The HiNF-P/p220NPAT Cell Cycle Signaling Pathway Controls Nonhistone Target Genes

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Abstract

HiNF-P and its cofactor p220NPAT are principal factors regulating histone gene expression at the G1-S phase cell cycle transition. Here, we have investigated whether HiNF-P controls other cell cycle- and cancer-related genes. We used cDNA microarrays to monitor responsiveness of gene expression to small interfering RNA-mediated depletion of HiNF-P. Candidate HiNF-P target genes were examined for the presence of HiNF-P recognition motifs, in vitro HiNF-P binding to DNA, and in vivo association by chromatin immunoprecipitations and functional reporter gene assays. Of 177 proliferation-related genes we tested, 20 are modulated in HiNF-P–depleted cells and contain putative HiNF-P binding motifs. We validated that at least three genes (i.e., ATM, PRKDC, and CK2) are HiNF-P dependent and provide data indicating that the DNA damage response is altered in HiNF-P–depleted cells. We conclude that, in addition to histone genes, HiNF-P also regulates expression of nonhistone targets that influence competency for cell cycle progression. [Cancer Res 2007;67(21):10334–42]

Introduction

HiNF-P is the gene regulatory end point for the cyclin E/cyclin-dependent kinase (CDK)-2/p220NPAT pathway that activates multiple histone H4 genes and supports chromatin packaging of nascent DNA (1–7). This pathway may be a key component of a broader cell cycle checkpoint (“S point”) defined, in part, by HiNF-P–responsive genes that are distinct from those encoding histones. The cell cycle regulatory role of HiNF-P in controlling histone gene expression is functionally coupled to formation of a HiNF-P/p220NPAT transcriptional coactivation complex (2). Whereas p220NPAT colocalizes with HiNF-P and histone genes at the G1-S phase transition when transcriptional activation occurs, this protein seems to be spatially restricted to two to four large subnuclear foci (related to Cajal bodies; refs. 2, 4–6). Our immunofluorescence data reveal that a significant fraction of HiNF-P is also located at a large number of other smaller subnuclear foci (2). We have shown that expression of HiNF-P is proliferation related (1, 8, 9). Depletion of HiNF-P decreases histone H4 mRNA levels in asynchronous cells and delays progression into S phase following serum stimulation of quiescent cells (1). These data show that HiNF-P is a critical component of the cell cycle–dependent mechanisms that respond to cyclin E/CDK2 to achieve competency for S-phase entry.

Whereas the HiNF-P/p220NPAT pathway plays a key role in S-phase entry, the majority of data on cyclin E/CDK2 signaling is centered on the target genes of E2F factors. The E2F proteins had been shown earlier to support S-phase entry and are activated following release from repressive RB-related pocket protein complexes through CDK-dependent phosphorylation at the restriction point (10, 11). E2F targets include a number of genes encoding enzymes that are required for nucleotide metabolism as well as proteins involved in DNA replication and cyclin subunits for CDKs. The recent realization that cyclin E/CDK2 signaling diverges into the E2F and HiNF-P pathways (2) necessitates examination of HiNF-P target genes to gain a more comprehensive understanding of regulatory events that control the onset of S phase.

To examine the role of HiNF-P in the transcription of nonhistone genes, we searched for other genes targeted by HiNF-P in asynchronous cells. Here, we have characterized HiNF-P target genes to identify nonhistone cell cycle–related pathways in which HiNF-P may participate. We have characterized genes that are modulated in response to HiNF-P small interfering RNA (siRNA) treatment. Genes directly regulated by HiNF-P were defined by in vitro binding assays, reporter gene assays, and chromatin immunoprecipitations that establish binding of HiNF-P to endogenous chromatin-embedded promoters. One principal result of this study is that HiNF-P interacts with divergently transcribed genes encoding proteins that are involved in DNA damage response pathways (e.g., ATM-p220NPAT and PKRDC-MCM4). We also show that HiNF-P deficiency impairs DNA repair. Based on these findings, we suggest that HiNF-P may have important cell cycle regulatory functions that are complementary to its role in controlling histone gene expression.

Materials and Methods

HiNF-P depletion by siRNA. For siRNA-mediated knockdown of HiNF-P mRNA, human T98G glioblastoma, human HeLa S3 cervical adenocarcinoma, or human U-2 OS osteosarcoma cells were transfected in six-well plates with either siCONTROL Non-Targeting siRNA#1 (Dharmacon), Silencer Negative Control #1 siRNA (Ambion, Inc.), or HiNF-P–specific double-stranded siRNA oligonucleotides (Ambion, Inc.) with Oligofectamine according to the manufacturer’s instructions (Invitrogen).

Cell cycle and cancer gene expression arrays. DNA-free total RNA samples isolated from control and HiNF-P siRNA–treated T98G cells were used for probe synthesis in the presence of [α-32P]dCTP according to the manufacturer’s protocol (SuperArray Bioscience Corp.). This probe was subsequently used to hybridize human cell cycle and cancer GEArray Q Series cDNA gene arrays following the instructions of the same manufacturer. Spot intensities were quantified with a Storm 840 PhosphorImager using ImageQuant 5.0 software (Molecular Dynamics) and normalized to the controls provided in the array. Hierarchical clustering was used to analyze the data, and cDNAs with normalized signal intensity values that differed by at least 1.4-fold between siCONTROL and HiNF-P siRNA oligonucleotides were further investigated.
cDNA synthesis and real-time quantitative PCR. Total RNA from sicontrol Non-Targeting siRNA (#1 (Dharmacon) and HiNF-P siRNA-treated HeLa cells was either subjected to DNase I digestion and purified by column chromatography (DNA-free RNA Kit, Zymo Research) or purified using the RNeasy Plus Mini Kit (Qiagen) and used to prepare cDNA with the SuperScript First-Strand Synthesis System (Invitrogen). Quantitation was determined using a 7000 sequence detection system (Applied Biosystems) and SYBR Green chemistry (SYBR Green PCR Master Mix; Applied Biosystems). The relative mRNA expression was determined by the ΔΔCt method. The following primer pairs were used for human mRNAs (in 5′-3′ direction): HiNF-P forward, TCCAGCCTGCTTACTGTCCT, and reverse, TCAGCTTGGTGTGGTAGCAG; H4/n forward AGCTGTCTATCGGGTTCTGGGTAGAAGCACGTCCGGGCCG; and a nonspecific competitor, RB1 (proximal), TTCCGCCCGCGGCGTCACGTCCGCGAGG; RB1 (distal), TATTGGCCAAGTCCGCTAAG; PRKDC-MCM4, CGCGGAGCCGACGG-GAATGATTCACCAA.

Chromatin immunoprecipitations. T98G cells were washed twice with ice-cold 1× PBS and subsequently cross-linked with 1% formaldehyde in 1× PBS for 10 min at room temperature with gentle agitation. To quench the cross-linking reaction, 0.125 M glycine in 1× PBS was added for 5 min. Cells were then washed twice with ice-cold 1× PBS, scraped in lysis buffer [50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1% NP40, 2× Complete protease inhibitor] and incubated on ice for 20 min. Lysates were sonicated to an average DNA size of 100 to 500 bp and then cleared by centrifugation at 14,000 rpm for 15 min at 4°C.Crude rabbit antiserum (3 μL) against HiNF-P, pre-bled serum (3 μL), or 2 μg of RNA polymerase II antibody were added and rotated at 4°C overnight. One-tenth volume of protein A/G beads (Santa Cruz Biotechnology) was added for 1 h at 4°C. Beads were then washed consecutively with the following buffers: low-salt [20 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1% Triton X-100, 2 ml/L EDTA, 1× Complete protease inhibitor], high-salt [20 mmol/L Tris-HCl (pH 8.0), 500 mmol/L NaCl, 1% Triton X-100, 2 ml/L EDTA], LiCl [10 mmol/L Tris-HCl (pH 8.0), 250 mmol/L LiCl, 1% deoxycholate, 1% NP40, 1 mmol/L EDTA], and three washes with Tri-DTA [10 mmol/L Tris-HCl (pH 8.0), 1 ml/L EDTA]. Protein/DNA complexes were eluted twice with elution buffer (1% SDS, 100 ml/L NaHCO3) at room temperature. One-tenth volume of 3 mol/L sodium acetate (pH 5.2) was added and samples were incubated at 65°C overnight to reverse cross-links. Genomic DNA was purified by phenol-chloroform extraction followed by isopropanol precipitation with 5 to 20 μg of glycogen carrier and dissolved in resuspension buffer (10 mmol/L Tris-HCl, pH 8.0).

Analysis of chromatin immunoprecipitations by real-time quantitative PCR. Quantitation of DNA from chromatin immunoprecipitation samples was achieved by real-time quantitative PCR using a 7000 sequence detection system (Applied Biosystems) and SYBR Green chemistry (SYBR Green PCR Master Mix; Applied Biosystems). The amount of DNA was determined using a standard curve and is expressed as percentage of input. PCR conditions were 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. We also carried out a dissociation protocol to ensure that a single peak was obtained. The following primer pairs were used for chromatin immunoprecipitation analyses: H4/n forward, AGCTGTCTATCGGGTTCTGGGTAGAAGCACGTCCGGGCCG; and a nonspecific competitor, RB1 (proximal), TTCCGCCCGCGGCGTCACGTCCGCGAGG; RB1 (distal), TATTGGCCAAGTCCGCTAAG; PRKDC-MCM4, CGCGGAGCCGACGG-GAATGATTCACCAA.

Electrophoretic mobility shift assay. Binding of HiNF-P to the specified oligonucleotides was assessed with in vitro transcribed/translated HiNF-P protein produced by the TNT Coupled Reticulocyte Lysate System (Promega). In vitro DNA binding reactions were done by combining 1 μl of in vitro transcribed/translated HiNF-P protein in 8 μl of protein buffer [final concentrations of 8 mmol/L HEPES pH 7.5, 0.08 mmol/L EDTA pH 8.0, 50 mmol/L KCl, 10% glycerol, 1× Complete protease inhibitor (Roche), 1 mmol/L NaF, and 1 mmol/L Na3VO4] with 10 μl of a DNA mixture containing 20 fmol of labeled, double-stranded oligonucleotide in DNA buffer (final concentrations of 0.1 μg/ml of salmon sperm DNA, 1 mmol/L DTT, 0.5 mmol/L MgCl2, 0.1 mmol/L MnCl2). Where indicated, unlabeled oligonucleotide competitor (in 1 μl) was added at 25- to 100-fold molar excess. Mixtures were incubated for 20 min at room temperature, and the protein/DNA complexes were then separated on a 4% (40:1) native polyacrylamide gel using 1× Tris-borate EDTA as running buffer at 4°C. Gels were dried and exposed to BioMax XAR (Kodak) films at –80°C. In affinity competition experiments, signal intensities of bound DNA [electrophoretic mobility shift assay (EMSA) bands] were quantified with an Alphalmager 2200 (Alpha Innotech Corp.) and expressed as percentage of wild-type binding in the absence of any specific competitor. Oligonucleotides used were in 5′-3′ direction) an optimized HiNF-P binding site and a nonspecific competitor, TTTCAATCTGGTCCGATACT; HiNF-P mutant, CTTCAGGTTTTGACAGGGGAATG, and reverse, GGCACTCATCCCCGTAGTAA; CKS2 forward, ACCGGCTATGTGTTACCC, and reverse, TGTGGTTCTGGCTCATGAAT; and RB1 forward, TTACCCTCTACGAGGTTAG, and reverse, AGTCCC-GAATGATTTCACCAA.

Differences in RNA levels between control and HiNF-P-specific siRNA treatments were elevated using general linear mixed models (12). Models were fit by restricted maximum likelihood estimation (13) using the packages. Statistical significance is defined as p < 0.05.
Lysates were measured for luciferase activity and normalized to Renilla (phRL-null) activity (dual-luciferase reporter assay system, Promega). Significant differences were determined in three independent experiments by Student's t test (**, P < 0.01).

Immunofluorescence microscopy and γ-irradiation of cells. U-2 OS cells were grown in six-well plates with or without coverslips (Fisher Scientific), seeded at a density of 80,000 per well, and treated after 24 h with siRNA oligonucleotides for 48 h as described above. DNA

**Figure 1.** Identification of HiNF-P target genes by siRNA depletion using cDNA arrays. A, DNA-free total RNA samples isolated from control and HiNF-P siRNA–treated T98G cells were used for synthesis of radioactive cDNA pools that were hybridized in duplicate to cell cycle and cancer gene arrays. A total of 10 and 18 HiNF-P–dependent genes were identified in cell cycle arrays and 6 and 32 genes in cancer arrays, which are consistently up-regulated (†) or down-regulated (‡). Subsets of these genes contain the HiNF-P core motif 5′-GTCCG-3′. HiNF-P protein depletion was confirmed by Western blotting. Bottom, protein samples of control (siC) and HiNF-P (siPB) siRNA oligonucleotide–treated T98G cells (CDK2 is shown as a loading control). B, cell cycle–related functions of six HiNF-P target genes that we selected for further analysis.
We determined that seven HiNF-P–responsive genes from core motif 5 of human histone H4 genes. Our search strategy focused on the HiNF-P consensus motif present in the cell cycle regulatory site depletion of HiNF-P modulates expression of 64 genes. To identify common to both arrays (Fig. 1). Of 177 independent genes, containing 96 cell cycle– or cancer-related genes; 15 genes are transition, these studies were done using asynchronous cells.

**Results**

**HiNF-P regulates expression of nonhistone genes.** To assess whether the histone gene activator HiNF-P can participate in regulation of nonhistone genes, we monitored a panel of cell growth regulatory genes for changes in expression in HiNF-P–depleted cells. HiNF-P uses the G1-S phase–related cofactor p220NPAT to activate histone H4 genes, but other cofactors may synergize with HiNF-P in other cell cycle stages. Because one cannot assume a priori that nonhistone targets are only regulated at the G 1-S transition, these studies were done using asynchronous cells.

Total RNA from siRNA-treated T98G cells was used to carry out real-time quantitative PCR analysis of total cellular RNA from HiNF-P–depleted cells. HeLa cells were treated with two distinct HiNF-P specific siRNA oligonucleotides at 25 nmol/L each. Each siRNA oligonucleotide reduces HiNF-P mRNA levels by 68% to 76% and leads to a ~44% decrease in mRNA levels for the two identical histone genes and p220NPAT, as well as CKS2 mRNA (Fig. 2 B), that represent prototypical HiNF-P–responsive genes (1–3, 20). We find that HiNF-P siRNA treatment reduces the levels of the ATM and PRKDC mRNAs, as well as CKS2 mRNA (Fig. 2 B), in a statistically significant manner, although minor changes observed in MCM4 and p220NPAT levels are not significant. Taken together, these results indicate that HiNF-P depletion regulates the expression of several nonhistone genes.

**Identification of HiNF-P binding sites in nonhistone genes.** To determine whether the six identified HiNF-P–responsive genes directly bind HiNF-P, we carried out EMSAs. We used putative HiNF-P recognition motifs located in the target gene promoters as probes. As previously established (17), HiNF-P binds with high affinity to its recognition motif in the H4/n gene (Fig. 3A). This HiNF-P protein/DNA complex is competed...
effectively by the unlabeled wild-type binding site but not by the corresponding mutant site or a nonspecific oligonucleotide. We assayed direct binding of HiNF-P to radiolabeled oligonucleotides containing each of the putative binding sites (Fig. 3A, right). We find that the sites derived from the PRKDC-MCM4 and RB1 (proximal) genes exhibit robust formation of a HiNF-P protein/DNA complex, comparable to that of the canonical HiNF-P target gene (histone H4/n-DNA complex, comparable to that of the canonical HiNF-P). (proximal) genes exhibit robust formation of a HiNF-P protein/DNA complex, comparable to that of the canonical HiNF-P.

In vivo interaction of HiNF-P with the shared regulatory regions of putative target genes. We carried out chromatin immunoprecipitation analyses to investigate in vivo interaction of HiNF-P with the endogenous promoters of the paired ATMP220NPAT and PRKDC-MCM4 gene loci, as well as the CKS2 locus, within the context of nucleosomal organization. As we have previously established (1–3), HiNF-P and RNA polymerase II both bind to the human histone H4/n promoter in actively proliferating cells (Fig. 4A). HiNF-P and RNA polymerase II each exhibit chromatin immunoprecipitation DNA enrichment for the H4/n locus that is ~30-fold above IgG background levels; because the primer set also amplifies a duplicate of the H4/n gene (i.e., H4/o; ref. 20), the values we measured are doubled relative to single-copy genes. We applied two probe sets that interrogate HiNF-P binding to each of the two sites within the intergenic regulatory region of the ATMP220NPAT locus: one set that examines the single HiNF-P element in the PRKDC-MCM4 locus and another set for the solitary CKS2 gene. We observed strong binding to both ATMP220NPAT.

Figure 3. Interaction of HiNF-P protein and its binding core motif in selected target genes. A, oligonucleotides for each target gene were labeled and used independently as probes to detect direct HiNF-P binding. Left, HiNF-P binding to a probe spanning its classic site in the histone H4/n gene is shown as control. Right, binding of HiNF-P to radiolabeled probes of nonhistone target genes. Competition experiments with unlabeled oligonucleotides were done at 100-fold molar excess. The arrowheads (P) indicate the HiNF-P/DNA complexes (short exposure, 10 h; long exposure, 96 h). B, alignment of sequences was done using Web-based resources (Kalign and Boxshade). MIZF protein is identical to HiNF-P; the MIZF consensus binding sequence is taken from Sekimata and Homma (19). The nonhistone HiNF-P consensus sequence represents an all-inclusive redundant motif that is based on all the validated HiNF-P binding sequences presented here with the exception of the distal site of RB1, which is a weak binding site. Symbols used: V, A or C or G; H, A or C or T; B, C or G or T; S, C or G; M, A or C; K, G or T; C, EMSAs were done with in vitro transcribed/translated HiNF-P protein and radiolabeled probes spanning putative HiNF-P binding sites. The arrowhead (P) indicates the HiNF-P/DNA complex. Competition assays were done with 100-fold molar excess of unlabeled oligonucleotides spanning the HiNF-P binding core motif. The HiNF-P probe sequence, its binding core motif (bold), and the mutated nucleotides (below) are shown at the bottom. D, titration experiments with competitor oligonucleotides reveal relative differences in strength of binding.
Novel Nonhistone HiNF-P Target Genes

Figure 4. In vivo binding of HiNF-P to nonhistone genes. A, chromatin immunoprecipitation assays were done in T98G cells with a HiNF-P antiserum and its corresponding pre-bleed (control for nonspecific binding) as well as RNA polymerase II (RNA pol II) antibody to define direct HiNF-P target genes. HiNF-P binding to its target gene promoters in chromatin immunoprecipitation samples was detected by real-time quantitative PCR using specific primer sets and expressed as a percentage of input. The known HiNF-P target gene histone H4/n is shown as a reference. HiNF-P and RNA polymerase II show an ~30-fold signal above background level (pre-bleed). Chromatin immunoprecipitation data are also presented for the ATM-p220NPAT (B), PRKDC-MCM4 (C), and CKS2 (D) loci. The promoter regions for each of these loci with the corresponding HiNF-P core motifs ( ● ) are indicated on top. Arrowheads, primer sets used in quantitative PCR to amplify HiNF-P target gene promoters.

and PRKDC-MCM4 loci with chromatin immunoprecipitation signals that are 8- to 11-fold above background (Figs. 4B and C). For comparison, in vivo binding of HiNF-P to the CKS2 locus is ~6-fold above the nonspecific signal observed for IgG (Fig. 4D). These chromatin immunoprecipitation data are in a similar range (i.e., 0.23-0.70% of input) as histone H4 genes (0.94% of input for H4/n). Therefore, our results validate the interaction of HiNF-P with these nonhistone genes and establish that they are direct targets of HiNF-P. Hence, HiNF-P controls the genes participating in cell cycle pathways that are distinct from genes required for histone gene expression.

HiNF-P/p220NPAT complex bidirectionally activates the divergently transcribed ATM-p220NPAT and PRKDC loci. To test whether HiNF-P, together with its coactivator p220NPAT, can regulate the promoters of either the ATM or p220NPAT gene, we generated luciferase reporter vectors in which the intergenic region separating the genes was inserted in both orientations. We find that forced coexpression of HiNF-P with these nonhistone genes and establish that they are direct targets of HiNF-P. Hence, HiNF-P controls the genes participating in cell cycle pathways that are distinct from genes required for histone gene expression.

The DNA damage pathway is altered in HiNF-P–depleted cells. To examine the physiologic consequences of HiNF-P controlling the ATM and PRKDC genes, we tested whether depletion of HiNF-P alters the DNA damage pathway. To assess DNA damage, we monitored phosphorylation of H2A.X protein (γH2A.X) in HiNF-P–depleted U-2 OS cells. Because γH2A.X concentrates at sites of DNA damage, reduction of γH2A.X levels and disappearance of γH2A.X nuclear foci together permit assessment of the recovery of cells from γ-irradiation–induced DNA lesions. Cells were treated with control and HiNF-P–specific siRNA oligonucleotides for 48 h before γ-irradiation (2 Gy) treatment. We monitored γH2A.X after 6 h of recovery and observed that depletion of HiNF-P elevates γH2A.X protein levels by 1.9-fold (Fig. 5A). Furthermore, examination of HiNF-P siRNA–treated cells by in situ immunofluorescence microscopy reveals that loss of HiNF-P increases the size and intensity of γH2A.X nuclear foci (Fig. 5C). Thus, the DNA damage pathway is altered in HiNF-P–depleted cells. Taken together, these results show that HiNF-P deficiency alters DNA damage pathways, perhaps in part by controlling the ATM and PRKDC genes.
Figure 5. Transcriptional activation of HiNF-P target gene promoter and alteration of the DNA damage pathway. A, transcriptional activation by the HiNF-P/p220NPAT complex of the promoters of the ATM, p220NPAT, and PRKDC genes. The empty pGL-luc vector was used as a negative control. Coexpression experiments were done with reciprocal amounts of expression vectors with or without cDNAs for HiNF-P and p220NPAT to maintain a constant level of DNA in each transfection. Firefly luciferase activity monitored as reporter readout was normalized to Renilla luciferase activity driven by a promoterless plasmid. Statistical significance was determined by the Student's t test for three independent experiments (**, P < 0.01). Alterations in the DNA damage response were monitored by evaluating γH2A.X protein levels by Western blotting and in situ immunofluorescence microscopy. B, quantitation of band intensities (Western blots, left) for two independent experiments after γ-irradiation and 6-h recovery. Cells were treated with control (NC) and HiNF-P–specific (siP) siRNA oligonucleotides to deplete HiNF-P (Western blot, bottom) 48 h before irradiation (2 Gy) treatment (*, P < 0.05). C, left, percentage of cells with low-intensity or high-intensity foci in U-2 OS cells treated with one of two HiNF-P (siPA and siPB) specific siRNA oligonucleotides or control siRNA (NC). Right, the three columns of images are representative microscopic fields of siRNA oligonucleotide–treated cells. The superimposed values represent percentages depicted on the graphs to the left. Images were captured as monochromatic light using MetaMorph software. The images on the top and bottom rows represent high-resolution images to show the appearance of γH2A.X in individual cells. The images in the middle two rows were further processed using Adobe Photoshop. To improve the visualization of our data, the images were inverted (to yield black signals on a white background) and the intensity distribution (“level”) was linearly adjusted; all images were processed in the same manner. Arrowheads, high-intensity γH2A.X foci. Dashed boxes surround cells that were shown in the top and bottom rows as dark-field images. Cells were treated for siRNA/γ-irradiation as described in (B). HiNF-P protein depletion was confirmed by Western blotting (top left); the blot shows protein samples of control (NC) and two HiNF-P siRNA–treated (siPA and siPB) U-2 OS cells (CDK2 is shown as a loading control).
Discussion

In this study, we have shown that the histone gene regulatory complex HiNF-P/p220NPAT can control cell cycle– and cancer-related gene targets beyond histones. By combined application of cDNA gene arrays, siRNA depletion, EMSAs, chromatin immunoprecipitations, and reporter gene assays, we have defined several genes that are directly regulated by HiNF-P/p220NPAT, including ATM (21–23), PRKDC (24, 25), and CKS2 (26). Each of these targets is either directly or indirectly related to competency for cell cycle progression, indicating novel cell growth control regulatory roles for the HiNF-P/p220NPAT complex.

HiNF-P regulation of the genes for ATM and PRKDC suggests functional linkage to pathways involved in repair DNA synthesis. The ATM gene encodes an important kinase that is stimulated after genomic insult and induces the p53-p21 DNA damage response pathway (27). Similarly, the PRKDC kinase participates in the nonhomologous end joining pathway that facilitates repair of double-strand DNA breaks to maintain genome integrity (28, 29). Our data show that HiNF-P deficiency alters the DNA damage response, which is reflected by increased levels of γH2AX protein as well as the intensity and size of γH2AX nuclear foci. Combined with the direct regulation of the ATM and PRKDC genes by HiNF-P, our findings suggest a new function for this transcription factor in DNA damage response pathways that may affect competency for cell cycle progression.

We have also provided evidence indicating that the CKS2 gene is a nonhistone target of HiNF-P. CKS2 belongs to a family of small proteins (9–18 kDa) with two human homologues (CKS1 and CKS2; related to p130) that modulate the levels and/or activities of the cyclin/cycin-dependent kinase complexes. CKS1 serves as an essential cofactor for the ubiquitin-mediated proteolysis of the CDK inhibitor p27Kip1 and p130, which are critical regulators of the G1-S phase transition (30, 31). CKS2 has been shown to be important in controlling the first metaphase-anaphase transition of mammalian meiosis (32). Oocytes must generate storage pools of maternal histone mRNAs in anticipation of the rapid cleavage stages that follow fertilization. HiNF-P is known to be expressed in oocytes and may thus be linked to CKS2-dependent regulatory events associated with mammalian meiosis.

This study has defined multiple recognition motifs in at least six nonhistone genes that interact with native HiNF-P in vivo and/or in vitro. Our functional siRNA results show that HiNF-P is rate-limiting for at least three of these genes (i.e., ATM, PRKDC, and CKS2). Furthermore, HiNF-P (1, 17, 33) is identical to the MIZF protein that was independently isolated in yeast two-hybrid screens (34). Sekimata and Homma (19) subsequently recognized that MIZF has sequence-specific DNA binding activity and defined the binding motif by CASTing analysis with bacterially produced recombinant glutathione S-transferase (GST)-MIZF/HiNF-P fusion protein. These studies showed that MIZF is able to bind and repress the RB promoter in HEK293 cells. In our study, we have confirmed that HiNF-P/MIZF interacts with two recognition motifs (proximal and distal) in the RB promoter. However, siRNA depletion reveals that HiNF-P is not rate-limiting for endogenous RB1 expression in T98G and HeLa cells. In addition, the H4 subtype consensus that we had earlier identified as the recognition element for HiNF-P (1, 3, 17, 33) is significantly longer than the MIZF consensus site defined by Sekimata and Homma (19). Here, using in vitro EMSAs and in vivo chromatin immunoprecipitation analysis with HiNF-P–specific antibodies, we have experimentally delineated a distinct nonhistone consensus element for HiNF-P. This motif is also larger than the MIZF consensus sequence, suggesting that endogenous HiNF-P/MIZF recognizes more extended sequence motifs than the recombinant GST-MIZF fusion protein.

In conclusion, our findings indicate that the cyclin E/CDK2/p220NPAT pathway participates in the control of several distinct cell cycle– and cancer-related genes. The identification of novel nonhistone target genes indicates that HiNF-P has regulatory roles beyond cell cycle control of histone genes at the G1-S phase transition. Our data support the concept that the response of both the E2F/RB and p220NPAT/HiNF-P pathways to cyclin E/CDK2 signaling activates two distinct regulatory programs that together support cell cycle progression.

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