adequate antibodies were not available for FOXJ3). The DNA fragments from each independent ChIP were sequenced independently. ChIP-seq of FOXK1 using the first antibody (AB G22; Santa Cruz Biotechnology) resulted in 58 million mapped sequence reads (2.1 million unmapped), and ChIP-seq using the second antibody (AB134550; Santa Cruz Biotechnology) resulted in 65 million mapped reads (2.2 million unmapped; Human Genome build 19). Enriched peaks were identified in each data set independently using MACS (Zhang et al., 2008; Liu et al., 2011; mfold 15, $p < 0.001$), which resulted in 7513 peaks in AB G22 and 6225 peaks in AB1344550. For a conservative estimate of FOXK1 binding, we focused our analysis on those sequences that were found in both experiments. Taking the intersection resulted in 4329 shared MACS peaks (Supplemental Table S2). Each includes the intersecting DNA region under the peaks, and we refer to these as “FOKK1 genomic loci.” We determined the distribution of these 4329 shared FOXK1 binding loci in the genome using the cis-regulatory element annotation system (CEAS; http://liulab.dfci.harvard.edu/CEAS; Zhang et al., 2008) implemented in Cistrome (www.cistrome.com). The 4329 genomic loci map to 2865 RefSeq mRNAs representing 1772 unique human genes. We found significant enrichment of genomic loci within 3 kb upstream of the transcriptional start site (6.1%, $p = 1.7 \times 10^{-53}$; Figure 7A), and 36.6% of the genomic loci were found in introns ($p = 1.5 \times 10^{-11}$). We found 51.7% of the FOXK1 loci in distal intergenic regions (defined in this analysis as >3 kb from the

![Figure 3: FOXM1, FOXJ3, and FOXK1 are necessary for periodic gene expression in synchronous U2OS cells.](image-url)
FIGURE 4: The cell cycle–dependent oscillations of the human PLK1 gene promoter are ablated after the knockdown of FOXK1 or FOXJ3. Luciferase activity was measured every 10 min over 72 h after release from the second thymidine arrest, concomitant with siRNA knockdowns as indicated. (A) The cell cycle–associated oscillations of luciferase activity driven by the human PLK1 gene promoter after transfection with a control siRNA. (B–E) U2OS cells treated with two independent siRNAs against FOXJ3 (B, C) or FOXK1 (D, E) showed decreased cell cycle–dependent oscillation of luciferase activity. All graphs are the mean of at least three samples; error bars are SE. The y-axes have been set to the same value based on luciferase levels in A.

FIGURE 5: FOXJ3 and FOXK1 knockdown results in accumulation of G1 cells and impaired proliferation. (A) Percentages of cells in each cell cycle phase as determined by PI staining. Cells were assayed 72 h after siRNA transfection. Both FOXJ3 and FOXK1 cells show an increase in the percentage of cells in G1. Error bars are SE from triplicate samples. All p values of treated cells compared with siControl #2 are <0.05 except FOXK1-1 (p = 0.0538). FlowJo was used to calculate the percentages of cells in each phase. (B) A 72-h proliferation curve shows a proliferation defect in U2OS cells treated with siRNAs targeting FOXJ3 or FOXK1. Error bars are SE of triplicate samples. Indicated p values are <0.05 (*), 0.01 (**), or 0.005 (***) when compared with the percentage of cells in G1 of the SiControl #2–treated cells. (C) Percentage of dead cells, as determined by trypan blue staining, from the proliferation curve in B.
FIGURE 6: Expression profiling of FOXJ3 and FOXK1 siRNA knockdowns in asynchronous U2OS cells. (A) Hierarchical clustering of the 1651 genes that changed at least 2.14-fold on three arrays relative to time zero. The first three columns are zero time points (blue line), the fourth is the zero average, and each time point for the indicated siRNA is indicated by the blue triangle (12, 24, 48, and 72 h). All three experiments were performed in parallel, and therefore the same zero time points are used for each siRNA knockdown. (B) Secreted extracellular matrix gene cluster that shows increased expression after FOXJ3 knockdown and decreased expression after FOXK1 knockdown. (C) The immediate early genes (Iyer et al., 1999; Chang et al., 2004). (D) FOXJ3 cluster shows a decrease in FOXJ3 mRNA, specifically in cells treated with the FOXJ3 siRNA, and includes a large number of zinc finger proteins. (E) Cell cycle cluster that shows decreased expression in both FOXJ3 and FOXX1 knockdowns. We find that 67.3% of the genes in this cluster are cell cycle regulated in HeLa cells.
FIGURE 7: FOXP1 binds to the promoters of an expansive set of genes critical for cell cycle progression. (A) CEAS analysis of the overlapping sequences found in the two antibody ChIP-seq analyses shows an enrichment in promoters (3000 base pairs upstream of the transcriptional start site) and a reduction of intronic FOXP1-binding loci relative to background. (B) Analysis of the 4329 overlapping FOXP1 loci for overrepresented sequence elements identifies two significantly enriched FOXP1-like DNA-binding motifs. We find that 83.4% of the FOXP1 genomic loci contain at least one of the motifs; 51.3% of the FOXP1 loci contain both motifs. (C) Comparison of all cell cycle-regulated genes from Whitfield et al. (2002) with FOXP1 loci shows a higher percentage of occupancy of the promoters of genes with peak expression in G1/S and S phase. (D) Comparison of the genes that result in a cell cycle arrest in one of four genome-wide siRNA screens (Mukherji et al., 2006; Root et al., 2006; Kittler et al., 2007; Rines et al., 2008) linked to FOXP1 ChIP-seq loci shows higher percentage of occupancy in genes that result in an S or G2 phase arrest. (E) Multiple FOXP1-binding loci (thick black bars) are found in the promoter and introns of the TFDP1 gene. FOXP1-like DNA-binding motifs found in each locus are indicated (thin black bars). (F) A single intronic FOXP1 binding locus with multiple FOXP1-like DNA-binding motifs (thin black bars) is found in DHFR. Direction of transcription is indicated for each gene. (G) Change in gene expression for TFDP1 mRNA in the siRNA knockdown experiment (Figure 6) shows a 2.6-fold drop in TFDP1 mRNA levels after FOXP1 knockdown. (H) The gene expression data for FOXP1 target genes DHFR, TFDP1, TYMS, and GSDMD are shown from the mock and FOXP1 siRNA knockdown. Each is linked to the FOXP1 genomic loci found by ChIP-seq. CEAS was used to determine the percentage coverage for each region relative to the TSS of gene models, TTS, or within the gene (right). Those that have peak expression levels in G1 or S as determined in Whitfield et al. (2002) or that result in a G1- or S-phase arrest when knocked down in one of three genome-wide siRNA screens (Mukherji et al., 2006; Root et al., 2006; Kittler et al., 2007) are indicated (the full list is available in Supplemental Tables S3 and S4).
transcription start site [TSS] or transcription termination site [TTTS],
similar to what would be expected by chance (50%). The percent-
age of FOXX1 loci in 5′ untranslated regions (UTR), coding exons, or
3′ UTRs of genes was minimal (Figure 7A).

To determine whether the genomic loci bound by FOXX1 were
enriched for the published FOXX1 consensus sequence motif
TAAACA (Tsai et al., 2006), we analyzed all 4329 FOXX1 loci for
enriched binding sites. We found that 83.4% of the FOXX1 loci con-
tain a FOXX1-like consensus motif. Repeat sequences in the ChiP-
seq intersecting sequences were masked using RepeatMasker
Open-3.0. (www.repeatmasker.org), and significant motifs with high
similarity to the FOXX1 motif were identified using the MEME Suite
of programs (Bailey et al., 2009). The MEME-Chip algorithm (Bailey,
2011; Machanick and Bailey, 2011) identified 15 motifs that were
overrepresented in repeat masked FOXX1 genomic loci. These mo-
tifs were then compared with the FOXX1 consensus motif using
STAMP (Mahony and Benos, 2007). A motif with consensus se-
quence AAKAAACA was the best match to the FOXX1 motif (with an
E value of 1.1 × 10−20) and found in 53% of the FOXX1 loci (Figure
7B). A second motif with consensus site ACAAYAY was the second-
best match (with an E value of 3.6 × 10−20) and was found in 81.6%
of the loci (Figure 7B). When the two motifs are considered together,
51.3% of the loci contain both motifs, and 83.4% of the loci contain
at least one of these two motifs.

Genes regulated by FOXX1 genomic loci
We sought to identify genes near FOXX1 genomic loci that are
cell cycle regulated in HeLa cells (Whitfield et al., 2002) or show a
cell cycle defect in a collation of genome-wide siRNA screens for
cell cycle phenotypes (Mukherji et al., 2006; Root et al., 2006; Kittler
et al., 2007; Rines et al., 2008). To determine whether there was an
enrichment of FOXX1 loci near cell cycle–regulated genes, we com-
pared the genes associated with FOXX1 loci as determined by Gene
Centered Annotation (GCA) to the list of cell cycle–regulated genes
in HeLa cells. We mapped the 1134 cell cycle–regulated clones of
Whitfield et al. (2002) to 688 unique Entrez Gene IDs. Of these
genes, 48 (7%) were linked to FOXX1 ChIP-seq loci by GCA (Supple-
mental Table S3). Analysis of the GCA gene models that have FOXX1
bound shows an increase in the percentage occupancy of promoters
(a measure of how much of each promoter was recovered by
ChiP-seq) for genes expressed in G1 and S phases (Figure 7C).

To determine whether there was a similar pattern in the siRNA
screen data sets, we identified those genes linked to FOXX1 loci that
also showed a cell cycle phenotype (arrest at a specific cell cycle
phase or endoreduplication) after knockdown. Of the 2585 genes
that showed a cell cycle arrest phenotype in at least one of the four
genome-wide siRNA screens (Mukherji et al., 2006; Root et al.,
2006; Kittler et al., 2007; Rines et al., 2008), 230 (9%) were linked by
GCA to a FOXX1 ChIP-seq locus (Supplemental Table S4). Again,
we found enrichment of genes that give an arrest in S phase but also
for genes that resulted in an arrest in G2. Of interest, genes that ar-
rest cells in G0/G1 have lower percentage occupancy than genes
that give an arrest in S, G2, or M phase (Figure 7D).

Those genes associated with FOXX1 ChIP loci that have peak
expression in G1/S include RUNX1, NPAT, C2orf90, and DDX12,
and those with peak expression in S phase include TMY5, DHFR,
ABCC5, EFHC1, MBD4, HIST1H2AC, and HIST1H4H, suggesting
that FOXX1 contributes to G1- and S-phase gene expression. Genes
with peak expression in G2 and M phases include TUBB, CDC27,
GAS6, KIF23, and CEP70. Those associated with FOXX1 ChIP-seq
loci whose genes gave S-phase arrest in genome-wide siRNA
screens included HIST1H2B, NPAT, GSDMD, RPS3A, and the E2F
binding partner TFDP1 (Figure 7E). Four of these genes (DHFR,
TYMS, GSDMD, and TFDP1) also showed decreased mRNA expres-
sion upon FOXX1 siRNA knockdown (Figure 7G-H). The full data are
available in Supplemental Tables S3 and S4.

TFDP1 shows a modest 2.6-fold decrease in mRNA expression
levels after FOXX1 knockdown (Figure 7, G and H). Knockdown of
TFDP1 by RNAi is known to result in cell cycle arrest (Masuhiro et al.,
2008) and is embryonic lethal when it is knocked out in mice (Kohn
et al., 2003). Consistent with this finding, our hE2F1 reporter failed
to oscillate after FOXX1 knockdown. To better resolve the location
of FOXX1 binding in the TFDP1 promoter, we examined the MACS
peaks in the two independent ChiP-seq experiments (Figure 7E).
We found FOXX1-binding loci in the TFDP1 promoter just upstream
of the TSS and within the second, third, and fourth introns of the
TFDP1 gene (Figure 7E and Supplemental Figure S4). Each of these
loci contained multiple FOXX1 consensus motifs as determined by
MEME. The FOXX1 ChIP-seq locus identified in the promoter by
MACS is 621 base pairs upstream of its TSS (Figure 7E) and contains
multiple FOXX1 motifs (Figure 7E).

In an attempt to phenocopy the FOXX1-knockdown phenotype
with a FOXX1-regulated gene, we knocked down TFDP1 in cells
expressing the PLK1-Luc reporter. As expected, the loss of TFDP1
expression ablates G2/M-specific luciferase activity driven by
the human PLK1 promoter (Supplemental Figure S3). Therefore the
loss of TFDP1, and potentially multiple other cell cycle–regulated
genes bound by and responsive to FOXX1, can result in a similar cell cycle
arrest upon siRNA knockdown.

We also examined DHFR, which shows peak expression in S
phase and has decreased mRNA levels 72 h after FOXX1 siRNA
knockdown (Figure 7H). We found a strong FOXX1 binding locus in
the second intron of the gene (Figure 7F) but no evidence for bind-
ing in the DHFR promoter. Consistent with this result, we found that
a DHFR promoter–luciferase reporter is not responsive to FOXX1
overexpression (see later discussion).

The 4329 FOXX1 genomic loci map to 1772 unique human
genes (most genes were associated with multiple FOXX1 genomic
loci), of which 1303 genes passed basic quality filtering in our
FOXX1 siRNA–knockdown DNA microarray analysis. To identify
genes linked to FOXX1 genomic loci that changed expression
upon FOXX1 siRNA knockdown, we divided genes into groups
using idealized expression vectors (see Materials and Methods)
representing decreased or increased expression in the FOXX1
siRNA–knockdown time course (Supplemental Figure S5). Exami-
nation of the highest-confidence groups shows that 37% (494
genes) have a specific decrease in expression after FOXX1 knock-
down relative to mock and 17.5% (228 genes) show an increase in
expression (Supplemental Figures S5 and S6). Therefore FOXX1
likely acts as both an activator and a repressor of gene expression,
derpending on context.

Thus we propose that FOXX1 is a key regulator of an expansive
set of critical cell cycle genes that includes TFDP1, DHFR, TYMS,
GSDMD, and the replication-dependent histone mRNAs. The elimi-
nation of either TFDP1 or GSDMD results in a G1- or S-phase arrest,
and loss of either could therefore result in the phenotype we initially
identified in the LumiCycle assay.

FOXX1 can transcriptionally activate key cell
cycle–regulated genes
To determine whether FOXX1 can activate target cell cycle promot-
ers identified by ChIP-seq, we determined the ability of FOXX1
overexpression to affect luciferase activity from reporters driven by
the GSDMD promoter (GSDMD-Luc, 1000 base pairs upstream of
suggesting that an intronic FOXK1-binding locus alone is not sufficient to drive transcription when placed upstream of the transcription start site.

**FO XK1 genomic loci can drive luciferase activity and are activated by FO XK1 overexpression**

To show that the FOXK1 genomic loci identified in our screen can drive transcription of a luciferase reporter and can be further activated by FOXK1 overexpression, we cloned five of our genomic loci that included only the DNA sequence from the intersection, upstream of luciferase in the absence of a basal promoter (pGL3-Basic). These five loci mapped within 3000 base pairs of the TSS for the genes TFDP1, RPS6KB1, ACAP3, Parp10, and Cep72. Loci ranged in size from 865 (Parp10) to 1097 base pairs (ACAP3) and contained one (RPS6KB1 and Parp10), two (TFDP1 and Cep72), or three (ACAP3) FOXK1-binding motifs as determined by MEME (Supplemental Figure S4). As previously mentioned, TFDP1 is an E2F binding partner required for the G1/S transition, RPS6KB1 encodes a member of the ribosomal S6 kinase family of serine/threonine kinases that increases protein synthesis and cell proliferation (Slattery et al., 2011), ACAP3 (also known as CENTB5) is a relatively uncharacterized GTPase regulator associated with neurodegenerative processes (Shubina et al., 2009), Parp10 is poly(ADP-ribose) polymerase that is phosphorylated by cdk2 in late G1 to S phase (Chou et al., 2006), and Cep72 is a centrosomal protein with a critical role in forming a bipolar spindle (Oshimori et al., 2009). Thus each of these proteins plays important functional roles in the cell division cycle.

To determine whether these putative regulatory loci could drive expression of luciferase in the absence of a basal promoter and whether expression of exogenous FOXK1 could activate or repress these reporter constructs, we cotransfected each into U2OS cells along with either wt FOXK1 or FOXK1 (H355A). All five of the reporters (TFDP1-Luc, RPS6KB1-Luc, ACAP3-Luc, Parp10-Luc, and Cep72-luc) resulted in expression of baseline luciferase activity over 120 h when cotransfected with wt FOXK1 but not with FOXK1 (H355A; Figure 9). The peak of induction occurred between 48 and 72 h posttransfection, and each of the four constructs showed a significant (p < 0.05) increase in luciferase activity between wt FOXK1 and FOXK1 (H355A; Table 1). The weakest induction was observed for Parp10-Luc, which showed a statistically significant

**TABLE 1: Luciferase assay p values.**

| Promoter construct | 0–24 h | 24–48 h | 48–72 h | 72–96 h | 96–120 h |
|--------------------|--------|---------|---------|---------|---------|
| GSDMD              | 0.599  | 0.021   | 0.014   | 0.019   | 0.034   |
| DHFR               | 0.919  | 0.638   | 0.400   | 0.432   | 0.527   |
| ACAP3              | 0.012  | 0.013   | 0.029   | 0.041   | 0.013   |
| Cep72*             | 0.004  | 0.003   | 0.001   | 0.002   | 0.063   |
| RPS6KB1            | 0.645  | 0.002   | 0.007   | 0.016   | 0.026   |
| TFDP1              | 0.727  | 0.003   | 0.006   | 0.065   | 0.099   |
| Parp10             | 0.764  | 0.004   | 0.265   | 0.782   | 0.641   |

Readings were binned and averaged in 24-h windows. One-way ANOVAs were calculated for wild-type FOXK1 vs. DNA binding–domain mutant FOXK1 (H355A) for the quadruplicate samples that were averaged in Figures 8 and 9. *Due to technical issues, Cep72 was performed in triplicate. Bold indicates p < 0.05.
endogenous FOXM1 activity, a second driven by the human PLK1 promoter to report the G2/M phase of the cell cycle, and a third that reports G1/S through the human E2F1 promoter. These cell cycle–controlled DNA elements faithfully report each respective phase of the cell cycle in synchronized U2OS cells based on the phasing of the luciferase rhythms in relation to the synchronization method, protein profiles, and FACS profiles after synchronization.

In addition, the Forkhead DNA-binding sites in the 6xDB reporter can be bound by FOXM1. We used these novel reporters to show that targeted reduction of FOXJ3 and FOXK1 expression by siRNA in synchronous U2OS cells ablates cell cycle–dependent luciferase activity in both reporter cell lines and confirmed the cell cycle phenotype by FACS analysis and growth curves.

In U2OS cells, FOXK1 is not cell cycle regulated at the protein level (unpublished data). This is not unprecedented, as cyclin-dependent kinases are not cell cycle regulated at the mRNA level (Whitfield et al., 2002), but instead their activity is regulated by their expression from 24 to 48 h ($p = 0.004$) but not from 48 to 72 h ($p = 0.265$).

Therefore five of the FOXK1-binding loci, including parts of the promoter of TFDP1, are sufficient to drive basal expression of luciferase and can be further activated by overexpression of the wt FOXK1. We find no induction when we use the DNA-binding mutant FOXK1 (H355A). In addition, the full promoter of a sixth target, GSDMD, which contained a FOXK1 ChIP-seq locus, is also responsive to wt FOXK1 but not FOXK1 (H355A), whereas the promoter of DHFR that did not contain a ChIP-seq locus was not responsive to wt FOXK1, showing specificity of the effect.

**DISCUSSION**

Here we described a novel method to follow cell cycle progression in live human cells with far greater precision than was previously possible and used this to identify novel cell cycle regulators. Three luciferase reporter constructs were used—one that monitors endogenous FOXM1 activity, a second driven by the human PLK1 promoter to report the G2/M phase of the cell cycle, and a third that reports G1/S through the human E2F1 promoter. These cell cycle–controlled DNA elements faithfully report each respective phase of the cell cycle in synchronized U2OS cells based on the phasing of the luciferase rhythms in relation to the synchronization method, protein profiles, and FACS profiles after synchronization. In addition, the Forkhead DNA-binding sites in the 6xDB reporter can be bound by FOXM1. We used these novel reporters to show that targeted reduction of FOXJ3 and FOXK1 expression by siRNA in synchronous U2OS cells ablates cell cycle–dependent luciferase activity in both reporter cell lines and confirmed the cell cycle phenotype by FACS analysis and growth curves.

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**FIGURE 9:** Wild-type FOXK1 but not mutant FOXK1 activates cell cycle promoters that contain FOXK1-binding motifs in live cells. FOXK1 (light line) activates the expression of luciferase from the ACAP3 (A), Cep72 (B), RPS6KB1 (C), or TFDP1 (D) promoter. Mutant FOXK1 (dark line) does not activate expression of luciferase from these promoters. (E) FOXK1 activates expression of the Parp10 promoter between 24 and 48 h posttransfection to a greater extent than mutant FOXK1. Each line is a representative experiment with at least three replicates. Error bars are SE of the mean. Time points were binned and averaged for every 24 h for a one-way ANOVA. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$. All $p$ values are given in Table 1.
well-characterized interactions with cyclins (Sherr, 2000). Therefore it is entirely plausible that there is a cell cycle phase-specific activation of FOXJ3 or FOXK1 due to other posttranslational modifications as such phosphorylation or cell cycle–regulated binding partners. Pointing to a possible role of FOXK1 in the cell cycle, mouse Mnf−/− (Foxk1) cells have dysregulated expression of cell cycle genes, and Mnf−/− mice are ~40% smaller than wild-type littermates and have impaired muscle repair (Garry et al., 2000). The ChIP-seq and gene expression data for FOXK1 allow us to propose a mechanism for its role in the cell cycle. Our data show that FOXK1 is required for the activation of an expansive set of cell cycle–regulated genes, many of which, when knocked out in published siRNA screens, result in a cell cycle arrest; these include TFDP1 and GSDMD. Thus there are numerous FOXK1 targets, any one of which might result in a cell cycle arrest when knocked out. Consistent with this, our attempts to rescue the FOXK1 cell cycle arrest phenotype by overexpressing TFDP1 failed to restore cell cycle–regulated gene expression (unpublished data). This result supports the idea that a combinatorial effect resulting from the loss of FOXK1 that includes multiple cell cycle genes results in the cell cycle arrest we observe in our screen.

Earlier publications in different model systems showed that FOXK1, when in a complex with the MADS-box SRF transcription factor or Sin3B, functions as a repressor of promoter activity (Yang et al., 2000; Freddie et al., 2007). Of interest, SRF contains a FOXK1-binding locus downstream of the TTS and shows decreased expression upon FOXK1 siRNA knockdown (Supplemental Figure S6). The repressive activity of FOXK1 when complexed with SRF is in contrast with the data presented here, which demonstrate that in the subset of genes analyzed, FOXK1 is an activator of transcriptional activity (Figures 8 and 9). It is likely that FOXK1 can be either an activator or a repressor, depending on promoter context and cell type (U2OS vs. RD18 or 293), and this is supported by our analysis of genes associated with FOXK1 genomic loci in the FOXK1 siRNA–knockdown time course, which shows genes with increased expression (e.g., are likely repressed by FOXK1) and decreased expression (e.g., FOXK1 activated as shown here). A simple mechanism for this duality may lie in the FOXK1 cofactors such as SRF, which impart a repressive function to the complex, whereas others result in activation. Of interest, FOXK1 does not appear to function as a pioneer factor, as it requires SRF for efficient recruitment to the α-actin promoter or the synthetic CArG construct (Freddie et al., 2007). This would imply that FOXK1 is more dependent on cofactors than other FOX genes, and its roles in gene expression may be much more varied, depending on complex composition.

Proposing a mechanism for the FOXJ3 cell cycle defect is more challenging, as there are no ChIP-seq data available, and current antibodies against FOXJ3 were insufficient for this purpose. The genes that cluster with FOXJ3 suggest that it may be involved in regulating a network of zinc finger–binding proteins that may affect gene expression themselves. What role these may have in cell cycle progression is unclear.

These results demonstrate that real-time luciferase assays can greatly enhance the precision and reduce the cost associated with the evaluation of potential novel regulators of cell cycle gene expression. This methodology will also facilitate development of synchronization protocols, as the need for sample collections at regular intervals over multiple cell cycles is not required. Luciferase monitoring is more cost-effective than traditional time-course experiments, fewer samples are required to achieve statistically significant data, and multiple conditions can be tested with replicates at a single time. With further refining, this technology may also prove to be a powerful tool in cancer drug assays. As a result of the assay’s nondestructive nature, using real-time luciferase assays will allow screening of compounds having a limited availability due to cost or origin and will also allow for multiple treatments of the same cell population in one experiment.

MATERIALS AND METHODS

Cell lines

U2OS cells were passaged at 37°C in a humidity-controlled, CO2-equilibrated incubator in DMEM (Life Technologies, Grand Island, NY) culture medium (CM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Pen Strep). All stable lines were passaged in a selection medium (SM) of CM and 1.5 μg/ml puromycin.

Stable U2OS hE2F1, 6xDB, and PLK1 luciferase lines were generated by cotransfection of a minimal E2F1 promoter driving luciferase (Johnson et al., 1994), a construct containing the 6xDB Forkhead consensus site (Laoukili et al., 2005), or the human PLK1 promoter (S119035, Switchgear Genomics, Menlo Park, CA), using 8 μg of plasmid with 2 μg of puromycin-resistant pBABE-puro with FuGENE 6 (Roche, Indianapolis, IN). Negative control cell lines were produced with the same protocol and plasmid insertion confirmed via PCR of the luciferase open reading frame (5′ATCCAGATCCACAACCCTTCG3′; 5′AGAACTGCCTGCGTGAGATT3′).

Cell line synchronization

Thymidine-thymidine synchronizations were performed with U2OS cells in 35-mm dishes (Corning, Lowell, MA) at 2 × 10^4 to 8 × 10^4 cells/plate. After 24 h, thymidine was added to a final concentration of 2.5 mM. Cells were held in thymidine for 18 h, washed with 1.0 ml of prewarmed, CO2-equilibrated, 1× phosphate-buffered saline (PBS), followed by the addition of fresh selection media. After growth for 8 h, 2.5 mM thymidine was added for 18 h, and cells were again washed and released into fresh selection media. Thymidine/nocodazole synchronizations were performed with U2OS cells at 4 × 10^4 cells/plate. After 24 h, cells were treated with 2.5 mM thymidine for 18 h, washed with 1× PBS once, and released into fresh medium with 100 ng/ml nocodazole. After 12 h, medium was removed, and floating mitotic cells were collected by centrifugation. The remaining cells were washed once with 1× PBS, and floating cells were resuspended in fresh medium and replated with the adherent population.

Real-time bioluminescence monitoring

Synchronous U2OS cells were washed with PBS and the SM replaced with 2 ml of assay medium (phenol red–free L15 [Life Technologies], 10% FBS, 1% Pen Strep, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and 0.1 mM luciferin). Tissue culture dishes were sealed with 38 circle #1 glass coverslips and silicone grease and transferred to the LumiCycle (Actimetrics, Wilmette, IL) at 36°C. Data analysis was performed with the LumiCycle Analysis software (Actimetrics). Where indicated, data were baseline subtracted using a 24-h running average. Time zero (t = 0) indicates time of release from cell cycle synchronization.

Promoter assays were performed by transfecting U2OS cells with 250 ng of each plasmid (see later description) using FuGENE 6 (Life Technologies) immediately before sealing the dishes and transferring them to the LumiCycle. Where possible, each promoter construct was placed in the LumiCycle so it would use the same photomultiplier tube. The p values were calculated using SPSS Statistics 19 (IBM, Armonk, NY) by performing a one-way analysis of variance (ANOVA) comparing the replicate wt FOXK1 transfections with the mutant FOXK1 (H355A) transfections. Luciferase readings were binned and averaged over each 24-h period.

Molecular Biology of the Cell
Plasmids

DHFR (S101166), PLK1 (S119035), and GSDMD (S112693) were purchased from Switchgear Genomics. The FOXK1 (2256) and DNA-binding mutant FOXK1 (2265) (Freddie et al., 2007) constructs were gifts from A. Sharrocks. 6xDB (Furuyama et al., 2000; Laoukili et al., 2005) was a gift from R. Medema, and the minimal hE2F promoter construct (Johnson et al., 1994) was a gift from W. D. Cress. FOXM1-ER was subcloned from an ER fragment (gift from M. Cole) and FOXM1-pBABE-puro, which was cloned from human cDNA. All plasmids were sequenced to confirm that there were no errors (Molecular Biology and Proteomics Core Facility, Dartmouth College).

FOXM1 target promoters were cloned based on the ChIP-seq loci as determined by MACS. Primers were designed to flank any FOXK1 motifs and to provide an amplicon between 800 and 1000 base pairs long (Primer 3; Rozen and Skaletsky, 2000). DNA fragments were amplified by PCR and cloned into Zero Blunt TOPO (Life Technologies), and then they were subcloned into the MCS of pGL3-basic (Promega, Madison, WI) using standard methods. All inserts were sequenced to confirm their identity (Molecular Biology and Proteomics Core Facility, Dartmouth College).

siRNA transfections

Cells were transfected with siRNA (100 nM) using Oligofectamine (Life Technologies). Bioluminescence assays were performed as described, except that siRNA transfection began 24 h before the first thymidine arrest (Supplemental Figure S1B). RNA was collected 48 h after transfection to measure knockdown efficiency of each siRNA by Taqman quantitative RT-PCR. All siRNAs were obtained from Dharmacon/Thermo Fisher Scientific (Lafayette, CO): FOXJ3 (#1, GGGAAGGUGUUAAAGGAAUU; #2, GUAGGAAACUGAAGGAAUU); FOXK1 (#1, GAGCAGUGGUUCUUGGAAAUU; #2, AGAACAAUGCCAAGUGAAAUU); FOXM1 (#1, GGACCACUUUCUCCUCACUUU; Wang et al., 2005); and siGENOME SMARTpool, human TPDP1 (7027). Human siGENOME Non-Targeting siRNA #2 was used as a negative control.

Western blot analysis

Antibodies to FoxM1 C-20 (1:500), cyclin B1 (H-433; 1:2000), and E2F1 (C-20; 1:500) were purchased from Santa Cruz Biotechnology. Antibodies were bound to a mix of protein A and protein G Dynabeads (Life Technologies) before an 18-h incubation at 4°C with the fragmented DNA. DNA was washed and cross-links reversed before purification with a QiAquick PCR purification kit (Qiagen, Valencia, CA). DNA concentrations were measured using Quuant-tiT PicoGreen (Life Technologies). A library was constructed from the bound fragments per manufacturer's protocols, and the DNA fragments from each independent ChIP were sequenced independently at the High Throughput Sequencing Facility, University of North Carolina at Chapel Hill (UNC; Chapel Hill, NC), using an Illumina Genome Analyzer II. Fastq files obtained from the Genome Analyzer for each run were mapped to human genome using Bowtie software (version 0.12.5), using the "best" flag to constrain reported alignments to those with the fewest mismatches and best read quality. ChIP-seq of FOXK1 using AB G22 resulted in 58 million mapped sequence reads (2.1 million unmapped), and ChIP-seq using AB134550 resulted in 65 million mapped reads (2.2 million unmapped, Human Genome build Hg19). Enriched peaks were identified independently in each data set using MACS version 1.3.7 (Zhang et al., 2008; mfold 15, p < 0.001), which resulted in 7513 peaks in AB G22 and 6225 peaks in AB1344550. As a conservative estimate of FOXK1 binding, we analyzed the intersection of the sequences under the peaks found in both experiments, resulting in 4329 shared FOXK1-binding loci. We determined the distribution of these 4329 shared FOXK1-binding loci in the genome using the Cis-regulatory Element Annotation System (CEAS; http://liulab.dfci.harvard.edu/CEAS; Zhang et al., 2008) implemented in Cistrome (www.cistrome.com). The full data are available from the Gene Expression Omnibus (GEO) repository GSE39138.

Identification of cis-acting regulatory elements

The 4329 shared loci were analyzed for cis-acting regulatory motifs. RepeatMasker Open-3.0 (www.repeatmasker.org) was used to screen input DNA for repeats and generate a modified version of the query sequence with masked repeats (replaced with N's). Sequence comparisons in RepeatMasker were performed by default search engine “cross match” and were run with “–nowol” parameter. To analyze masked ChIPseq data and discover significant motifs with high similarity to FOXK1 site and their potential partners, we used MEME Suite (Bailey et al., 2009). Specifically, we analyzed sequences resulting from the intersection of the MACS peaks from the two experiments by MEME-Chip (Machanick and Bailey, 2011), which is designed specifically for motif analysis in large ChIP-seq data sets. MEME-Chip was run via Web server with default settings except for the following: motif distribution, any number of repetitions; optimum motif width, 6–10 base pairs; maximum number of motifs to find, 10.

To identify the best matches to known FOXK1 sites among motifs predicted by MEME-Chip, we used STAMP (Mahony and Benos, 2007). We compared the output from MEME-Chip to a manually constructed database that contained a position-weight matrix that represented the published FOXK1 motif (TAAACA; Tsai et al., 2006).

ChIP-seq

FOXM1 chromatin immunoprecipitations were performed with Upstate Biotechnology/Millipore (Billerica, MA) ChIP assay kit protocol. Primers were obtained from Life Technologies and were as follows: 6xDB primer 1 (forward, AGCGTGTGTAGCTTGTTAAG; reverse, CGAATTCGAACACACGAGAT); 6xDB primer 2 (forward, CCACACGTGTACCTAAAA; reverse, GCCCGCCTGTTGCTT- CATT), and GAPDH (forward, CAATTCCCATCTCAGTGT; reverse, TAGTGGCGGCGCCACTTATT).

FOXK1 ChIP-seq was carried out as previously described (Lupien et al., 2008) using antibodies sc-133592 (G22) and sc-134550 (H140) against FOXK1 (Santa Cruz Biotechnology). Briefly, HeLa cells were fixed with 1% formaldehyde before the DNA was sonicated to a fragment length of 200–600 base pairs with a Bioruptor (Diagenode, Sparta, NJ). Antibodies were bound to a mix of protein A and protein G Dynabeads (Life Technologies) before an 18-h incubation at 4°C with the fragmented DNA. DNA was washed and cross-links reversed before purification with a QiAquick PCR purification kit (Qiagen, Valencia, CA). DNA concentrations were measured using Quuant-tiT PicoGreen (Life Technologies). A library was constructed from the bound fragments per manufacturer's protocols, and the DNA fragments from each independent ChIP were sequenced independently at the High Throughput Sequencing Facility, University of North Carolina at Chapel Hill (UNC; Chapel Hill, NC), using an Illumina Genome Analyzer II. Fastq files obtained from the Genome Analyzer for each run were mapped to human genome using Bowtie software (version 0.12.5), using the "best" flag to constrain reported alignments to those with the fewest mismatches and best read quality. ChIP-seq of FOXK1 using AB G22 resulted in 58 million mapped sequence reads (2.1 million unmapped), and ChIP-seq using AB134550 resulted in 65 million mapped reads (2.2 million unmapped, Human Genome build Hg19). Enriched peaks were identified independently in each data set using MACS version 1.3.7 (Zhang et al., 2008; mfold 15, p < 0.001), which resulted in 7513 peaks in AB G22 and 6225 peaks in AB1344550. As a conservative estimate of FOXK1 binding, we analyzed the intersection of the sequences under the peaks found in both experiments, resulting in 4329 shared FOXK1-binding loci. We determined the distribution of these 4329 shared FOXK1-binding loci in the genome using the Cis-regulatory Element Annotation System (CEAS; http://liulab.dfci.harvard.edu/CEAS; Zhang et al., 2008) implemented in Cistrome (www.cistrome.com). The full data are available from the Gene Expression Omnibus (GEO) repository GSE39138.

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A motif was considered to be a match to a FOXK1 site if STAMP E value was $< 1 \times 10^{-4}$. STAMP was run with default settings. To find significant spacing patterns between FOXK1 matches and other motifs predicted by MEME-ChIP, we used SpaMo (Whittington et al., 2011). SpaMo was run in command line mode with the following parameters: "-margin 100 -shared 1." Motifs that formed significant spacing patterns with FOXK1 matches were run via STAMP against TRANSFAC, version 11.3 (Matys et al., 2003), to identify potential FOXK1 cofactors. To extract all instances of each individual motif, we used FIMO in command line mode with the modified p value output threshold of $1 \times 10^{-3}$.

**RNA isolation and microarray hybridization**

Asynchronous U2OS cells were transfected with siRNAs targeting FOXJ3 or FOXK1 with Oligofectamine, following the manufacturer’s protocols. Experiments were performed in a time-course design. Three time-zero points were collected from untransfected cells. Time points were then harvested in parallel from the mock-transfected and the FOXJ3 and FOXK1 siRNA–transfected cells at 12, 24, 48, and 72 h after transfection. Cellular RNA was isolated using the RNeasy Plus Kit (Qiagen). Total RNA (200 ng) was amplified using the Quick-Amp Labeling Kit (Agilent Technologies, Santa Clara, CA) and hybridized to Agilent Technologies Whole Human Genome Oligonucleotide arrays (4 × 44k) following the manufacturer’s protocol, except for the labeling reaction, for which volumes were reduced by half. Microarrays were scanned using a GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA). Spot pixel intensities were determined by the GenePix Pro 5.1 software. Poor-quality spots were manually flagged and excluded from further analysis. Arrays were stored in the UNC Microarray Database.

Gene expression data were retrieved for each microarray from the UNC microarray database. Only spots with intensity over background of >1.5 were used for further analysis. Genes missing >20% of their data were excluded from further analysis; only genes with three observations with a log2 absolute value of ≥1.1 (a 2.14-fold change) across all three time-course experiments were selected. Gene selection and hierarchical clustering were performed using Cluster 3.0 (Eisen et al., 1998). The 24-h time point for the FOXK1 siRNA was excluded from the analysis due to a technical failure. Data were zero transformed by normalizing each time course to the average of the three zero time points. Data were visualized using Java TreeView software (Saldanha, 2004).

Functional annotation of gene clusters was performed using DAVID (Dennis et al., 2003; Huang da et al., 2009) with the following categories: cog_ontology, sp_pir_keywords, up_seq_feature, goterms_bp, fbat, BBID, Biocarta, KEGG_pathway. Only categories with p < 0.05 are shown in Supplemental Table S1. The p values corrected for multiple hypothesis testing are also provided.

To identify genes associated with FOXK1 ChIP-seq loci that also showed a change in expression upon FOXK1 knockdown, we normalized the expression values of genes associated with FOXK1 loci (as determined by CEAS) to a maximum value between −1 and 1. We then constructed “ideal patterns” of expression, where perfect induction would have a 1 for each time point, perfect repression would be signify by −1, and unregulated would be indicated by 0. Because there are three expression categories and two treatment conditions, we set up $2^3 = 8$ bins, one per pattern. For example, the “ideal pattern” for a gene that is repressed after FOXK1 siRNA treatment and unregulated after mock treatment is four zeros followed by four ones (00001111). We then normalized the ideal patterns in the same way as the gene expression data. To assign each gene to a bin, we computed the dot product of the normalized expression data for each gene to each of the eight possible ideal patterns to generate an “ideal pattern” score. We ordered each bin by the “ideal pattern” score and displayed the data using Java TreeView (Supplemental Figure S5).

**FACS determination of cell cycle phase**

Cell synchrony was monitored by flow cytometry of propidium iodide–stained cells (Whitfield et al., 2002). Percentage of cells in each cell cycle phase was determined with FlowJo, version 7.5 (Tree Star, Ashland, OR).

**Growth curve**

U2OS cells expressing the E2F1 construct were plated at a density of $6 \times 10^5$ cells/well in 12-well dishes. After 24 h of growth, 100 nM siRNA was transfected with Oligofectamine, and three wells were counted using trypan blue for each time point.

The full data figures in both red/green and blue/yellow formats are available from a website maintained by the authors at http:// whitfieldlab.dartmouth.edu/lumicycle.

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**REFERENCES**

Bailey TL (2011). DREME: motif discovery in transcription factor ChIP-seq data. Bioinformatics 27, 121653–1659.

Bailey TL, Boden M, Buske FA, Frith M, Grant CE, Clementi L, Ren J, Li WW, Noble WS (2009). MEME SUITE: tools for motif discovery and searching. Nucleic Acids Res 37 (Web Server issue), W202–W208.

Bar-Joseph Z, Siegfried Z, Brandes M, Brors B, Lu Y, Eils R, Dynlacht BD, Simon I (2008). Genome-wide transcriptional analysis of the human cell cycle identifies genes differentially regulated in normal and cancer cells. Proc Natl Acad Sci USA 105(3), 955–960.

Chandriani S, Frengen E, Cowling VH, Pendergrass SA, Perou CM, Whitfield ML, Cole MD (2009). A core MYC gene expression signature is prominent in basal-like breast cancer but only partially overlaps the core serum response. PLoS One 4, e6693.

Chang HY, Sneddon JB, Alizadeh AA, Sood R, West RB, Montgomery K, Chi JT, van de Rijn M, Botstein D, Brown PO (2004). Gene expression signature of fibroblast serum response predicts human cancer progression: similarities between tumors and wounds. PLoS Biol 2, E7.

Cho RJ, Huang M, Campbell MJ, Dong H, Steinmetz L, Sapinoso L, Hampton G, Elledge SJ, Davis RW, Lockhart DJ (2001). Transcriptional regulation and function during the human cell cycle. Nat Genet 27, 48–54.

Chou HY, Chou HT, Lee SC (2006). CDK-dependent activation of poly(ADP-ribose) polymerase member 10 (PARP10). J Biol Chem 281, 15201–15207.

Dennis G, Jr, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC, Lempicki RA (2003). DAVID: Database for Annotation, Visualization, and Integrated Discovery. Genome Biol 4, P3.

Eisen MB, Spellman PT, Brown PO, Botstein D (1998). Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci USA 95, 14863–14868.

Freddie CT, Ji Z, Marais A, Sharrocks AD (2007). Functional interactions between the Forkhead transcription factor FOXK1 and the MADS-box protein SRF. Nucleic Acids Res 35, 5203–5212.

Fu Z, Malureanu L, Huang J, Wang W, Li H, van Deursen JM, Tindal DJ, Chen J (2008). Pkl1-dependent phosphorylation of FoxM1 regulates a transcriptional programme required for mitotic progression. Nat Cell Biol 10, 1076–1082.

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Furuyama T, Nakazawa T, Nakano I, Morii N (2000). Identification of the differential distribution patterns of mRNAs and consensus binding sequences for mouse DAF-16 homologues. Biochem J 349, 629–634.

Gamsby JJ, Loros JJ, Dunlap JC (2009). A phylogenetically conserved DNA damage response resets the circadian clock. J Biol Rhythms 24, 193–202.

Garry DJ, Meeson A, Etterman J, Zhao Y, Pang P, Bassel-Duby R, Williams RS (2000). Myogenic stem cell function is impaired in mice lacking the forkhead/winged helix protein MNF. Proc Natl Acad Sci USA 97, 5416–5421.

Huang da W, Sherman BT, Lempicki RA (2009). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 4, 44–57.

Huang H, Tindall DJ (2007). Dynamic FOXO transcription factors. J Cell Sci 120, 2479–2487.

Hwang A, Maity A, McKenna WG, Muschel RJ (1999). Cell cycle-dependent regulation of the cyclin B1 promoter. J Biol Chem 270, 28419–28424.

Iyer VR et al. (1999). The transcriptional program in the response of human fibroblasts to serum. Science 283, 83–87.

Johnson DG, Ohtani K, Nevin JR (1994). Autoregulatory control of EF1 expression in response to positive and negative regulators of cell cycle progression. Genes Dev 8, 1514–1525.

Kaestner KH, Knochel W, Martinez DE (2000). Unified nomenclature for the winged helix/forkhead transcription factors. Genes Dev 14, 142–146.

Kaida A, Sawai N, Sakaguchi K, Miura M (2011). Fluorescence kinetics in HeLa cells after treatment with cell cycle arrest inducers visualized with Fucci (fluorescent ubiquitination-based cell cycle indicator). Cell Biol Int 35, 359–363.

Kittler R et al. (2007). Genome-scale RNAi profiling of cell division in human tissue culture cells. Nat Cell Biol 9, 1401–1412.

Kohn MJ, Bronson RT, Harlow E, Dyson NJ, Yamasaki L (2003). Dp1 is required for extra-embryonic development. Development 130, 1295–1305.

Laoukili J, Alvarez-Fernandez M, Stahl M, Medema RH (2008). FoxM1c is degraded at mitotic exit in a Cdh1-dependent manner. Cell Cycle 7, 2720–2726.

Laoukili J, Kooistra MR, Bras A, Kauw J, Kerkhoven RM, Morrison A, Clevers H, Medema RH (2005). FoxA1 translates epigenetic signatures into transcriptional activity and cell cycle progression via interaction with DP-1. J Biol Chem 280, 2720–2726.

Lemasters JJ, Hackenbrock CR (1977). Kinetics of product inhibition during kinetics of product inhibition during enzymatic reactions. J Biol Chem 252, 7801–7809.

Liu T (2010). Identification of genes encoding the SCF (Skp2-Cks1) ubiquitin ligase. Mol Cell Biol 25, 10875–10894.

Liu XS, Brown M (2008). FoxA1 causes centrosome amplification and mitotic catastrophe. Genes Dev 22, 1289–1295.

Lukashin AV, Schonegge M, Knoch W (1997). Interaction between ubiquitin-protein ligase SCFSkp2 and E2F-1 underlies the regulation of FoxM1c by cyclin E/CDK2. FEBS Lett 580, 1716–1722.

Luo J, Alvarez-Fernandez M, Stahl M, Medema RH (2008). FoxM1 is degraded at mitotic exit in a Cdh1-dependent manner. Cell Cycle 7, 2720–2726.

Machanick P, Bailey TL (2011). MEME-ChIP: motif analysis of large DNA datasets. Bioinformatics 27, 1696–1697.

Mahony S, Benos PV (2007). STAMP: a web tool for exploring DNA-binding motif similarities. Nucleic Acids Res 35 (Web Server issue), W253–W258.

Marti A, Wirbelauer C, Scheffner M, Krek W (1999). Structure of the human FOXK1a-DNA complex and its implications on the winged helix/forkhead proteins. J Biol Chem 281, 17400–17409.

Matsya V et al. (2010). TRANSFAC: transcriptional regulation, from patterns to profiles. Nucleic Acids Res 38, 374–378.

Mukherji M et al. (2006). Genome-wide functional analysis of human cell cycle regulators. Proc Natl Acad Sci USA 103, 14819–14824.

Oshimori N, Li X, Ohsugi M, Yamamoto T (2009). Cep72 regulates the localization of key centrosomal proteins and proper bipolar spindle formation. EMBO J 28, 2068–2076.

Rines DR et al. (2008). Whole genome functional analysis identifies novel components required for mitotic spindle integrity in human cells. Genome Biol 9, R44.

Robertson JB, Stowers CC, Boczkow, Johnson CH (2008). Real-time luminescence monitoring of cell-cycle and respiratory oscillations in yeast. Proc Natl Acad Sci USA 105, 17988–17993.

Root DE, Hacohen N, Hahn WC, Lander ES, Sabatini DM (2006). Genome-scale loss-of-function screening with a lentiviral RNAi library. Nat Methods 3, 715–719.

Rozen S, Skalskay H (2000). Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 132, 365–386.

Saldanha AJ (2004). Java TreeView—extensible visualization of microarray data. Bioinformatics 20, 3246–3248.

Sherr CJ (2000). The Pecozeller Lecture: cancer cell cycles revisited. Cancer Res 60, 3689–3695.

Shibutani ST, de la Cruz AF, Tran V, Turbyfill WJ, Reis T, Edgar BA, Duronio RJ (2008). Intrinsic negative cell cycle regulation provided by PIP box- and Cul4Cdt2-mediated destruction of E2F1 during S phase. Dev Cell 15, 890–900.

Shubina DM, Suchkova IO, Slominskaia NA, Alenina N, Bader M, Patkin EL (2009). CENT85 gene expression in human and mouse [in Russian]. Mol Biol (Mosk) 43(3), 407–413.

Slattery ML, Lundgreen A, Herrick JS, Wolff RK (2011). Genetic variation in RP56K1A, RP56K2A, RP56K81, and PDK1 and risk of colon or rectal cancer. Mutat Res 706, 13–20.

Stacey DW, Hitomi M (2008). Cell cycle studies based upon quantitative image analysis, Cytoometry A 73, 270–278.

Talalai P, Huang CY, Chang CH, Sun YJ, Chuang WJ, Hsiao CD (2006). Crystal structure of the human FOXK1a-DNA complex and its implications on the divergence binding specificity of winged helix/forkhead proteins. J Biol Chem 281, 17400–17409.

Wang IC, Chen YJ, Hughes D, Petrovic V, Major ML, Park JH, Tan Y, Ackerson T, Costa RH (2005). Forkhead box M1 regulates the transcription of the cyclin B1 promoter. J Biol Chem 270, 28419–28424.

Wierstra I, Alves J (2007). FOXM1, a typical proliferation-associated transcription factor. Biol Chem 388, 1257–1274.

Xie J, Wang Y, Liu AC, Takahashi JS, Kay SA (2004). Bioluminescence imaging of individual fibroblasts reveals persistent, independently phased circadian rhythms of clock gene expression. Curr Biol 14, 2289–2295.

Whitfield ML et al. (2002). Identification of genes periodically expressed in the human cell cycle and their expression in tumors. Mol Biol Cell 13, 1977–2000.

Whittington T, Frith MC, Johnson J, Bailey TL (2011). Inferring transcription factor complexes from ChIP-seq data. Nucleic Acids Res 39, e98.

Wierstra I, Alves J (2007). FOXM1, a typical proliferation-associated transcription factor. Biol Chem 388, 1257–1274.

Wonsey DR, Follettie MT (2005). Loss of the forkhead transcription factor MYCN in human epidermal nevus cells. J Invest Dermatol 124, 648–654.

Worthington CR, Lyles MA (1999). Reduction of circadian clock in human cells. Cell 139, 199–210.

Yamaguchi N, Hanazawa S (2008). SOCS-3 inhibits E2F/DP-1 transcription factor FOXM1c by cyclin E/CDK2. FEBS Lett 580, 1716–1722.

Yoo SH et al. (2004). PERIOD2: LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. Proc Natl Acad Sci USA 101, 5339–5346.

Zalmas LP, Zhao X, Graham AL, Fisher R, Reilly C, Coutts AS, La Thangue NB (2008). DNA-damage response control of E2F7 and E2F8. EMBO Rep 9, 252–259.

Zhang EE et al. (2008). Genome-wide RNAi screen for modifiers of the circadian clock in human cells. Cell 139, 199–210.

Zhang Y et al. (2008). Model-based analysis of ChIP-Seq (MACS). Genome Biol 9, R137.