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CDK inhibitors selectively diminish cell cycle controlled activation of the histone H4 gene promoter by p220^{NPAT} and HiNF-P

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Abstract

Cell cycle progression into S phase requires the induction of histone gene expression to package newly synthesized DNA as chromatin. Cyclin E stimulation of CDK2 at the Restriction point late in G1 controls both histone gene expression by the p220^{NPAT}/HiNF-P pathway and initiation of DNA replication through the pRB/E2F pathway. The three CDK inhibitors (CKIs) p21^{CIP1/WAF1}, p27^{KIP1} and p57^{KIP2} attenuate CDK2 activity. Here we find that γ -irradiation induces p21^{CIP1/WAF1} but not the other two CKIs, while reducing *histone H4* mRNA levels but not *histone H4* gene promoter activation by the p220^{NPAT}/HiNF-P complex. We also show that p21^{CIP1/WAF1} is less effective than p27^{KIP1} and p57^{KIP2} in inhibiting the CDK2 dependent phosphorylation of p220^{NPAT} at subnuclear foci and transcriptional activation of *histone H4* genes. The greater effectiveness of p57^{KIP2} in blocking the p220^{NPAT}/HiNF-P pathway is attributable in part to its ability to form a specific complex with p220^{NPAT} that may suppress CDK2/cyclin E phosphorylation through direct substrate inhibition. We conclude that CKIs selectively control stimulation of the *histone H4* gene promoter by the p220^{NPAT}/HiNF-P complex.

Keywords

cell cycle; histone genes; transcription; chromatin; proliferation

At the G1/S phase transition of the human cell cycle, DNA replication is initiated and histone gene expression is induced to package nascent DNA. At the Restriction point that precedes the G1/S boundary, growth factor dependent signaling pathways activate cyclin E and its cognate Cyclin Dependent Kinase 2 (CDK2) (Sherr and Roberts, 2004; Blagosklonny and Pardee, 2002). Cyclin E/CDK2 complexes control phosphorylation of two distinct regulatory pathways to support the synthesis of DNA or histone proteins. One pathway is initiated by phosphorylation of the retinoblastoma protein pRB1 which releases E2F proteins that stimulate transcription of a number of genes to support the initiation and progression of DNA synthesis during S phase (e.g., *DHFR*, *TK*, *DNA polymerase α*) (Nevins, 2001; Dyson,

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1998). Equally important, cyclin E/CDK2 controls the activity of the histone gene transcription factor HiNF-P through phosphorylation of its co-activator p220^{NPAT}, and this complex coordinately regulates *histone H4* gene expression in somatic cells (Ma et al, 2000;Zhao et al, 2000;Mitra et al, 2003;Miele et al, 2005;Holmes et al, 2005;Mitra et al, 2007;Pauli et al, 1987;van Wijnen et al, 1992) and human embryonic stem cells (Ghule et al, 2007;Becker et al, 2007;Becker et al, 2006). HiNF-P and p220^{NPAT} co-localize at Cajal Body-related subnuclear foci together with histone genes and factors that support the processing of histone gene transcripts (Miele et al, 2005;Zhao et al, 2000;Ma et al, 2000;Shopland et al, 2001;Ghule et al, 2007). In addition, HiNF-P and p220^{NPAT} are components of broader regulatory networks of protein/protein interaction and target genes involved in cell cycle control (Medina et al, 2007;Xie et al, 2007;Miele et al, 2007;Medina et al, 2006).

CDK2 activity is regulated by direct binding to one of three CDK inhibitory proteins (CKIs) p21^{CIP1/WAF1} (CDKN1A), p27^{KIP1} (CDKN1B) and p57^{KIP2} (CDKN1C) that have distinct biological roles in mammalian development (Harper et al, 1993;el-Deiry et al, 1994;Luo et al, 1995;Sherr and Roberts, 1999;Nakayama and Nakayama, 1998;Matsuoka et al, 1995;Zhang et al, 1998;Zhang et al, 1999;Zhang et al, 1997;Reynaud et al, 1999). The general roles of p21^{CIP1/WAF1} and p27^{KIP1} in mediating cell cycle arrest during differentiation or DNA damage responses have been extensively investigated, but the function of p57^{KIP2} has been more enigmatic (Baumbach et al, 1987). The expression of p57^{KIP2} in vivo is more restricted than that of p27^{KIP1} and p21^{CIP1/WAF1} due to CpG methylation dependent imprinting (Kondo et al, 1996;Matsuoka et al, 1995;Matsuoka et al, 1996). Loss of p57^{KIP2} expression in mice and humans may increase susceptibility to specific tumors (Caspary et al, 1999;Zhang et al, 1997), and the p57^{KIP2} gene is transcriptionally silenced in several cancers (Canalli et al, 2005;Lodygin et al, 2005;Kikuchi et al, 2002;Li et al, 2002). Structural similarities between CKIs (e.g., N-terminal cyclin binding domain) reflect biochemical redundancy in blocking CDK2 and the shared ability to attenuate cell growth and mediate checkpoint control. However, the structure of p57^{KIP2} is distinct, because it contains a C-terminal proline-alanine extension (PAPA repeat) (Matsuoka et al, 1995). While all three CKIs can inhibit CDK activity, p57^{KIP2} may have unique properties that have not yet been appreciated.

In this study, we compare the inhibitory function of p21^{CIP1/WAF1}, p27^{KIP1} and p57^{KIP2} in the cyclin E/CDK2/p220^{NPAT}/HiNF-P/histone gene-regulatory pathway that supports entry into S phase. Our data suggest that CKIs exhibit selectivity in their ability to inhibit signaling at the histone H4 promoter through the p220^{NPAT}/HiNF-P complex, a principal CDK2 substrate that operates in parallel to the pRB/E2F pathway at the G1/S phase transition.

EXPERIMENTAL PROCEDURES

Cell Culture and Transient Transfections

Cos7 cells were co-transfected with HiNF-P responsive promoters (i.e., *histone H4*) fused to luciferase reporters and expression vectors encoding the indicated proteins (e.g., p57^{KIP2}, p220^{NPAT} and HiNF-P) using FuGENE6 (Miele et al, 2005;Mitra et al, 2003). Luciferase activity was measured within 24 h. Vectors for human Myc-tagged p57^{KIP2} (Roger Watson, Imperial College of London, UK), mouse p57^{KIP2} (James Cross, University of Toronto), human Skp2 and a Skp2 F-box deletion mutant (Skp2 Δ F), and mouse p27^{KIP1} (Jack Pledger, University of South Florida, Tampa, Florida) were the generous gifts of the indicated individuals. Adenovirus p57^{KIP2} was kindly provided by Matthew Stewart (University of Illinois).

Treatments with p57^{KIP2} siRNA smart pool duplexes or universal controls (Dharmacon RNA Technologies, Chicago, IL) were performed at 12–16 h after co-transfection of H4/luciferase reporters. At 48 h, we examined luciferase activity using a luminometer and p57^{KIP2} levels by immunoblotting with ~15–20 µg total protein separated by 10% SDS-PAGE (Miele et al, 2005; Mitra et al, 2003). Immunoprecipitations were obtained with ~100 µg of whole cell extract protein, the indicated antibodies and protein A/G-agarose after overnight incubation at 4°C. Samples were then separated by 9% or 10% SDS-PAGE followed by western blotting and chemiluminescence detection.

Expression Analysis and Reporter Gene Assays

For reporter gene assays with irradiated cells, we plated U2OS cells at a density of 1.1×10^5 cells per well in six-well plates. The next day, co-transfections were performed using FuGENE6 (Roche) with the same wild-type histone H4 promoter luciferase reporter construct (200 ng) as above and expression vectors for HiNF-P (25 ng) or p220^{NPAT} (200 ng) or the corresponding empty vector as described previously (Miele et al, 2005; Mitra et al, 2003) while maintaining the same total amount of DNA in every transfection. Cells were irradiated by exposure to 5 or 12 Gy γ -irradiation at 24 h after transfection. At 4 or 16 h after irradiation, cell lysates were analyzed for luciferase activity and normalized to *Renilla* (phRL-null, 5 ng per well) using the dual-luciferase reporter assay system (Promega, Madison, WI).

Reporter gene experiments were also performed with normal diploid human WI-38 cells. These cells were plated at a density of 1.6×10^5 /well in six-wells plates and transiently transfected at day 2 after plating at a cell density of ~30% with wild-type histone H4 promoter luciferase reporter construct, and co-transfected with the expression vectors HiNF-P, p220^{NPAT} or p57 as described above. The same total amount of DNA (2.5 µg) was maintained in every transfection. Lipofectamine LTX (Invitrogen) was used as a transfection agent in combination with PLUS reagent (Invitrogen) and transfection was performed in the absence of FBS and antibiotics. After 16 h medium was changed to normal growth medium with FBS, and cells were lysed in 1x PLB lysis buffer (Promega) after a total of 40 h transfection time. Cell lysates were analyzed for luciferase activity and normalized to *Renilla* (phRL-null) with dual-luciferase reporter assay system (Promega).

For protein analyses, cell lysates obtained from reporter gene assays were diluted in SDS sample buffer and loaded on a 4–15% ready gel precast gel (Bio-Rad). HiNF-P was detected with the 802 antibody (1:2,000 dilution) (Miele et al, 2005; Mitra et al, 2003) and p21^{CIP1/WAF1} was visualized with a commercially available antibody (sc-756; 1:1,000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA). Tubulin was used as an internal control. Peroxidase labeled goat-anti-rabbit antibody (Santa Cruz) was used as secondary antibody and visualized with enhanced chemiluminescence (ECL) chemistry (PerkinElmer, Waltham, MA).

The levels of mRNAs for human histone *HIST2H4* (*H4/n*), p21^{CIP1/WAF1}, p27^{KIP1} and p57^{KIP2}, *CDK2* and *GAPDH* were detected by quantitative real-time reverse transcriptase PCR (qRT-PCR). Purified total RNA using Trizol (Invitrogen) from triplicate experiments of reporter gene assays was subjected to DNase I digestion, and cDNA was prepared with the iScript cDNA synthesis kit (Bio-Rad). Relative quantitation was determined using a 7000 sequence detection system (Applied Biosystems) with SYBR Green chemistry (Applied Biosystems). The relative mRNA expression was calculated with the $\Delta\Delta CT$ method. Real-time primer sequences for *H4/n*, p27 and *CDK2* were published previously (Medina et al, 2007; Becker et al, 2007). The following primer pairs were used for human mRNA (in 5'-3' direction): p21 forward 5'-GGA AGA CCA TGT GGA CCT GT and reverse 5'-GGC GTT TGG AGT GGT AGA AA; p57 forward 5'-AAG AGA TCA GCG

CCT GAG AA and reverse 5'-TGG GCT CTA AAT TGG CTC AC; GAPDH forward 5'-ATG TTC GTC ATG GGT GTG AA and reverse 5'-TGT GGT CAT GAG TCC TTC CA.

We also examined gene expression in total RNA that was extracted and purified from mouse embryonic fibroblasts (MEFs) isolated from wild type *p57* (WT), heterozygous *p57* null (HET) and homozygous *p57* null (KO) mice. The relative mRNA expression of mouse *HiNF-P*, *Hist2H4*, *HistH4/m* and *Hist1H4/f*, *p57*, *p27* and *p21* was calculated using the $\Delta\Delta CT$ method with *HPRT* as an internal control. The following mouse primer sequences were used: *p57* forward: 5'-GTC TGA GAT GAG TTA GTT TAG AGG and reverse 5'-TGC TAC ATG AAC GAA AGG TC; *p27* forward 5' TCT AAA GCC CAC TTA TAA CCC AG and reverse 5'-CCT GTG CCA TCT CTA TAT TCC T; *p21* forward 5'-CTT CTC CCA TTT CTT AGT AGC AG and reverse 5'-CCA CGG TAT TCA ACA CTG AG; *HiNF-P* forward 5'-ATG TTT GCC AAC AAC ACC AA and reverse 5'-GCC TCT CTG TGG CAA ATC TC; *Hist2h4* forward 5'-CCA GCT GGT GTT TCA GAT TAC A and reverse 5'-ACC CTT GCC TAG ACC CTT TC; *Hist1h4m* forward 5'-GAG CAG TAC AGT TTT GTC TTC ATC A and reverse 5'-CGT GAT GCC CTG GAT GTT AT; *Hist1h4f* forward 5'-CAA CTC AGT GCT CCA TAG CC and reverse 5'-GGT GAT GCC CTG GAT GTT AT; *Hprt1* forward 5'-CAG GCC AGA CTT TGT TGG AT and reverse 5'-TTG CGC TCA TCT TAG GCT TT.

Immunofluorescence Microscopy

Cells grown on gelatin-coated coverslips (Fisher Scientific, Springfield, NJ) were examined by in situ immunofluorescence microscopy 24 h after transfection (Miele et al, 2005). Cells were washed with cold saline, fixed with 3.7% formaldehyde for 10 min on ice, and permeabilized with 0.1% Triton X-100 for 20 min. Coverslips were blocked with serum albumin prior to antibody staining and incubated at 37°C for 1 h with the following antibodies using 1:1,000 dilutions (unless indicated otherwise): mouse Flag (1:10,000; Sigma St. Louis, MO) and p220^{NPAT} (BD Biosciences, San Jose, CA) monoclonals and rabbit polyclonals against phospho-Thr1270 and phospho-Thr1350 of p220^{NPAT} (Ma et al, 2000). Cells were incubated at 37°C for 1 h with Alexa 488 goat anti-rabbit or Alexa 594 goat anti-mouse (Molecular Probes, Eugene, Oregon). Cells were stained with 4',6-diamidino-2-phenylindole (DAPI) (5 mg/ml) for 5 min, mounted to slides and examined by an Axioplan 2 epifluorescence microscope (Zeiss, Thornwood, NY) attached to a charge-coupled device camera.

RESULTS

Irradiation reduces *histone* mRNA levels but not *histone* gene promoter activation by the p220^{NPAT}/HiNF-P complex

The CDK2 mediated phosphorylation of p220^{NPAT} as the co-activator of HiNF-P ensures the transcriptional activation of histone genes in conjunction with the onset of S phase. Therefore, it is necessary to understand how the activity of this transcriptional complex responds to inhibition of CDK activity that prevents cells from replicating DNA. *Histone* mRNA levels are rapidly degraded following inhibition of DNA synthesis (Baumbach et al, 1987; Stein et al, 1992). Indeed, we observe a rapid decrease of histone gene expression (e.g., *histone H4/n*) upon γ -irradiation of U2OS cells at a non-lethal dose (5 Gy), while *CDK2* and *GAPDH* mRNA levels are not affected (Fig. 1). Similar results for all parameters were obtained with a 12 Gy dose (data not shown). γ -Irradiation evokes a DNA damage response as reflected by an increase in *p21^{CIP1/WAF1}* mRNA levels at both 4 hr and 16 hr after irradiation, while the mRNA levels of *p27^{KIP1}* decrease modestly and those of *p57^{KIP2}* remain relatively constant (Fig. 1).

Transfection assays with *histone H4* promoter/Luciferase reporter gene constructs show that γ irradiation at 5 Gy does not affect activation of the *histone H4* gene promoter by HiNF-P and p220^{NPAT}, although p21^{CIP1/WAF1} protein levels are clearly upregulated at both the 4 and 16 hr time points (Fig. 2). Similar results were obtained upon increasing the radiation dose to 12 Gy (data not shown). Hence, physiological induction of p21^{CIP1/WAF1} during a genotoxic stress response contributes to a reduction of *histone* mRNA accumulation but does not impinge on the CDK2 dependent transcriptional activation of *histone* genes by the p220^{NPAT}/HiNF-P complex. Our findings are in keeping with the longstanding observation that histone mRNA accumulation is dictated by both transcriptional and post-transcriptional mechanisms and that mRNA destabilization will override transcriptional activation (Baumbach et al, 1987; Stein et al, 1992).

Selective inhibition of p220^{NPAT} phosphorylation by CKIs at Subnuclear Foci

The finding that elevation of p21^{CIP1/WAF1} gene expression during a DNA damage response is not potent enough to block the activity of the p220^{NPAT}/HiNF-P transcriptional complex is unexpected. The data indicate that p220^{NPAT} phosphorylation may occur despite a reduction in cellular CDK kinase activity upon elevation of p21^{CIP1/WAF1} levels. Therefore, we compared the potency of p21^{CIP1/WAF1} in relation to the other two CKIs in regulating the in situ phosphorylation of p220^{NPAT} by CDK2 at subnuclear foci.

Phosphorylation of p220^{NPAT} by cyclin E/CDK2 at the G1/S boundary occurs on at least two distinct phospho-epitopes (T1270 and T1350) and is essential for activation of histone genes by HiNF-P (Ma et al, 2000; Mitra et al, 2003). Actively proliferating Cos7 cells typically have three to six p220^{NPAT} foci (Fig. 3A). Phosphorylation of p220^{NPAT} at both phospho-epitopes is observed in ~80–90% of the cells and predominantly in cells with more than three foci (Figs. 3B and 3C, and data not shown). These data are consistent with the cell cycle specific phosphorylation of p220^{NPAT} during late G1 that persists throughout S and G2, as well as the expected doubling of p220^{NPAT} foci during S phase that has been observed in other cell types (Ma et al, 2000).

The focal organization of p220^{NPAT} is sustained upon introduction of exogenous p57^{KIP2}, p27^{KIP1} or p21^{CIP1/WAF1} (Fig. 3A). Forced expression of CKIs in each case reduces phosphorylation at both CDK2 related epitopes in transfected cells, but not in adjacent untransfected cells (Figs. 3B, 3C). Importantly, p57^{KIP2} and p27^{KIP1} appear to be more effective than p21^{CIP1/WAF1} in blocking p220^{NPAT} phosphorylation at T1270. We observe that none of the cells transfected with p57^{KIP2} and almost none of the p27^{KIP1} expressing cells (1% positive, 99% negative cells) are positive for phospho-T1270, while p21^{CIP1/WAF1} expressing cells show residual immuno-reactivity with the phospho-T1270 antibody (15% positive, 85% negative cells) (Fig. 3B). Moreover, p57^{KIP2} completely abrogates phosphorylation at T1350, while p27^{KIP1} (12% positive cells) and p21^{CIP1/WAF1} (23% positive cells) do not (Fig. 3C). Our data suggest that p57^{KIP2} is more effective in blocking p220^{NPAT} phosphorylation in situ than the other two CKIs.

We tested the specificity of p57^{KIP2} to block p220^{NPAT} phosphorylation at subnuclear foci using p57^{KIP2} mutants (Fig. 4A). Both human and mouse wild type proteins are equally effective in blocking p220^{NPAT} phosphorylation (Figs. 4A, 4B). The CC and CCT mutants of p57^{KIP2} (see diagram in Fig. 6C below) are defective in cyclin binding and do not affect phosphorylation of p220^{NPAT} at T1270 or T1350 (Fig. 4A). Mutant p57^{KIP2}-T that lacks a CDK phosphorylation site required for Skp2 dependent degradation (Hattori et al, 2000) is equally effective as wild type (Fig. 4). Thus, in situ inhibition of p220^{NPAT} apparently requires the functional cyclin binding domain of p57^{KIP2}.

The structure of p57^{KIP2} differs from p27^{KIP1} by the presence of a C-terminal proline-alanine extension (PAPA repeat) (Matsuoka et al, 1995) that is similar but not entirely identical in mouse and human. Despite only partial conservation of the C-terminus, both human and mouse p57^{KIP2} are similarly effective in blocking p220^{NPAT} phosphorylation (Figs. 4A, 4B). To examine the contribution of the C-terminus, we prepared a chimera in which the C-terminus of human p57^{KIP2} is fused to the N-terminal cyclin binding domain of p27^{KIP1}. The p27^{KIP1}-p57^{KIP2} chimera is as effective as wild type p57^{KIP2} in blocking T1270 and T1350 phosphorylation of p220^{NPAT} (Fig. 4B). Hence, our data suggest that the selective ability of human p57^{KIP2} to prevent p220^{NPAT} phosphorylation is mediated in part by its unique C-terminus.

p57^{KIP2} is the most effective inhibitor of the Cyclin E/CDK2/p220^{NPAT}/HiNF-P pathway

Phosphorylation of p220^{NPAT} is inhibited by the three CKIs in part due to reduced CDK2 kinase activity as measured using histone H1 as a substrate (Fig. 5A). Under our experimental conditions, p27^{KIP1} is a stronger inhibitor of CDK2 activity than p57^{KIP2} or p21^{CIP1/WAF1} (Fig. 5A). Hence, the relative intrinsic strength by which CKIs inhibit CDK2 kinase activity does not appear to correlate directly with their ability to reduce phosphorylation of the two epitopes of p220^{NPAT}.

We tested the functional effects of the three CKIs on HiNF-P/p220^{NPAT} co-activation using *histone H4* gene reporter assays (Fig. 5B). Forced expression using limited amounts of expression vector elevates the levels of p57^{KIP2}, p27^{KIP1} and p21^{CIP1/WAF1} (Fig. 5B), but only p57^{KIP2} elevation represses the HiNF-P/p220^{NPAT} dependent stimulation of *H4* gene transcription at the doses shown here (Fig. 5B). We note that p21^{CIP1/WAF1}, p27^{KIP1} and p57^{KIP2} can each block *histone H4* gene promoter activity in a dose-dependent manner when exogenously expressed at higher levels, although p57^{KIP2} still remains more effective than p27^{KIP1} or p21^{CIP1/WAF1} (data not shown). Excessive non-physiological levels of CKIs will result in a general block of CDK2 activity thus indiscriminately suppressing the phosphorylation of p220^{NPAT} and preventing activation of transcription by the p220^{NPAT}/HiNF-P complex. We also examined the effect of the F-box protein Skp2, which promotes p57^{KIP2} degradation (Kamura et al, 2003), on activation of the *H4* gene promoter. Co-expression of Skp2 decreases p57^{KIP2} and restores the ability of p220^{NPAT} and HiNF-P to stimulate the *H4* promoter, while a Skp2 F-box mutant (Skp2 Δ F, which is defective in promoting p57^{KIP2} degradation) does not (data not shown). In addition, HiNF-P and/or p220^{NPAT} enhanced reporter gene expression under control of multimerized HiNF-P binding sites is consistently inhibited by p57^{KIP2}, but not when HiNF-P elements are mutated (Fig. 5C). Taken together, our data indicate that p57^{KIP2}, p27^{KIP1} and p21^{CIP1/WAF1} exhibit differences in their ability to inhibit the p220^{NPAT}/HiNF-P dependent stimulation of the *histone H4* promoter (summarized in Fig. 5D). The preferential effectiveness of p57^{KIP2} in blocking *H4* gene transcription is consistent with our previous observation that exogenous HiNF-P does not activate *H4* gene transcription in cell types that express high levels of endogenous p57^{KIP2} (Mitra et al, 2006).

p57^{KIP2} complexes with p220^{NPAT}

Because p57^{KIP2} is more effective than p27^{KIP1} or p21^{CIP1} in blocking HiNF-P/p220^{NPAT} co-activation, we postulated that p57^{KIP2} may act beyond merely inhibiting CDK2 kinase activity and have molecular specificity for p220^{NPAT}. Immuno-precipitation experiments reveal that p220^{NPAT} forms a complex with wild type p57^{KIP2} (Fig. 6). Mutants of p220^{NPAT} that are defective in interactions with HiNF-P (i.e., LisH mut, LoxP1 mut) remain capable of binding to p57^{KIP2}. However, the p220^{NPAT}- Δ CDK2 mutant, which cannot be phosphorylated by CDK2 (Wei et al, 2003) and is transcriptionally inactive, does not bind to p57^{KIP2} (Fig. 6A). Furthermore, the cyclin binding defective p57^{KIP2}-CCTmutant (Hattori et

al, 2000) does not interact with p220^{NPAT} (Fig. 6B, top panel). Hence, CDK2 phosphorylatable amino acids in the C-terminal half of p220^{NPAT} and the N-terminal cyclin binding domain of p57^{KIP2} both contribute to the p220^{NPAT}/p57^{KIP2} interaction.

We examined which functions of p57^{KIP2} are required to neutralize co-activation of the HiNF-P/p220^{NPAT} complex (Fig. 6C). Cyclin binding domain mutants of p57^{KIP2} (CC and CCT) do not block enhancement by HiNF-P and p220^{NPAT} in reporter gene assays. However, the p57^{KIP2}-T mutant that is defective for Skp2 dependent degradation (Hattori et al, 2000) effectively blocks promoter co-stimulation by HiNF-P and p220^{NPAT}. Hence, functional inhibition of p220^{NPAT} correlates with the abilities of p57^{KIP2} to block CDK2 activity through a cyclin/CDK interaction, to participate in a complex with p220^{NPAT}, and to prevent phosphorylation of both T1270 and T1350 of p220^{NPAT}.

We also investigated the role of the unique C-terminus of p57^{KIP2} by analyzing the functional effects of p27^{KIP1}-p57^{KIP2} and p57^{KIP2}-p27^{KIP1} chimeras on *H4* gene transcription (Fig. 6D). The p27^{KIP1}-p57^{KIP2} chimera is as effective as wild type p57^{KIP2} in blocking the activation of *H4* gene transcription by p220^{NPAT} and HiNF-P, while neither the p57^{KIP2}-p27^{KIP1} chimera nor wild type p27^{KIP1} is inhibitory at the concentrations we tested. Hence, the cyclin binding function and the C-terminus of p57^{KIP2} are both necessary for inhibiting histone gene transcription.

Modulation of p57^{KIP2} levels alters the co-activation potential of HiNF-P/p220^{NPAT} and histone *H4* gene expression

Exogenous HiNF-P does not activate *H4* gene transcription in cells that express high levels of endogenous p57^{KIP2} (Mitra et al, 2006), perhaps because of the formation of inactive complexes containing HiNF-P, p220^{NPAT}, p57^{KIP2} and perhaps other components (see Discussion). Therefore, we assessed whether removal of endogenous p57^{KIP2} would alter the activity of HiNF-P and/or p220^{NPAT} and convert HiNF-P/p220^{NPAT} complexes into functional activators of *H4* gene transcription. The results show that treatment with p57^{KIP2} siRNA reduces endogenous p57^{KIP2} mRNA and increases *histone H4* gene expression in HeLa S3 cells, suggesting that p57^{KIP2} may control the co-activation potential of HiNF-P and p220^{NPAT} (Fig. 7).

Inhibition of p220^{NPAT}/HiNF-P co-activation by p57^{KIP2} in normal diploid cells

Because the above studies were performed with tumor-derived cell lines, the question arises whether p57^{KIP2} suppresses *histone H4* gene expression or activation of the *histone H4* gene promoter by the p220^{NPAT}/HiNF-P complex in cells with normal cell growth characteristics. In one set of experiments, we examined expression of several representative mouse *histone H4* genes in embryonic fibroblasts from wild type mice and mice with heterozygous or homozygous null mutations in the mouse p57^{Kip2} gene (Fig. 8A). The results show that loss of either one or two p57^{Kip2} alleles abolishes p57^{Kip2} gene expression as expected, with modest compensatory changes in the expression of p21^{Cip/Waf}. However, we did not observe changes in the expression of the three mouse *histone H4* genes we examined nor in the expression of mRNAs for *HiNF-P* or *HPRT*. Hence, loss of p57^{Kip2} mRNA expression does not alter the accumulation of *histone H4* mRNAs. This finding is consistent with results presented in Figure 1 that reveal that diminished *histone H4* gene transcription is not necessarily reflected by a change in histone mRNA levels (e.g., due to simultaneous changes in histone mRNA stability). We performed nuclear run-on analysis with MEFs with heterozygous or homozygous null mutations in p57^{Kip2} to test whether loss of this CKI changes *histone H4* gene transcription. However, the experimental variation in cell growth rates of different MEF preparations appeared to dominate the outcome and we were not able

to ascertain genotype-specific changes in transcription rates using this approach (data not shown).

In a final set of experiments, we studied the effect of p57^{KIP2} protein on a human *histone H4* gene promoter construct in normal diploid human fibroblasts (WI38 cells) (Fig. 8B). The results show that p57^{KIP2} suppresses *histone H4* gene promoter activation by p220^{NPAT} and HiNF-P. We conclude that p57^{KIP2} can control the transcriptional output of the Cyclin E/CDK2/p220^{NPAT}/HiNF-P signaling pathway, but this regulatory level does not immediately influence accumulation of *histone H4* mRNAs.

DISCUSSION

The cyclin E/CDK2 dependent phosphorylation of pRB and p220^{NPAT} ensures that E2F and HiNF-P can activate their respective target genes, thus mechanistically separating the onset of histone production from DNA replication at the G1/S phase transition (Fig. 9). Release of E2F from pRB can be inhibited by p57^{KIP2}, p27^{KIP1} or p21^{CIP1/WAF1} with each preventing phosphorylation of pRB by blocking the activity of CDK2/Cyclin E kinase (Sherr and Roberts, 2004). Our study shows that activity of the p220^{NPAT}/HiNF-P transcriptional co-activation complex is also directly controlled by CKIs. CKIs prevent phosphorylation by CDK2 of at least two phospho-epitopes of p220^{NPAT} that stimulate the functional activity of the p220^{NPAT}/HiNF-P complex. However, our studies suggest that p57^{KIP2} is more potent than p27^{KIP1} or p21^{CIP1/WAF1} in blocking their *in situ* phosphorylation of p220^{NPAT} at Cajal Body-related subnuclear foci.

Interestingly, p57^{KIP2} has weaker intrinsic CDK2 inhibitory activity than p27^{KIP1} and our data suggest p57^{KIP2} may compensate for weaker CDK2 inhibition by forming a complex with its substrate p220^{NPAT}. The question arises how p57^{KIP2} but not p27^{KIP1} or p21^{CIP1/WAF1} can selectively recognize p220^{NPAT}. The C-terminal sequences (e.g., PAPA repeats) of p57^{KIP2} differ from the other two CKIs (p27^{KIP1} and p21^{CIP1/WAF1}), and a chimeric protein that contains the C-terminus of p57^{KIP2} fused to the cyclin binding domain of p27^{KIP1} is as effective as the wild type p57^{KIP2} protein in blocking p220^{NPAT}/HiNF-P activity. The unique structure of p57^{KIP2} may provide the requisite specificity for direct interactions with p220^{NPAT} and thus endow p57^{KIP2} with its ability to suppress the function of p220^{NPAT} more effectively. However, CKIs are unstructured in solution when they do not interact with their cognate cyclin/CDK complexes (Lacy et al, 2004; Adkins and Lumb, 2002). Therefore, it is conceivable that p57^{KIP2} may interact with p220^{NPAT} through a cyclin/CDK protein bridge with the unique C-terminus of p57^{KIP2} stabilizing the ternary complex. Interestingly, both p57^{KIP2} and p220^{NPAT} are CDK2 substrates and contain cyclin binding motifs which could permit formation of larger complexes and/or an exchange of components (e.g., cyclin E or CDK2). Consistent with this model, the cyclin binding motif and unique C-terminus of p57^{KIP2}, as well as the CDK2 phosphorylation sites of p220^{NPAT}, are each required for the formation of complexes containing these two proteins.

It remains to be established whether the regulation of the p220^{NPAT}/HiNF-P complex occurs only at the level of protein/protein interactions or may also reflect promoter recruitment. We have been unable to detect p57 on the *H4* gene promoter, possibly for technical reasons (e.g., detection of promoter-bound p57 may require multiple protein/DNA and protein/protein cross-links). Similarly, it will be of future interest to examine whether phosphorylation of p220^{NPAT} at the T1270 and T1350 phospho-epitopes affects recruitment of p220^{NPAT} to the *H4* promoter. However, it is clear from our previous studies (Miele et al, 2005; Holmes et al, 2005; Mitra et al, 2007) that recruitment of both HiNF-P and p220^{NPAT} to *histone H4* gene promoters is detected in both T98G cells where p57 levels are below the

level of detection, and in HeLa cells that express robust levels of p57. Thus, it appears that recruitment of HiNF-P and p220^{NPAT} to *H4* gene promoters is independent of p57^{KIP2}.

We have previously shown that exogenous HiNF-P cannot activate *H4* gene transcription if endogenous levels of p57^{KIP2} are high (Mitra et al, 2006). Consistent with these findings, the data presented here indicate that p57^{KIP2} is the most effective CKI in suppressing gene activation by the p220^{NPAT}/HiNF-P complex and operates via the HiNF-P binding motif in the cell cycle domain of *histone H4* gene promoters. Furthermore, Skp2-dependent degradation and siRNA induced deficiency of p57^{KIP2} can each alleviate inhibition of the p220^{NPAT}/HiNF-P pathway in cells that express p57^{KIP2}. Depletion of p57^{KIP2} levels by siRNA also alters the relative expression of different *histone H4* gene copies. Taken together, we propose that one of the biological functions of p57^{KIP2} in vivo is to control the activity of p220^{NPAT} as a co-activator of the HiNF-P mediated stimulation of *histone H4* gene promoter activity.

The greater effectiveness of p57^{KIP2} to block the function of the HiNF-P/p220^{NPAT} complex on the *H4* gene promoter is consistent with cell type specific differences in the expression of this CKI in relation to the other two CKI members. For example, during myoblast differentiation, p57^{KIP2} is upregulated in parallel with p21^{CIP1/WAF1}, while p57^{KIP2} and p27^{KIP1} are selectively expressed in differentiated osteoblasts (Drissi et al, 1999;Urano et al, 1999). In both mesenchymal lineages, the elevated expression of p57^{KIP2} will support efficient inhibition of *histone H4* gene transcription at the onset of quiescence during differentiation. However, the majority of proliferating cells express p57^{KIP2} only at very low levels and its function in blocking *histone H4* gene expression may be mostly restricted to quiescent cells. In comparison, the physiological elevation of p21^{CIP1/WAF1} during the DNA damage response in proliferating cells may preferentially permit continued signaling through the CDK2 responsive p220^{NPAT}/HiNF-P pathway but not the E2F/RB pathway to allow histone gene transcription during DNA repair.

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The abbreviations used are

HiNF-P	histone nuclear factor P
CDK	cyclin dependent kinase
NPAT	nuclear protein, ataxia-telangiectasia locus
CIP	CDK inhibitory protein
KIP	kinase inhibitory protein
CKI	CDK inhibitor
pRB	retinoblastoma protein
E2F	adenovirus early gene 2 transcription factor
skp2	S phase kinase associated protein 2
SDS	sodium dodecyl sulfate

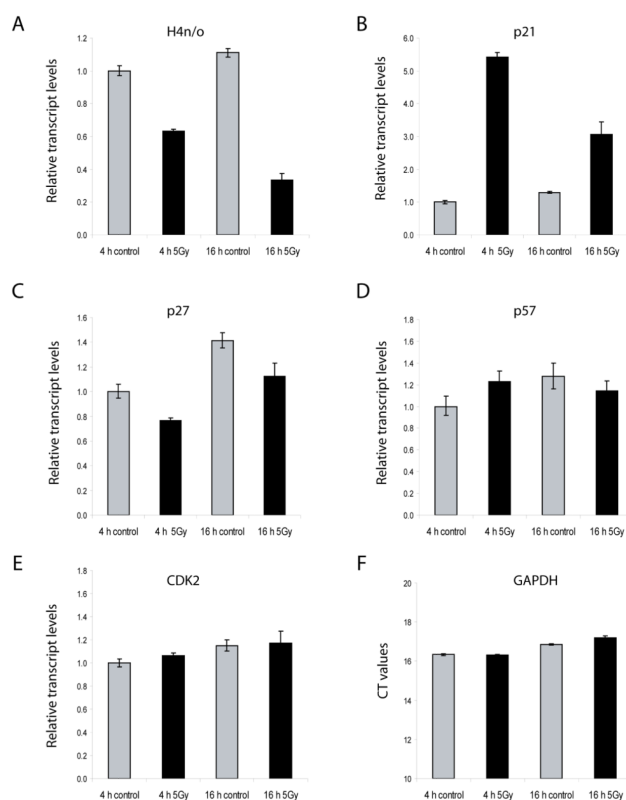
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
HPRT	hypoxanthine guanine phosphoribosyl transferase

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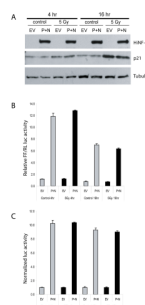
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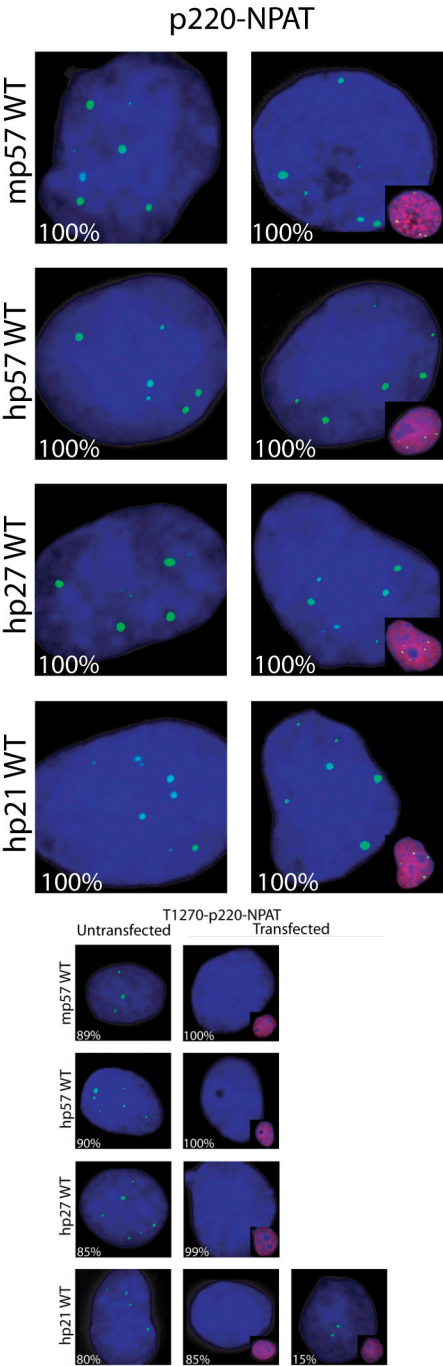
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**Fig. 1.**

γ -Irradiation reduces *histone H4* gene expression while selectively modulating the expression of *p21^{CIP1/WAF1}* and *p27^{KIP1}*. U2OS cells were exposed to 5 Gy irradiation at 24 h after transfection. At 4 or 16 h after irradiation, total RNA was extracted and purified from triplicate experiments. The relative mRNA expression of *H4n*, *p21^{CIP1/WAF1}*, *p27^{KIP1}*, *p57^{KIP2}* and *CDK2* was calculated with the $\Delta\Delta$ CT method using *GAPDH* as an internal control. For *GAPDH*, the CT values were plotted and these values did not change appreciably upon irradiation.

**Fig. 2.**

Co-activation of the *histone H4/n* gene by HiNF-P and p220^{NPAT} occurs independently of γ irradiation. U2OS cells were transiently transfected with a wild-type *histone H4* promoter luciferase reporter construct and co-transfected with the expression vectors for HiNF-P (P), or p220^{NPAT} (N), or empty expression vector (EV). For irradiation experiments, cells were exposed to 5 Gy at 24 h after transfection. At 4 or 16 h after irradiation, cell lysates were analyzed for (A) protein levels of HiNF-P and p21^{CIP1/WAF1} by western blotting protein and (B, C) for luciferase activity. Firefly (FF) luciferase activity was normalized to *Renilla* (RL) luciferase activity in panel B. In panel C, these values are normalized to the empty vector. Error bars indicate standard deviations from triplicate experiments.



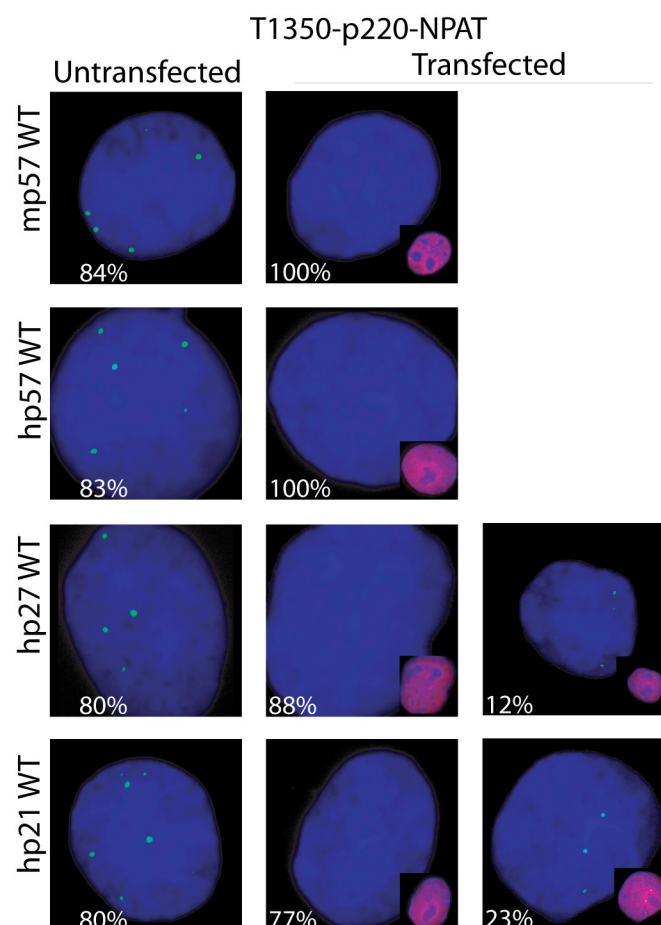


Fig. 3. p57^{KIP2} selectively regulates the phosphorylation of p220^{NPAT} at Cajal bodies. (A–C) Immunofluorescence microscopy was performed on Cos7 cells with antibodies detecting endogenous p220^{NPAT} (A), or CDK2 dependent phosphorylation at two distinct epitopes [i.e., T1270 (B) or T1350 (C)] in cells transfected with wild type human p57^{KIP2}, p27^{KIP1} or p21^{CIP1/WAF1} (right columns) or untransfected cells (left columns). Immunofluorescence signals (green) for total p220^{NPAT}, or the T1270 and T1350 phospho-epitopes are merged with DAPI signals (blue). The insets show merged image of cells transfected with wild type p57^{KIP2}, p27^{KIP1} or p21^{CIP1/WAF1} (red; combined with DAPI to yield purple). The percentages on each panel indicate the number of positive or negative cells.

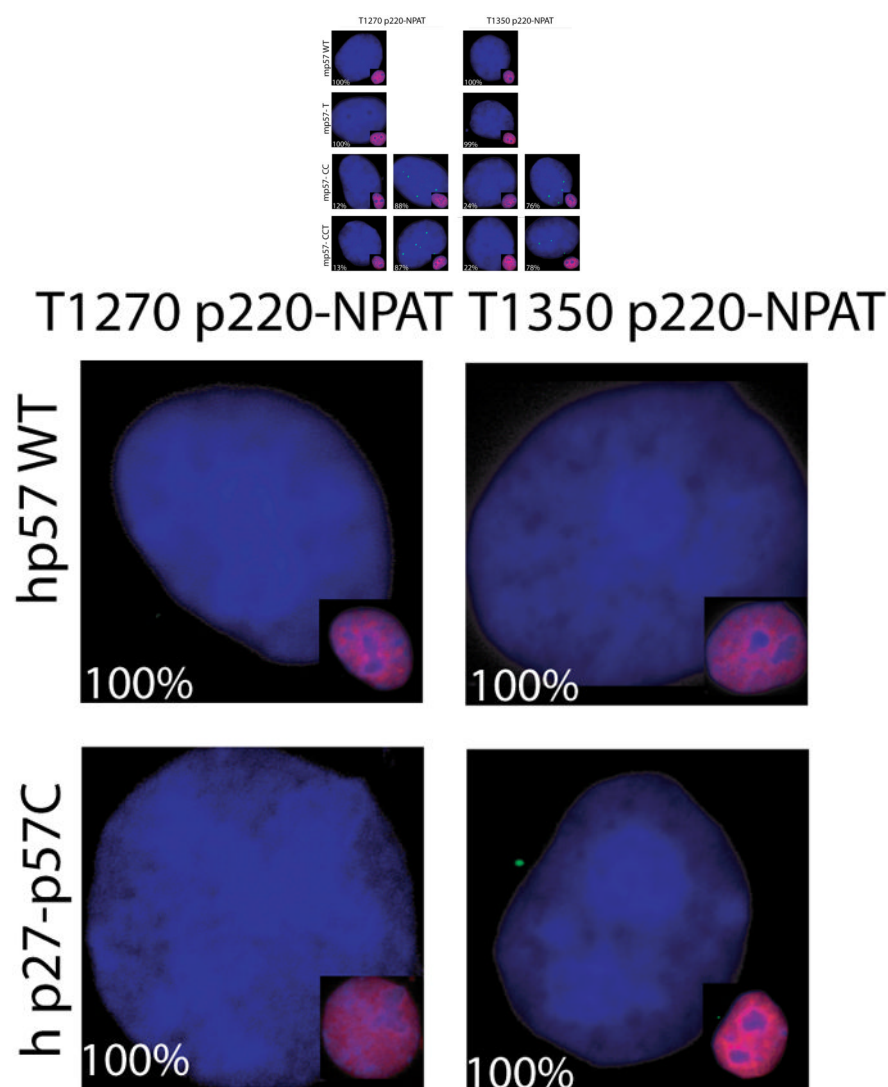


Fig. 4. Mutant p57^{KIP2} is defective in regulating in situ phosphorylation of p220^{NPAT}. (A–C) Immunofluorescence microscopy detecting the T1270 (A) or T1350 (B) epitopes (green) was performed as described in Fig. 1 using cells transfected with wild type mouse p57^{KIP2} or three different mutants (i.e., p57^{KIP2}-T, -CC, or -CCT) (A,B) or human p57^{KIP2} compared with a human p27^{KIP1}-p57^{KIP2} chimera (C). Insets show merged images of p57^{KIP2} and DAPI signals in transfected cells. The percentages on each panel indicate the number of cells with a microscopic phenotype.

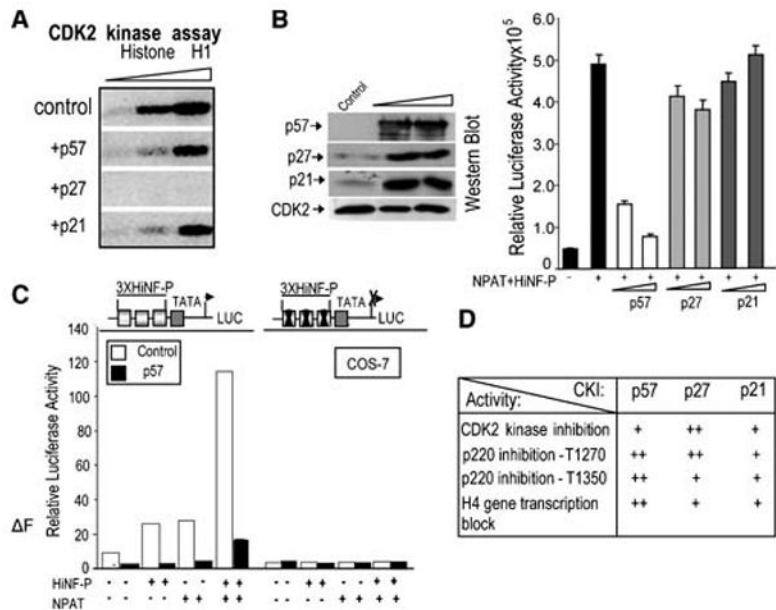


Fig. 5. Inhibition of CDK2 kinase activity and *H4* gene transcription by p57^{KIP2}. (A) In Cos7 lysates, p27^{KIP1} is more potent than p57^{KIP2} or p21^{WAF1/CIP1} in blocking CDK2 activity. CDK2 kinase activity was measured using γ -³²P-ATP and histone H1 as a substrate in CDK2 immunoprecipitates (respectively, 2, 5 and 10 μ l of beads) obtained with lysates of mock-transfected cells or cells expressing p57^{KIP2}, p27^{KIP1} or p21^{WAF1/CIP1}. (B) Selective inhibition of HiNF-P/p220^{NPAT} signaling at the *H4* promoter by p57^{KIP2}. Co-transfection experiments with a wild type *H4* promoter-luciferase reporter gene in the absence (first bar) or presence of vectors for HiNF-P (200 ng/well) or p220^{NPAT} (150 ng/well) (remaining bars) in cells expressing exogenous CKIs (25 or 50 ng vector) as indicated. *H4* promoter-luciferase activities were measured within 24 h after transfection and plotted as a function of p57^{KIP2} vector concentration (right panel). Western blot analysis (left panel) was used to examine CKI expression in whole cell lysates (20 μ g protein). Note that Cos7 cells do not express endogenous p57^{KIP2}. (C) The HiNF-P binding site is sufficient for p57^{KIP2} dependent inhibition of the *H4* gene promoter. Cos7 cells were co-transfected with Luciferase reporters controlled by wild type or mutant HiNF-P elements fused to a minimal TATA box promoter. Relative promoter activity was assessed in the presence (+) or absence (–) of vectors expressing HiNF-P, p220^{NPAT} or p57^{KIP2} (25 ng/ml) as above. (D) Summary of data presented in Figs. 1 to 3 with + signs indicating the relative effectiveness of CKIs to affect the activities in the left column.

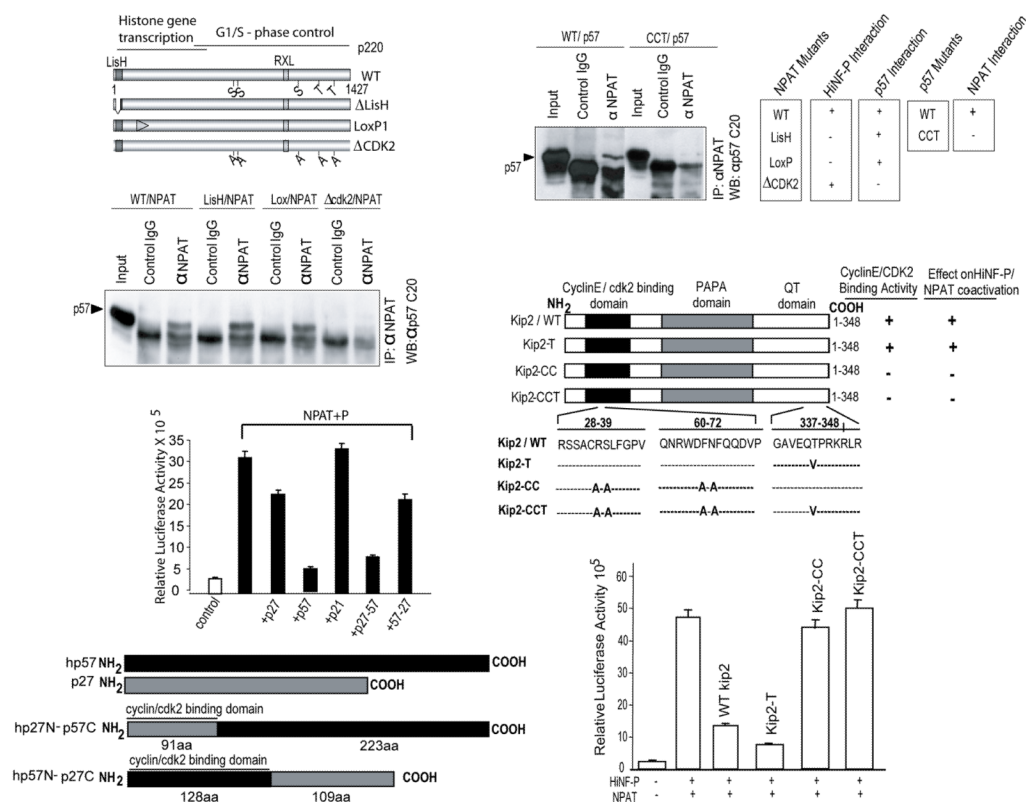
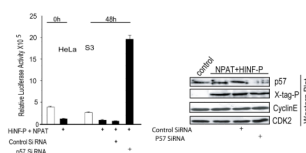


Fig. 6. p57^{KIP2} and p220^{NPAT} form specific complexes. (A) Wild type p220^{NPAT} forms a complex with p57^{KIP2} via residues involved in CDK2 phosphorylation. Immuno-complexes were obtained from Cos7 cells transfected with expression vectors for p57^{KIP2} (25 ng/well) and wild type or mutant p220^{NPAT} proteins (200 ng/well). Whole cell protein (~100 μg) was precipitated with anti-p220^{NPAT} antibody (1 μg) antibody, and analyzed by western blotting using an anti-rabbit-p57^{KIP2} antibody (1:3,000 dilution; secondary goat anti-rabbit IgG antibody= 1:5,000 dilution). Wild type p220^{NPAT} and two p220^{NPAT} mutants (LisH, deletion of aa 3-38; LoxP1: alanine substitutions between aa 121-145, respectively) interact with p57^{KIP2}, but the p220^{NPAT}-ΔCDK2 mutant with alanine substitution in five C-terminal CDK2 phosphorylation sites (S/T) does not. (B) The N-terminal cyclin binding of wild type p57^{KIP2} supports interactions with p220^{NPAT}. Wild type p57^{KIP2} and a p57^{KIP2} mutant with amino acid substitutions in the cyclin binding domain (see C) were expressed in Cos7 cells, and immunoprecipitates were obtained as described above (see A). Complexes with p220^{NPAT} are only formed with wild type p57^{KIP2} but not with p57^{KIP2}-CCT as indicated. The immunoprecipitation results presented here were correlated with those obtained for HiNF-P and p220^{NPAT} previously (lower panel). (C) Inhibition of *H4* gene transcription requires the cyclin binding domain of p57^{KIP2}. Co-activation assays for p220^{NPAT}/HiNF-P were performed with cells co-transfected with vectors expressing wild type or mutant p57^{KIP2} (i.e., T, CC and CCT; 25 ng/well) and luciferase activity for each mutant is plotted. (D) As in Panel C, but using reciprocal in the C-termini of p57^{KIP2} and p27^{KIP1} are swapped.

**Fig. 7.**

Rescue of HiNF-P co-activation by p57^{KIP2} siRNA and changes in endogenous *histone H4* gene expression upon p57^{KIP2} modulation. Treatment of HeLa cells that express endogenous p57^{KIP2} with siRNA specific for p57^{KIP2} restores co-activation of HiNF-P and p220^{NPAT}. HeLa cells were transfected with the *H4* promoter-luciferase reporters in the presence (+) or absence of p220^{NPAT} and HiNF-P expression vectors. After ~12–16 h, cells were treated with (+) control siRNA or p57^{KIP2} siRNA (100 nM). Luciferase activity was measured after 48 h. Selective siRNA mediated deficiency of p57^{KIP2} relative to Cyclin E, CDK2 and HiNF-P was confirmed by immunoblotting.

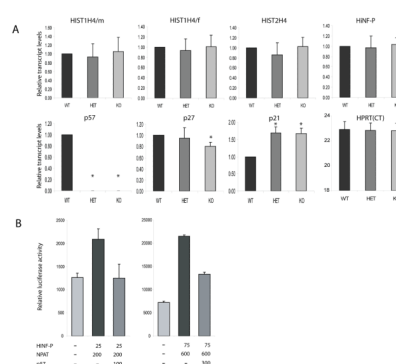


Fig. 8. $p57^{\text{Kip2}}$ suppresses *histone H4* promoter activity in normal diploid human cells. (A) Total RNA from wild type $p57$ (WT), heterozygous $p57$ null (HET) and homozygous $p57$ null (KO) mouse embryonic fibroblasts was examined for mRNA expression of mouse *HiNF-P*, *Hist2H4*, *HistH4/m*, *Hist1H4/f*, $p57$, $p27$ and $p21$ using quantitative RT-PCR. Values were calculated with the $\Delta\Delta\text{CT}$ method using *HPRT* as an internal control. We did not observe significant changes in the expression of *Hist2H4*, *Hist1H4/m* and *Hist1H4/f* histone in HET and KO cells compared to WT cells. As expected $p57$ mRNA is absent in KO and HET cells (HET cells are functionally null for $p57$ due to imprinting), while expression of $p21$ mRNA in KO and HET cells is significantly higher than in WT cells. Student's t-test (unpaired, 2-tailed, non-parametric) was used to calculate significance by comparing gene expression in WT to either HET or KO cells. Asterisks (*) indicate P-values <0.05). (B) WI-38 human diploid fibroblasts were plated at a density of 1.6×10^5 /well in six-well plates and transiently transfected at day 2 after plating at a cell density of ~30% with wild-type *histone H4* promoter luciferase reporter construct. Cells were co-transfected with the indicated amounts of expression vectors for *HiNF-P* (P), $p220^{\text{NPAT}}$ (N) and $p57$, or an empty vector (EV). Experiments were performed with either 200 ng (left graph) or 600 ng (right graph) of firefly luciferase reporter gene construct. The promoterless *Renilla* luciferase control plasmid was also different (25 ng, left graph; 75 ng, right graph), but total amount of DNA was maintained at 2.5 μg in every transfection.

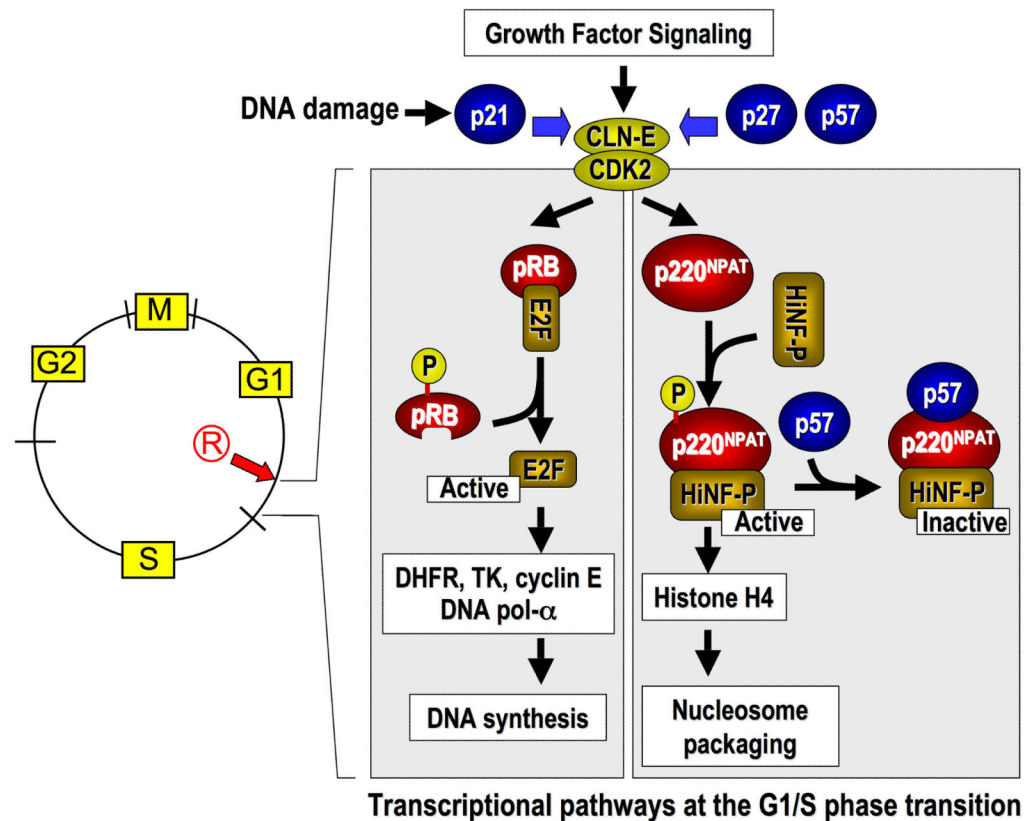


Fig. 9.

Control of the HiNF-P/p220^{NPAT} pathway by p57^{KIP2}. *Histone H4* gene transcription is controlled by the HiNF-P/p220^{NPAT} complex that is activated in parallel to the E2F/pRB pathway that controls transcription of genes involved in nucleotide metabolism and DNA synthesis. Both pathways are responsive to growth factor dependent induction of CDK2/cyclin E at the R-point, and thus sensitive to the levels of CDK inhibitors. The findings in this study suggest that while p57^{KIP2} is not a strong CDK inhibitor, it can effectively inhibit CDK phosphorylation of p220^{NPAT} through direct protein/protein interactions. It is possible that p57^{KIP2} can be recruited to histone gene promoters through interaction with the HiNF-P/p220^{NPAT} complex.