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NPM3, a Member of the Nucleophosmin/Nucleoplasmin Family, Enhances Activator-Dependent Transcription†

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ABSTRACT: The chromatin is comprised of repeating subunits that make up the nucleosome which is composed of an octamer of histones: H3, H4, H2A, and H2B. The replication-dependent and -independent nucleosome assembly occurs in an ordered fashion and is aided by cellular proteins such as histone chaperones and chromatin remodelers. Previously, we found that the histone chaperone NPM1 activates transcription from the chromatin template. Here we report that NPM3, a member of the nucleophosmin/nucleoplasmin family, lacks intrinsic histone chaperone activity, inhibits histone assembly activity of NPM1 in vitro, and dramatically enhances transcription in a cellular system.

Histone chaperones make up a group of histone interacting proteins, which are involved in nucleosome assembly as well as disassembly during several physiological processes like replication, transcription, and repair (*1*). Recently, we have shown that multifunctional human histone chaperone NPM1 enhances transcription from the chromatin template (*2*). NPM2 and NPM3 are the other two members of the nucleophosmin/nucleoplasmin family (*3*). In the past few years, NPM1 has attracted a great deal of attention. Several groups have discovered that it plays diverse roles in the cells, including roles in ribosome biogenesis, centrosome duplication, transcription regulation, and histone chaperone function. NPM1 regulates cell proliferation and is overexpressed in several human cancers (*4*). NPM2 was reported to bind to histones and regulate chromatin assembly in *Xenopus laevis* oocytes (*3*). NPM3 was known to be associated with histone tail peptides in mouse embryonic stem cells, and its overexpression led to enhanced cell proliferation (*5*). In humans, NPM3 is homologous with the N-terminal and acidic stretch region of NPM1. It was found that both NPM1 and NPM3 were associated in a cellular complex (*6*). Here we report that NPM3 interacts with all the individual core histones and could also activate transcription in a cellular system. Though NPM3 lacks inherent histone transfer ability, it modulates the histone chaperone function of its interacting partner, NPM1, in vitro.

The homology between the core regions of NPM1 and NPM3 suggested the possible similarity in their functions which have not been explored. To study the ability of NPM3 to interact with core histones, we performed Ni-NTA pull-down assays with bacterially expressed NPM3-His_6 (for details, see Materials and Methods in the Supporting Information and Figure S1) and core histones (purified from HeLa cells). The results show that NPM3 interacts with all the core histones even at a salt concentration of 250 mM (Figure 1, lanes 3−5). However, at a salt concentration of 300 mM, the interaction with H2B and H2A was substantially weakened (Figure 1, lane 6). The interaction of core histones with Ni-NTA beads was considered as a negative control (Figure 1, lane 2). To determine the specificity of the interaction, Ni-NTA pull-down assays were conducted with NPM3 and bacterially expressed individual core histones. We found that NPM3 could pull down all the histones (H3, H2B, H2A, and H4) with varying efficiencies (Figure S2 of the Supporting Information, lane 3 vs lane 2) at a salt concentration of 200 mM. The histone interaction ability of NPM3 suggests that it could be a putative histone chaperone.

Human NPM3 interacts with NPM1 through its N-terminal oligomerization domain and regulates NPM1-mediated RNA binding and ribosome biogenesis function (*6*). It is known that NPM1 interacts with core histones and also possesses histone chaperone activity. NPM1 functions are modulated by its post-translational modification status and its interacting partners (*1*). We investigated whether NPM3 possesses intrinsic histone chaperone activity and/or alters the NPM1 functional activities. The histone chaperone assay measures the ability of a protein to deposit nucleosome onto a DNA template, using a free histone pool, and thereby introduce negative supercoils in the process (*7*). We performed the histone chaperone assay with increasing concentrations of NPM1 in the presence of core histones and *Drosophila* topoisomerase I (dTopoI) and monitored the appearance of supercoiled DNA (Figure 2A). NPM1 could facilitate nucleosome assembly in a dose-dependent manner as revealed by the gradual increase in the level of supercoiled plasmid DNA (Figure 2B, lanes 5−9). Incubation of relaxed DNA with either core histones or NPM1 was taken as a negative control (Figure 2B, lanes 3 and 4), where only minimal supercoiling was observed with core histones alone (lane 3). Possibly, this could be due to nonspecific, inherent ionic interaction of core histones with the relaxed DNA; hence, the observed supercoiling is taken as the basal level in these studies. A similar chaperone assay was conducted with NPM3 by keeping the reaction conditions identical (to those of NPM1). Interestingly, NPM3 failed to exhibit any chaperone activity which was conspicuous by the absence of any supercolied plasmid DNA (Figure 2C, lanes 5−10). These data suggest that the unique C-terminal domain of NPM1 is essential for the chaperone function (*8*) which is lacking in NPM3. We then went on to test the effect of NPM3 on the histone chaperone function of NPM1 (Figure 2D). At first, the reaction conditions were standardized so that we

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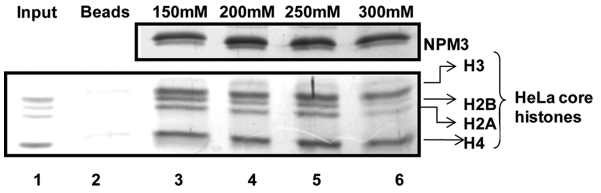

FIGURE 1: NPM3 interacts with core histones. His_6-tagged NPM3 (1 μg) was incubated with HeLa core histones (4 μg) at salt concentrations of 150 (lane 3), 200 (lane 4), 250 (lane 5), and 300 mM (lane 6). Beads were incubated with core histones at 150 mM (lane 2); 800 ng of core histones was taken as input (lane 1). The protein profiles were visualized with CBB staining.

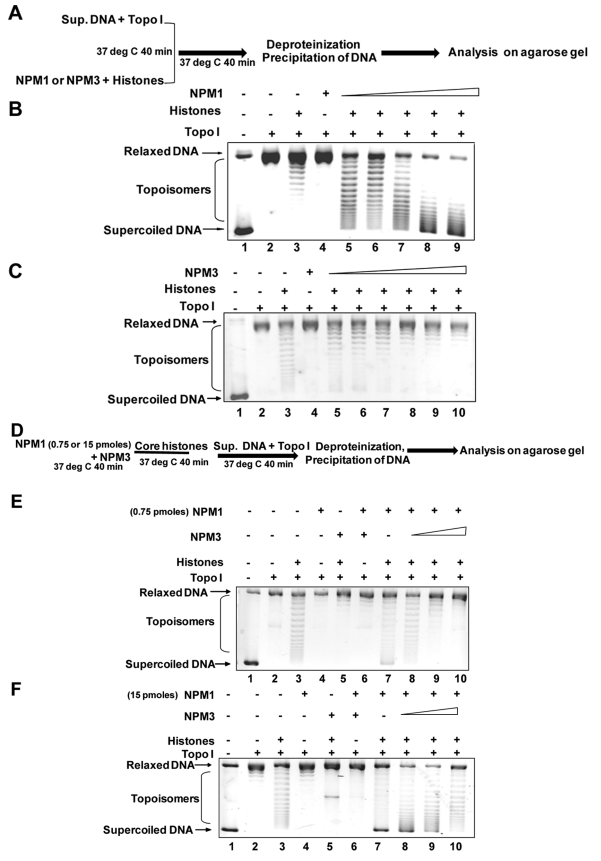

FIGURE 2: NPM3 does not possess an intrinsic histone chaperone activity. (A and D) Schematic depiction of the histone chaperone assay. (B) The histone chaperone assay was conducted with 500 ng of histones and increasing concentrations of NPM1: lane 1, supercoiled DNA; lanes 2−9, DNA relaxed by TopoI; lanes 3 and 5−9, core histones; lane 4, NPM1; lanes 5−9, 5, 2.5, 10, 15, and 20 pmol of NPM1, respectively. (C) The chaperone assay was conducted with increasing concentrations of NPM3: lane 1, supercoiled DNA; lanes 2−10, DNA relaxed by TopoI; lanes 3 and 5−10, core histones; lane 4, NPM3; lanes 5−10, 2.5, 5, 10, 15, 20, and 25 pmol of NPM3, respectively. (E) The chaperone assay was conducted with 0.75 (E) or 15 pmol (F) of NPM1 in the presence of increasing concentrations of NPM3: lane 1, supercoiled DNA; lanes 2−10, DNA relaxed by TopoI; lanes 3, 5, and 7−10, core histones; lane 4, NPM1; lane 5, NPM3; lane 6, NPM3 with NPM1; lanes 7−10, NPM1; lanes 8−10, 5, 10, and 15 pmol of NPM3, respectively.

could observe a significant appearance of supercoiled DNA over and above the basal level in the presence of NPM1 (0.75 pmol). Keeping the amount of NPM1 constant, we increased the concentration of NPM3 in the chaperone assay. Interestingly, we observed that NPM3 could potently inhibit the NPM1-mediated histone chaperone activity in a dose-dependent manner (in Figure 2E, compare lane 7 vs lanes 8−10). A similar pattern of inhibition was seen even at the maximum dose of NPM1 (15 pmol, where NPM1 has its highest chaperone activity) (in Figure 2F, compare lane 7 vs lanes 8−10). These results were also verified with the change in the order of incubation, where an increasing concentration of NPM3 was preincubated with core histones followed by the addition of NPM1 (0.75 pmol) (Figure S3A of the Supporting Information), and it was found that NPM3 inhibits the histone chaperone ability of NPM1 (in Figure S3B, compare lane 7 vs lanes 8−10). Next we preincubated NPM1 (0.75 pmol) with core histones and added increasing concentrations of NPM3 (Figure S4A of the Supporting Information). A similar inhibition of NPM1 chaperone activity was observed upon NPM3 addition (Figure S4B, lanes 8−10). Collectively, these data indicate that NPM3 represses the histone chaperone function of NPM1. This regulatory role of NPM3 in NPM1 function could have wide-ranging consequences in several cellular processes.

NPM3 is distributed in the nucleolus and also in the nucleoplasm of the cells. NPM3 together with NPM1 is translocated to the nucleoplasm upon being treated with actinomycin D (*6*). NPM1 is known to be involved in the activation of RNA polymerase II-driven transcription (*8*). These observations led us to investigate the possible role of NPM3 in transcription regulation. Gal4-VP16 (activator)-dependent reporter gene expression analysis was performed, where either FLAG-NPM1 or FLAG-NPM3 was transiently transfected alone or in combination. Interestingly, we found that overexpression of NPM3 enhanced the reporter gene expression severalfold (Figure 3, lane 3) which was comparable to that of NPM1 (Figure 3, lane 2). Surprisingly, we found that coexpression of NPM1 and NPM3 resulted in a dramatic increase in the level of reporter gene expression (in Figure 3, compare lane 1 vs lane 4). Since the cotransfection of NPM1 and NPM3 constructs also enhanced the protein expression level of NPM1 and NPM3, the enhancement of transcription could be global in nature (in Figure 3, compare lanes 2 and 3 vs lane 4, Western blot). Mechanistically, the elevated level of protein expression could be a consequence of increased ribosome biogenesis activity due to the overexpression of NPM1. To address this issue, a nucleo-cytoplasmic shuttling defective mutant of NPM1 (FLAG-NPM1dL) (*9*) was used in a reported gene expression assay. Interestingly, FLAG-NPM1dL could also induce reporter gene expression to an extent similar to that of wild-type FLAG-NPM1 (in Figure S5 of the Supporting Information, compare lane 3 vs lane 2). Furthermore, when cotransfected with FLAG-NPM3 (in Figure S5, compare lane 6 with lane 5), a nucleo-cytoplasmic shuttling defective mutant could also efficiently enhance transcription. Taken together, these observations suggest that elevated levels of protein expression could be due to transcriptional activation, rather than translational activation. This conclusion is further strengthened by the observation that NPM3 overexpression inhibited ribosome biogenesis (*6*).

The molecular mechanism of synergistic transcriptional activation caused by NPM1 and NPM3 could be a complex phenomenon. The sequestration of histones by NPM3 upon chaperone-mediated eviction of the histone from the chromatin template could be one possibility of keeping the chromatin open for a longer time for transcriptional activation. However, we have found that in a histone interaction assay, NPM1 has a relatively better histone interacting ability than NPM3 (Figure S6 of the

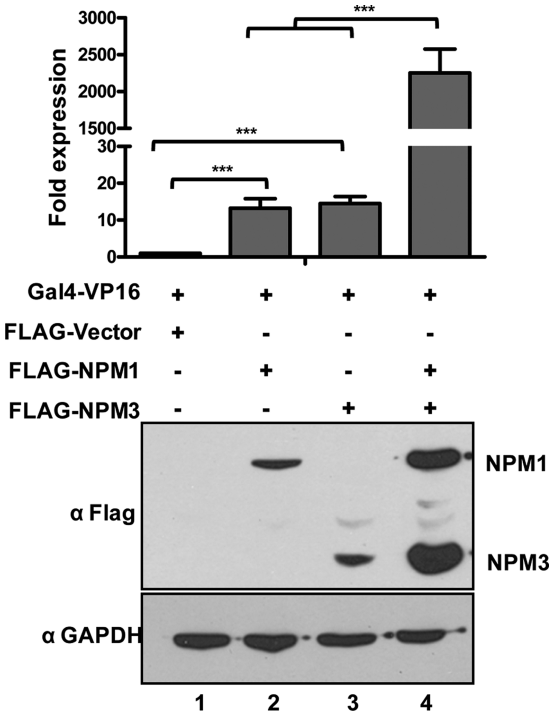

FIGURE 3: NPM3 enhances activator-dependent transcription. The Gal4-VP16 responsive luciferase assay (pG10luc) was performed with either 1 μg of FLAG vector alone (lane 1), FLAG-NPM1 (lane 2), FLAG-NPM3 (lane 3), or FLAG-NPM1 with FLAG-NPM3 (lane 4) in HEK293T cells transiently transfected with pG10luc (100 ng), Gal4-VP16 (10 ng), and pCMV-β-gal (100 ng). Values in terms of fold expression over vector are means $\pm$ the standard deviation in triplicate. The data were analyzed with a paired t test (***$P < 0.001$). The equal expression level of both FLAG-NPM1 and FLAG-NPM3 of a representative experiment was verified by Western blotting with an anti-Flag antibody (Sigma).

Supporting Information). Since NPM3 is a potent inhibitor of NPM1-mediated histone assembly activity, the possibility of reduced histone assembly as a cause of hyperactivation of transcription cannot be ruled out. Furthermore, NPM1 and NPM3 together may also enhance chromatin remodeling, causing the activation of transcription. In agreement with this hypothesis, it was observed that the overexpression of NPM3 increases the level of proliferation of mouse embryonic stem cells, presumably through chromatin remodeling (*5*).

A recent observation suggests that a histone interacting protein, Alien, facilitates the histone deposition function of NAP1, which causes the repression of chromatin-mediated transcription (*10*). In our study, NPM3 repressed the histone chaperone function of NPM1 (Figure 2), which culminated in the observed enhancement of gene expression in vivo (Figure 3) probably by making the chromatin template more amenable to transcription. These data broaden the regulatory circuit of NPM1 and assign a new role to its interacting partner, NPM3. This could be considered as one of the significant findings amidst growing reports of the involvement of NPM1 in various types of cancer where it functions as an oncogene (*2*, *4*) as well as in altered physiological cellular processes. These results imply that the critical balance between NPM1 and NPM3 levels is essential for cellular homeostasis and any deregulation would contribute to associated malignant transformation and uncontrolled proliferation of the cells. However, the fine modulation of NPM1 and NPM3 interaction by post-translational modifications such as acetylation and phosphorylation could also have several functional implications, which needs to be addressed.

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SUPPORTING INFORMATION AVAILABLE

Details of the experimental procedures and Figures S1−S6. This material is available free of charge via the Internet at http://pubs.acs.org.

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