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HiNF-P Is a Bifunctional Regulator of Cell Cycle Controlled Histone H4 Gene Transcription

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Abstract Cell cycle progression beyond the G_1/S phase transition requires the activation of a transcription complex containing histone nuclear factor P (HiNF-P) and nuclear protein mapped to ataxia telangiectasia ($p220^{NPAT}$) in response to cyclin dependent kinase 2 (CDK2)/cyclin E signaling. We show here that the potent co-activating properties of HiNF-P/$p220^{NPAT}$ on the histone H4 gene promoter, which are evident in the majority of human cell types, are sporadically neutralized in distinct somatic cell lines. In cells where HiNF-P and $p220^{NPAT}$ do not activate the H4 gene promoter, HiNF-P instead represses transcription. Our data suggest that the cell type specific expression of the cyclin-dependent kinase inhibitory (CKI) protein $p57^{KIP2}$ inhibits the HiNF-P dependent activation of the histone H4 promoter. We propose that, analogous to E2F proteins and other cell cycle regulatory proteins, HiNF-P is a bifunctional transcriptional regulator that can activate or repress cell cycle controlled genes depending on the cellular context. J. Cell. Biochem. 101: 181–191, 2007. © 2006 Wiley-Liss, Inc.

Key words: CDK inhibitor; p57; KIP2; HiNF-P; NPAT; transcription factor; cell cycle; histone

Entry into S phase necessitates the induction of multiple gene regulatory programs that promote both DNA synthesis and the concomitant obligatory assembly of newly replicated DNA and histone proteins into chromatin. The onset of DNA replication and histone gene expression are each controlled by functionally and mechanistically distinct regulatory modules (i.e., pRB/E2F and $p220^{NPAT}$/HiNF-P, respectively) that respond to cyclin E/CDK2. Cyclin E/CDK2 phosphorylation of the tumor suppressor pRB releases transcription factor E2F, which controls expression of many target genes involved in the enzymology of nucleotide metabolism and/or DNA synthesis (e.g., DHFR, TK, DNA polymerase alpha) [Dyson, 1998; Nevins, 2001]. Furthermore, cyclin E/CDK2 stimulates the activity of the HiNF-P/$p220^{NPAT}$ co-activation complex that coordinately controls the histone H4 gene family [Ma et al., 2000; Zhao et al., 2000; Mitra et al., 2003; Holmes et al., 2005; Miele et al., 2005], a set of 15 genes encoding the most highly conserved nucleosomal protein [Stein et al., 1984]. Consistent with the critical coupling of histone gene expression and DNA replication, deficiency of HiNF-P [Mitra et al., 2003] or CDK2 [Berthet et al., 2003; Martin et al., 2005] severely delays progression through S phase, while genetic ablation of $p220^{NPAT}$ blocks entry into S phase [Ye et al., 2003] and normal embryonic development [Di Fruscio et al., 1997].

The discovery of HiNF-P as the critical transcription factor for histone H4 gene transcription [Mitra et al., 2003] emerged from a large number of studies that used the human histone H4/n gene (pF0108; locus HIST2H4 at 1q21) as a paradigm to study transcriptional control at the G_1/S phase transition (reviewed in [Stein et al., 1992, 1996]). HiNF-P is one of three factors that bind to a highly conserved domain (H4 subtype specific element) in the histone H4 gene promoter [van Wijnen et al., 1991, 1992;

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Mitra et al., 2003; Holmes et al., 2005]. Apart from HiNF-P that we identified as a novel multi-zinc finger protein [Mitra et al., 2003], the H4 subtype specific element interacts with interferon regulatory factors (IRFs) [Vaughan et al., 1995; Xie et al., 2001], and the CCAAT displacement protein (CDP-cut) which forms a complex with $p105^{RB1}$, cyclin A, and CDK1 [van Wijnen et al., 1989, 1994, 1996; Aziz et al., 1998b; Gupta et al., 2003]. The H4 subtype specific element is directly adjacent to the TATA-element and this geometric arrangement is conserved among functionally expressed vertebrate histone H4 genes [Holmes et al., 2005]. Importantly, deletion of the H4 subtype specific element abrogates correct cap-site initiation of histone H4 mRNA transcripts in vivo [Kroeger et al., 1987]. Thus, the multiple protein/DNA interactions at site II may directly regulate the TATA box protein dependent initiation of histone H4 gene transcription.

Studies examining in vivo protein/DNA interactions in the H4/n promoter have shown that both the H4 subtype specific element and the adjacent TATA box are contained within a large genomic DNaseI footprint (site II) [Pauli et al., 1987; Hovhannisyan et al., 2003]. Occupancy of site II correlates with active transcription of histone H4 genes and site II is rendered vacant when histone H4 gene expression is shut down during differentiation [Stein et al., 1989; Hovhannisyan et al., 2003]. Loss of site II protein/DNA interactions at the H4 locus is mediated in part by down-regulation of the cognate proteins [Holthuis et al., 1990; van den Ent et al., 1993; Hovhannisyan et al., 2003]. Functional studies have revealed that site II contributes to basal promoter activity, and is required and sufficient (together with a TATA box) for cell cycle regulated transcription [Kroeger et al., 1987; Ramsey-Ewing et al., 1994; Birnbaum et al., 1995b; Aziz et al., 1998a]. The activity of site II is dramatically enhanced by an upstream element (Site I) that binds transcription factors SP-1, YY1, ATF-1, and HiNF-A/HMG-Y [van Wijnen et al., 1989; Birnbaum et al., 1995a,b; Guo et al., 1997; van der Meijden et al., 1998; Last et al., 1999]. Hence, the interplay of multiple transcription factors at the H4 promoter determines the level at which the gene is transcribed.

Histone genes must be actively transcribed in all proliferating cell types, and the question arises whether the same molecular mechanisms are operative in each cell type to promote H4 gene transcription. In this study, we show that the trans-activation potential of the H4 subtype specific transcription factor HiNF-P, which is evident in the majority of cell types, is sporadically rendered ineffective in selected cell types. Our finding suggests that the proliferation-specific transcription of histone H4 genes may be mediated by alternative mechanisms in which HiNF-P operates as an activator or a repressor, perhaps depending on the growth phenotype of the cell.

MATERIALS AND METHODS

Cell Culture

Transfection experiments were performed with a panel of cell lines, including HPV-18 transformed human cervical carcinoma HeLa cells, human Saos-2 osteosarcoma cells, SV40 transformed monkey kidney COS-7 fibroblasts, human T98G glioblastoma cells, SV40 T antigen transformed human 293 T embryonic kidney cells, human IMR-90 female fetal diploid fibroblasts, and human WI-38 fetal lung diploid fibroblasts, all of which were obtained from the America Type Culture Collection (Rockville, MD). Rat ROS 17/2.8 osteosarcoma cells were a kind gift of Dr. Gideon Rodan. Saos-2 cells were maintained in McCoy's 5A medium (Gibco/Invitrogen, Carlsbad, CA) supplemented with 15% fetal bovine serum, 2 mM L-glutamine. HeLa, T98G, 293T, and COS-7 cells were cultured in Delbecco's modified Eagle's medium (DMEM; Gibco/Invitrogen) supplemented with 10% FBS, whereas ROS 17/2.8 cells were maintained in F12 (Gibco/Invitrogen) medium containing 5% FBS. All cell culture mediums were supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and cells are maintained in humidified 5% CO_2 incubator.

Transfections and Reporter Gene Assays

Cells were plated at a density of $2–2.5 \times 10^5$ cells/ml and incubated overnight at 37°C before transfection. Histone gene promoter constructs fused to either the chloramphenical acetyl transferase (CAT) or the luciferase reporter gene have been described previously [Ramsey-Ewing et al., 1994; Mitra et al., 2003]. Expression vectors for Xpress-tagged human HiNF-P, $p220^{NPAT}$, and Myc-tagged $p57^{KIP2}$ (generous gift of Roger Watson, Imperial College of London, UK) were described in a

previous study [Mitra et al., 2003]. About 2 μg plasmid DNA was transfected in each well of a 35 mm plate using Fugene6 (Roche). Cells were harvested after 24 h and lysed by adding 200 μl of lysis buffer (Promega) to each well of a six-well plate and incubated for 10 min at room temperature. Extracts were collected and centrifuged for 10 min to collect the supernatants which were then used for reporter gene assays (CAT or Luc Assay). Relative promoter activity of CAT or Luc promoter-reporter is presented as percent conversion or fold activity.

Extract Preparation and Western Blot Analysis

Whole cell extracts were prepared from the indicated cell lines which were plated at a density of $2-2.5 \times 10^5$/ml and allowed to grow for 36 h. Cells were harvested and pellet of about $4.5-5 \times 10^7$ cells were suspended in 0.5 ml lysis buffer (20 mM HEPES pH 7.5, 0.5 M KCl, 0.2 mM EDTA pH 8.0, 0.5 mM PMSF, 0.5 mM DTT, 0.1% NP-40, and 20% glycerol). Cell suspensions were quickly frozen and incubated at 37°C to complete lysis. Whole cell extracts (i.e., 30 μg protein lysate) were centrifuged at 10,000*g* for 10 min and the supernatants were collected for analysis of endogenous proteins by western blotting following 10% SDS–PAGE. Lysates from transfected cells (~15–20 μg protein per sample) were analyzed for expression of exogenous proteins using epitope tags (see below). For immunoblot experiments, PVDF membranes (Millipore, UK) were washed with phosphate buffered saline (PBS) with 0.5% NP-40. After secondary antibody treatment, blots were developed by chemiluminescence (Amersham Biosciences).

RT-PCR and Southern Blotting

Purified total RNA was isolated from asynchronously growing cells (Trizol, Invitrogen) and subjected to RT-PCR as described by the manufacturer (Roche). We used primer set described by Tokino et al. [1996] to detect the expression of $p57^{KIP2}$ mRNA. The same RNA sample was also used to detect expression of histone H4 [Holmes et al., 2005], HiNF-P [Mitra et al., 2003], and glyceraldehydes-3-phosphate dehydrogenase (GAPDH). Aliquots (~10 μl) from each sample were analyzed by agarose gel electrophoresis to visualize cDNA amplification. To confirm selective amplification of $p57^{KIP2}$ cDNAs from RNA samples, small aliquots of the RT-PCR products were separated by gel electrophoresis and subjected to Southern blot analysis using a human $p57^{KIP2}$ cDNA as probe.

RESULTS

HiNF-P Is a Bifunctional Transcriptional Regulator

We have previously purified and characterized HiNF-P as a critical transcriptional regulator that controls the E2F independent induction of human histone H4 gene transcription at the G_1/S phase transition. HiNF-P operates as the ultimate link of the cyclin E/CDK2/$p220^{NPAT}$/HiNF-P gene regulatory cascade [Mitra et al., 2003; Miele et al., 2005] through a highly conserved and multipartite element (site II-CCE or H4 subtype specific consensus element) that is contiguous to the histone H4 TATA box [Pauli et al., 1987; van Wijnen et al., 1992]. As expected, forced expression of HiNF-P results in a robust (up to fivefold) and dose-dependent activation of histone H4 promoter activity in human Saos-2 cells (Fig. 1A). Strikingly, the histone H4 promoter is not activated but rather is suppressed by HiNF-P in human HeLa S3 cells (Fig. 1A). For comparison, in both Saos-2 and HeLa S3 cells, there is no appreciable effect of forced expression of HiNF-P on the DHFR gene promoter, which is known to be regulated by an E2F dependent mechanism (Fig. 1A). Furthermore, transfection experiments with H4 promoter point mutants show that mutation of the HiNF-P site, but not of recognition motifs for other site II binding proteins (i.e., HiNF-M/IRF2 or HiNF-D/CDP) [Vaughan et al., 1995; van Wijnen et al., 1996; Gupta et al., 2003], abolishes the transcriptional repression by HiNF-P (Fig. 1C). Deletion of regulatory elements upstream from the HiNF-P element (i.e., removal of Site I) reveals that site II with its embedded HiNF-P binding site is sufficient for gene repression (Fig. 1B). Because HiNF-P is a transcriptional activator in Saos-2 cells and a repressor in HeLa cells, we propose that HiNF-P is a bifunctional transcription factor and its promoter-selective gene regulatory activity is cell type dependent.

$p220^{NPAT}$ Is Functionally Inactive in HeLa Cells

Histone H4 gene activation at the G_1/S phase transition requires a direct physical association between HiNF-P and its co-activator $p220^{NPAT}$

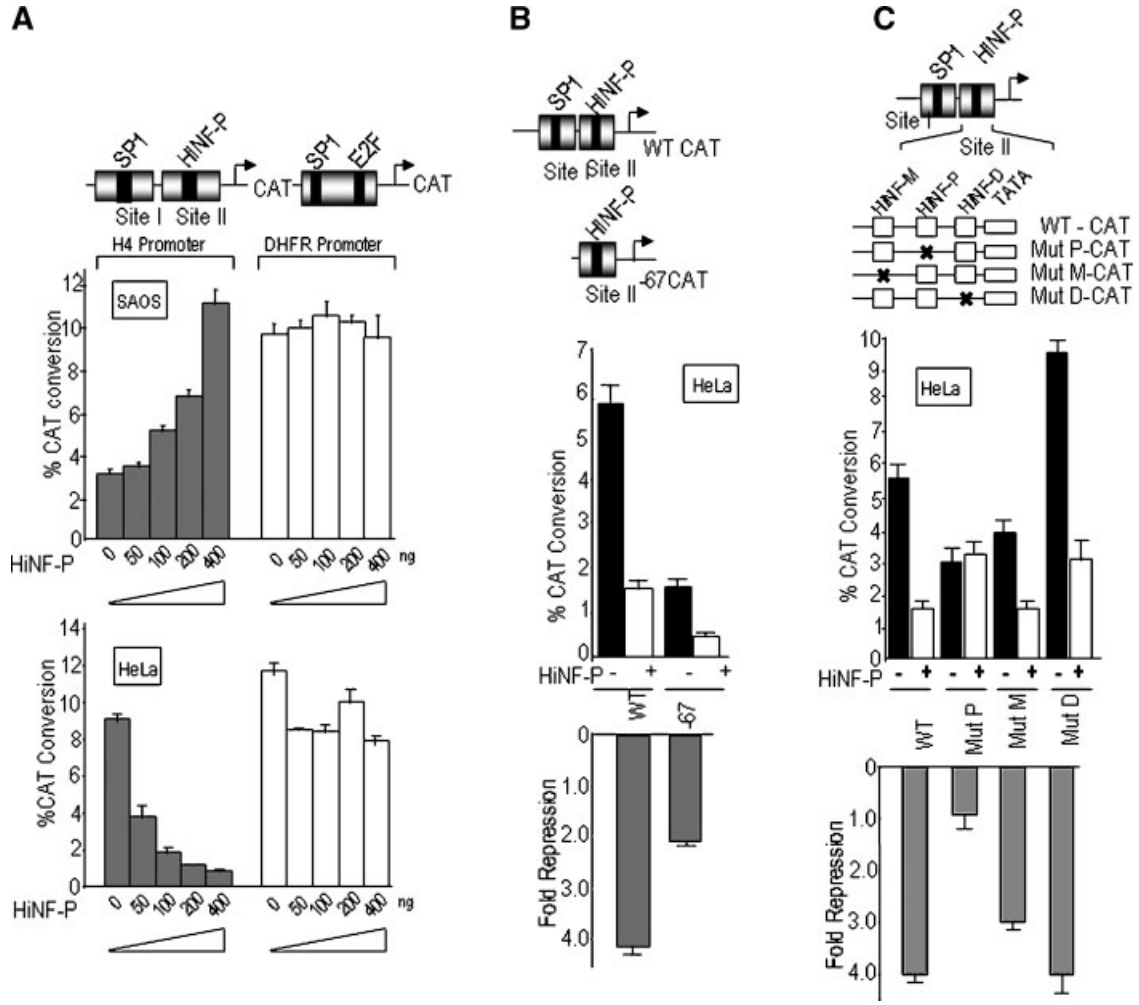

Fig. 1. HiNF-P is a bifunctional regulator of H4 gene transcription. **A**: Dose-response curves with increasing amounts of HiNF-P expression vector reveal cell type specific effects of HiNF-P on the activity of the histone H4 but not the DHFR promoter. Actively proliferating Saos-2 and HeLa cells were transiently transfected with CAT constructs containing promoters for histone H4 or DHFR in the presence of the indicated amounts of HiNF-P expression vector (in ng). The bar graphs show the relative CAT activity for the histone H4 (**left**; gray bars) and DHFR (**right**; open bars) promoters in Saos-2 (**upper panel**) and HeLa cells (**lower panel**). Key elements in the H4 and DHFR promoters are indicated in the schematic at the top [van Wijnen et al., 1992; Jensen et al., 1997]. The data represent the average of three independent triplicate experiments. **B**: The H4 promoter cell cycle element site II is sufficient for HiNF-P dependent transcriptional inhibition. Asynchronously growing HeLa cells were transfected with CAT constructs (diagram above the bar graphs) containing the wild-type H4 promoter (-215/CAT) or a truncated promoter (-67/CAT) [Ramsey-Ewing et al., 1994] in the absence (−; black bars) or presence of the (+; open bars); HiNF-P expression vector (200 ng/well). The bar graphs indicate relative CAT activity (**upper panel**) or the fold repression relative to the control (**lower panel**). **C**: Down-regulation of H4 promoter activity in HeLa cells requires an intact HiNF-P element. HiNF-P mediated down-regulation of H4 promoter/CAT reporter genes with point mutations in site II (see **top panel**) is not observed when the recognition motif of HiNF-P is mutated (MutP-mutant CAT). Mutation of the HiNF-M (MutM-CAT) or HiNF-D (MutD-mutant CAT) binding site has no effect on regulation of H4 promoter activity. The bar graphs indicate relative CAT activity (**upper panel**) or the fold repression relative to the control (**lower panel**).

[Mitra et al., 2003; Miele et al., 2005], which is a substrate of cyclin E/CDK2 [Zhao et al., 1998, 2000; Ma et al., 2000; Wei et al., 2003]. We therefore tested whether co-activation by $p220^{NPAT}$ is compromised in HeLa S3 cells. Forced expression of HiNF-P or $p220^{NPAT}$ in COS7 cells increases H4 gene promoter activity by at least four- to five-fold, and co-expression of both proteins causes a 20-fold synergistic induction of promoter activity (Fig. 2A). In striking contrast to the results with COS7 cells (Fig. 2A) and Saos-2 cells [Mitra et al., 2003], $p220^{NPAT}$ is not capable of either stimulating H4 gene transcription on its own or functioning as a co-activator of HiNF-P in HeLa S3 cells (Fig. 2B). Hence, HiNF-P dependent repression

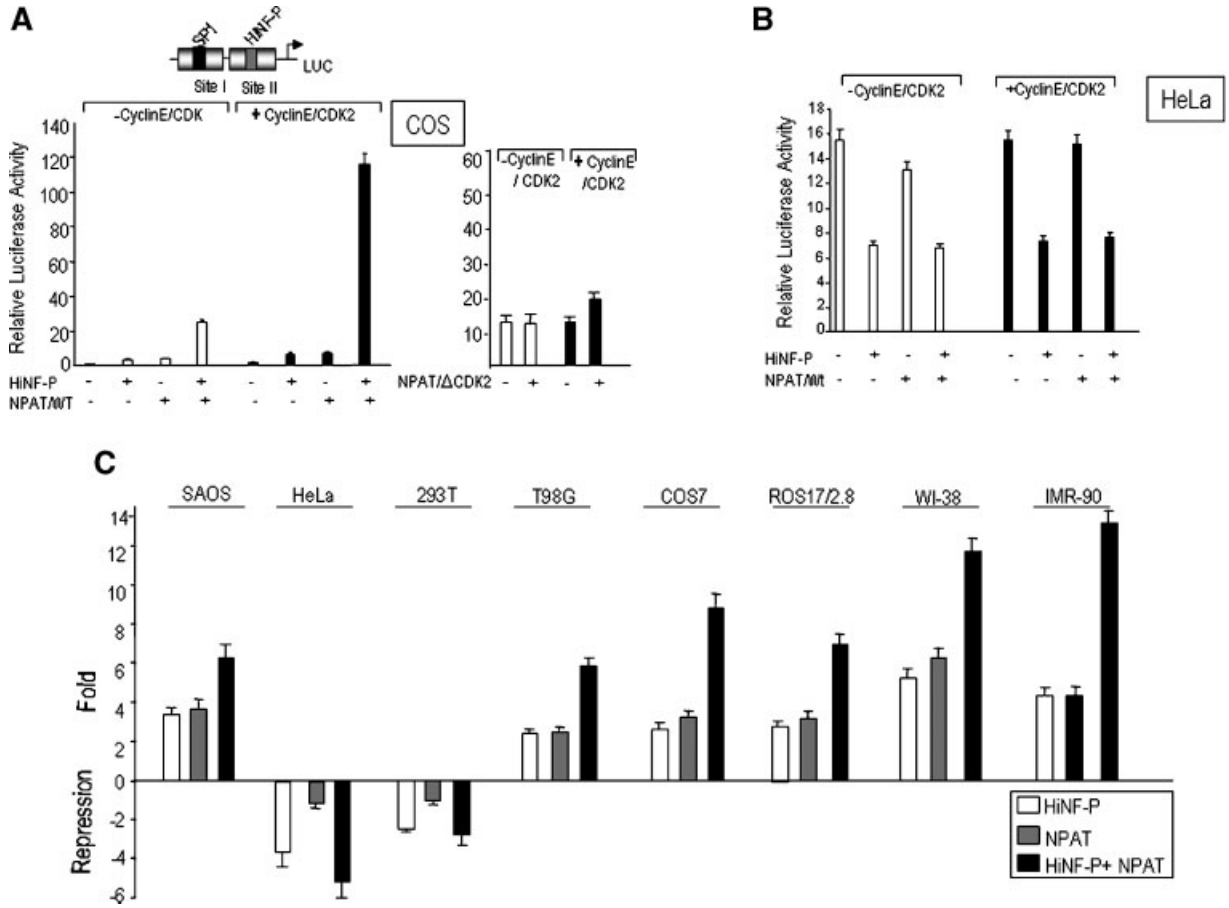

Fig. 2. Cyclin E/CDK2 stimulation of HiNF-P and p220^{NPAT} is cell type dependent. **A**: Cyclin E/CDK2 stimulates co-activation of H4 gene transcription by HiNF-P and wild-type p220^{NPAT} but does not stimulate ΔCDK2 p220^{NPAT}. COS-7 cells were co-transfected with a histone H4/luciferase reporter gene and different combinations of expression vectors [presence (+) or absence (−)] for HiNF-P (200 ng/well), p220^{NPAT} (150 ng/well), and/or cyclin E together with CDK2 (500 ng of each per well). Luciferase activity reflecting histone H4 promoter activity was measured 24–30 h after transfection. **B**: Forced expression of cyclin E and CDK2 does not alleviate HiNF-P mediated repression of H4 gene transcription and inactivation of p220^{NPAT}. Transfections were performed using proliferating HeLa cells and data were plotted using the same symbols as described in **Panel A**. **C**: HiNF-P dependent repression of histone H4 gene transcription is cell type specific. Actively proliferating cells (HeLa, Saos-2, T98G, COS-7, ROS 17/2.8, 293T, and WI-38) were transfected with wild-type H4 promoter/luciferase reporter gene constructs together with expression vectors for HiNF-P (200 ng/well) and/or p220^{NPAT} (300 ng/well) as indicated by plus (present) and minus (absent) symbols. Promoter upon activity was measured as relative luciferase activity and values are presented as fold activation relative to the values obtained for the promoter-less Luciferase construct (arbitrarily set as 1). H4 gene transcription is enhanced by forced expression of HiNF-P (open bars), p220^{NPAT} (gray bars) or both (black bars) in all cell lines except HeLa and 293T cells.

correlates with neutralization of the co-activation function of p220^{NPAT}.

Co-expression of cyclin E/CDK2 normally stimulates HiNF-P and/or p220^{NPAT} dependent transactivation by three- to five-fold in COS7 (Fig. 2A) or Saos-2 cells [Mitra et al., 2003]. This stimulatory effect of cyclin E/CDK2 on H4 gene transcription is not evident with a p220^{NPAT}mutant that lacks cyclin E/CDK2 phosphorylation sites (p220^{NPAT}ΔCDK2) (Fig. 2A, right panel). More importantly, supplementation of HeLa S3 cells with co-expressed cyclin E/CDK2 does not endow p220^{NPAT} with competency for co-activation (Fig. 2B). Thus, while CDK2 phosphorylation sites in p220^{NPAT} are critical for the activation function of the HiNF-P/p220^{NPAT} complex, increased levels of cyclin E/CDK2 alone are not sufficient to overcome inhibition of this complex in HeLa cells.

HiNF-P/p220^{NPAT} Mediated Activation of Histone H4 Gene Transcription Is Cell Type Specific

Because HiNF-P and p220^{NPAT} form a co-activation complex [Mitra et al., 2003; Miele

et al., 2005], we assessed whether there are differences in the fundamental molecular properties of these two proteins in HeLa S3 and Saos-2 cells. The relative expression of HiNF-P mRNA and protein, as well as its DNA binding activity are not appreciably different between HeLa S3 and Saos-2 cells (data not shown). Furthermore, sequence analysis of HiNF-P cDNAs and immunofluorescence microscopy with HiNF-P antibodies reveal that both cell types express a wild-type HiNF-P protein with normal nuclear localization. Immunoprecipitation and chromatin immunoprecipitation experiments revealed that HiNF-P and $p220^{NPAT}$ can form a complex that interacts with histone H4 gene promoters in both HeLa S3 and Saos-2 cells ([Mitra et al., 2003; Miele et al., 2005], and data not shown). The absence of differences in these basic parameters of the HiNF-P/$p220^{NPAT}$ complex suggests that other factors suppress its activation potential in HeLa S3 cells.

We examined the prevalence of HiNF-P/$p220^{NPAT}$ inactivation in several established mammalian cell lines with distinct growth phenotypes. Similar to human Saos-2 osteosarcoma cells, we find that HiNF-P activates the H4 promoter and that $p220^{NPAT}$ is capable of co-activating H4 gene transcription in several tumor-derived or transformed cell types (Fig. 2C). In contrast, HiNF-P represses the H4 promoter in human 293T embryonic kidney cells while $p220^{NPAT}$ does not stimulate H4 gene transcription (Fig. 2C). Importantly, the HiNF-P/$p220^{NPAT}$ complex enhances H4 gene transcription in normal diploid WI-38 and IMR-90 fibroblasts (Fig. 2C). Thus, the activity of the cyclin E/CDK2/$p220^{NPAT}$/HiNF-P pathway is intrinsically stimulatory in the majority of cell types, but not in specific tumor or transformed cells (e.g., HeLa S3 and 293T).

HiNF-P Is Inactivated in Cells Expressing Elevated Levels of $p57^{KIP2}$

Because previous results suggested that expression of $p57^{KIP2}$ above its normal physiological level in 293T cells can inhibit $p220^{NPAT}$ activity [Ma et al., 2000], we tested whether the repressive activity of HiNF-P and the transcriptional inertness of $p220^{NPAT}$ is controlled by endogenous levels of $p57^{KIP2}$. While HeLa S3 and 293T cells express clearly detectable levels of $p57^{KIP2}$ protein as assessed by immunoblotting, $p57^{KIP2}$ levels are below our detection limit in several cell types (Fig. 3A) which we have shown to exhibit HiNF-P/$p220^{NPAT}$

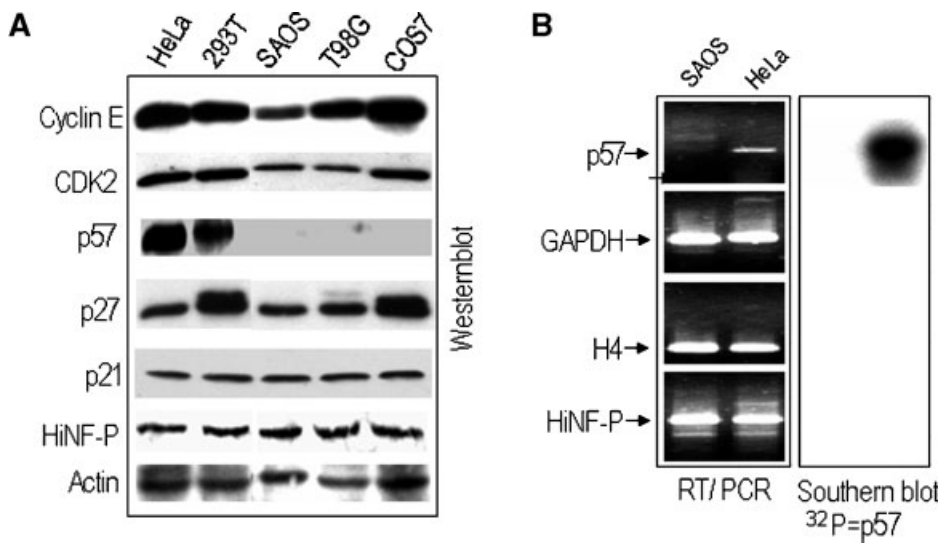

Fig. 3. The cell type specific repression of histone H4 gene transcription by HiNF-P correlates with endogenous $p57^{KIP2}$ levels. **A**: Whole cell lysates (about 20 μg protein) of actively proliferating cell lines (HeLa, 293T, Saos-2, T98G, and COS7) were analyzed on SDS–PAGE and analyzed by western blotting using the indicated antibodies. Compared other cell lines, the endogenous level of $p57^{KIP2}$ is high in HeLa and 293T cells. **B**: Total RNA was isolated form actively growing HeLa and Saos-2 cell and used in RT-PCR to monitor expression of mRNAs for $p57^{KIP2}$, GAPDH, histone H4, and HiNF-P. Expression of $p57^{KIP2}$ was observed only in HeLa cells but not in Saos-2 cells, but GAPDH, histone H4, and HiNF-P are easily detected in both cell lines (**left panel**). To validate selective expression of $p57^{KIP2}$, we subjected the amplified $p57^{KIP2}$ product to Southern blot analysis using $p57^{KIP2}$ cDNA as a probe (**right panel**).

co-activation (see Fig. 2C). Importantly, the levels of the closely related $p27^{KIP1}$ protein or the third member of the Kip/Cip class of CDK inhibitors (i.e., $p21^{CIP1}$) are comparable in the three cell types. Corroborating the protein results, RT-PCR analysis of RNA isolated from Saos-2 and HeLa S3 cells shows that $p57^{KIP2}$ mRNA is expressed in HeLa S3 cells, but below detection in Saos-2 cells (Fig. 3B). Our data suggest that the endogenous expression of $p57^{KIP2}$ may dramatically influence the activity of the cyclin E/CDK2/$p220^{NPAT}$/HiNF-P pathway to control transcription of cell cycle dependent H4 genes.

$p57^{KIP2}$ Suppresses HiNF-P/$p220^{NPAT}$ Activation of H4 Gene Transcription

We examined whether exogenous $p57^{KIP2}$ can modulate basal H4 gene promoter activity under control of endogenous histone gene transcription factors in Saos-2 cells that do not express $p57^{KIP2}$. Expression of $p57^{KIP2}$ decreases the basal activity of the wild-type H4 promoter, but not that of the corresponding HiNF-P mutant promoter or the promoter of the unrelated osteocalcin (OC) gene (Fig. 4A). This finding establishes that $p57^{KIP2}$ operates directly through the HiNF-P binding site. The $p57^{KIP2}$ dependent suppression of H4 promoter activity is observed with minimal doses of the transfected $p57^{KIP2}$ expression construct (i.e., 25 ng/well) following a relatively brief transfection period (i.e., 16 h). Cells transfected with as much as fourfold higher amounts of the $p57^{KIP2}$ construct (i.e., 100 ng) exhibit a normal cell cycle distribution as evidenced by flow cytometry (Fig. 4B), although cell cycle arrest can be induced at higher doses of $p57^{KIP2}$ (data not shown). Our data suggests that the ability of $p57^{KIP2}$ to suppress the HiNF-P dependent activation of the H4 promoter can apparently be distinguished from its ability to inhibit cell cycle progression.

DISCUSSION

In this study, we find that the ability of HiNF-P to activate or repress histone H4 gene transcription differs among various cell types. Elevated levels of the CDK inhibitor $p57^{KIP2}$ correlate with the suppressive potential of HiNF-P, and our data show that forced expression of $p57^{KIP2}$ reduces the HiNF-P dependent activity of the histone H4 promoter. We propose that HiNF-P is a bifunctional regulator of histone gene transcription and that $p57^{KIP2}$ levels may directly or indirectly contribute to the transcriptional activity of HiNF-P.

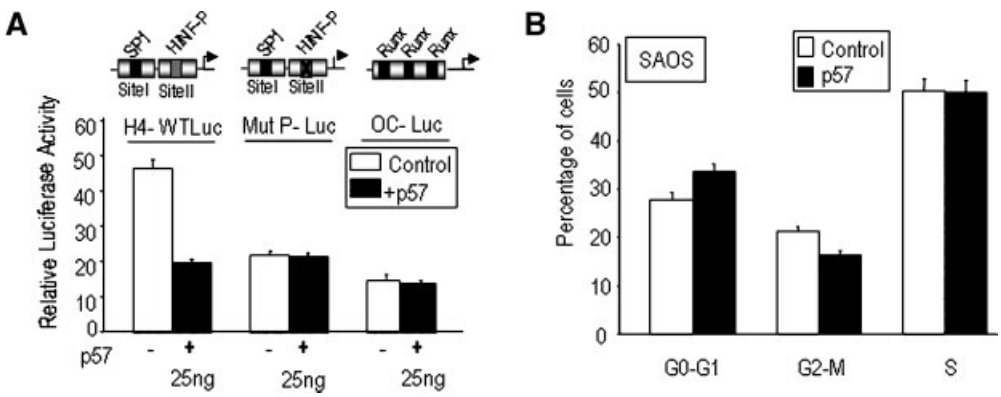

Fig. 4. Inhibition of HiNF-P/$p220^{NPAT}$ co-activation of H4 gene transcription by $p57^{KIP2}$. **A**: Selective inhibition of basal H4 promoter activity by $p57^{KIP2}$. Saos-2 cells that are $p57^{KIP2}$ deficient were transfected with a minimal amount of $p57^{KIP2}$ expression vector (25 ng) together with wild-type or HiNF-P mutant H4 promoter/luciferase reporter gene constructs (50 ng) or an osteocalcin (OC) promoter-luciferase (OC-Luc) construct (as indicated above the bar graphs). The OC gene is expressed in osteosarcoma cells and its promoter is used as an additional control to assess absence of effects of $p57^{KIP2}$ on a HiNF-P independent wild-type promoter. The expression of $p57^{KIP2}$ causes a $\sim$ two- to three-fold down-regulation of wild-type H4 promoter activity driven by endogenous HiNF-P, but $p57^{KIP2}$ has no effect when the HiNF-P site is mutated. No effect of $p57^{KIP2}$ is observed for the unrelated OC gene. The values represent the average of a representative triplicate experiment. **B**: Down-regulation of H4 promoter upon $p57^{KIP2}$ overexpression is not due to an overt block in cell cycle progression. Saos-2 cells described in **Panel A** were transiently transfected with 100 ng/well of $p57^{KIP2}$ expression vector (i.e., four times the amount used in Panel A). Cells were harvested 20 h after transfection (i.e., less than one cell cycle) and subjected to FACS analysis. The bar graphs show the percentage of cells present in different stages of the cell cycle in the absence (open bars) or presence of $p57^{KIP2}$ (closed bars).

Although HiNF-P lacks consensus CDK phosphorylation sites (unpublished observations), the HiNF-P co-activator $p220^{NPAT}$ is phosphorylated by cyclin E/CDK2, and the CDK sites in $p220^{NPAT}$ are required for its co-stimulatory activity as a component of the HiNF-P/$p220^{NPAT}$ complex [Ma et al., 2000; Zhao et al., 2000; Mitra et al., 2003; Holmes et al., 2005; Miele et al., 2005]. Hence, the possibility arises that levels of the CDK inhibitor $p57^{KIP2}$ may control HiNF-P activity by blocking the cyclin E/CDK2 dependent phosphorylation of $p220^{NPAT}$.

The inverse coupling between $p57^{KIP2}$ levels and HiNF-P dependent activation of histone H4 gene transcription may have several biological ramifications. First, because elevation of $p57^{KIP2}$ impairs the HiNF-P dependent induction of histone gene expression, and because efficient expression of histones is necessary for normal packaging of newly replicated DNA, deficiency of $p57^{KIP2}$ in tumor cells should confer a selective growth advantage. Consistent with this concept, $p57^{KIP2}$ expression is frequently silenced or inactivated during tumorigenesis in cancers of the lung, breast, prostate, and pancreas, as well as lymphoblastic and myeloid leukemias [Matsuoka et al., 1995; Hatada et al., 1996; Kondo et al., 1996; Orlow et al., 1996; Zhang et al., 1997; Kikuchi et al., 2002; Kobatake et al., 2004; Arai and Miyazaki, 2005; Hoffmann et al., 2005; Lodygin et al., 2005]. Recent studies by Nimer and colleagues revealed that $p57^{KIP2}$ is the only cyclin-dependent kinase inhibitor that is induced by transforming growth factor (TGF)-β in hematopoietic cells, and that $p57^{KIP2}$ deficient hematopoietic cells proliferate more rapidly than normal cells [Scandura et al., 2004]. In contrast, $p57^{KIP2}$ is negatively regulated by TGF-β via the ubiquitin-proteasome pathway in osteoblastic cells to stimulate proliferation [Nishimori et al., 2001]. Our results permit the interpretation that the cytostatic versus growth promoting effects of TGF-β may be related in part to $p57^{KIP2}$ dependent inhibition of HiNF-P activation potential and consequently to inhibition of histone gene expression.

Our data and those of others show that some tumor or transformed cell types (e.g., HeLa S3 and 293T) exhibit high levels of $p57^{KIP2}$, yet still exhibit robust cell growth. The inverse relationship between $p57^{KIP2}$ levels and HiNF-P activation potential clearly suggests that compensatory mechanisms are operative in tumor cells with elevated $p57^{KIP2}$ levels to cope with reduced activity or inhibition of the cyclin E/CDK2/$p220^{NPAT}$/HiNF-P pathway. For example, the activities of the site II binding proteins IRF-2 or CDP-cut may participate in cell cycle regulation and compensate for inactivation of HiNF-P [Aziz et al., 1998a; Vaughan et al., 1998]. In addition, $p57^{KIP2}$ containing tumor cells that are by default poised to proliferate may relinquish control at the level of histone gene promoters. Indeed, constitutively transcribed histone genes can be controlled post-transcriptionally by modulating histone mRNA stability through an autoregulatory mechanism [Peltz et al., 1989; Morris et al., 1991; Dominski and Marzluff, 1999].

HiNF-P was convincingly identified by DNA affinity chromatography, peptide sequencing and immuno-reactivity as a 65 kD multi-Zn finger protein that activates histone H4 gene transcription and recognizes the H4 site II consensus motif [van Wijnen et al., 1992; Mitra et al., 2003]. While our studies were in progress, HiNF-P was independently identified as a protein, referred to as MIZF, which is capable of interacting with the methylated DNA binding protein 2 (MBD2) in yeast two hybrid assays and forms a complex with MBD2 and HDAC1 [Sekimata et al., 2001]. However, our previous studies have clearly shown that HiNF-P cannot bind to H4-site II when it is methylated at just one CpG doublet in an essential sub-motif (5′ GTCCG) within the 20-bp HiNF-P consensus element [van Wijnen et al., 1992]. Subsequent studies by the Homma group characterized MIZF as a repressor protein that binds to this same 5′GTCCG sub-motif, but their experiments were done exclusively in 293T cells [Sekimata et al., 2001; Sekimata and Homma, 2004]. As we have shown here, the modest repressive function of HiNF-P/MIZF in 293T cells is attributable to the high endogenous level of $p57^{KIP2}$.

The observation that HiNF-P is a bifunctional regulator of replication dependent H4 gene transcription is analogous to findings for the E2F class of transcription factors [Dimova and Dyson, 2005], as well as the other two H4-site II binding proteins, IRF2(HiNF-M) and CDP-cut (DNA binding subunit of HiNF-D). For example, IRF2 can repress gene transcription, but can also function as a dose-dependent

trans-activator [Palombella and Maniatis, 1992; Vaughan et al., 1995; Xie et al., 2001]. Furthermore, CDP-cut has been shown to activate or repress cell cycle controlled genes in different physiological settings [Gupta et al., 2003; Truscott et al., 2003, 2004]. The bifunctionality of all three site II binding proteins, HiNF-P, IRF-2, and CDP-cut may increase the versatility by which cells support cell cycle control of histone gene transcription in distinct biological contexts. This versatility may provide a mechanistic safeguard to maintain the cellular competency to express histone proteins during S phase to support the packaging of newly replicated DNA.

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REFERENCES

Arai S, Miyazaki T. 2005. Impaired maturation of myeloid progenitors in mice lacking novel Polycomb group protein MBT-1. EMBO J 24:1863–1873.

Aziz F, van Wijnen AJ, Stein JL, Stein GS. 1998a. HiNF-D (CDP-cut/CDC2/cyclin A/pRB-complex) influences the timing of IRF-2 dependent cell cycle activation of human histone H4 gene transcription at the G1/S phase transition. J Cell Physiol 177:453–464.

Aziz F, van Wijnen AJ, Vaughan PS, Wu S, Shakoori AR, Lian JB, Soprano KJ, Stein JL, Stein GS. 1998b. The integrated activities of IRF-2 (HiNF-M) CDP/cut (HiNF-D) and H4TF-2 (HiNF-P) regulate transcription of a cell cycle controlled human histone H4 gene: Mechanistic differences between distinct H4 genes. Mol Biol Rep 25:1–12.

Berthet C, Aleem E, Coppola V, Tessarollo L, Kaldis P. 2003. Cdk2 knockout mice are viable. Curr Biol 13:1775–1785.

Birnbaum MJ, van Wijnen AJ, Odgren PR, Last TJ, Suske G, Stein GS, Stein JL. 1995a. Sp1 trans-activation of cell cycle regulated promoters is selectively repressed by Sp3. Biochemistry 34:16503–16508.

Birnbaum MJ, Wright KL, van Wijnen AJ, Ramsey-Ewing AL, Bourke MT, Last TJ, Aziz F, Frenkel B, Rao BR, Aronin N, Stein GS, Stein JL. 1995b. Functional role for Sp1 in the transcriptional amplification of a cell cycle regulated histone H4 gene. Biochemistry 34:7648–7658.

Di Fruscio M, Weiher H, Vanderhyden BC, Imai T, Shiomi T, Hori TA, Jaenisch R, Gray DA. 1997. Proviral inactivation of the Npat gene of Mpv 20 mice results in early embryonic arrest. Mol Cell Biol 17:4080–4086.

Dimova DK, Dyson NJ. 2005. The E2F transcriptional network: Old acquaintances with new faces. Oncogene 24:2810–2826.

Dominski Z, Marzluff WF. 1999. Formation of the 3′ end of histone mRNA. Gene 239:1–14.

Dyson N. 1998. The regulation of E2F by pRB-family proteins. Genes Dev 12:2245–2262.

Guo B, Stein JL, van Wijnen AJ, Stein GS. 1997. ATF1 and CREB trans-activate a cell cycle regulated histone H4 gene at a distal nuclear matrix associated promoter element. Biochemistry 36:14447–14455.

Gupta S, Luong MX, Bleuming SA, Miele A, Luong M, Young D, Knudsen ES, van Wijnen AJ, Stein JL, Stein GS. 2003. The tumor suppressor pRB functions as a co-repressor of the CCAAT displacement protein (CDP/cut) to regulate cell cycle controlled histone H4 transcription. J Cell Physiol 196:541–546.

Hatada I, Ohashi H, Fukushima Y, Kaneko Y, Inoue M, Komoto Y, Okada A, Ohishi S, Nabetani A, Morisaki H, Nakayama M, Niikawa N, Mukai T. 1996. An imprinted gene p57KIP2 is mutated in Beckwith-Wiedemann syndrome. Nat Genet 14:171–173.

Hoffmann MJ, Florl AR, Seifert HH, Schulz WA. 2005. Multiple mechanisms downregulate CDKN1C in human bladder cancer. Int J Cancer 114:406–413.

Holmes WF, Braastad CD, Mitra P, Hampe C, Doenecke D, Albig W, Stein JL, van Wijnen AJ, Stein GS. 2005. Coordinate control and selective expression of the full complement of replication-dependent histone H4 genes in normal and cancer cells. J Biol Chem 280:37400–37407.

Holthuis J, Owen TA, van Wijnen AJ, Wright KL, Ramsey-Ewing A, Kennedy MB, Carter R, Cosenza SC, Soprano KJ, Lian JB, et al. 1990. Tumor cells exhibit deregulation of the cell cycle histone gene promoter factor HiNF-D. Science 247:1454–1457.

Hovhannisyan H, Cho B, Mitra P, Montecino M, Stein GS, van Wijnen AJ, Stein JL. 2003. Maintenance of open chromatin and selective genomic occupancy at the cell-cycle-regulated histone H4 promoter during differentiation of HL-60 promyelocytic leukemia cells. Mol Cell Biol 23:1460–1469.

Jensen DE, Black AR, Swick AG, Azizkhan JC. 1997. Distinct roles for Sp1 and E2F sites in the growth/cell cycle regulation of the DHFR promoter. J Cell Biochem 67:24–31.

Kikuchi T, Toyota M, Itoh F, Suzuki H, Obata T, Yamamoto H, Kakiuchi H, Kusano M, Issa JP, Tokino T, Imai K. 2002. Inactivation of p57KIP2 by regional promoter hypermethylation and histone deacetylation in human tumors. Oncogene 21:2741–2749.

Kobatake T, Yano M, Toyooka S, Tsukuda K, Dote H, Kikuchi T, Toyota M, Ouchida M, Aoe M, Date H, Pass HI, Doihara H, Shimizu N. 2004. Aberrant methylation of p57KIP2 gene in lung and breast cancers and malignant mesotheliomas. Oncol Rep 12:1087–1092.

Kondo M, Matsuoka S, Uchida K, Osada H, Nagatake M, Takagi K, Harper JW, Takahashi T, Elledge SJ, Takahashi T. 1996. Selective maternal-allele loss in human lung cancers of the maternally expressed p57KIP2 gene at 11p15.5. Oncogene 12:1365–1368.

Kroeger P, Stewart C, Schaap T, van Wijnen A, Hirshman J, Helms S, Stein G, Stein J. 1987. Proximal and distal regulatory elements that influence in vivo expression of a cell cycle-dependent human H4 histone gene. Proc Natl Acad Sci USA 84:3982–3986.

Last TJ, van Wijnen AJ, Birnbaum MJ, Stein GS, Stein JL. 1999. Multiple interactions of the transcription factor YY1 with human histone H4 gene regulatory elements. J Cell Biochem 72:507–516.

Lodygin D, Epanchintsev A, Menssen A, Diebold J, Hermeking H. 2005. Functional epigenomics identifies genes frequently silenced in prostate cancer. Cancer Res 65:4218–4227.

Ma T, Van Tine BA, Wei Y, Garrett MD, Nelson D, Adams PD, Wang J, Qin J, Chow LT, Harper JW. 2000. Cell cycle-regulated phosphorylation of p220(NPAT) by cyclin E/Cdk2 in Cajal bodies promotes histone gene transcription. Genes Dev 14:2298–2313.

Martin A, Odajima J, Hunt SL, Dubus P, Ortega S, Malumbres M, Barbacid M. 2005. Cdk2 is dispensable for cell cycle inhibition and tumor suppression mediated by p27(Kip1) and p21(Cip1). Cancer Cell 7:591–598.

Matsuoka S, Edwards MC, Bai C, Parker S, Zhang P, Baldini A, Harper JW, Elledge SJ. 1995. p57KIP2, a structurally distinct member of the p21CIP1 Cdk inhibitor family, is a candidate tumor suppressor gene. Genes Dev 9:650–662.

Miele A, Braastad CD, Holmes WF, Mitra P, Medina R, Xie R, Zaidi SK, Ye X, Wei Y, Harper JW, van Wijnen AJ, Stein JL, Stein GS. 2005. HiNF-P directly links the cyclin E/CDK1/p220NPAT pathway to histone H4 gene regulation at the G1/S phase cell cycle transition. Mol Cell Biol 25:6140–6153.

Mitra P, Xie RL, Medina R, Hovhannisyan H, Zaidi SK, Wei Y, Harper JW, Stein JL, van Wijnen AJ, Stein GS. 2003. Identification of HiNF-P, a key activator of cell cycle controlled histone H4 genes at the onset of S phase. Mol Cell Biol 23:8110–8123.

Morris TD, Weber LA, Hickey E, Stein GS, Stein JL. 1991. Changes in the stability of a human H3 histone mRNA during the HeLa cell cycle. Mol Cell Biol 11:544–553.

Nevins JR. 2001. The Rb/E2F pathway and cancer. Hum Mol Genet 10:699–703.

Nishimori S, Tanaka Y, Chiba T, Fujii M, Imamura T, Miyazono K, Ogasawara T, Kawaguchi H, Igarashi T, Fujita T, Tanaka K, Toyoshima H. 2001. Smad-mediated transcription is required for transforming growth factor-beta 1-induced p57(Kip2) proteolysis in osteoblastic cells. J Biol Chem 276:10700–10705.

Orlow I, Iavarone A, Crider-Miller SJ, Bonilla F, Latres E, Lee MH, Gerald WL, Massague J, Weissman BE, Cordon-Cardo C. 1996. Cyclin-dependent kinase inhibitor p57KIP2 in soft tissue sarcomas and Wilms'tumors. Cancer Res 56:1219–1221.

Palombella VJ, Maniatis T. 1992. Inducible processing of interferon regulatory factor-2. Mol Cell Biol 12:3325–3336.

Pauli U, Chrysogelos S, Stein G, Stein J, Nick H. 1987. Protein-DNA interactions in vivo upstream of a cell cycle-regulated human H4 histone gene. Science 236:1308–1311.

Peltz SW, Brewer G, Groppi V, Ross J. 1989. Exonuclease activity that degrades histone mRNA is stable when DNA or protein synthesis is inhibited. Mol Biol Med 6:227–238.

Ramsey-Ewing A, van Wijnen AJ, Stein GS, Stein JL. 1994. Delineation of a human histone H4 cell cycle element in vivo: The master switch for H4 gene transcription. Proc Natl Acad Sci USA 91:4475–4479.

Scandura JM, Boccuni P, Massague J, Nimer SD. 2004. Transforming growth factor beta-induced cell cycle arrest of human hematopoietic cells requires p57KIP2 up-regulation. Proc Natl Acad Sci USA 101:15231–15236.

Sekimata M, Homma Y. 2004. Sequence-specific transcriptional repression by an MBD2-interacting zinc finger protein MIZF. Nucleic Acids Res 32:590–597.

Sekimata M, Takahashi A, Murakami-Sekimata A, Homma Y. 2001. Involvement of a novel zinc finger protein, MIZF, in transcriptional repression by interacting with a methyl-CpG-binding protein, MBD2. J Biol Chem 276: 42632–42638.

Stein GS, Stein JL, Marzluff WF. 1984. Histone Genes. New York: John Wiley & Sons, Inc.

Stein G, Lian J, Stein J, Briggs R, Shalhoub V, Wright K, Pauli U, van Wijnen A. 1989. Altered binding of human histone gene transcription factors during the shutdown of proliferation and onset of differentiation in HL-60 cells. Proc Natl Acad Sci USA 86:1865–1869.

Stein GS, Stein JL, van Wijnen AJ, Lian JB. 1992. Regulation of histone gene expression. Curr Opin Cell Biol 4:166–173.

Stein GS, Stein JL, van Wijnen AJ, Lian JB. 1996. Transcriptional control of cell cycle progression: The histone gene is a paradigm for the G1/S phase and proliferation/differentiation transitions. Cell Biol Int 20:41–49.

Tokino T, Urano T, Furuhata T, Matsushima M, Miyatsu T, Sasaki S, Nakamura Y. 1996. Characterization of the human p57KIP2 gene: Alternative splicing, insertion/deletion polymorphisms in VNTR sequences in the coding region, and mutational analysis. Hum Genet 97:625–631.

Truscott M, Raynal L, Premdas P, Goulet B, Leduy L, Berube G, Nepveu A. 2003. CDP/Cux Stimulates Transcription from the DNA Polymerase alpha Gene Promoter. Mol Cell Biol 23:3013–3028.

Truscott M, Raynal L, Wang Y, Berube G, Leduy L, Nepveu A. 2004. The N-terminal region of the CCAAT displacement protein (CDP)/Cux transcription factor functions as an autoinhibitory domain that modulates DNA binding. J Biol Chem 279:49787–49794.

van den Ent FM, van Wijnen AJ, Last TJ, Bortell R, Stein JL, Lian JB, Stein GS. 1993. Concerted control of multiple histone promoter factors during cell density inhibition of proliferation in osteosarcoma cells: Reciprocal regulation of cell cycle-controlled and bone-related genes. Cancer Res 53:2399–2409.

van der Meijden CMJ, Vaughan PS, Staal A, Albig W, Doenecke D, Stein JL, Stein GS, van Wijnen AJ. 1998. Selective expression of specific histone H4 genes reflects distinctions in transcription factor interactions with divergent H4 promoter elements. Biochim Biophys Acta 1442:82–100.

van Wijnen AJ, Wright KL, Lian JB, Stein JL, Stein GS. 1989. Human H4 histone gene transcription requires the proliferation- specific nuclear factor HiNF-D. Auxiliary roles for HiNF-C (Sp1- like) and HiNF-A (high mobility group-like). J Biol Chem 264:15034–15042.

van Wijnen AJ, Ramsey-Ewing AL, Bortell R, Owen TA, Lian JB, Stein JL, Stein GS. 1991. Transcriptional element H4-site II of cell cycle regulated human H4 histone genes is a multipartite protein/DNA interaction

site for factors HiNF-D, HiNF-M, and HiNF-P: Involvement of phosphorylation. J Cell Biochem 46:174–189.

van Wijnen AJ, van den Ent FM, Lian JB, Stein JL, Stein GS. 1992. Overlapping and CpG methylation-sensitive protein-DNA interactions at the histone H4 transcriptional cell cycle domain: Distinctions between two human H4 gene promoters. Mol Cell Biol 12:3273–3287.

van Wijnen AJ, Aziz F, Grana X, De LA, Desai RK, Jaarsveld K, Last TJ, Soprano K, Giordano A, Lian JB, Stein JL, Stein GS. 1994. Transcription of histone H4, H3, and H1 cell cycle genes: Promoter factor HiNF-D contains CDC2, cyclin A, and an RB-related protein. Proc Natl Acad Sci USA 91:12882–12886.

van Wijnen AJ, van Gurp MF, de Ridder MC, Tufarelli C, Last TJ, Birnbaum M, Vaughan PS, Giordano A, Krek W, Neufeld EJ, Stein JL, Stein GS. 1996. CDP/cut is the DNA-binding subunit of histone gene transcription factor HiNF-D: A mechanism for gene regulation at the G1/S phase cell cycle transition point independent of transcription factor E2F. Proc Natl Acad Sci USA 93:11516–11521.

Vaughan PS, Aziz F, van Wijnen AJ, Wu S, Harada H, Taniguchi T, Soprano KJ, Stein JL, Stein GS. 1995. Activation of a cell-cycle-regulated histone gene by the oncogenic transcription factor IRF-2. Nature 377:362–365.

Vaughan PS, van der Meijden CMJ, Aziz F, Harada H, Taniguchi T, van Wijnen AJ, Stein JL, Stein GS. 1998. Cell cycle regulation of histone H4 gene transcription requires the oncogenic factor IRF-2. J Biol Chem 273:194–199.

Wei Y, Jin J, Harper JW. 2003. The cyclin E/Cdk2 substrate and Cajal body component p220(NPAT) activates histone transcription through a novel LisH-like domain. Mol Cell Biol 23:3669–3680.

Xie R, van Wijnen AJ, van der Meijden CMJ, Luong MX, Stein JL, Stein GS. 2001. The cell cycle control element of histone H4 gene transcription is maximally responsive to interferon regulatory factor pairs IRF-1/IRF-3 and IRF-1/IRF-7. J Biol Chem 276:18624–18632.

Ye X, Wei Y, Nalepa G, Harper JW. 2003. The cyclin E/Cdk2 substrate p220(NPAT) is required for S-phase entry, histone gene expression, and Cajal body maintenance in human somatic cells. Mol Cell Biol 23:8586–8600.

Zhang P, Liégeois NJ, Wong C, Finegold M, Hou H, Thompson JC, Silverman A, Harper JW, DePinho RA, Elledge SJ. 1997. Altered cell differentiation and proliferation in mice lacking p57KIP2 indicates a role in Beckwith-Wiedemann syndrome. Nature 387:151–158.

Zhao J, Dynlacht B, Imai T, Hori T, Harlow E. 1998. Expression of NPAT, a novel substrate of cyclin E-CDK2, promotes S-phase entry. Genes Dev 12:456–461.

Zhao J, Kennedy BK, Lawrence BD, Barbie DA, Matera AG, Fletcher JA, Harlow E. 2000. NPAT links cyclin E-Cdk2 to the regulation of replication-dependent histone gene transcription. Genes Dev 14:2283–2297.