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The Interactome of the Histone Gene Regulatory Factor HiNF-P Suggests Novel Cell Cycle Related Roles in Transcriptional Control and RNA Processing

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Abstract HiNF-P is a recently identified histone H4 subtype specific transcriptional regulator that associates with the conserved cell cycle control element in the proximal promoter regions of histone H4 genes. HiNF-P interacts with the global histone gene regulator and direct cyclin E/CDK2 substrate $p220^{NPAT}$ to potently upregulate histone H4 gene transcription at the G1/S phase transition in response to cyclin E/CDK2 signaling. To gain insight into the function of HiNF-P in a broader cellular context, we performed a yeast two-hybrid screen to identify its novel interacting proteins. In this study, we detected 67 candidate HiNF-P interacting proteins of varying cellular functions. We have identified multiple RNA associated proteins, including the splicing co-factor SRm300. HiNF-P and SRm300 interact in yeast two-hybrid, co-immunoprecipitation, and co-immunofluorescence assays. Our screen also identified several gene regulators that associate with HiNF-P including THAP7. HiNF-P and THAP7 interact in mammalian cells and THAP7 abrogates HiNF-P/p220 mediated activation of histone H4 gene transcription, consistent with its known role as a transcriptional repressor. Finally, we identified several proliferation related proteins including Ki-67 and X transactivated protein 2 (XTP2) which may be functioning with HiNF-P in cell cycle regulation. The HiNF-P interactome indicates that HiNF-P is a multifunctional gene regulator with a large functional network and roles beyond cell cycle-dependent histone gene regulation. J. Cell. Biochem. 102: 136–148, 2007.

Key words: cell cycle; histone; Cajal body; transcription; Zn finger; CDK2; cyclin; RNA processing

Cell cycle-dependent regulation of histone genes is coupled with DNA replication during S phase. Histone gene transcription is upregulated concomitantly with the onset of replication to provide adequate histone proteins to package the nascent DNA into chromatin ([Stein et al., 1975; Marashi et al., 1982]; and reviewed in Osley [1991]). Transcriptional control of the histone H4/n (FO108, HIST2H4) gene has been extensively studied. This gene is regulated in a cell cycle-dependent manner independent of E2F by DNA/protein interactions at a multipartite proximal promoter element, Site II ([Ramsey-Ewing et al., 1994; van Wijnen et al., 1996]; and reviewed in Osley [1991]). Histone nuclear factors (HiNFs) are transcription factors associating with this site [Kroeger et al., 1987; van Wijnen et al., 1991; Ramsey-Ewing et al., 1994; Vaughan et al., 1995]. They are HiNF-M/IRF-2, the HiNF-D complex which includes CDP/cut as the DNA binding subunit, and HiNF-P a recently identified zinc finger protein [Mitra et al., 2003] that was independently characterized as an MBD-2 interacting zinc finger protein (MIZF) [Sekimata et al., 2001].

Our laboratory has identified HiNF-P as a principal activator of histone H4 gene transcription [Mitra et al., 2003]. We have shown that HiNF-P physically interacts with the histone gene coactivator, $p220^{NPAT}$, and links the cyclin E/CDK2 pathway directly to histone gene

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upregulation at the G1/S phase transition. HiNF-P associates with Site II at a histone H4 subtype specific consensus element and recruits $p220^{NPAT}$ to this site [Miele et al., 2005]. HiNF-P is necessary for efficient cell cycle progression and for optimal histone H4 gene expression during S phase [Mitra et al., 2003]. HiNF-P may also regulate other cell cycle related genes, including Rb and p57 that contain HiNF-P consensus binding elements ([Sekimata and Homma, 2004] and unpublished data).

To gain further insight into components of combinatorial control at the G1/S phase cell cycle transition that are mediated by HiNF-P, we performed an unbiased yeast two-hybrid screen. Among the 67 candidate interacting proteins of HiNF-P were proteins involved in RNA processing and metabolism, known and putative gene regulators, proliferation-related proteins, and unknown proteins. Here we present validation and characterization of a subset of these proteins which suggest additional roles for HiNF-P.

MATERIALS AND METHODS

Expression Plasmids

Full-length HiNF-P, HiNF-P zinc finger domain (ZF domain), and the C-terminus of HiNF-P (Cterm domain) were cloned into the BamHI/XhoI site in pEG202, creating LexA/HiNF-P fusion proteins. Full-length HiNF-P was subcloned from a CMV-driven HiNF-P expression plasmid [Mitra et al., 2003]. The ZF domain of HiNF-P was cloned by PCR with CMV-full-length HiNF-P as the template using the following PCR primers: Forward 5′-CGG GAT CCC GCC GCC TCC TGG GAA AG-3′ and Reverse 5′-CCG CTC GAG CCA CTT GAA CTG GTG-3′. The Cterm domain of HiNF-P was cloned by PCR with CMV-full-length HiNF-P as template using the following PCR primers: Forward 5′-CGG GAT CCC GCC CTC AGG GCA-3′ and Reverse 5′-CCG CTC GAG AAC CAT CTG GAT CTC TGG-3′.

Full-length THAP7 was cloned in pcDNA 3.1/HisC by PCR of cDNA prepared from HeLa total RNA isolated by Trizol reagent (Invitrogen, Carlsbad, CA) and the Superscript First Strand Synthesis RT-PCR system (Invitrogen). The following primers were used, for THAP7: Forward 5′-CGC GGA TCC ATG CCG CGT CAC TGC-3′ and Reverse 5′-CCG CTC GAG GGC CAT GCT GCT GCT-3′.

Yeast Two-Hybrid Screen

The yeast two-hybrid screen was performed using full-length LexA/HiNF-P as the bait. A HeLa-S3 cDNA library used for our studies was prepared in the pJG4-5 vector (generously provided by Dr. R. Brent, Mass General Hospital, Boston, MA). In addition a HeLa cDNA library (Invitrogen) prepared in the pYesTrp2 vector was also screened (generously provided by Dr. Elizabeth Luna, University of Massachusetts Medical School, Worcester, MA). Bait and library constructs were transformed into the yeast strain EGY48 (Invitrogen) using the lithium acetate method. EGY48 is integrated with the auxotrophic marker Leucine 2 (LEU2) and transformed with the LacZ reporter plasmid pSH18-34, both of which contain upstream LexA operator sequences. A two-hybrid interaction was detected by yeast growth on medium deficient for leucine or by detection of LacZ reporter activity on medium supplemented with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). The yeast two-hybrid screen was performed as described [Ausubel et al., 1997].

Confirming Positives

A candidate yeast two-hybrid approach was used to confirm positive interactions and to determine which region of HiNF-P interacts with the prey. LexA/HiNF-P, LexA/ZF, or LexA/C-term were transformed into the EGY48 yeast strain as indicated above. Positives interacting proteins were categorized according to their Gene Ontology functional classifications (www.geneontology.org).

Western Blot Analysis

Expression of LexA/HiNF-P and deletion constructs was confirmed by Western blot analysis. Yeast protein lysates were prepared in lysis buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 10% glycerol, and 1× Complete protease inhibitor (Roche, Indianapolis, IN). Cells were lysed in 200 μL lysis buffer and glass beads then vortexed 5–10 min at 4°C. The lysates were centrifuged for 5 min and the supernatants were denatured by adding 1/3 vol of 4× SDS buffer (40% glycerol, 200 mM Tris-HCl pH 6.8, 8% SDS, 0.4% bromophenol blue, and 400 mM DTT) and boiled for 5 min. Anti-LexA antibody (Invitrogen) was used for detection of the proteins on the

immunoblot at a dilution of 1:5,000. Proteins isolated from co-immunoprecipitation analysis (described below) were resuspended in 2× SDS buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol, and 200 mM DTT) and subsequently boiled for 5 min. Antibodies used for detection on immunoblots were chicken anti-HiNF-P (1:5000), anti-Xpress (1:5,000, Invitrogen), and anti-GFP (1:5,000, Zymed, San Francisco, CA). Horseradish peroxidase-conjugated secondary antibodies were used at a dilution of 1:10,000 (Santa Cruz Biotechnology, Santa Cruz, CA) and bands were visualized by using the Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer Life Sciences, Shelton, CT).

Cell Culture and Transient Transfections

HeLa and SaOS cells were obtained from American Type Culture Collection (Manassas, VA). HeLa cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. SaOS cells were maintained in McCoy's 5A medium (Invitrogen) supplemented with 15% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Culture conditions were 37°C in a 98% humidified and 5% CO_2 incubator. HeLa cells or SaOS cells were plated at a density of 0.65×10^6 cells/100 mm plate or 0.12×10^6 cells/well of a 6-well plate. Twenty-four hours later cells were transfected with the indicated amount of various plasmids using FuGENE 6 protocol (Roche, Indianapolis, IN), and 24 h later the cells were harvested for the appropriate assay.

Immunofluorescence Microscopy

SaOS cells were seeded on coverslips coated with 0.5% gelatin at a density of 0.12×10^6 cells/well and were prepared for in situ immunofluorescence microscopy as described previously [Zaidi et al., 2003]. Briefly, cells were washed twice with ice cold 1× PBS and subsequently fixed with 3.7% formaldehyde in 1× PBS on ice for 10 min. Cells were then washed once with ice cold 1× PBS and permeabilized with 0.1% Triton-X 100 in 1× PBS, then rinsed twice with PBS and once with 1× PBS containing 0.5% BSA (PBSA) followed by antibody staining. The following antibodies were used: rabbit polyclonal anti-HiNF-P 1:500, anti-Xpress (Invitrogen) 1:500, anti-Ki-67 (Sigma-Aldrich, St. Louis, MO) 1:500, anti-coilin (BD Biosciences Pharmingen, San Diego, CA) 1:1,000, and anti-SRm300 (generously provided by Dr. Jeffrey Nickerson, UMass Medical School, Worcester, MA). SRm300 was also detected by overexpression of SRm300 fused to GFP in SaOS cells (generously provided by Dr. Jeffrey Nickerson). Secondary antibodies were used at a dilution of 1:800 (Alexa 488 goat anti-rabbit and Alexa 594 goat anti-mouse; Invitrogen). Images were captured using a charge-coupled device camera attached to an epifluorescence Zeiss Axioplan 2 microscope (Zeiss, Inc., Thorwood, NY) and processed using Metamorph Imaging Software (Universal Imaging, Downingtown, PA).

Immunoprecipitations

In vitro transcribed and translated proteins were prepared using the TNT coupled reticulocyte lysate system (Promega, Madison, WI) either labeled with ^{35}S-methionine (yeast two-hybrid clones) or unlabeled (HiNF-P). Five microliters of both recombinant proteins were used for each immunoprecipitation and diluted in 500 μL IP buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, and 1× Complete protease inhibitors (Roche)). Proteins were incubated together for 1 h rotating at 4°C. About 2 μg anti-HiNF-P antibody was added and samples were rotated at 4°C for 1 h. Then 50 μL protein A/G beads (Santa Cruz Biotechnology) were added for 1 h and subsequently washed three times with IP buffer. Samples were then separated by 10% SDS–PAGE using ProtoGel solutions (National Diagnostics, Atlanta, GA) and the gels were dried and exposed to X-ray film. Co-immunoprecipitation experiments in HeLa cells were performed using cells overexpressing HiNF-P and THAP7 or SRm300/GFP which were lysed in IP buffer. Lysates were cleared by centrifugation for 10 min at 14,000 rpm at 4°C. Two micrograms of the indicated antibody (anti-HiNF-P, anti-SRm300 (provided by Dr. Jeffrey Nickerson), anti-Xpress (Invitrogen), or control IgG (Santa Cruz Biotechnology) were added and lysates were rotated at 4°C for 1–2 h. Then 50 μL protein A/G beads (Santa Cruz Biotechnology) were added for 1 h and subsequently washed three times with IP buffer. Proteins were then separated by SDS–PAGE

using ProtoGel solutions (National Diagnostics) and subjected to Western blot analysis as described above.

Luciferase Assays

SaOS cells were seeded at 0.12×10^6 cells/well of a 6-well plate and transfected 24 h later using FuGENE 6 reagent (Roche, Indianapolis, IN). Cells were transfected with 150 ng empty vector plasmid or CMV-HiNF-P, 200 ng CMV-$p220^{NPAT}$, either 100 ng or 400 ng THAP7/XP as indicated, and 100 ng histone H4/n wild-type promoter/Luciferase construct [Xie et al., 2001]. Luciferase assays were performed using the Luciferase Assay Kit (Promega) according to manufacturer's specifications and a Monolight 2010 Luminometer (Analytical Luminescence Laboratory, San Diego, CA).

Chromatin Immunoprecipitations

HeLa cells were seeded at 0.65×10^6 cell/100 mm plate and transfected after 24 h with CMV-THAP7 or untransfected. Twenty-four hours post-transfection cells were washed twice with ice-cold 1× PBS and subsequently crosslinked with 1% formaldehyde in 1× PBS for 10 min at room temperature with gentle agitation. To quench the crosslinking reaction 0.25% glycine in 1× PBS was added for 5 min. Cells were then washed twice with ice-cold 1× PBS and scraped in lysis buffer (50 mM Tris-Cl pH 8.1, 150 mM NaCl, 1% v/v NP-40, 2× Complete protease inhibitor [Roche]), and incubated on ice for 20 min. Lysates were sonicated to an average DNA size of 100–500 bp and then cleared by centrifugation at 14,000 rpm for 15 min at 4°C. Two micrograms of the indicated antibodies (anti-Xpress [Invitrogen]), anti-HiNF-P, anti-$p220^{NPAT}$ [BD Biosciences Pharmingen], normal IgG [Santa Cruz Biotechnology]) 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 mM Tris-Cl pH 8.1, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 1× Complete protease inhibitor), high salt (20 mM Tris-Cl pH 8.1, 500 mM NaCl, 1% Triton X-100, 2 mM EDTA), LiCl (10 mM Tris-Cl pH 8.1, 250 mM LiCl, 1% deoxycholate, 1% NP-40, 1 mM EDTA) and three washes with TE (10 mM Tris-Cl pH 8.1, 1 mM EDTA). Protein/DNA complexes were eluted twice with elution buffer (1% SDS, 100 mM $NaHCO_3$) at room temperature. One-tenth volume of 3 M NaOAC pH 5.2 was added and samples incubated at 65°C overnight to reverse crosslinks. DNA was purified by phenol/chloroform extraction followed by isopropanol precipitation with 5–20 μg glycogen carrier and resuspended in resuspension buffer (10 mM Tris-Cl pH 8.1). ChIP samples were subjected to quantitative PCR analysis using SybrGreen chemistry (Applied Biosystems, Inc., Foster City, CA) and an ABI Sequence Detection System with histone H4/n specific primers: Forward 5′-AGCTGTCTATCGGGCTCCAG-3′ and Reverse 5′-CCTTTGCCTAAGCCTTTTCC-3′.

RESULTS AND DISCUSSION

Identification of Potential HiNF-P Interacting Proteins by Yeast Two-Hybrid Screening

To identify HiNF-P interacting proteins, we performed yeast two-hybrid assays. HiNF-P contains nine C_2H_2 zinc fingers in its N-terminus (ZF domain) and a C-terminus (Cterm domain) devoid of zinc fingers (Fig. 1A). We generated three chimeric proteins in which full-length HiNF-P, the ZF domain, and the Cterm domain were fused to the LexA DNA binding domain (LexA/HiNF-P, LexA/ZF, and LexA/Cterm). Each construct was first tested for absence of activation of the auxotrophic marker *Leucine 2* (*LEU2*) and the LacZ reporter gene under the control of multiple upstream LexA operator sites in the yeast strain EGY48. Although the three LexA fusion proteins were expressed as confirmed by Western blot (Fig. 1B), the clones did not grow on leucine-free media and did not show blue staining on X-gal containing medium (Fig. 1C). Thus, HiNF-P does not self activate the two selectable marker genes.

We carried out two independent yeast two-hybrid screens using two distinct HeLa cDNA libraries in which random cDNAs are fused to the B42 activation domain. With LexA/HiNF-P as bait, we identified more than 70 positive clones out of 1×10^6 transformants that exhibit growth on media lacking leucine and a blue color on media containing X-gal. Further analysis revealed that these clones represent 67 distinct putative HiNF-P interacting proteins. These proteins were classified into different functional and/or structural categories based on their Gene Ontology annotations (Fig. 1D). The groupings include known and putative gene

regulators, RNA metabolism proteins, uncharacterized proteins, cell cycle related, metabolic, and signaling proteins. A subset of these genes (e.g., mitochondrial proteins, proteosome subunits, and ribosomal subunits L27 and L38a) are frequently detected in yeast two-hybrid assays [Hengen, 1997] and may represent false positives that were not further analyzed (data not shown). The spectrum of the HiNF-P interactome suggests that HiNF-P may have broad cellular functions beyond control of histone H4 gene expression.

HiNF-P is associated with subnuclear bodies where the regulatory machinery for the cell cycle-dependent transcription and processing of histone RNA transcripts is organized and assembled. Therefore, we selected for further analysis nine candidate HiNF-P interacting proteins that fall into three classes that may be relevant to the function of HiNF-P. The first group includes RNA binding proteins (e.g., SRm300), the second group contains known and putative gene regulators (e.g., THAP7, MRG15, ADO23, and NPDC-1), and the third group encompasses proteins associated with cell cycle control (e.g., Ki-67, X transactivated protein 2 (XTP2), MDC1, and IDN3). As discussed below, each of these candidates was further examined by yeast two-hybrid deletion analyses, immunoprecipitations, in situ immunofluorescence microscopy, and/or reporter gene transcription assays.

Our yeast two-hybrid screen also identified a number of anonymous proteins, signaling proteins, and metabolic proteins that lack identifiable domains or motifs to provide insight into their cellular functions (data not shown). Such putative HiNF-P interacting proteins involved in cell signaling or metabolism may reflect functions of HiNF-P that have evolved independently of its role in histone gene regulation or indicate dimensions to cell cycle control that have yet to be explored. Additional studies will be necessary to characterize the functions of this set of factors.

HiNF-P Interacts With Multiple RNA Processing Proteins Including the Splicing Complex Protein, SRm300

Our yeast two-hybrid screen identified seven proteins involved in RNA metabolism and RNA processing (Supplementary Table 1). Included in this category are components of the U2 and U5 SnRNP complexes as well as RNA splicing factors such as SPF45. We focused on the serine/arginine (SR)-related nuclear matrix protein of 300 kDa (SRm300) because it was isolated as two independent overlapping clones (Fig. 2A). SRm300 is a member of a splicing complex with its partner protein SRm160 that associates with pre-mRNA by a U1 snRNP-specific pathway and supports splicing reactions [Blencowe et al., 1998, 2000]. We determined the region of HiNF-P that interacts with SRm300 by using deletion mutants in yeast two-hybrid assays. The results show that both SRm300 clones interact with LexA/HiNF-P and LexA/ZF fusion proteins but not the LexA/Cterm protein (Fig. 2B). Our data indicate that SRm300 binds to the N-terminal ZF domain of HiNF-P.

To validate the yeast two-hybrid results in mammalian cells, we performed co-immunoprecipitations with HeLa cells using exogenously expressed SRm300/GFP and HiNF-P. A highly specific anti-HiNF-P antibody immunoprecipitates SRm300, and conversely, anti-GFP antibodies recognizing the SRm300/GFP fusion protein immunoprecipitate HiNF-P (Fig. 2C). Thus, HiNF-P and SRm300 are interacting in mammalian cells.

We examined the cellular localization of SRm300 and HiNF-P using in situ immunofluorescence microscopy. SRm300 is distributed in a speckled nuclear pattern typical of RNA splicing factors. HiNF-P is also a nuclear protein with a punctate distribution. Despite distinct nuclear patterns, there is significant overlap of HiNF-P foci with SRm300 speckles (Fig. 3A). Using immunofluorescence microscopy we analyzed whether SRm300 is localized to cellular foci that support histone gene regulation. First, we examined SRm300 localization with respect to Cajal bodies and $p220^{NPAT}$ foci. Immunofluorescence microscopy shows that a small percentage of SRm300 foci reproducibly co-localize with $p220^{NPAT}$ and the Cajal body specific protein coilin in HeLa cells (Fig. 3B). Further, triple labeling experiments show that a subset of HiNF-P and SRm300 localize near to Cajal bodies (Fig. 3C). Our results suggest that these factors may participate in the same molecular process.

We used chromatin immunoprecipitation experiments to examine if SRm300 is in complex with HiNF-P and $p220^{NPAT}$ at the histone H4 promoter. HiNF-P, $p220^{NPAT}$, and RNA polymerase II are present at the histone H4/n promoter (data not shown) as previously

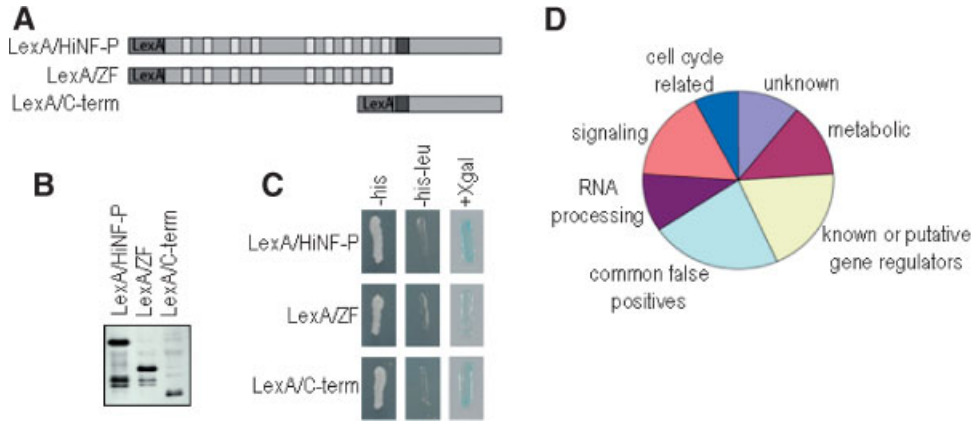


Fig. 1. LexA/HiNF-P fusion proteins do not self-activate reporter genes. **A**: Full-length HiNF-P as well as the zinc finger (ZF) domain and C-terminus (Cterm) of HiNF-P were cloned into a yeast expression vector to create fusion proteins with the LexA DNA binding domain. HiNF-P consists of nine zinc finger domains (white boxes) and a highly conserved region in the Cterm domain (black box). **B**: LexA/HiNF-P, LexA/ZF, and LexA/Cterm expression was analyzed by Western blot. Anti-LexA antibodies were used to detect the fusion proteins. **C**: LexA/HiNF-P, LexA/ZF, and LexA/Cterm were assayed for their ability to self-activate the dual reporter system. The LexA fusion proteins contain the selectable marker gene *HIS1* while the reporters are the *LEU2* and *LacZ* genes. The LexA/HiNF-P fusion proteins do not self activate the reporters based on the lack of growth on leucine deficient media and absence of dark blue color on media containing Xgal. **D**: A yeast two-hybrid screen was performed using LexA/HiNF-P as bait. Transformants (1×10^6) yielded 67 candidate HiNF-P interacting proteins. These candidates were classified based upon their Gene Ontology annotations.

reported [Miele et al., 2005]. However, highly specific SRm300 antibodies are unable to precipitate the histone H4/n promoter region (data not shown). Thus, SRm300 apparently does not occupy the promoters of histone H4 genes. The HiNF-P/SRm300 complex may support a post-transcriptional mechanism; for example, interaction between the two proteins may support the processing of histone pre-mRNAs at Cajal bodies or non-histone mRNAs in subnuclear splicing domains.

Histone pre-mRNAs are intronless and do not undergo traditional splicing reactions, but are subject to endonucleolytic cleavage that creates a non-polyadenylated mature 3′-end adjacent to a stem-loop structure [Dominski and Marzluff, 1999; Dominski et al., 2005]. Recent data suggest that traditional splicing factors may contribute to 3′-end formation of histone mRNAs [Dominski et al., 2005]. Our finding that SRm300 and HiNF-P interact is consistent with this idea and further suggests that 3′-end processing of histone mRNAs is closely coupled to transcription.

HiNF-P Interacts With Known and Putative Gene Regulators, Including the Transcriptional Repressor, THAP7

Our yeast two-hybrid screen revealed 13 known and putative gene regulators

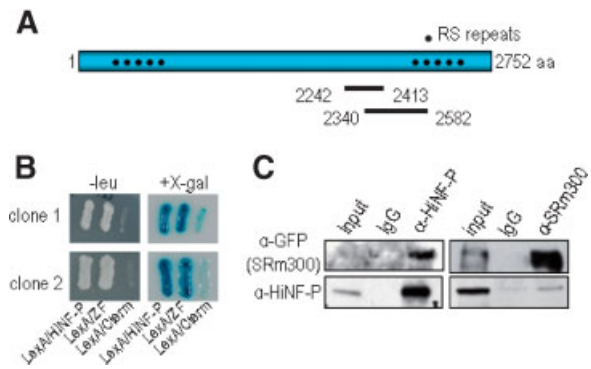


Fig. 2. HiNF-P interacts with SRm300. **A**: The splicing protein SRm300 was identified as two overlapping clones from a yeast two-hybrid screen, indicated by the black bars. SRm300 contains multiple arginine/serine (RS) repeats indicated by black circles. **B**: LexA/HiNF-P, LexA/ZF, and LexA/Cterm were co-transformed with the two independent yeast clones of SRm300 and assayed for reporter gene activation. SRm300 interacts with LexA/HiNF-P and LexA/ZF as indicated by growth on leucine deficient media and a blue color on media supplemented with X-gal. **C**: Co-immunoprecipitation experiments were performed to determine if HiNF-P and SRm300 interact in mammalian cells. HiNF-P and SRm300 fused to the green fluorescent protein (SRm300/GFP) were co-transfected into HeLa cells. Co-IPs were performed using anti-HiNF-P or anti-SRm300 antibodies and analyzed by Western blot using anti-GFP or anti-HiNF-P antibodies. IgG lanes represent non-specific antibody controls and input lanes represent 5% of the total protein used in each immunoprecipitation.

as potential partner proteins of HiNF-P (Supplementary Table 2). We focused on THAP7, a recently characterized zinc finger protein involved in transcriptional repression [Macfarlan et al., 2005]. THAP7 contains a highly conserved THAP zinc finger domain, a C2CH signature zinc finger in its N-terminus, a proline-rich central domain, and a basic C-terminus [Macfarlan et al., 2005]. Based on the THAP7 clone isolated from the yeast two-hybrid screen, the putative HiNF-P binding domain encompasses the THAP domain (Fig. 4A). To determine which region of HiNF-P is involved in this interaction, we performed yeast two-hybrid assays with THAP7 and the LexA/HiNF-P deletion mutants as described above. Our results show HiNF-P interacts through the N-terminal zinc finger domain with the THAP domain of THAP7 (Fig. 4B).

We validated our yeast two-hybrid data by co-immunoprecipitations and in situ immunofluorescence microscopy using mammalian cells. THAP7 and HiNF-P proteins exogenously expressed in HeLa cells were used in immunoprecipitations. Our results show that immunoprecipitation of Xpress-tagged THAP7 results in co-precipitation of HiNF-P (Fig. 4C). In situ immunofluorescence microscopy demonstrates that THAP7 is a nuclear protein with a punctate distribution. that exhibits significant overlap with HiNF-P, as well as $p220^{NPAT}$ foci in HeLa cells. These microscopy results suggest that THAP7 and HiNF-P (Fig. 4D, left panel), as well as THAP7 and $p220^{NPAT}$ (Fig. 4D, right panel) associate in situ at selective sites within the nucleus. These results suggest that THAP7 may form distinct complexes with HiNF-P and its co-activator $p220^{NPAT}$ in vivo. Therefore, we examined whether THAP7 regulates histone gene transcription in reporter gene assays. HiNF-P, $p220^{NPAT}$, and increasing amounts of THAP7 were expressed in Cos7 cells and assayed for their ability to transactivate a histone H4/n gene promoter/luciferase construct. THAP7 inhibits the synergistic co-activation of histone H4 transcription by HiNF-P and $p220^{NPAT}$ (Fig. 4E). This finding corroborates the known function of THAP7 as a transcriptional repressor [Macfarlan et al., 2005], and identifies the HiNF-P dependent regulation of histone H4 genes as a possible repression target for THAP7.

Three additional HiNF-P interacting gene regulatory factors were examined by yeast two-hybrid deletion analyses to delineate the region of HiNF-P involved in binding. The Morf Related Gene 15 (MRG-15) interacts with the N-terminal Zn finger region (Fig. 5A), while AD023 (Fig. 5B) and NPDC-1 (Fig. 5C) interact with the C-terminal region of HiNF-P. MRG15 is a chromodomain protein that associates with Rb and E2F to activate the promoter of the Myb-B gene [Leung et al., 2001], a cell cycle-dependent regulator of cell growth [Lam and Watson, 1993]. The clone identified in the original yeast two-hybrid screen encodes the chromodomain of MRG15 (Fig. 5A) and chromodomains are structural modules observed in enzymes that promote arginine methylation of histone proteins. The possibility arises that HiNF-P associates with MRG15 to repress target gene transcription by altering the methylation status of histones and/or that HiNF-P itself is methylated on arginines that are abundant in its N-terminus.

AD023, which interacts with the C terminal domain of HiNF-P (Fig. 5B), is an anonymous protein that was isolated as two individual overlapping clones, both coinciding with a structural module first observed in the eukaryotic initiation factor 4G (eIF4G) designated as the middle domain of eIF4G (MIF4G). This domain contains alpha helical repeats and associates with DNA as well as RNA [Marintchev and Wagner, 2005]. NPDC-1, which also interacts with the HiNF-P C-terminus (Fig. 5C), is a neural specific transcriptional regulator that drives neuronal differentiation by sequestering E2F-1, type D cyclins, and Cdk2 [Sansal et al., 2000] thereby inhibiting transcription of E2F-1 target genes. The HiNF-P binding region in NPDC-1 is within the helix-loop-helix (HLH) and PEST domains as indicated by the original clone from the yeast two-hybrid screen. Taken together, the isolation of THAP7, MRG15, ADO23, and NPDC1 protein segments as putative interaction surfaces with either the N- or C-terminal regions of HiNF-P indicates that these interactions are selective and may reflect novel functions of HiNF-P in transcriptional control of proliferation-related target genes.

HiNF-P Interacts With Proliferation Specific Proteins Ki-67 and XTP2

HiNF-P is required for cell cycle-dependent regulation of histone H4 genes. Therefore, we

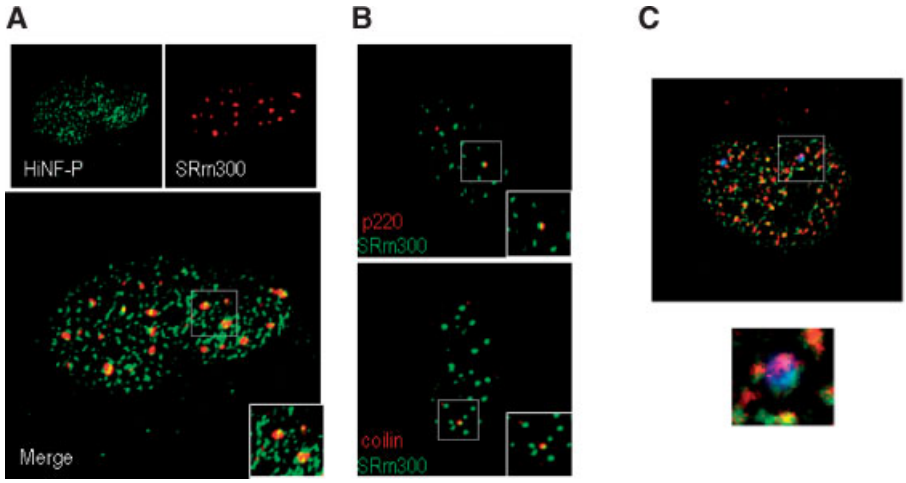
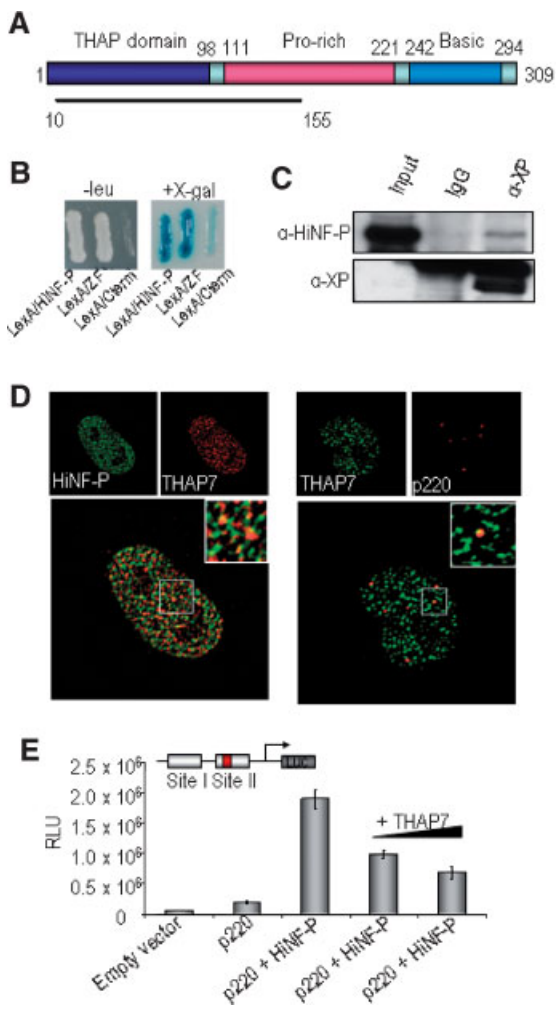


Fig. 3. HiNF-P and SRm300 co-localize in situ. **A**: Subnuclear localization of HiNF-P (green) and SRm300 (red) was assessed by in situ immunofluorescence microscopy. The merged image shows areas of co-localization detected as a yellow signal. The white box represents the area enlarged to show detail. **B**: Co-localization studies were performed with $p220^{NPAT}$ (red) and SRm300 (green) (**top**) or the Cajal body specific protein, coilin (red) and SRm300 (green) (**bottom**) to determine if SRm300 is localized to support a role in histone gene expression. Our data show SRm300 co-localizes with $p220^{NPAT}$ and coilin as shown by a yellow signal in the merged images. The white box represents the area enlarged to show detail. **C**: Triple immunofluorescence microscopy was performed to detect HiNF-P (green), SRm300 (red), and coilin (blue).



were particularly interested in characterizing the interactions of HiNF-P with cell cycle related proteins. Our yeast two-hybrid assays revealed potential interacting proteins of HiNF-P that are proliferation-related, including Ki-67, XTP-2, MDC-1, and IDN3 (Supplementary Table 3).

Fig. 4. HiNF-P interacts with THAP7 in a yeast two-hybrid assay and in mammalian cells. **A**: THAP7 was identified as a candidate HiNF-P interacting protein by a yeast two-hybrid screen (black bar). THAP7 contains a THAP family signature zinc finger domain (dark blue box), a proline-rich central domain (pink box), and a C-terminal basic region (light blue box). **B**: LexA/HiNH-P, LexA/ZF, and LexA/Cterm were co-transformed and assayed for reporter gene activation as indicated above. THAP7 interacts with the ZF domain of HiNF-P as shown by growth on leucine deficient media and a blue color on media supplemented with X-gal. **C**: Immunoprecipitations with HeLa cells transfected with HiNF-P and THAP7 fused to the Xpress epitope were assayed by Western blot using anti-HiNF-P and anti-Xpress antibodies. Input lanes represent 5% of the total protein used for each immunoprecipitation. IgG lanes are nonspecific antibody controls. **D**: Subnuclear localization of HiNF-P (green) and THAP7 (red) (**left panel**) or $p220^{NPAT}$ (red) and THAP7 (green) (**right panel**) was analyzed by in situ immunofluorescence microscopy using anti-HiNF-P, anti-Xpress, and anti-$p220^{NPAT}$ antibodies. **E**: THAP7 abrogates HiNF-P and $p220^{NPAT}$ synergistic activation of the histone H4 promoter in Cos7 cells. The reporter constructs contains Site I and Site II regulatory domains. The HiNF-P binding site is present in Site II (red box).

Ki-67 was isolated in two independent, overlapping clones in our yeast two-hybrid screen. Ki-67 is a proliferation specific protein expressed throughout the cell cycle but not present in quiescent (G_0) cells. It has been classically used as a proliferation marker in histopathology although its exact cellular function is unknown [Scholzen and Gerdes, 2000]. The HiNF-P binding domain of Ki-67 derived from the regions obtained in our yeast two-hybrid screen is comprised of two repetitive sequences and a bipartite nuclear targeting sequence (Fig. 6A). There are 16 repeated elements, each of approximately 122 amino acids, spanning the entire length of this protein. The function of these repeats is unclear and they are only present in the human form. In addition, Ki-67 contains a forkhead-associated (FHA) domain that has no known function but is found in other cell cycle related proteins such as RAD53 in *S. cerevisiae* [Scholzen and Gerdes, 2000] (Fig. 6A). To determine which region of HiNF-P interacts with Ki-67, we used HiNF-P deletion mutants in yeast two-hybrid assays (Fig. 6B). The results reveal that Ki-67 interacts with the C-terminus of HiNF-P.

To confirm our yeast two-hybrid interactions, we performed co-immunoprecipitation experiments with recombinant HiNF-P and a Ki-67 derived polypeptide encoded by clone 1 from our yeast two-hybrid library. Both proteins were produced by coupled in vitro transcription and translation using reticulocyte lysates. Our data show that HiNF-P specific antibodies are capable of immunoprecipitating this Ki-67 domain identified in the yeast assay (Fig. 6C). Assuming that reticulocyte lysates do not contain endogenous bridging proteins, our data strongly suggest HiNF-P and Ki-67 interact directly. We examined whether HiNF-P and Ki-67 co-localize in situ using immunofluorescence microscopy in HeLa cells. As noted above, HiNF-P is distributed in a typical punctate pattern that is dispersed throughout the nucleus, while Ki-67 is predominantly present in the nucleoli [Bullwinkel et al., 2006]. Merged images show very limited signal overlap around the periphery of the nucleoli (Fig. 6D). These data are consistent with functions of HiNF-P and Ki-67 that diverge significantly at a spatial level. However, a specific interaction around the nucleolus may be related to the putative cell growth regulatory functions of both proteins.

Our yeast two-hybrid screen identified a protein that was originally isolated in a screen of proteins upregulated when cells overexpress the hepatitis B virus X protein. This virally induced cellular protein is referred to as Hepatitis Bx transactivated protein 2 (XTP2). XTP2 was detected in our screen by three individual clones, two of which overlap in the C-terminus and one of which spans the extreme N-terminus (Fig. 7A). XTP2 does not contain any discernible domains or motifs, but amino acids 1807–1998 exhibit significant sequence similarity with a region in the CDK inhibitor p57/KIP2/WAF1, which is characterized by repetitive prolines and alanines (PAPA repeats) [Matsuoka et al., 1995]. One of the two C-terminal yeast clones overlaps with this region that is similar to p57 (Fig. 7A). This is of particular interest, as we have shown that p57 inhibits the cyclin E/CDK2 dependent phosphorylation of $p220^{NPAT}$ and blocks the transcriptional activity of the HiNF-P/$p220^{NPAT}$ co-activation complex [Mitra et al., 2007].

Based on the possibility that XTP2 may have a HiNF-P dependent role in cell cycle

Fig. 7. HBXTP2 interacts with HiNF-P by yeast two-hybrid assays. **A**: XTP2 was isolated in three independent clones by a yeast two-hybrid screen (black bars). One clone overlaps with the p57 homologous region (yellow box). **B**: XTP2 interacts with the Cterm domain of HiNF-P as assayed by activation of the reporter genes in yeast two-hybrid assays. **C**: ^{35}S radiolabeled XTP2 recombinant protein and unlabeled recombinant HiNF-P were combined and subjected to co-immunoprecipitation assays. IgG represents a non-specific antibody control and the input lane is 5% of the total protein used for each immunoprecipitation.

Fig. 8. HiNF-P interacts with multiple proliferation related proteins including MDC-1 and NPDC-1. **A**: HiNF-P interacts with the DNA repair protein MDC-1 in yeast two-hybrid assays. MDC-1 contains a FHA domain (yellow box), a PST domain (light gray box), and a BRCA C-terminus (BRCT) domain (dark gray box) (**upper panel**). MDC-1 interacts with the Cterm domain of HiNF-P as shown by the activation of the reporter genes (**lower panel**). **B**: IDN3 was isolated in one independent clone from a yeast two-hybrid screen (black bar, **upper panel**). IDN3 contains a HEAT repeat (light green box). IDN3 interacts with the Cterm domain of HiNF- P in a yeast two-hybrid assay as show by growth on leucine deficient media and a dark blue color on +X-gal media (**lower panel**).

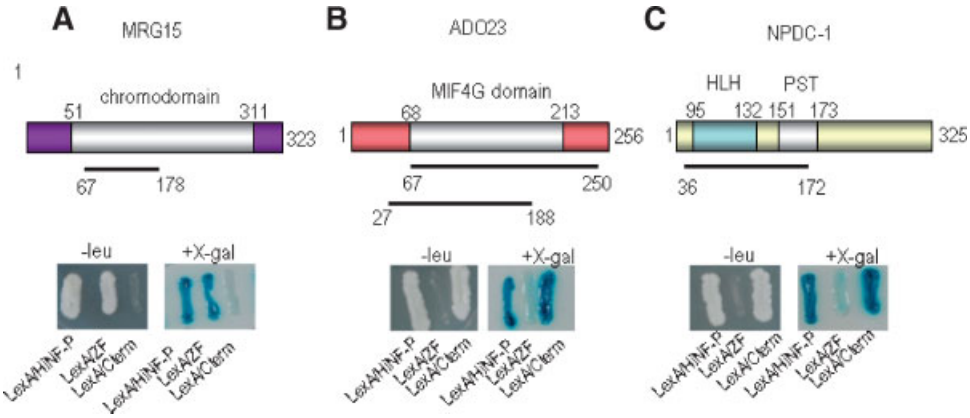


Fig. 5. HiNF-P interacts with multiple known and putative gene regulators in yeast two-hybrid assays. **A**: A yeast two-hybrid screen of interacting proteins of HiNF-P identified the gene regulator MRG15. The clone isolated (black bar) overlaps with the chromodomain (light gray bar). MRG15 interacts with the ZF region of HiNF-P as shown by growth on leucine deficient media and a dark blue color on media supplemented with X-gal. **B**: AD023, an uncharacterized protein, was identified in two independent clones (black bars) that overlap with the MIF4G domain (gray bar). AD023 interacts with the C-terminus of HiNF-P as shown by activation of reporter genes. **C**: Neural specific protein NPDC-1 interacts with the Cterm domain of HiNF-P. NPDC-1 contains a HLH domain (blue bar) and a PST domain (gray bar).

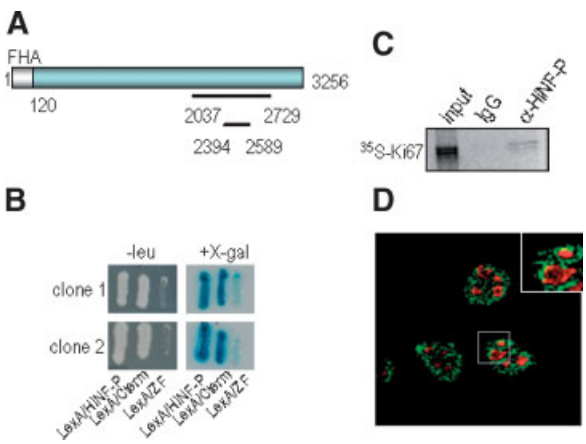


Fig. 6. Proliferation specific protein Ki-67 is a candidate HiNF-P interacting protein. **A**: Ki-67 was identified as a candidate HiNF-P interacting protein by two independent overlapping clones (black bars). Ki-67 contains a FHA domain in the N-terminus (gray box). **B**: Both Ki-67 clones interact with the Cterm domain of HiNF-P as shown by activation of reporter genes in yeast two-hybrid assays. **C**: ^{35}S-radiolabeled Ki-67 and unlabeled HiNF-P recombinant proteins were mixed and subjected to co-immunoprecipitation assays with HiNF-P antibodies. IgG lanes represent a non-specific antibody control and input lanes are 5% of the total protein used for each immunoprecipitation. **D**: Subnuclear localization of HiNF-P (green) and Ki-67 (red) was assayed by in situ immunofluorescence microscopy in mammalian cells. The white box represents the enlarged area.

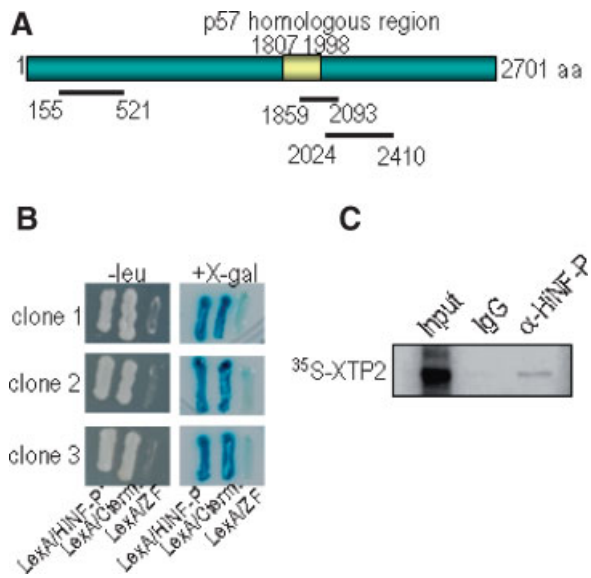


Fig. 7.

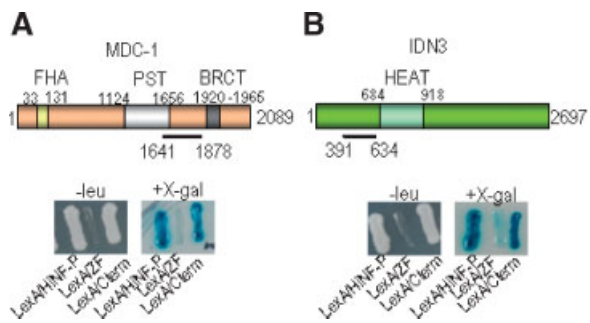


Fig. 8.

regulation, we examined which region of HiNF-P is required for the interaction. Using deletion analyses in combination with yeast two-hybrid assays, we determined that all three XTP2 fragments identified in the original screen interact with the C-terminus of HiNF-P (Fig. 7B). This finding suggests that XTP2 contains two distinct interaction surfaces that can independently bind to HiNF-P. Immunoprecipitation experiments with in vitro synthesized recombinant XTP2 protein (encoded by clone 2 from our yeast two-hybrid screen) and recombinant HiNF-P show that anti-HiNF-P antibodies were able to immunoprecipitate ^{35}S-radiolabelled recombinant XTP2 (Fig. 7C). Thus, HiNF-P and XTP2 are capable of interacting directly.

Our screen also identified one protein, MDC-1, involved in DNA repair pathways (Fig. 8A). Similar to Ki-67, MDC-1 contains a FHA domain, but also a BRCA1 C-terminus (BRCT) domain, and a PST domain. The HiNF-P interaction domain of MDC-1 is near the PST domain, based on the partial cDNA identified in the initial yeast two-hybrid screen (Fig. 8A, **upper panel**). Yeast two-hybrid deletion analyses showed that MDC-1 interacts with the C-terminus of HiNF-P (Fig. 8A, **lower panel**). MDC-1 is involved in the intra-S phase damage checkpoint and associates with the M/R/N complex, histone H2AX, and ATM [Goldberg et al., 2003]. It is well known that DNA damage blocks histone gene expression, and recent data suggest that DNA irradiation damage blocks cyclin E/CDK2 activity and phosphorylation of p220^{NPAT} [Ye et al., 2003; Su et al., 2004]. The MDC-1/HiNF-P interaction may predict the existence of novel pathway in control of gene expression following DNA damage.

Our yeast two-hybrid screen also identified IDN3 or delangin (Fig. 8B), which is encoded by the NIPBL gene, a homologue of the Nipped-B protein in *Drosophila*. In the fly, Nipped-B is involved in promoter-enhancer communication and in Notch signaling during development [Rollins et al., 1999]. Mutations in a highly conserved region of IDN3 are associated with Cornelia de Lange syndrome, a human inherited multisystem developmental disorder that is characterized by facial and limb deformities with growth and cognitive retardation [Krantz et al., 2004]. IDN3 contains a number of interesting protein motifs (e.g., HEAT repeat) and is ubiquitously expressed with the highest levels in heart, skeletal muscle and liver [Krantz et al., 2004]. The HiNF-P interaction domain (amino acids 391–634) as determined by our yeast two-hybrid screen is devoid of any repetitive elements or known motifs (Fig. 8B, **upper panel**), and this 391–634 polypeptide interacts with the C-terminus of HiNF-P (Fig. 8B, **lower panel**).

CONCLUSIONS

We have identified 67 potential interacting proteins of the histone gene regulator, HiNF-P by an unbiased yeast two-hybrid approach. We have shown that HiNF-P interacts with a number of RNA binding and processing factors including SRm300, an SR protein component of a splicing complex. We also identified a number of known and putative gene regulators that might be functioning as HiNF-P transcriptional co-factors, including THAP7. In addition, known and unknown proteins involved in cell cycle regulation, and proteins involved in signaling and metabolism have been identified as HiNF-P interactors via yeast two-hybrid. We have demonstrated that a number of these proteins interact with the ZF domain of HiNF-P indicating the zinc fingers present in HiNF-P are multi-functional. Another subset interacts with the Cterm domain, which contains a highly conserved motif [Mitra et al., 2003]. These candidate HiNF-P interacting proteins contain a diverse range of known motifs and domains including chromodomains and forkhead-associated domains. The HiNF-P interactome provides insight into components of cell cycle control that are responsive to signaling networks that converge at p220^{NPAT} subnuclear bodies to integrate regulatory cues for gene expression at the G1/S phase transition. In addition, the spectrum of HiNF-P interacting proteins suggests that HiNF-P is a multi-gene regulatory factor that participates in biological control beyond cell cycle-dependent histone gene expression.

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