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The Multifunctional Protein Nucleophosmin (NPM1) Is a Human Linker Histone H1 Chaperone

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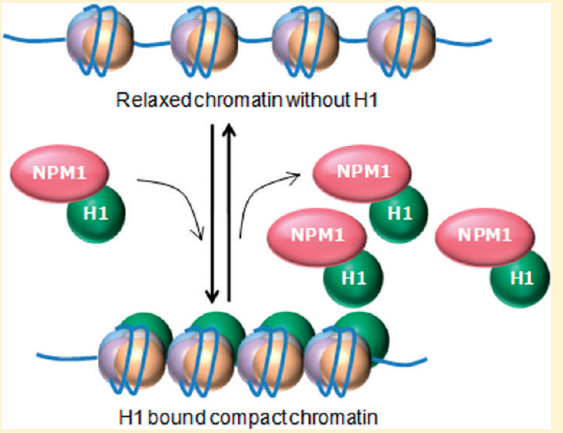
Supporting Information

ABSTRACT: Linker histone H1 plays an essential role in chromatin organization. Proper deposition of linker histone H1 as well as its removal is essential for chromatin dynamics and function. Linker histone chaperones perform this important task during chromatin assembly and other DNA-templated phenomena in the cell. Our in vitro data show that the multifunctional histone chaperone NPM1 interacts with linker histone H1 through its first acidic stretch (residues 120−132). Association of NPM1 with linker histone H1 was also observed in cells in culture. NPM1 exhibited remarkable linker histone H1 chaperone activity, as it was able to efficiently deposit histone H1 onto dinucleosomal templates. Overexpression of NPM1 reduced the histone H1 occupancy on the chromatinized template of HIV-1 LTR in TZM-bl cells, which led to enhanced Tat-mediated transactivation. These data identify NPM1 as an important member of the linker histone chaperone family in humans.



Linker histone H1 plays important roles in the dynamics of chromatin organization. Although the exact molecular mechanisms of linker histone H1 function are not fully understood, evidence from several reports suggests that the different variants of H1, in addition to chromatin packing, are also involved in specific cellular functions.[1] Histone H1 is incorporated into chromatin in both replication-dependent and -independent manners. Like core histones, linker histone H1 also needs specific histone chaperones for its precise recruitment to chromatin at any given point of the cell cycle. Histone chaperones are a group of proteins that directly interact with histones and are implicated in histone storage, transport, and deposition.[2] Different core histones are deposited by distinct histone chaperones. For example, during replication, the H2A−H2B dimer appears to be deposited by NAP1 while the H3−H4 tetramer is deposited by CAF-1.[2] There are several other core histone chaperones that are involved in dynamic histone exchange, as well as in the assembly and disassembly during transcription and DNA repair, typical examples being Spt16, nucleolin, FKBP, and NPM1.[2] However, there are only two linker histone H1 chaperones, NAP1[3] and NASP,[4] reported so far in humans.

Nucleophosmin (NPM1/B23), a member of the nucleoplasmin family of histone chaperones, is a highly dynamic nucleolar protein.[5] It is multifunctional in nature and has roles in ribosome biogenesis,[6] centrosome duplication,[7,8] maintenance of genomic stability, and embryonic development.[9] It is known to be a molecular chaperone[10] and functions as a nuclear export chaperone for the mammalian ribosome.[11] NPM1 shares 50% homology with the *Xenopus laevis* nucleoplasmin at its N-terminus.[12] Nucleoplasmin is a well-established histone chaperone and helps in the assembly of histones onto DNA. It binds sperm nuclear basic proteins (SNBPs) and facilitates the decondensation and remodeling of paternal chromatin following fertilization.[5] Nucleoplasmin has been shown to bind to linker histones and remove linker histones from sperm and somatic chromatin during fertilization and early development.[13,14] Similarly, nucleophosmin was found to interact with core histones H3, H2B, and H4 and also possess histone chaperone activity that resides in its

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unique C-terminal domain.[15,16] It was found to regulate RNA polymerase I[17] and RNA polymerase II transcription through its histone chaperone activity.[16,18] Moreover, it has recently been reported that the regulation of nucleolar chromatin by NPM1 depends on its RNA binding activity and its recruitment by transcription factor UBF.[19] NPM1 along with NPM3, another member of the nucleoplasmin family, has been shown to synergistically activate transcription.[20]

We found that NPM1 interacts with all the somatic linker histone variants without any apparent preference. Interaction studies with deletion constructs of H1.3 and NPM1 show that the first acidic stretch of NPM1 (residues 120−132) and the C-terminal domain of H1 are critical for the interaction. NPM1 deposits histone H1 onto a dinucleosomal template as efficiently as the bona fide linker histone H1 chaperone, NAP1. In addition, overexpression of NPM1 leads to a reduction in the amount of histone H1 associated with the HIV-1 LTR promoter and enhances HIV-1 Tat-mediated transactivation.

MATERIALS AND METHODS

Expression and Purification of Recombinant Proteins and HeLa Core Histones. Recombinant C-terminally His_6-tagged NPM1 internal deletion mutants, namely, NPM1Δ120−125-His_6, and NPM1Δ120−132-His_6 were cloned by site-directed mutagenesis (Stratagene) using full-length His_6-tagged NPM1. His_6-tagged recombinant NPM1, NPM1 (amino acids 1−200), NPM1 (amino acids 1−147), and NPM1 (amino acids 1−119) were expressed in *Escherichia coli* and purified as described previously.[16] C-terminally His_6-tagged recombinant linker histone H1 variants (H1.1−H1.5) and different deletion constructs of H1.3 (H1.3ΔC, H1.3ΔN, and H1.3ΔNC) were cloned by PCR-based subcloning using primers complementary to the respective 5′ and 3′ ends of the full-length (FL) human GFP-tagged linker histone H1 variants (kind gift from M. Hendzel). The amplicons were inserted between NcoI and XhoI restriction sites of the pET28b vector (Novagen). The His_6-tagged recombinant proteins were purified using nickel-NTA (Ni-nitrilotriacetic acid) agarose (Novagen) (details available on request). Recombinant GST-NPM1 was subcloned by PCR-based subcloning using primers complementary to the respective 5′ and 3′ ends of the full-length (FL) human NPM1-His_6. The amplicons were inserted between NcoI and XhoI restriction sites of the pGEXCD vector, and GST-NPM1 Sepharose beads were prepared as described previously.[21] The His_6-tagged recombinant PC4 was purified using nickel-NTA (Ni-nitrilotriacetic acid) agarose (Novagen) as described previously.[22] Human core histones were purified from the HeLa nuclear pellet as described previously.[23] Recombinant core histones (*Xenopus*) H2B and H3 were purified from inclusion bodies, by denaturation in 8 M urea followed by renaturation as described previously.[24] Purification of HeLa H1 was conducted as described previously.[25] The mammalian N-terminally FLAG-tagged NPM1 construct was cloned by PCR-based subcloning using primers complementary to the respective 5′ and 3′ ends of the full-length (FL) human NPM1-His_6. The amplicons were inserted between HindIII and XbaI restriction sites of the pCMV-FLAG vector (Sigma). Mammalian FLAG-tagged NPM1 (internal deletion mutant (NPM1Δ120-132-His_6), H1 interaction defective) was subcloned using His_6-tagged internal deletion mutants of NPM1.

Stable Cell Line. NPM1 cDNA was cloned in the pOZ-FH-N retroviral vector fused to an N-terminal FLAG tag epitope, and HeLa S3 cells were transduced. Cells positive for FLAG-NPM1 were also coexpressed with the surface IL2 receptor protein (IL2R), which facilitated multiple rounds of selection by anti-IL2R magnetic sorting to yield 95% stably expressing cells.

In Vivo and in Vitro Histone Interaction Assays. The in vivo NPM1−linker histone H1 interactions were investigated by performing M2-agarose pull-down assays using FLAG-NPM1-transfected HEK293T whole-cell extracts prepared in RIPA buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.1% sodium deoxycholate, 1% SDS, and 1 mM EDTA containing 50 μg/mL aprotinin and 50 μg/mL leupeptin], followed by immunoblotting by the anti-linker histone H1 polyclonal antibody.

Further in vivo NPM1−H1 interaction was confirmed in a stable cell line. Briefly, the soluble nuclear extract of HeLa S3 cells stably expressing FLAG-NPM1 was prepared as described previously,[26] and the salt concentration was adjusted to 200 mM KCl. The nuclear extracts were incubated with M2-anti-FLAG resin (Sigma) for 4 h at 4 °C, which was then washed three times with wash buffer 250 [20 mM Hepes (pH 7.5), 0.2 mM EDTA, 0.5 mM DTT, 10% glycerol, and 250 mM KCl] and three times with wash buffer 125 [20 mM Hepes (pH 7.5), 0.2 mM EDTA, 0.5 mM DTT, 10% glycerol, and 125 mM KCl]. The purified complexes were eluted from the resin by incubation with FLAG peptide (1 μg/μL) for 30 min at room temperature, resolved via a sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) gel, and analyzed by Western immunoblotting.

The linker histone H1 interaction ability of NPM1 was further characterized by incubation of 10 μL of Ni-NTA beads with 1 μg of His_6-NPM1 or GST-NPM1 (conjugated with GST Sepharose) and 4 μg of HeLa H1, calf thymus H1, PC4, His_6-tagged linker histone H1 variants, and recombinant (*Xenopus*) individual histones H2B and H3 in a final volume of 300 μL in biochemical buffer containing 300 mM KCl (HeLa H1, calf thymus H1, H3, and H2B), 200 mM KCl (H1 variants), and 150 mM PC4 (supplemented with 30 mM imidazole) at 4 °C for 2 h. The beads were washed three times with 1 mL of the incubation buffer each time. The GST Sepharose or Ni-NTA agarose pull-down complexes were analyzed by SDS−PAGE followed by Coomassie staining. Control experiments were performed with 10 μL of Ni-NTA beads or 1 μg incubated with 4 μg of HeLa H1, calf thymus H1, PC4, individual recombinant H2B, H3, and recombinant linker histone H1 variants in the same buffer. To map the domain of histone H1 involved in the interactions with NPM1, glutathione *S*-transferase (GST) pull-down assays were performed as described previously.[27] His_6-tagged deletion mutants of linker histone H1.3, namely, H1.3ΔC (C-terminal domain deleted), H1.3ΔN (N-terminal domain deleted), and H1.3ΔNC (N-terminal and C-terminal domains deleted) were cloned, expressed, and purified (details available on request), and interaction studies were conducted with GST-NPM1 in the presence of 200 mM NaCl. For scoring the interaction, GST pull-down assays were performed followed by probing with an anti-His monoclonal antibody (Sigma). The domain of NPM1 interacting with H1 was identified by performing Ni-NTA pull-down assays with 4 μg of HeLa H1 or calf thymus H1 incubated with 1 μg of NPM1 C-terminal or internal deletion mutants. The pulled down complexes were analyzed via SDS−PAGE followed by Coomassie staining.

Isothermal Titration Calorimetry. Isothermal titration calorimetry (ITC) measurements were taken using a MicroCal ITC200 instrument (MicroCal, Inc.). Aliquots (2 μL) of NPM1 at a concentration of 21 μM were injected from the syringe into the cell of 240 μL filled with 2 μM H1. Titrations were conducted in 20 mM Tris-HCl (pH 7.5), 0.2 mM EDTA, and 100 mM KCl buffer. To minimize the contribution of dilution to binding heat, the protein solutions were dialyzed against the same buffer prior to the

ITC experiments. Injections were made at intervals of 120 s, and the duration of each injection was 0.4 s. To ensure proper mixing after each injection, a constant stirring speed of 1000 rpm was maintained during the experiment. The control experiment was performed via injection of buffer in the ITC cell containing H1.2 (Figure S1 of the Supporting Information). All molarity calculations of NPM1 were conducted according to its reported oligomeric molecular mass 240 kDa,[28] while H1 was taken as a monomer. The heat change versus the molar ratio of the titrated products was plotted and analyzed using the manufacturer's software, which yielded the stoichiometry (n, in terms of the number of molecules of NPM1 per H1.2) and the dissociation constant (K_d).

Dinucleosome Reconstitution, EMSA, and DNase I Footprinting. The dinucleosome construct was cloned using the construct of 33 × 200−601 chromatin template plasmid from D. Rhodes.[29] Briefly, the chromatin templates were partially digested, and the DNA approximately representing the dinucleosome repeat size was gel purified from the ladder of different sized oligomers that were cloned into a plasmid vector. For radiolabeling, the dinucleosome fragment (423 bp), containing 44 and 32 bp outer linkers and a 53 bp middle linker, was excised from the plasmid using XbaI and EcoRI, purified, and Klenow labeled on the EcoRI-generated 3′ overhang. The dinucleosome was reconstituted by the salt dialysis method.[30] The dinucleosomal substrate was used for the deposition of linker histone H1 in the absence or presence of NPM1, the NPM1 mutant, and the NAP1 chaperone at a 2:1 chaperone:H1 ratio.[3] All molarity calculations were made by taking into account the oligomeric form of NPM1, the dimeric form of NAP1, and the monomeric form of H1. The reaction was conducted in binding buffer (15 mM Tris-HCl, 0.3 mM EDTA, 0.2 mM DTT, 2% glycerol, and 25 mM NaCl) for 30 min at 30 °C and the mixture subsequently loaded on a 2% agarose gel, and bands were visualized by autoradiography. NPM1-His_6 or NPM1Δ120−132-His_6 mutant-assisted H1-saturated dinucleosomes, as determined by the band shift assay (lanes 6, 8, and 14 of Figure 4A), were used to perform the DNase I footprinting experiments as described previously[31] and compared with either the footprint of intact dinucleosomes lacking H1 or the H1 specific footprint using NAP1 as previously reported.[3]

Immunofluorescent Staining. TZM-bl cells were grown on polylysine-coated coverslips for 24−48 h in the presence of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells were transfected with the FLAG vector or F-NPM1 or F-NPM1Δ120−132 mutant, and 24 h post-transfection, they were fixed with 4% paraformaldehyde and permeabilized with 1% Triton X-100. Anti-FLAG (Sigma) was used as the primary antibody, and Alexa 633-conjugated anti-mouse (Invitrogen) antibodies were used as secondary antibodies. The nucleus was stained using 1 μg/mL propidium iodide (Sigma) and 10 μg/mL RNase A (Qiagen) to eliminate the cytoplasmic staining of RNA by propidium iodide. The Alexa and propidium iodide fluorescence was visualized using a Carl Zeiss confocal laser scanning microscope (LSM 510 META) using LSM 5 Image Examiner.

Luciferase Assay. TZM-bl cells are HeLa cell derivatives engineered to express integrated reporter genes for firefly luciferase and *E. coli* β-galactosidase under control of an HIV long-terminal repeat sequence. The cells were cultured in DMEM containing 10% FBS and antibiotics. In TZM-bl cells, expression of the reporter gene is induced in trans by viral Tat protein. We exploited this sensitive reporter assay in our experiments and measured Tat-regulated luciferase reporter gene expression following transient transfection. An equal number of cells (0.8×10^4 cells/well) was seeded in a 12-well dish. The cells were cotransfected with Tat and FLAG-vector, FLAG-NPM1, or FLAG-NPM1Δ120−132. Twenty-four hours post-transfection, the spent medium was removed; the cells were washed twice with 1× PBS and lysed with cell cuture lysis reagent (Promega). Following this, the Bright-Glo luciferase assay system (Promega) was used to quantify the luciferase activity using a Berthhold luminometer.

Chromatin Immunoprecipitation. Expression of the reporter gene under the control of the LTR promoter was induced in TZM-bl cells by cotransfection of Tat with FLAG-vector, FLAG-NPM1, or FLAG-NPM1Δ120−132. The ChIP assay was performed as described previously.[32] Briefly, after transfection, cross-linking was conducted with 1% formaldehyde followed by cell lysis in SDS lysis buffer [1% SDS, 10 mM EDTA, and 50 mM Tris-HCl (pH 8)]. After sonication of the chromatin (six times for 10 s at a power setting of 91%), cold dilution buffer [0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl (pH 8), and 167 mM NaCl] was added along with preblocked protein G-Sepharose (Amersham Pharmacia) and sheep-raised anti-H1 polyclonal antibody (Abcam) or anti-H2B polyclonal antibody and the mixture kept for binding overnight. The sonicated samples were precleared prior to immunoprecipitation. Beads were successively washed with low-salt buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8), and 150 mM NaCl], high-salt buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8), and 500 mM NaCl], LiCl buffer [250 mM LiCl, 1% NP40, 1% NaDOC, 1 mM EDTA, and 10 mM Tris-HCl (pH 8)], and TE [10 mM Tris-HCl (pH 8) and 1 mM EDTA]. Elution buffer (0.2% SDS and 100 mM $NaHCO_3$) along with 200 mM NaCl was added to the washed beads, and the bead solution was kept overnight at 65 °C. The next day, 0.1 mg/mL proteinase K (Invitrogen) and 0.04 mg/mL RNase A (Qiagen) were added to the bead solution, and the mixture was incubated for 2 h at 55 °C. The immunoprecipitated samples were deproteinized, ethanol-precipitated, and used for real-time PCR analysis. The following region specific primer sets were used for the real-time PCR analysis:[33]

primer	region	primer sequence
Nu1f (forward)	Nuc-1	AGTAGTGTGTGCCCGTCTGT
Nu1r (reverse)		TTGGCGTACTCACCAGTCGC

RESULTS AND DISCUSSION

NPM1 Is Associated with Linker Histone H1 in Vivo. To identify the proteins associated with NPM1 in vivo, we transiently transfected HEK293T cells with FLAG-NPM1 (F-NPM1) expression vector. The expression of F-NPM1 was confirmed by Western blotting (Figure 1A). Whole cell extract (WCE) was prepared from the transfected cells, and F-NPM1 was immunoprecipitated using anti-FLAG antibody (Figure 1B). The immunoprecipitated complex was resolved via sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) along with recombinant NPM1 and histones purified from HEK293T cells followed by Coomassie staining of the gel. Interestingly, doublet bands exhibiting mobility similar to that of linker histone H1 were observed (in Figure 1B, compare lane 3 to lane 4). The doublet bands were absent in the control immunoprecipitate, where WCE from empty vector-transfected cells was used (Figure 1B, lane 2). To confirm the identity of the doublet bands, we performed Western blotting of the immunoprecipitated samples using specific polyclonal antibodies against H1 (Figure 1C and Figure S1 of the Supporting Information).

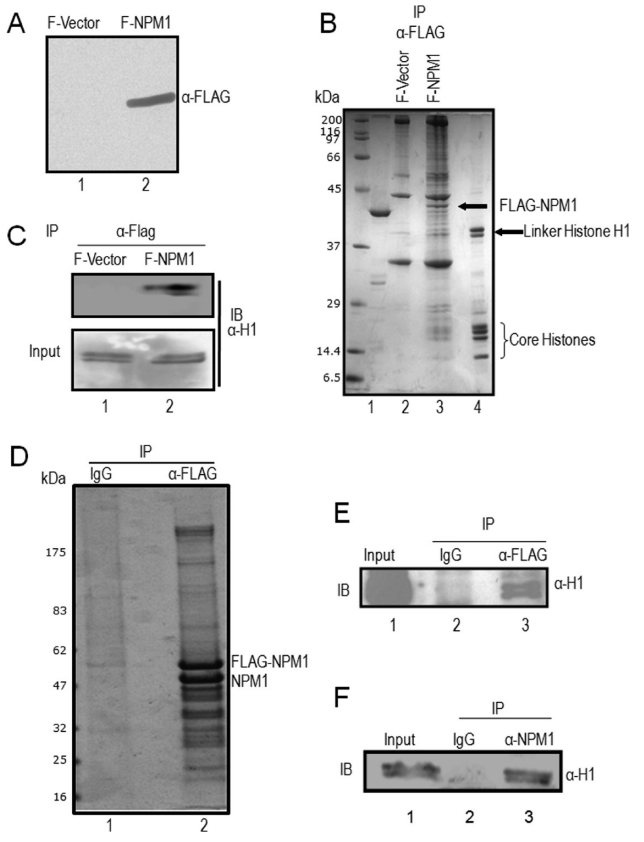

Figure 1. Linker histone H1 is associated with NPM1. (A) F-NPM1 (lane 2) or empty FLAG (F) vector control (lane 1) was transfected into HEK293T cells, and whole cell extracts (WCE) from transfected cells were run on 12% SDS−PAGE. Immunoblotting was performed by using anti-FLAG antibodies. (B) WCE prepared from either F-NPM1 or empty F-vector-transfected cells were used to immunoprecipitate F-NPM1 with anti-FLAG antibodies. The immunoprecipitated samples were run on 12% SDS−PAGE, and the gel was stained with Coomassie blue: lane 1, purified recombinant His_6-tagged NPM1; lanes 2 and 3, immunoprecipitated samples from WCE prepared from F-vector (control) and F-NPM1 expression vector-transfected cells, respectively; lane 4, total histones. The arrows indicate the position of FLAG-NPM1 and linker histone H1. The position of the core histones is also indicated. (C) Western blot analysis of the F-NPM1 complex immunoprecipitated from F-NPM1 (lane 2)- or empty vector (lane 1)-transfected HEK293T WCE using anti-FLAG antibodies. The blot was subjected to Western blot analysis using anti-H1 polyclonal antibodies. The input panel shows 10% of the input fraction used for immunoprecipitation. (D) Immunoprecipitation of F-NPM1 expressed in HeLa stable cell line. Nuclear extract prepared from HeLa stable cell line was used to immunoprecipitate F-NPM1 by using anti-FLAG antibodies (lane 2) or preimmune serum (lane 1). The samples were analyzed on a 4 to 12% gradient PAGE gel and visualized by Coomassie blue staining. The positions of F-NPM1 and NPM1 are indicated. (E) Western blot analysis of the material described in panel D. The blot was subjected to Western blotting analysis using polyclonal anti-H1 antibody. Lane 1 contained 10% of the input fraction used for immunoprecipitation. (F) Same as panel E, but the endogenous NPM1 was immunoprecipitated with the monoclonal antibody against NPM1. Lane 1 contained 10% of the input fraction used for immunoprecipitation.

The data show that F-NPM1 was associated with histone H1 in the cells (Figure 1C).

This result was further confirmed by using a stable cell line expressing F-NPM1. The F-NPM1 complex was immunopurified from these cells, resolved on a gradient SDS−PAGE gel, and visualized with Coomassie blue staining. Both endogenous NPM1 and F-NPM1 were detected in this complex (Figure 1D, lane 2). Western blotting analysis showed that F-NPM1 was associated with histone H1 (Figure 1E, lane 3), a result in agreement with those of the transient transfection experiments. As expected, in the control experiments with cells not expressing F-NPM1, association of H1 with the preimmune IgG-coated agarose beads was not observed (in Figure 1E, compare lane 2 to lane 3). Monoclonal antibodies were also used to immunoprecipitate endogenous NPM1, and Western blotting showed that histone H1 was present in the immunoprecipitate (Figure 1F). Taken together, these results suggest that NPM1 is associated with linker histone H1 in vivo.

Residues 120−132 of the First Acidic Stretch of NPM1 Are Critical for Interaction with Linker Histone H1. To further characterize the interaction of NPM1 with linker histone H1, in vitro interaction assays were performed using commercially available calf thymus H1 and/or highly purified HeLa H1 (Figure S2A of the Supporting Information, lanes 2 and 3) with His_6-NPM1 in the presence of 300 mM salt. In agreement with the in vivo immunoprecipitation assays, we found that NPM1 could pull down HeLa H1 (in Figure 2A, compare lane 3 to lane 2) and calf thymus H1 (data not shown).

In mammals, five somatic subtypes of H1 (H1.1−H1.5), a terminally differentiated expressed isoform (H1.0), two tissue specific variants (H1 testis and H1 oocyte),[1] and H1x (poorly characterized) variant have been identified.[34,35] These linker histone H1 variants play important, diverse functional roles in cells. For example, H1.2 is involved in repair,[36] whereas H1.5 interacts with HP1 proteins to establish facultative heterochromatin.[37] We were interested in determining whether NPM1 has any specific preference for any of these linker histone H1 variants. For this, His_6-tagged somatic linker H1 variants (H1.1−H1.5) were also cloned, expressed, and purified from *E. coli* (Figure S2B of the Supporting Information). We also subcloned, expressed, and purified GST-fused NPM1 from *E. coli* (Figure 2B, panel I). The pull-down assays revealed that GST-NPM1 could interact with all the linker histone H1 variants, without any apparent preference, whereas GST alone did not pull down any variant (in Figure 2B, panel II, compare lane 3 to lane 2). The interactions were further confirmed by Western blotting analysis of pull-down complexes using anti-His_6 monoclonal antibodies (in Figure 2C, compare lanes 1−5 to lanes 6−10). To rule out nonspecific charge-based interaction, we also performed a similar GST pull-down assay using a positively charged nuclear protein, His_6-PC4, which has an isoelectric point (pI) comparable with that of histone H1 (Figure 2B). The results show that GST-NPM1 could not pull down PC4 (in Figure 2B, compare lane 3 to lane 2). Collectively, these results suggest that NPM1 directly interacts with all the somatic variants of linker histone H1. To determine the strength of the interaction between H1 and NPM1, isothermal titration calorimetry studies were performed with recombinant NPM1 and H1.2. The results show that NPM1 binds with H1.2 with a dissociation constant of approximately 1×10^{-6} M at 1.5 ± 0.1 binding sites of oligomeric NPM1 on H1.2 (Figure 2D).

Subsequently, we went on to identify the domains of H1 and NPM1 involved in the direct interaction between these two proteins. Different domains of linker histone H1 have been shown to contain specific binding sites on the nucleosome. Single-base resolution mapping studies of the histone H1−DNA interaction have shown that the globular domain of linker histone H1 binds to the minor groove located at the center of the nucleosomes and contacts a 10 bp region of DNA localized symmetrically with respect to the nucleosomal dyad,[38] whereas

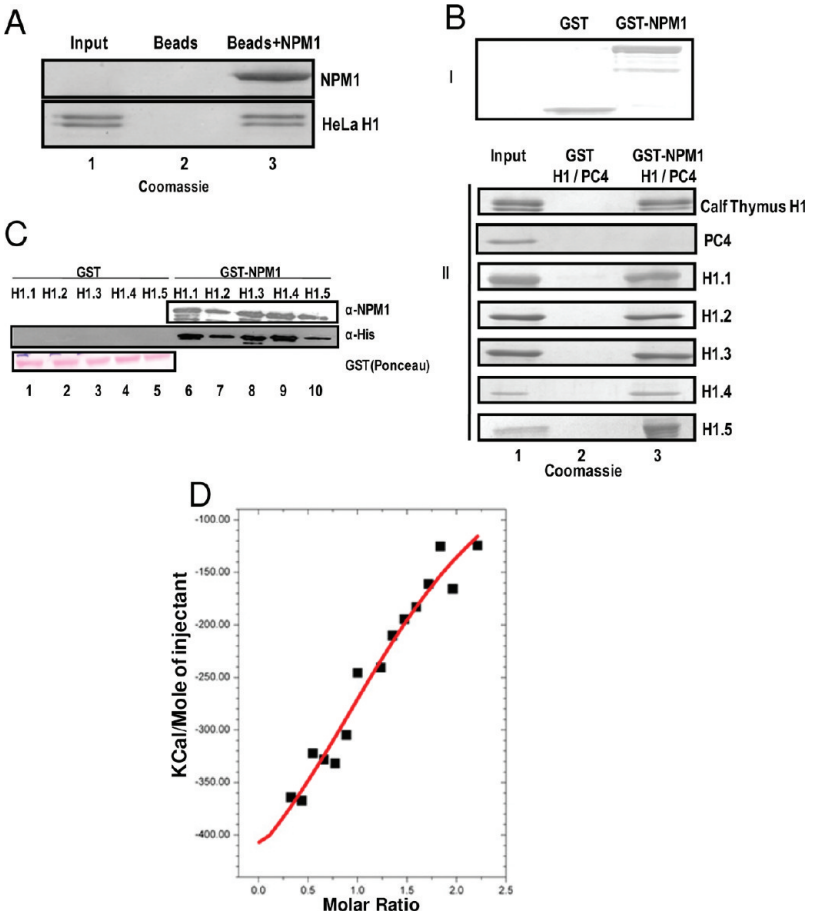

Figure 2. NPM1 interacts with all the linker histone H1 variants in vitro. (A) The in vitro interactions of histone H1 and NPM1 were assayed by incubating 1 μg of His_6-tagged NPM1 (His_6-NPM1) bound to Ni-NTA beads with 4 μg of HeLa H1 (lane 3). Interaction with Ni-NTA beads alone was used as a control (lane 2). The complexes were pulled down and resolved via 12% SDS−PAGE, and proteins were visualized by CBB staining. (B) Different variants of linker histone H1 or human transcriptional coactivator PC4 (4 μg) was incubated with GST-NPM1 (lane 3) or only GST (lane 2) in the in vitro pull-down assays. The protein complexes were analyzed via 12% SDS−PAGE. (C) GST or GST-NPM1 pull-down complexes were also subjected to Western blot analysis using a monoclonal antibody against His_6 (Sigma) (for detection of H1) and a monoclonal anti-NPM1 antibody (Invitrogen) (for detection of NPM1). (D) ITC was conducted via titration of H1 (2 μM) vs NPM1 (21 μM) at 18 °C. The one-site binding model of the isotherm is shown.

the C-terminal domain (involved in chromatin condensation) seals the entering and exiting nucleosomal DNA.[39] We made different deletion constructs of H1.3, which included His_6-tagged ΔC (C-terminal domain deleted), ΔN (N-terminal domain deleted), and ΔNC (N-terminal and C-terminal domains deleted), expressed them in bacteria, and purified them (Figure 3A). We then used Ni-NTA pull-down assays to examine the ability of NPM1 to interact with these H1.3 mutants. Western blotting analysis showed that NPM1 interacts with the ΔN mutant as efficiently as full-length H1.3 (in Figure 3B, compare lane 6 to lane 8). In contrast, ΔC and ΔNC mutants of H1.3 failed to interact with NPM1 (in Figure 3B, compare lanes 7 and 9 to lane 6), suggesting that the C-terminal domain of the linker histone H1 is crucial for the interaction with NPM1. However, the possible role of the globular domain in the context of the C-terminal domain cannot be ruled out for the NPM1−H1.3 interaction. Collectively, the interaction data indicate a plausible role for NPM1 in the physiological recruitment of linker histone H1 to the nucleosome by preventing charge-mediated disordered association of linker histone H1 with DNA. This also raises the possibility that the acidic region of NPM1 could be the key domain for the association with linker histone H1.

NPM1 has a unique organization of different functional domains (Figure 3C): an N-terminal oligomerization domain[5] rich in hydrophobic amino acids, which interacts with H3 and H4; a central acidic domain interspersed by a bipartite nuclear localization signal; and a unique C-terminal domain that is indispensable for its core histone chaperone activity.[16] To identify the exact region of NPM1 that associates with linker histone H1, we have used different His_6-tagged C-terminal deletion mutants,[16] which were constructed by sequentially deleting the unique C-terminal domain (NPM1 1−200), the second acidic stretch and nuclear localization signal (NPM1 1−147), and up to the first acidic stretch (NPM1 1−119). These mutants were used for the Ni-NTA pull-down assay to study their ability to interact with linker histone H1. The mutants (NPM1 1−200 and NPM1 1−147) could interact with HeLa H1 to the same extent as the full-length protein (in Figure 3C, panel II, compare lanes 4 and 5 to lane 3). Of note, the N-terminal oligomerization domain (NPM1 1−119) did not interact with HeLa H1 (in Figure 3C, panel II, compare lane 6 to lane 3), suggesting that NPM1 interacts with linker histone H1 through its first acidic stretch. To confirm this observation, we constructed two additional His_6-tagged internal acidic stretch 1 deletion mutants of NPM1, NPM1Δ120−125 (amino acids 120−125 deleted) and

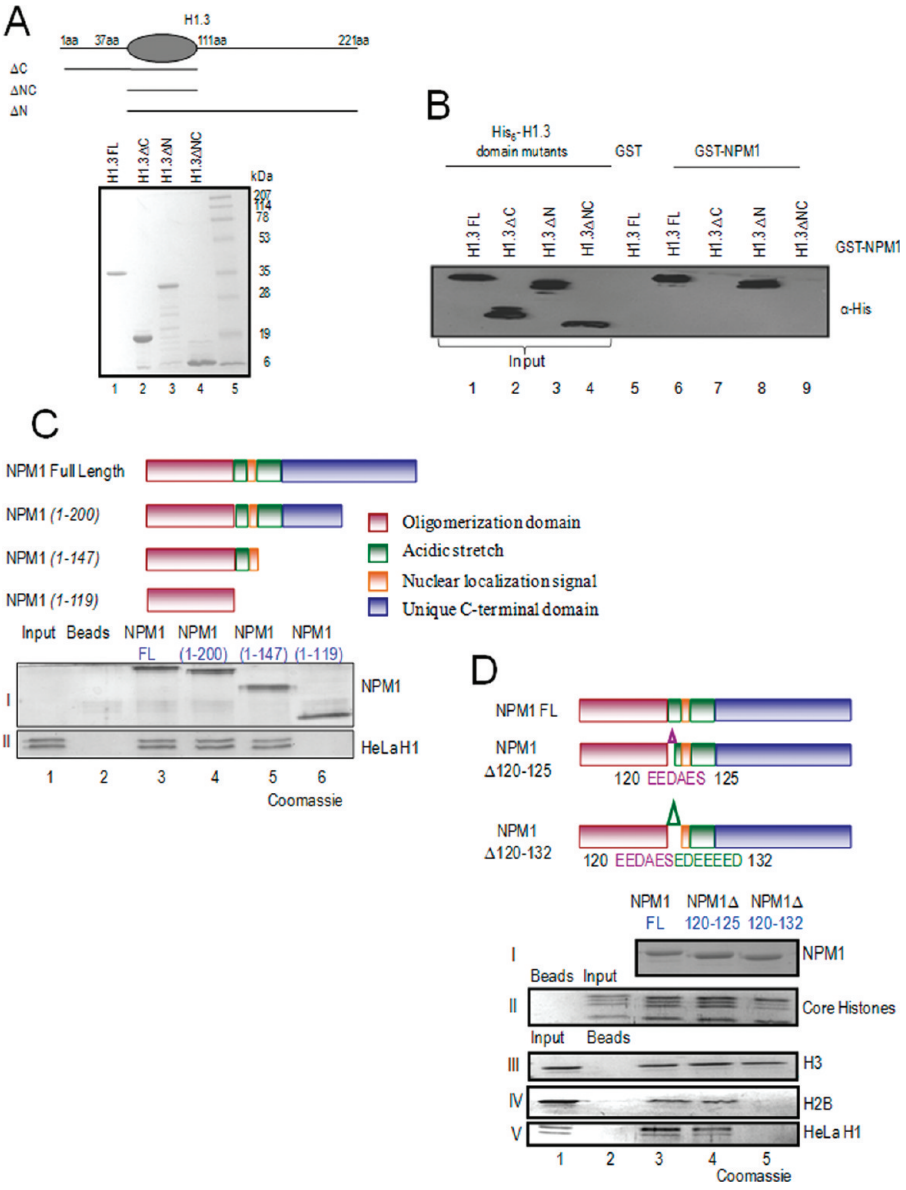

Figure 3. First acidic stretch (domain) of NPM1 that interacts with the C-terminal domain of linker histone H1. (A) Expression profile of different deletion mutants of histone H1.3. A diagrammatic representation of the deletion constructs is also presented (top). (B) These mutants of linker histone H1.3 were incubated with GST-NPM1 (lanes 6−9) and subjected to a pull-down assay followed by Western blot analysis using an anti-His_6 antibody. GST alone was incubated with full-length H1.3 as a negative control (lane 5). The input represents 20% of prebound H1 proteins (lanes 1−4). (C) Diagrammatic representation of full-length and C-terminal deletion mutants of NPM1 (top). One microgram of His_6-NPM1 or various His_6-tagged C-terminal deletion mutants (panel I) bound to Ni-NTA beads were incubated with 4 μg of HeLa H1 (panel II). (D) Diagrammatic representation of full-length and internal deletion mutants of NPM1 (top). One microgram of His_6-NPM1 or various His_6-tagged internal deletion mutants (panel I) bound to Ni-NTA beads were incubated with 4 μg of HeLa core histones (panel II), recombinant H3 (panel III), recombinant H2B (panel IV), or HeLa H1 (panel V). The complexes were pulled down and resolved via 12% SDS−PAGE, and proteins were visualized by CBB staining.

NPM1Δ120−132 (amino acids 120−132 deleted). These deletion mutants were expressed and purified from *E. coli* (Figure 3D, panel I). We performed Ni-NTA pull-down assays to test the ability of these mutants to interact with linker histone H1. NPM1Δ120−125 interacted with core histones, histone H3, H2B, and HeLa H1 as efficiently as the full-length NPM1 (in Figure 3D, panels II−IV, compare lane 4 to lane 3). In contrast, NPM1Δ120−132 interacted efficiently with histone H3 and H4 in HeLa core histones and recombinant histone H3 (in Figure 3D, panel II and III, compare lane 4 to lane 3). However, it failed to bind recombinant histone H2B and HeLa linker histone H1 (in Figure 3D, panels IV and V, compare lane 5 to lane 3). Taken together, these data suggest that the first acidic stretch of NPM1 (amino acids 120−132) interacts with the C-terminal domain of H1. These observations are in accordance with a previous report that showed that the well-known histone chaperone NAP1 interacts with B4 (linker histone H1 variant in *Xenopus* eggs) through its acidic domain.[3]

NPM1 Mediates Proper Deposition of Linker Histone H1 onto the Dinucleosomal Template. The direct involvement in the deposition of linker histone H1 onto a bona fide linker DNA between nucleosomes was assayed by using a well-positioned dinucleosomal template consisting of 601 nucleosome positioning

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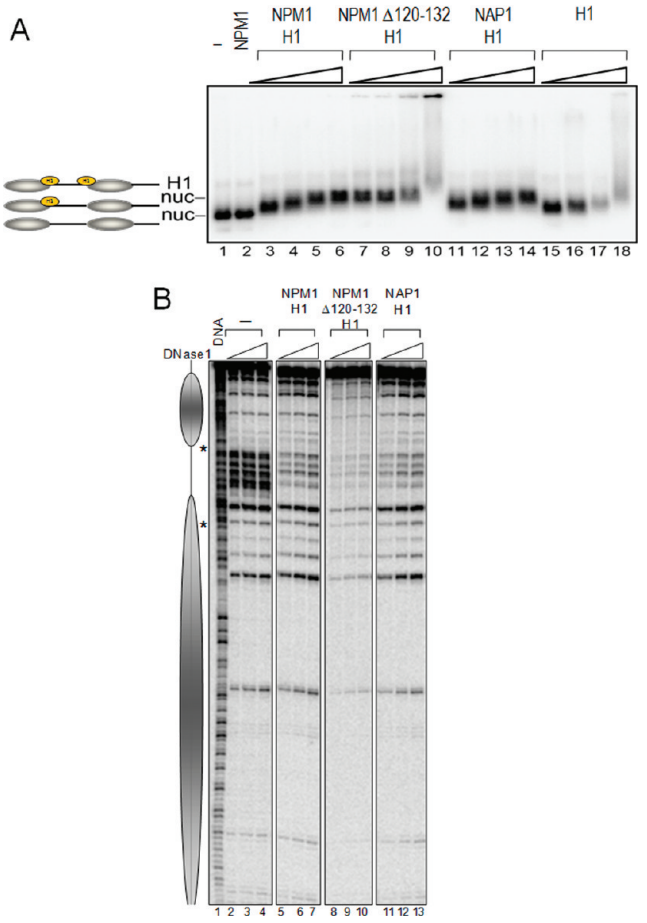

Figure 4. NPM1 facilitates proper deposition of histone H1 onto a dinucleosomal template. (A) The H1-interacting first acidic stretch of NPM1 is essential for the deposition of the linker histone onto a dinucleosomal template. NPM1−H1 (lanes 3−6), NPM1Δ120−132−H1 (lanes 7−10), and NAP1−H1 (lanes 11−14) complexes at a ratio of 2:1 (chaperone:H1) were ectopically added in increasing concentrations to a fixed amount of reconstituted dinucleosomal template. H1:nucleosome ratios of 0.8, 1.2, 1.8, and 2.5 were used. Only histone H1 at increasing concentrations was incubated with the dinucleosomal template as a control (lanes 15−18). The nucleosomal complexes were resolved on an agarose gel and subjected to autoradiography. The positions of dinucleosomes with different amounts (numbers) of linker histone H1 are indicated. (B) DNase I footprinting of dinucleosomes. Either NPM1, the NPM1Δ120−132−H1 complex, or NAP1 was used to deposit H1 on the dinucleosomes. The end-radiolabeled (γ-^{32}P) dinucleosomes were incubated with increasing concentrations of DNase I; the digested DNA was purified and run on an 8% PAGE gel under denaturing conditions: lanes 2−4, DNase I digestion patterns of the control (nucleosomes without H1); lanes 5−7, 8−10, and 11−13, DNase I digestion patterns of H1-containing nucleosomes, deposited by NPM1, the NPM1Δ120−132−H1 complex, and NAP1, respectively; lane 1, digestion pattern of free DNA. On the left is shown a schematic presentation of the dinucleosome. Note the specific protection against DNase I of the linker DNA in the dinucleosome containing NPM1- and NAP1-deposited H1. Asterisks denote the region of linker DNA.

sequences[29] (Figure 4A). The proper assembly of H1 between the two nucleosomes was visualized by agarose gel electrophoresis, in which the specific mobility of assembled chromatosomes could be clearly distinguished from either aggregated or smeared species containing an unassembled nucleoprotein complex.[3] In addition, a slow-moving properly formed species of complex could be observed. The band mobility became slower in the presence of a larger amount of the NPM1−H1 complex, indicating that linker histone H1 has been properly deposited in the linker regions of dinucleosomes (Figure 4A, lanes 3−6). Similar results were observed when the well-established linker histone H1 chaperone (NAP1) and H1 complex (NAP1−H1) were incubated with the dinucleosomal template (Figure 4A, lanes 11−14). However, the linker histone H1 interaction deficient NPM1 mutant (NPM1Δ120−132) could not assist the deposition of linker histone H1 as efficiently as wild-type NPM1 (Figure 4A, lanes 7−10). At higher concentrations of the NPM1Δ120−132−H1 complex, the entire complex aggregated and most of it could not even enter the gel (Figure 4A, lane 10). However, at lower concentrations of the NPM1Δ120−132−H1 complex (Figure 4A, lane 7), a slowly migrating band close to that of the dinucleosomes with two properly deposited H1 molecules could be seen (Figure 4A, lane 6). This could be attributed to the fact that in the absence of the chaperone function of NPM1, H1 is deposited nonspecifically on the dinucleosome, which gives a slower mobility even though only one H1 is deposited. Incubation of linker histone H1 alone with dinucleosomal template formed nonspecific nucleoprotein aggregates, as depicted by the presence of smears in the agarose gel (Figure 4A, lanes 15−18). This is in agreement with the previous reports that showed that linker histone H1 alone could not be incorporated into the dinucleosomal template. Altogether, these data suggest that NPM1 possesses a potent linker H1 chaperone activity comparable to that of NAP1.

To determine how NPM1-mediated H1 recruitment affects the structure of the dinucleosome, we subjected the dinucleosome and H1 complexes (from lanes 6, 8, and 14 of Figure 4A) to DNase I footprinting analysis. Cleavage of the well-positioned nucleosomes resulted in the typical 10-base ladders outside of the linker DNA region (Figure 4B, lanes 2−13). In contrast, DNA alone did not produce a similar pattern (Figure 4B, lane 1). The control dinucleosomes (without H1) exhibited a high accessibility of the linker DNA (lanes 2−4). Incorporation of linker histone H1 mediated by either NPM1 or NAP1 led to a high level of reduction of DNase I cleavage of linker DNA but did not affect the accessibility of the nucleosomal DNA to DNase I (in Figure 4B, compare lanes 5−7 and 11−13 to lanes 2−4). On the other hand, dinucleosomes incubated with the NPM1Δ120−132−H1 complex did not show any specific protection of the linker DNA (Figure 4B, lanes 8−10). In fact, an overall protection of the dinucleosome was observed, showing a nonspecific deposition of H1 (on core particle as well as linker DNA) on the dinucleosomes by the NPM1Δ120−132−H1 complex. The profile shown is very similar to the DNase I profile of the dinucleosome, when H1 is added alone without any chaperone (Figure S3 of the Supporting Information). Taken together, these results suggest that NPM1 through its first acidic stretch facilitates proper deposition of linker histone H1 onto dinucleosomes, which results in a specific interaction of histone H1 with linker DNA.

Histone chaperones are a group of proteins that dynamically interact with histones and assist both assembly and disassembly of nucleosomes on the DNA template. Overexpression of a histone chaperone may facilitate the removal of histones from the chromatinized template rather than their deposition on DNA and thereby may activate the transcription in the higher-order chromatin context. To address the linker histone H1 chaperone activity of NPM1 in the cellular context, we investigated the ability of NPM1 to remove histone H1 from a transcription template and measured the level of transcription upon

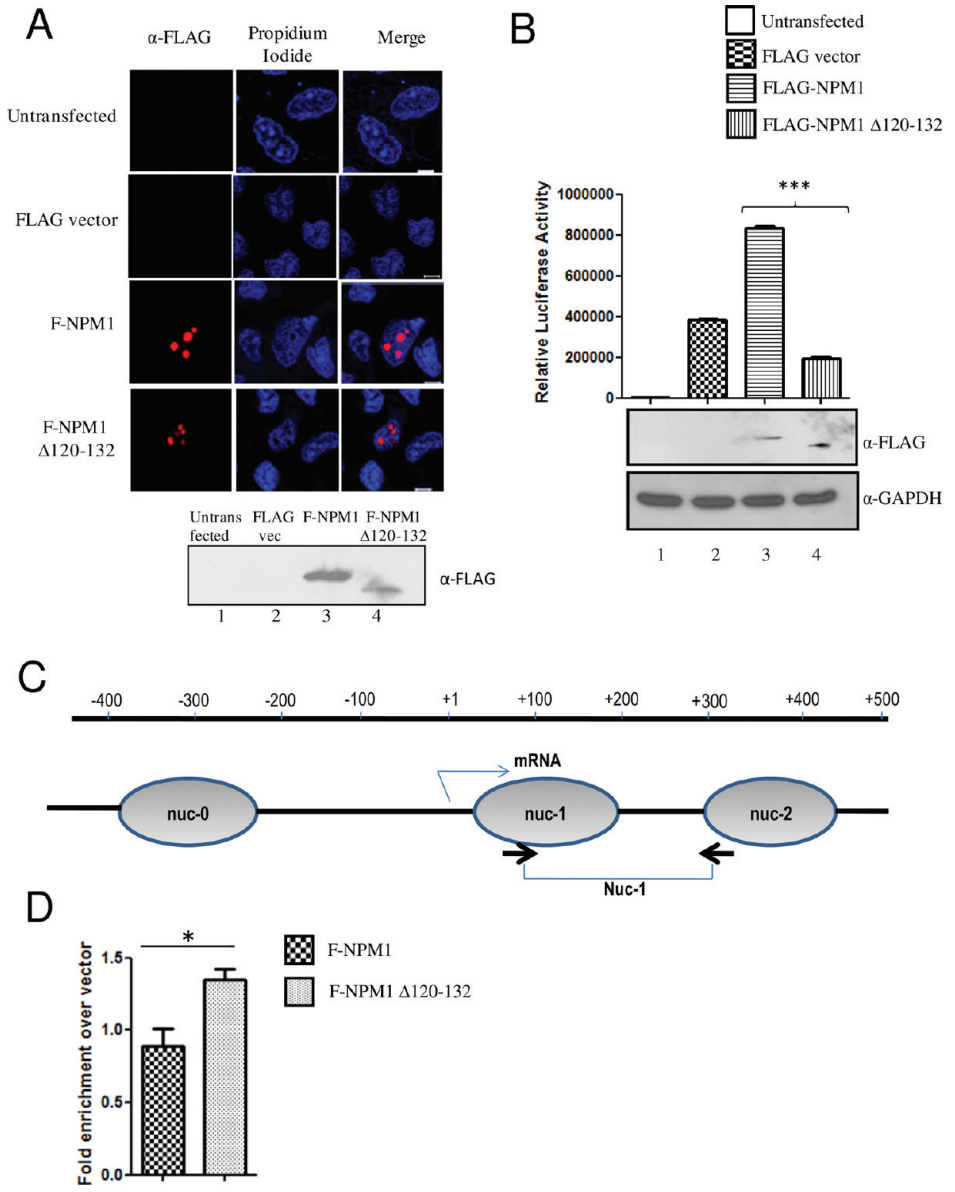

Figure 5. NPM1 alleviates H1-mediated repression during Tat-induced viral transactivation. (A) Localization of FLAG-tagged constructs in TZM-bl cells. Cells were transfected as indicated and were stained using anti-FLAG antibodies. Nucleus was stained using propidium iodide. The scale bar is 5 μm. The bottom panel represents the Western blotting analysis for expression of F-NPM1 and F-NPM1Δ120−132. (B) TZM-bl (stably integrated HIV-LTR upstream of the luciferase gene) cells were transfected with the constructs for the FLAG vector, FLAG-NPM1, or H1 interaction defective mutant FLAG-NPM1Δ120−132 and HIV-1 Tat, as indicated. The histogram shows means of three independent experiments (±standard deviation) depicting transactivation in terms of relative luminescence units per second. The data were analyzed with a Student's *t* test (***$P < 0.0001$). The bottom panel shows the expression profile of F-NPM1 and F-NPM1Δ120−132 and the loading control across the luciferase assay lysates. (C) Schematic representation of the chromatinized HIV-LTR.[33] (D) Recruitment of linker histone H1 to the HIV-LTR (at nuc-1) in the presence of Tat and the FLAG vector, FLAG-NPM1, or H1 interaction defective mutant FLAG-NPM1Δ120−132 analyzed in TZM-bl cells by chromatin immunoprecipitation (ChIP). The histogram represents the fold enrichment of H1 over FLAG vector control as probed by ChIP and followed by quantitative PCR. Values in terms of fold expression over vector are means ± the standard deviation for triplicate runs. The data were analyzed with a Student's *t* test (*$P < 0.05$).

overexpression of NPM1. For this purpose, we used TZM-bl (for details, see Materials and Methods) that contains an integrated HIV proviral promoter controlling the expression of the luciferase reporter gene. The TZM-bl cells are permissive to HIV-1, HIV-2, and SIV infection. This cell line closely represents the physiological state as the viral genome is chromatinized, and this model system could be used to study the effect of overexpression or knockdown of various factors on the transcription of the luciferase gene. The FLAG-tagged mammalian construct of NPM1Δ120−132 in the pFLAG-CMV vector was constructed, and its expression was checked in TZM-bl cells using immunofluorescence and Western blotting studies. Moreover, the cellular localization of F-NPM1Δ120−132 was compared with that of the wild type. It was found that the mutant protein localized predominantly to the nucleolus as the native NPM1 (Figure 5A). To gain insights into the NPM1-mediated derepression of the HIV-1 promoter by removing H1, we performed luciferase assays in TZM-bl cells in the presence of wild-type F-NPM1 and H1

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interaction defective mutant, F-NPM1Δ120−132 (Figure 5B). Cotransfection of Tat and F-NPM1 significantly enhanced the Tat-mediated transactivation as observed by the luciferase reporter activity compared to that of Tat alone (in Figure 5B, compare lane 3 to lane 2). Interestingly, F-NPM1Δ120−132 repressed the Tat-mediated transactivation (in Figure 5B, compare lane 4 to lane 3). In fact, a mild repression of transactivation was observed as compared to the activation in the presence of Tat (in Figure 5B, compare lane 4 to lane 2). This result could be attributed to the higher level of expression of F-NPM1Δ120−132 or hetero-oligomers formed by the NPM1Δ120−132 mutant with the native NPM1, which possibly gives a dominant negative effect. Western blot analysis was performed to show equal expression of the wild-type mutant proteins across the luciferase lysates (Figure 5B, bottom panel). To eliminate the possibility that the differences in luciferase readings are due to an alteration in cell proliferation caused by transfection of different constructs, we performed a cell proliferation assay with different combinations of transfections (Figure S4 of the Supporting Information). However, we could not find any difference in the proliferation of cells upon transfections within a 24 h period, the time period used for all the experiments (Figure S4 and Table S1 of the Supporting Information). On the basis of these observations, we hypothesize that ectopic overexpression of NPM1 may be involved in the removal or disassembly of histone H1 and thereby enhancement of transcription from the HIV-LTR promoter.

To investigate the possible removal of H1 from HIV-LTR, the viral promoter, we performed a chromatin immunoprecipitation assay using anti-H1 polyclonal antibodies. The organization of the nucleosome within the viral promoter has been depicted schematically (Figure 5C). The chromatin structure in the proviral 5′ LTR is composed of two distinct nucleosomes, nuc-0 and nuc-1, which flank the cis-acting regulatory elements of the viral promoter (Figure 5C). In the provirus, these nucleosomes define two large nucleosome-free areas. (i) The first one is composed of the core promoter and the enhancer region, and (ii) the second one is downstream of the 5′ LTR (reviewed in ref 40). These two open regions are separated by the single nucleosome, nuc-1, which is specifically and rapidly destabilized during transcriptional activation. nuc-1 is in the proximity of the transcription start site, and its disruption during transcriptional activation establishes the vital role of chromatin in the repression of HIV-1 transcription during latency.[41] To determine whether transcriptional activation by NPM1 reduces the linker histone H1 occupancy at the viral promoter in vivo, we cotransfected the FLAG vector, FLAG-NPM1, or FLAG-NPM1Δ120−132 constructs with Tat in the TZM-bl cells. After being transfected for 24 h, cells were subjected to chromatin immunoprecipitation analysis (ChIP) using anti-H1 polyclonal antibodies. The immunoprecipitated DNA fragments were analyzed by quantitative PCR using primers specific to the nuc-1 region of HIV-LTR (Figure 5D). The results suggest that overexpression of NPM1 reduced the H1 occupancy in the vicinity of nuc-1 as compared to the overexpression of the H1 interaction defective NPM1 mutant (F-NPM1Δ120−132) (Figure 5D). The data are the values normalized with the vector-transfected control. Collectively, these data indicate that NPM1 mediates removal of linker histone H1 and contributes to transactivation. These results argue for the significant role of NPM1 as a linker histone H1 chaperone apart from its coactivator function as a core histone chaperone.

Linker histone H1 along with core histones organizes the DNA into chromatin in an ordered fashion. Cells need an efficient molecular mechanism for the removal, recruitment, and displacement of linker histone H1 depending on the physiological state of the cell. Therefore, H1 specific chaperones are very important. In humans, apart from NAP1[3] and NASP,[4] no other well-established linker histone H1 chaperone is known. Identification of such an important function of NPM1 could be highly significant. NPM1 interacts with a majority of the somatic linker histone H1 variants. How NPM1 is possibly involved in the regulation of H1 dynamics, not only in the normal cellular function but also under stress condition, especially in cancer as well as during differentiation, remains to be investigated.

■ ASSOCIATED CONTENT

Supporting Information. Additional experimental procedures and supplementary Figures S1−S4. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

NPM1, nucleophosmin 1; HIV, human immunodeficiency virus; LTR, long terminal repeats; NAP-1, nucleosome assembly protein 1; CAF-1, chromatin assembly factor 1; NASP, nuclear autoantigenic sperm protein; FKBP, FK506 binding protein; UBF, upstream binding factor; NPM3, nucleoplasmin 3; HP1, heterochromatin protein 1.

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