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ORIGINAL ARTICLE

RNA aptamers interfering with nucleophosmin oligomerization induce apoptosis of cancer cells

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Nucleophosmin (NPM) is a multifunctional protein involved in both proliferation and apoptosis. Importantly, NPM negatively regulates p53 and is frequently overexpressed in a wide variety of cancers. To identify inhibitory molecules of NPM, we used an *in vitro* selection method termed systematic evolution of ligands by exponential enrichment (SELEX) to select RNA aptamers that bind to NPM with high affinity and specificity. The selected RNA aptamers bind to the central acidic region of NPM and affect its oligomerization both *in vitro* and *in vivo*. Remarkably, expression of NPM-specific aptamers causes mislocalization of NPM in the nucleoplasm rather than in the nucleolus, suggesting that NPM oligomerization is important for its proper localization. Moreover, p14ARF is mislocalized in the nucleoplasm and p53 is upregulated in cells expressing NPM aptamers. In addition, cancer cells expressing NPM aptamers not only undergo apoptosis on their own, but are more susceptible to apoptosis induced by DNA-damaging agents as well. These results suggest that interfering with NPM oligomerization can inhibit NPM function and aptamers targeting NPM can serve as potential lead for developing anticancer drugs.
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Introduction

Nucleophosmin (also referred to as NPM, B23, Numatrin or NO38) is an abundant phosphoprotein predominantly localized in the nucleolus. NPM can bind to DNA and RNA and possesses RNase activity that cleaves the second internal transcribed spacer (ITS2) in the 32S pre-rRNA to generate the matured 28S rRNA. Downregulation of NPM inhibits pre-rRNA processing (Savkur and Olson, 1998; Itahana *et al*., 2003). By interacting with several other nucleolar proteins such as nucleolin and ribonucleoproteins, NPM primarily functions to control ribosome biogenesis. In addition, NPM acts as a molecular chaperon by shuttling between the nucleolus, nucleoplasm and cytoplasm in a cell-cycle-dependent manner (Borer *et al*., 1989; Szebeni *et al*., 1995), which likely prevents the aggregation of nucleolar proteins, stimulates the import of proteins containing nucleolar localization signals (NuLS) into the nucleolus as well as facilitates the export of proteins from the nucleus to cytoplasm.

Although NPM was initially identified as an important player of ribosome biogenesis, subsequent studies have revealed that it is involved in a wide range of cellular processes. NPM is critical to the proper duplication of centrosomes and maintenance of genomic stability (Okuda *et al*., 2000). Mice deficient in *Npm1* exhibit unrestricted centrosome duplication accompanied with accumulation of DNA damage and p53-dependent apoptosis (Colombo *et al*., 2005; Grisendi *et al*., 2005). In fact, NPM interacts with several key factors involved in p53-mediated cellular response to genotoxic stress. For example, NPM is upregulated in response to genotoxic stress and can directly bind to p53 to affect its transcriptional activity through different mechanisms (Colombo *et al*., 2002; Yang *et al*., 2002; Maiguel *et al*., 2004). Moreover, NPM interacts with the tumor suppressor p14ARF, a positive regulator of p53 (Korgaonkar *et al*., 2005). p14ARF prevents the degradation of p53 by interacting with Mdm2 to suppress Mdm2-mediated p53 ubiquitination, which leads to increased expression of p53 target genes responsible for either cell-cycle arrest or apoptosis (Gjerset and Bandyopadhyay, 2006). Because both NPM and Mdm2 can bind to the N terminus of p14ARF, NPM likely competes with Mdm2 for association to p14ARF, which sequesters p14ARF in the nucleolus and inhibits its function in antagonizing Mdm2 (Korgaonkar *et al*., 2005). In addition, NPM serves as a nuclear PI(3,4,5)P3 receptor and interacts with active CAD/DFF40 to inhibit apoptotic DNA fragmentation (Ahn *et al*., 2005). Altogether, these findings indicate that NPM is an antiapoptotic protein that can suppress either p53-dependent or -independent apoptosis. In agreement with this notion, it has been found that NPM is highly expressed in proliferating cells and is frequently overexpressed in a wide variety of solid

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tumors including gastric, colon, ovarian and prostate carcinomas. In hematopoietic tumors, NPM is often mutated or aberrantly localized in the cytoplasm (Grisendi *et al*., 2006). It is therefore conceivable that NPM is an ideal target for pharmaceutical intervention in the treatment of various cancers.

Aptamers are small synthetic RNA or single-stranded DNA molecules that can bind to other target molecules such as protein and RNA with high affinity and specificity. With the technique named systematic evolution of ligands by exponential enrichment (SELEX), aptamers binding to different proteins can be routinely selected from synthetic combinatorial libraries (Nimjee *et al*., 2005). Because aptamers normally fold up into unique tertiary structures, they can bind to specific regions or domains of target proteins. Aptamers are therefore very useful reagents for probing the biological function of a specific protein domain, or for dissecting distinct functions of highly homologous proteins. Importantly, the inhibitory effects of aptamers on their target proteins make them excellent lead compounds for diagnosis or therapy of many human diseases including cancers. For example, the aptamer against a specific isoform of human vascular endothelial growth factor has been successfully developed into drug to cure age-related macular degeneration (Apte, 2008). The anti-nucleolin aptamer AS1411, which can inhibit DNA replication and induce cell-cycle arrest and apoptosis, is now in phase II clinical trials for cancer therapy (Ireson and Kelland, 2006). A large number of aptamers against cancer-related antigens, such as prostate-specific membrane antigen, mucin 1, Tenacin-C, nuclear factor-κB and $\alpha_v\beta_3$ integrin, are being tested for better understanding of tumor pathogenesis, and for diagnostic and therapeutic purposes of cancer treatment (Nimjee *et al*., 2005). In addition, aptamers are used in combination with other technologies in developing novel therapeutic strategies. For example, aptamers can be conjugated with nanoparticles and toxins to yield better targeting and inhibition of tumors (Levy-Nissenbaum *et al*., 2008). Furthermore, by conjugating with siRNA, cancer antigen-specific aptamers can help better delivery of siRNA into tumor cells to silence gene expression (Chu *et al*., 2006; McNamara *et al*., 2006).

Because NPM is an ideal target for cancer therapeutics, we hope to identify small-molecule compounds to inhibit its antiapoptotic functions. As the first step toward this goal, we took advantage of the SELEX method to generate RNA aptamers against NPM and obtained four aptamers from a synthetic combinatorial library. We used one of these aptamers, which possesses the highest binding affinity to NPM, to perturb its functions in cancer cells. We found that the aptamer 1A1 and its truncated form 1A1(1–40) bound to the central acidic region of NPM and interfered with its oligomerization both *in vitro* and in cancer cells. Expression of these aptamers led to mislocalization of NPM in the nucleoplasm rather than in the nucleolus in several human cancer cell lines. Moreover, cancer cells expressing NPM aptamers underwent apoptosis, and were more susceptible to apoptosis induced by DNA-damaging agents. Our results suggest that interfering with NPM oligomerization can inhibit its function and aptamers targeting NPM can serve as lead compounds for developing anticancer drugs.

Results

Isolation and characterization of NPM-specific aptamers

To select RNA aptamers for NPM, we carried out a SELEX procedure consisting of an electric mobility shift assay (EMSA) and a dot-blot assay. Briefly, an RNA library was generated by *in vitro* transcription of an oligonucleotide library containing a central region of 49 randomized nucleotides flanked by constant sequences at both ends and a bacterial T7 promoter at the 5′ end (Yang *et al*., 2006). For each round of selection, recombinant NPM ($NPMHis_6$; Figure 1a) was incubated with ^{32}P-labeled RNA library to yield RNA/protein complexes, which were subsequently isolated by either EMSA or dot-blot assay. RNAs bound to NPM were then recovered and reverse-transcribed into cDNAs for further PCR amplification to yield a new oligonucleotide library encoding RNAs of higher binding affinity to NPM. After 11 rounds of SELEX, the RNA–NPM binding affinity reached a platform (Figure 1b). Then cDNAs encoding these RNA aptamers were cloned into a vector for sequencing. Sequence analysis of 16 randomly picked cDNA clones identified four different RNA species, among which the aptamer 1A1 is the most abundant one (11 of 16) (Figure 1c). The aptamers 1B2 (3 of 16) and 1G1 (1 of 16) differ slightly from 1A1 by only one or two nucleotides, whereas the aptamer 2B9 is totally different from 1A1 (Figure 1c). Dot-blot analysis indicated that the aptamers 1A1 and 1G1 bound more strongly to NPM than the aptamers 1B2 and 2B9 (Figure 1d). To determine whether NPM-specific aptamers were enriched during our selection, we used the central region of the aptamer 1A1 lacking the constant regions of aptamers to probe cDNAs obtained from each round of selection by Southern blotting. As shown in Figure 1e, 1A1 was sharply enriched after nine rounds of selection, indicating that our SELEX procedure successfully enriched specific aptamers for NPM.

Because the aptamer 1A1 is the predominant RNA molecule obtained in our selection (Figure 1), we performed subsequent studies using this aptamer. First we performed secondary structure predication using the Mfold program and found that 1A1 contains two stem loops (Figure 2a). We then examined which stem loop is important for 1A1 to bind to NPM by testing the interactions between GST-NPM and 1A1 truncation mutants generated by serial deletions from the 3′ end. Our results indicate that the nucleotides 1–40, which form the first stem loop of 1A1, were sufficient and necessary for its binding to NPM (Figures 2a and b). Consistently, disruption of this stem-loop structure by deleting the first 17 nucleotides abrogated its NPM-binding capacity (Figure 2b). Moreover, we used EMSA to determine the binding affinity of 1A1 and 1A1(1–40)

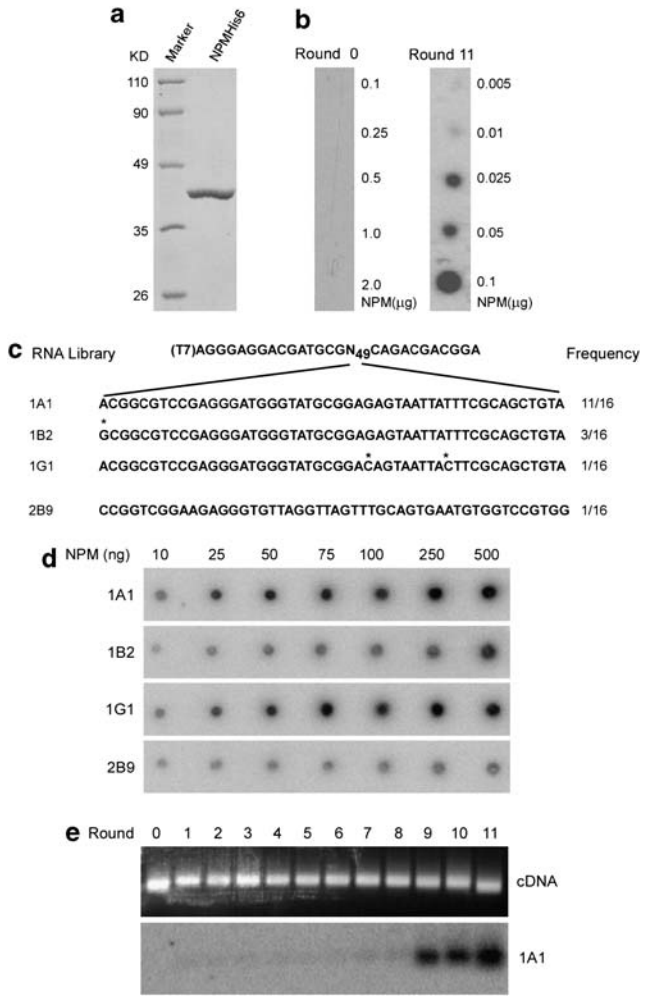

Figure 1 Selection of nucleophosmin (NPM) aptamers. (**a**) A Coomassie blue-stained gel showing purified $NPMHis_6$ used for systematic evolution of ligands by exponential enrichment (SELEX). (**b**) Binding of RNA library to NPM detected by dot-blot assay after 11 rounds of selection. $NPMHis_6$ of indicated amount was dotted on nitrocellulose membrane and incubated with ^{32}P-labeled RNA. Bound RNA was detected by autoradiography. (**c**) Sequences of selected NPM aptamers. The sequences shown are cDNA sequences corresponding to the 49-nucleotide variable region of the RNA aptamers selected from a random RNA library containing the T7 promoter sequence (AATACGACTCACTAT AG). N49 designates the 49 random nucleotides in the variable region of the RNA library. Frequency indicates the number of clones showing the same sequence among 16 randomly selected cDNA clones from the 11th round. Asterisks indicate different nucleotides in 1B2 and 1G1 from 1A1. (**d**) Dot-blot assay showing binding of individual aptamer to NPM. (**e**) Southern blot analysis of the enrichment of 1A1 in each round of selection. ^{32}P-labeled 1A1 cDNA lacking both 5′ and 3′ constant regions was used to probe the cDNAs (top) of each round of selection and signals were detected by autoradiography (bottom).

to NPM (Figure 2c). The dissociation constant (K_d) for 1A1–NPM complexes measured by this assay was $\sim$33 nM, whereas the K_d for 1A1(1–40)/NPM complexes was $\sim$30 nM (Figure 2d), indicating that these two aptamers bound to NPM with similar affinity.

We next determined the region of NPM required for its binding to the aptamer 1A1. Previously it has been shown that NPM contains several distinct functional domains, including the N-terminal oligomerization domain (amino acids 1–113), the central acidic region (amino acids 114–186) and the C-terminal nucleic acid-binding region (amino acids 187–294) (Figure 2e; Hingorani *et al*., 2000). We therefore examined the interaction between ^{32}P-labeled 1A1 and different domains of NPM using EMSA (Figure 2f). As shown in Figure 2f, the full-length NPM and $NPM_{114-186}$ bound almost equally well to 1A1; however, 1A1 did not bind to NPM_{1-113} or $NPM_{187-294}$, indicating that the central acidic region is sufficient and necessary for NPM to bind to 1A1 (Figure 2f).

NPM aptamers interfere with NPM oligomerization

NPM normally oligomerizes to form pentamer or decamer, which is important for its cellular activities (Namboodiri *et al*., 2004). To assess the effect of 1A1 on NPM function, we tested whether 1A1 could affect NPM oligomerization. For this purpose, $NPMHis_6$ was first incubated with GST-NPM immobilized on glutathione-Sepharose beads in the presence or absence of 1A1 and 1A1(1–40), then $NPMHis_6$ associated with GST-NPM was pulled down and detected by western blotting. Our results indicate that the association of $NPMHis_6$ with GST-NPM was significantly reduced in the presence of either 1A1 or 1A1(1–40) in a concentration-dependent manner (Figure 3a, right). By contrast, a control aptamer that did not bind to NPM failed to show any obvious effect on the interaction between GST-NPM and $NPMHis_6$ (Figure 3a, right). These results suggest that 1A1 likely interfered with the oligomerization of NPM. To further prove this, we expressed and purified GST-NPM/$NPMHis_6$ complexes in bacteria (Figure 3b, left) and tested whether 1A1 could release $NPMHis_6$ from the GST-NPM/$NPMHis_6$ complexes immobilized on glutathione-Sepharose beads. As shown in Figure 3b (right), the aptamers 1A1 and 1A1(1–40), but not the control aptamer, released significant amount ($\sim$20%) of $NPMHis_6$ from the GST-NPM/$NPMHis_6$ complexes, confirming that 1A1 can indeed affect NPM oligomerization.

Because NPM was found to interact with the tumor suppressor p53, we tested whether 1A1 could perturb their interaction. To do so, we prepared GST-p53/$NPMHis_6$ complexes immobilized on glutathione-Sepharose beads (Figure 3c, left), and used both 1A1 and 1A1(1–40) aptamers to release $NPMHis_6$ from the complexes. However, both aptamers failed to release $NPMHis_6$ from the GST-p53/$NPMHis_6$ complexes, suggesting that 1A1 did not affect NPM/p53 interaction (Figure 3c, right). Thus, the aptamers 1A1 and 1A1 (1–40) appeared to affect NPM oligomerization specifically.

NPM localization is changed in cells expressing NPM aptamers

The findings that the aptamers 1A1 and 1A1(1–40) bound to the central acidic region of NPM and affected its oligomerization *in vitro* promoted us to further

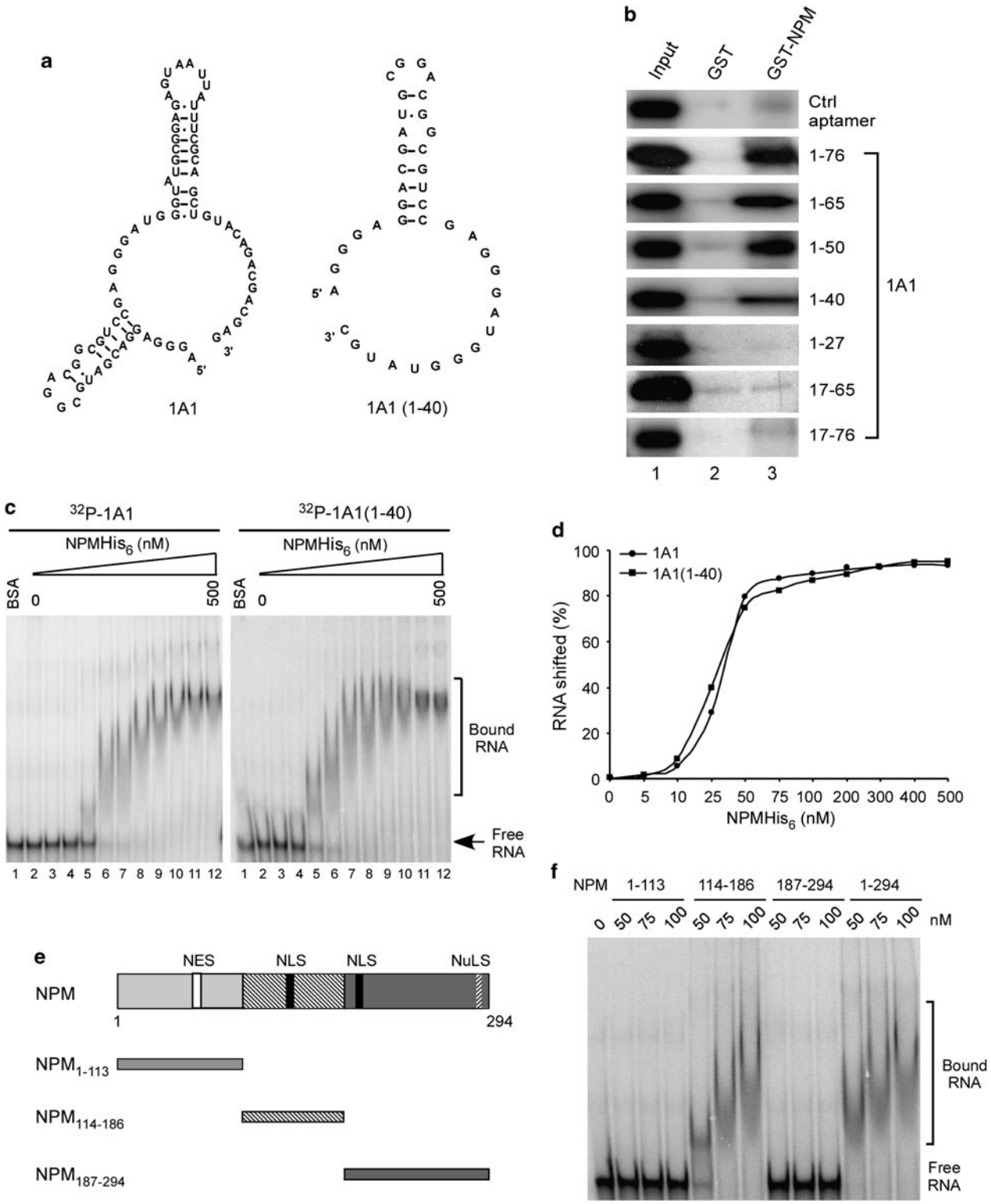

Figure 2 Characterization of nucleophosmin (NPM)/aptamer interaction. (**a**) Predicted secondary structures of the aptamers 1A1 and 1A1 (1–40). (**b**) The binding of 1A1 and its truncation mutants to NPM. ^{32}P-labeled RNAs were incubated with glutathione *S*-transferase (GST) or GST-NPM immobilized on glutathione-Sepharose beads and bound RNAs were resolved on 7.5% denature gel and detected by autoradiography. Lanes 1 indicates 1/10 of input aptamer. (**c**) Electric mobility shift assay (EMSA) analysis of the binding of 1A1 (left) and 1A1(1–40) (right) to NPMHis$_6$. ^{32}P-labeled RNA was incubated with bovine serum albumin (BSA; 500 nM, lane 1) or increasing amount of NPMHis$_6$ (lanes 2–12 indicate 0, 5, 10, 25, 50, 75, 100, 200, 300, 400 and 500 nm of NPMHis$_6$, respectively) at 30 °C for 30 min and resolved on 7.5% native gel. (**d**) NPM–aptamer binding curves derived from (**c**). (**e**) Schematic representation of NPM full-length and truncation proteins. The nuclear export signal (NES), nuclear localization signal (NLS) and nucleolar localization signal (NuLS) are indicated. (**f**) EMSA analysis of the binding of 1A1 to different domains of NPM. EMSA was performed as in (**c**).

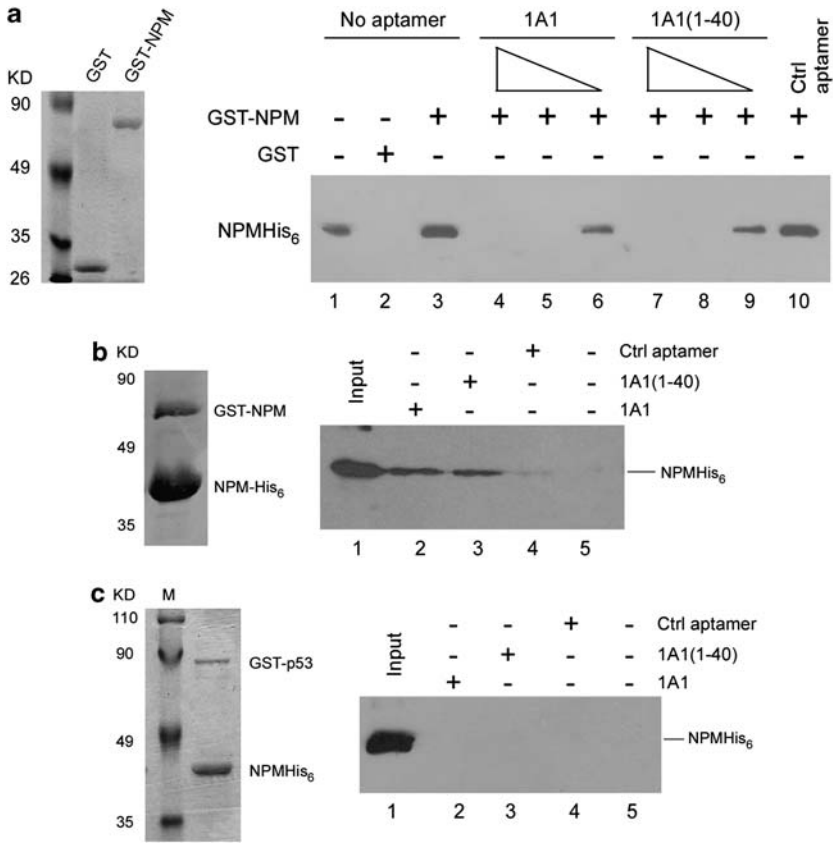

Figure 3 Nucleophosmin (NPM)-specific aptamers affect NPM oligomerization *in vitro*. (**a**) NPM aptamers interfere with the interaction between GST-NPM and $NPMHis_6$. $NPMHis_6$ (5 μM) preincubated with *in vitro* transcribed aptamers (5 μM in lanes 4, 7 and 10; 2.5 μM in lanes 5 and 8; 1 μM in lanes 6 and 9) was incubated with glutathione *S*-transferase (GST) or GST-NPM (5 μg) immobilized on glutathione-Sepharose beads. After extensive wash, bound proteins were resolved on 10% SDS–polyacrylamide gel electrophoresis (PAGE) and detected by using anti-His_6 antibody. The left panel shows the purified proteins and right panel shows the protein interactions in the presence of aptamers. Lane 1 indicates 10% of input $NPMHis_6$. (**b**) NPM aptamers release $NPMHis_6$ from the GST-NPM/$NPMHis_6$ complexes. GST-NPM/$NPMHis_6$ complexes (5 μg) immobilized on glutathione-Sepharose beads (left) was incubated with *in vitro* transcribed aptamers (1 μM) and half the $NPMHis_6$ released into the supernatant was detected by using anti-His_6 antibody (right). Lane 1 indicates 30% of $NPMHis_6$ from the GST-NPM/$NPMHis_6$ complexes. (**c**) NPM aptamers do not release $NPMHis_6$ from the GST-p53/$NPMHis_6$ complexes. The left panel shows the GST-p53/$NPMHis_6$ complexes and the right panel shows the release of $NPMHis_6$ from the GST-p53/$NPMHis_6$ complex as performed as in (**b**).

explore whether these aptamers had a similar effect in cells. First, we examined whether these aptamers interacted with NPM in cells by transiently expressing them in MCF-7 cells under the control of the U6 snRNA promoter (nucleotide −265 to +27) and its transcriptional terminator, which were shown to direct the expression of RNA in the nucleus (Good *et al*., 1997). Using anti-NPM antibody, we performed immunoprecipitation and examined NPM-associated aptamers. Both 1A1 and 1A1(1–40), but not the control aptamer, were co-immunoprecipitated with NPM, suggesting that these aptamers and NPM interacted specifically in cells (Figure 4a). Next, we investigated whether these aptamers could affect the oligomerization of endogenous NPM. We used native polyacrylamide gel to resolve nuclear lysates prepared from MCF-7 and HeLa cells expressing 1A1, 1A1(1–40) or a control aptamer, and detected the oligomeric and monomeric forms of NPM with western blotting. As shown in Figure 4b, the amount of NPM oligomer was strongly reduced in cells expressing 1A1 and 1A1(1–40) compared to that in cells expressing the control aptamer. Together, these results indicate that NPM-specific aptamers interfered with the oligomerization of endogenous NPM.

To assess the cellular effect of 1A1 and 1A1(1–40) on NPM, we examined the subcellular localization of NPM in cells stably expressing these aptamers. As reported previously (Grisendi *et al*., 2006), the endogenous NPM was found to localize mainly in the nucleolus in MCF-7 cells expressing the control aptamer. In contrast, in cells expressing either 1A1 or 1A1(1–40), NPM was excluded from the nucleolus and exhibited an even distribution pattern in the nucleoplasm (Figure 4c). Similar mislocalization of NPM in the nucleoplasm was observed in HeLa and SGC7901 cells expressing these NPM-specific aptamers (data not shown). Taken together, these results suggest that the binding of aptamers to NPM disrupted its oligomerization, leading to mislocalization of NPM in the nucleoplasm rather than in the nucleolus.

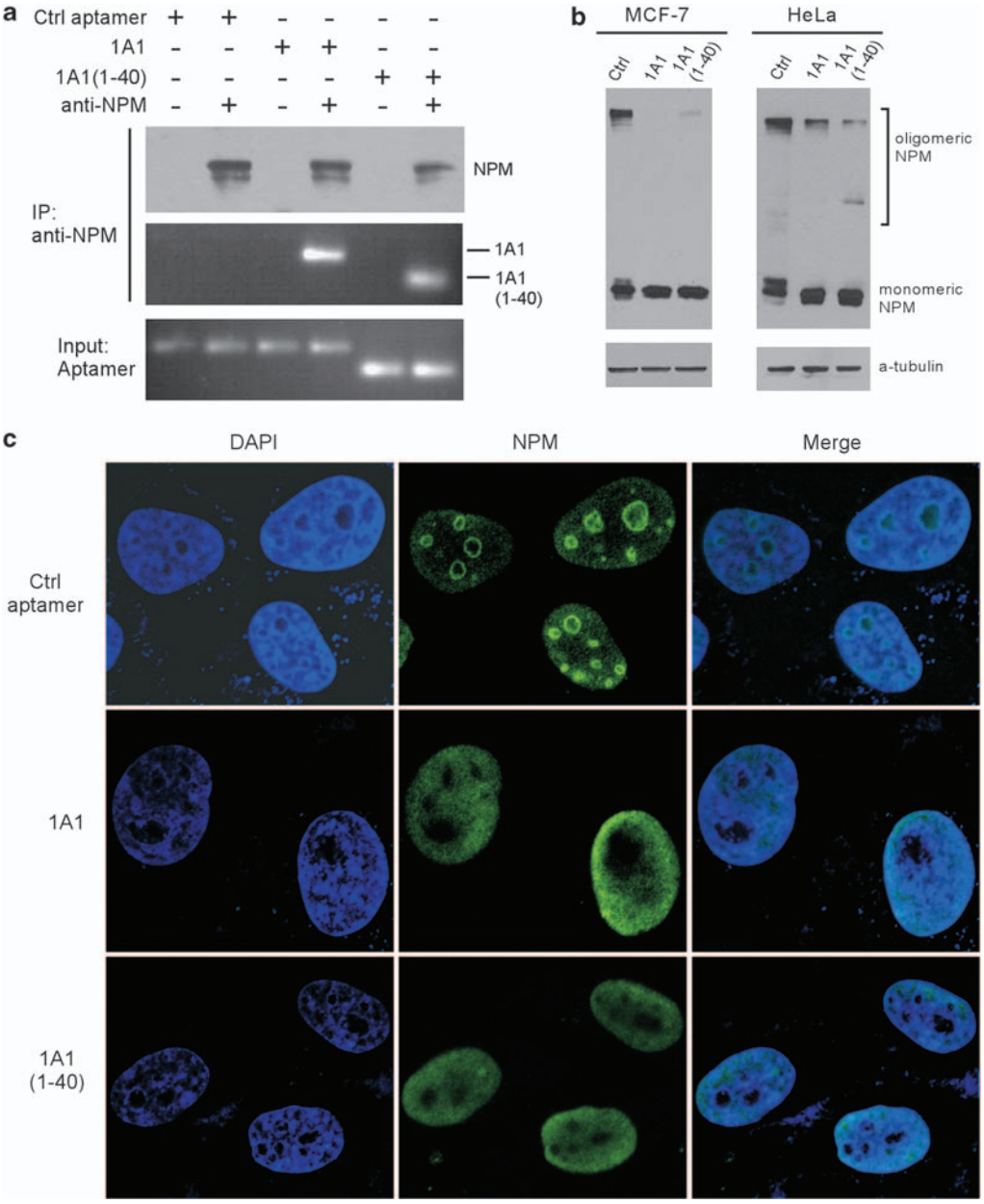

Figure 4 Nucleophosmin (NPM) aptamers interfere with the oligomerization and subcellular localization of NPM in cancer cells. (**a**) NPM aptamers interact with NPM in MCF-7 cells. NPM was immunoprecipitated by using anti-NPM antibody from MCF-7 cells transiently expressing 1A1 and 1A1(1–40). The immunoprecipitated NPM was detected by western blot assay (top) and aptamers associated with NPM were detected by reverse transcription (RT)–PCR (middle). The input of aptamers in cell lysate is shown in the bottom. (**b**) NPM aptamer affects NPM oligomerization. Nuclear lysates from MCF-7 and HeLa cells expressing NPM aptamers were resolved on native polyacrylamide gel and detected by western blotting using anti-NPM antibody. (**c**) NPM delocalized from the nucleoli in cells stably expressing NPM aptamers. The endogenous NPM in MCF-7 cells stably expressing NPM aptamers was detected by immunostaining using anti-NPM antibody and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG. Nuclei were stained by 4′,6-diamidino-2-phenylindole (DAPI).

NPM aptamers affect p14ARF localization and upregulate p53

Because NPM aptamers induced mislocalization of NPM in the nucleoplasm, we wanted to know whether the nucleolar localization of p14ARF was also changed, because NPM interacts with p14ARF and sequesters the latter in the nucleolus (Korgaonkar *et al*., 2005). To answer this, we transiently expressed Flag-tagged p14ARF in HeLa cells stably expressing 1A1, 1A1(1–40) or the control aptamer. In cells expressing the control aptamer, p14ARF was predominantly localized in the nucleolus (Figure 5a), which is consistent with previous reports (Korgaonkar *et al*., 2005). In cells expressing 1A1 and 1A1(1–40), however, p14ARF lost its nucleolar localization and adopted an even distribution pattern in the nucleoplasm, suggesting that aptamer-induced NPM mislocalization likely resulted in similar mislocalization of p14ARF. As nucleoplasmic p14ARF can promote

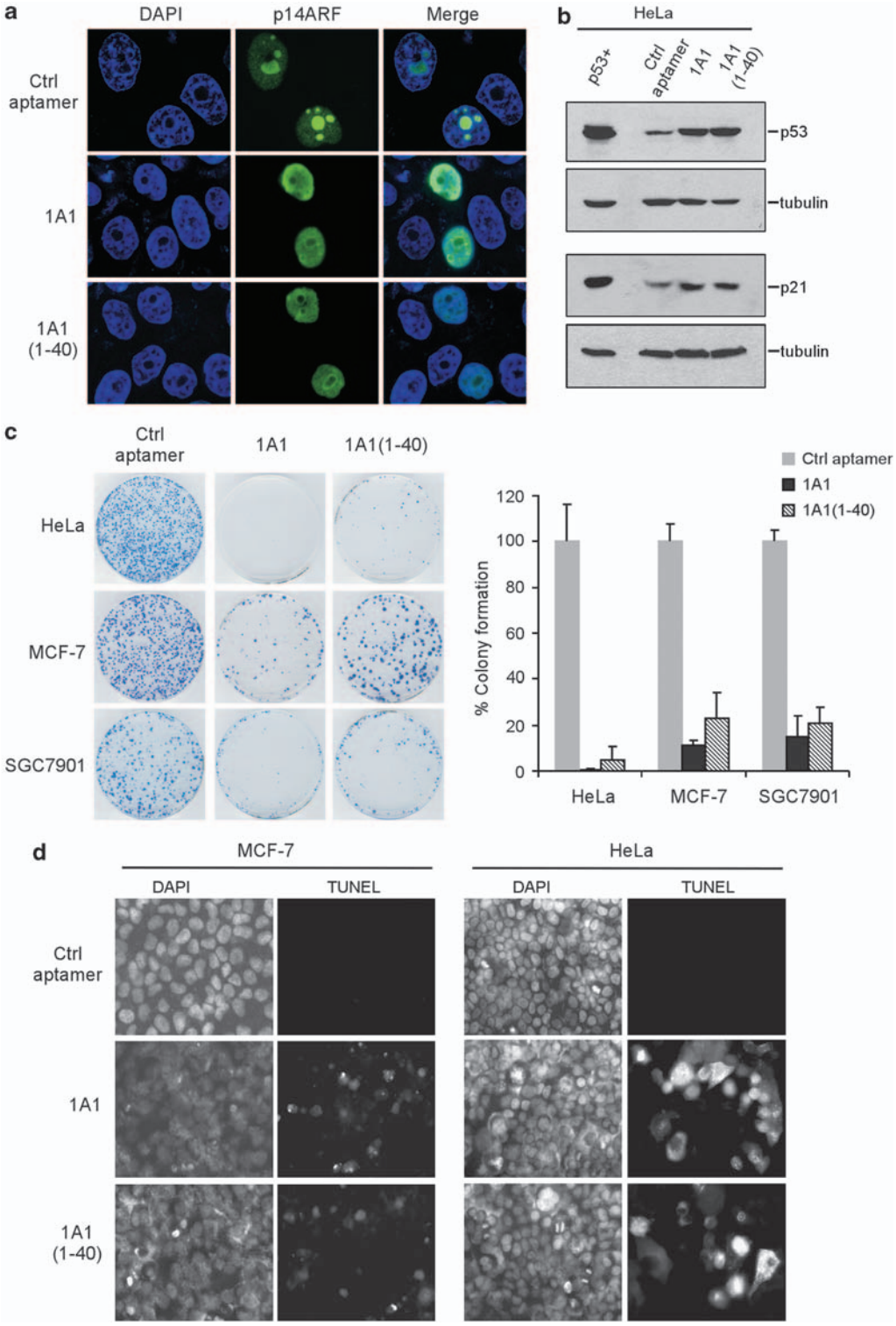

Figure 5 Nucleophosmin (NPM) aptamers affect p14ARF, p53 and induce apoptosis. (**a**) Subcellular localization of Flag-tagged p14ARF in HeLa cells stably expressing NPM aptamers. HeLa cells stably expressing 1A1 and 1A1(1–40) were transiently transfected with mammalian vector expressing Flag-tagged p14ARF. After 36 h, the localization of Flag-p14ARF was detected by immunostaining using anti-Flag antibody. Nuclei were stained by 4′,6-diamidino-2-phenylindole (DAPI). (**b**) Western blot showing protein levels of p53 and p21 in HeLa cells stably expressing NPM aptamers. p53 + indicates reinforced expression of p53 by transfection of a p53-expressing vector in HeLa cells containing a control aptamer. Tubulin was used as loading control. (**c**) Colonogenic survival of HeLa, MCF-7 and SGC7901 cells stably expressing NPM aptamers. The left panel shows representative pictures of colony formation in three cancer cell lines. The right panel shows relative formation of colonies in cells stably expressing NPM aptamers. Colony formation was performed in triplicate and normalized to that of control aptamer for each cell line. (**d**) Terminal transferase dUTP nick-end labeling (TUNEL)-staining of apoptotic DNA in MCF-7 and HeLa cells stably expressing NPM aptamers. Nuclei were stained by DAPI.

p53 stability by antagonizing Mdm2 (Gjerset and Bandyopadhyay, 2006), we further examined whether the expression of p53 was elevated in these NPM aptamer-expressing cells. In HeLa cells stably expressing 1A1 and 1A1(1–40), p53 protein level was obviously increased as compared to that in cells expressing the control aptamer. Consistently, the protein level of the p53 target gene *p21* was significantly increased in cells expressing these NPM aptamers (Figure 5b).

Expression of NPM aptamers induces apoptosis

Our findings demonstrated that the aptamers 1A1 and 1A1(1–40) bound to NPM in cells and resulted in its mislocalization, which in turn caused the nucleoplasmic localization of p14ARF and the upregulation of p53. Next, we examined whether elevated apoptosis occurred in cancer cells expressing these aptamers. About 24–27% of cell deaths were observed in HeLa cells stably expressing 1A1 and 1A1(1–40), which was comparable with that caused by siRNA of NPM (33%). In comparison, NPM aptamer-expressing MCF-7 and SGC7901 cells showed a lower but significant proportion of cell death (5–12%). These findings suggest that NPM aptamers likely induced apoptosis. To prove this further, we performed a colonogenic survival assay in which cells were seeded at a very low density (see Materials and methods). Our results revealed that cells expressing 1A1 and 1A1(1–40) formed much fewer colonies than those expressing the control aptamer (Figure 5c). Furthermore, terminal transferase dUTP nick-end labeling (TUNEL) staining was performed to detect the apoptotic DNA fragmentation in these aptamer-expressing cells. Obvious TUNEL-positive nuclei were observed in MCF-7 and HeLa cells expressing 1A1 or 1A1(1–40), which were barely seen in cells expressing the control aptamer, indicating that apoptotic DNA fragmentation occurred in these cells (Figure 5d). Collectively, these results demonstrated that expression of NPM aptamers induced apoptosis in these cancer cells.

NPM aptamers synergize with DNA-damaging agents to induce apoptosis

Finally, we tested whether NPM aptamers could synergize with other chemicals to induce apoptosis by treating aptamer-expressing cells with different concentrations of cisplatin and etoposide, two most frequently used apoptosis-inducing drugs acting through DNA damage. In HEK293 and HeLa cells transiently expressing the aptamers 1A1 or 1A1(1–40), a low concentration (0.1 μM) of either cisplatin or etoposide induced significantly more apoptosis than in cells expressing the control aptamer, and such synergistic effect on cell killing became more prominent at a higher concentration (1.0 μM) of both drugs (Figures 6a–d), suggesting that NPM-specific aptamers can synergize with DNA-damaging agents to induce apoptosis in both non-cancer and cancer cells. Similarly, MCF-7 and SGC7901 cells stably expressing aptamers 1A1 and 1A1(1–40) showed an obvious increase in apoptosis compared to the control aptamer-expressing cells when treated with either cisplatin or etoposide at several different concentrations (Figures 6e–h), indicating that NPM-specific aptamers sensitized these cells to DNA-damage-induced apoptosis.

Discussion

In the present study, we used the SELEX assay to select small RNA molecules that can bind specifically to the antiapoptotic protein NPM with high affinity. From a starting synthetic RNA library containing about 10^{11} different molecules, we obtained four RNA aptamers, of which the majority shared very similar nucleotide sequences. Interestingly, the major RNA species obtained from our selection, the aptamer 1A1, bound to the NPM central acidic region but not to the C terminus that was shown to bind nucleic acids nonspecifically (Wang *et al*., 1994). This might result from that the NPM C terminus binds to nucleic acids with relatively low affinities whereas the SELEX procedure mainly enriched those aptamers folding up into unique structures capable of binding to the central region of NPM with much higher affinities. Remarkably, the aptamers 1A1 and 1A1(1–40) could interfere with the oligomerization of NPM as the interaction between GST-NPM and $NPMHis_6$ was obviously reduced by 1A1 and 1A1(1–40), and both aptamers released $NPMHis_6$ protein from the GST-NPM/$NPMHis_6$ protein complex. Moreover, the oligomerized form of NPM was strongly reduced in cells expressing these two aptamers. Therefore, despite that the N-terminal region (amino acids 1–113) of NPM was reported to oligomerize into pentamer or decamer (Namboodiri *et al*., 2004), our results suggest that the central region (amino acids 114–186) is important for the oligomerization of the intact NPM.

Nucleophosmin is primarily localized in the nucleolus. However, expression of the aptamers 1A1 and 1A1 (1–40) led to mislocalization of NPM in the nucleoplasm in several cancer cell types. It was less likely that these aptamers act by blocking the NuLS (amino acids 288–290) of NPM because they did not bind to the NPM C-terminal part containing the NuLS. Rather, these aptamers bound to the central region and affected the oligomerization of NPM, suggesting that oligomerization is required for NPM to localize to the nucleolus properly. These findings are consistent with a recent report that point mutations affecting NPM oligomerization abolish its nucleolar localization (Enomoto *et al*., 2006). Intriguingly, although NPM is mislocalized in the nucleoplasm in NPM aptamer-expressing cells, the pre-rRNA processing in these cells seemed not obviously affected (data not shown), suggesting that the nucleolar NPM is not essential for rRNA processing. Conversely, the aptamer-induced relocation of NPM likely caused p14ARF to localize to the nucleoplasm instead of the nucleolus. It was reported previously that NPM binds to and sequesters p14ARF in the nucleolus, which inhibits the function of p14ARF in promoting p53 stabilization

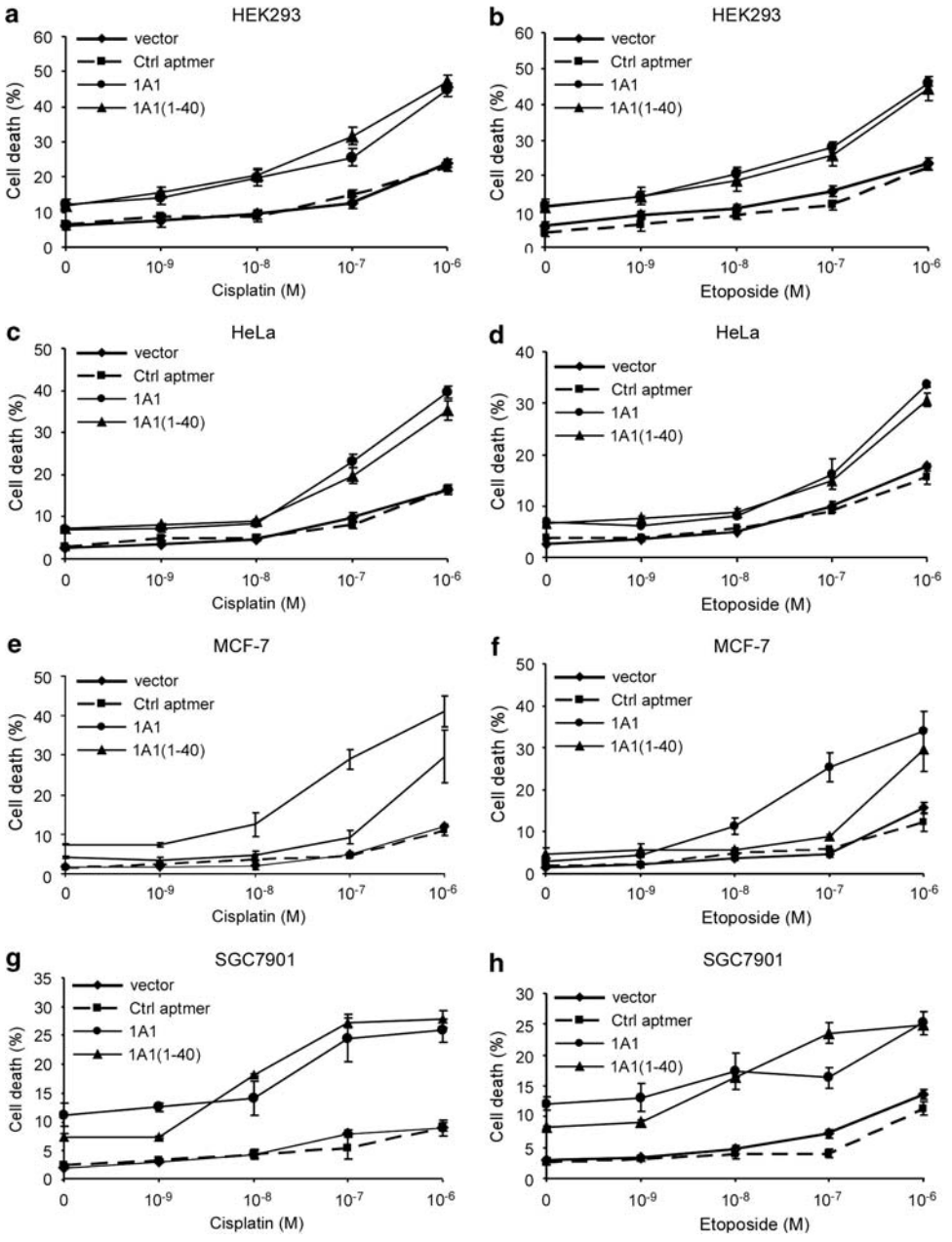

Figure 6 Nucleophosmin (NPM) aptamers synergize with DNA-damaging agents to induce cell death. (**a**–**d**) NPM aptamers sensitized HEK293 and HeLa cells to apoptosis induced by cisplatin and etoposide. HEK293 (**a**, **b**) and HeLa cells (**c**, **d**) were transfected with vectors expressing 1A1 and 1A1(1–40); 24 h later, cisplatin or etoposide at indicated concentration was added to the cells. Cell viability was determined by Trypan blue staining 48 h after addition of drugs. (**e**–**h**) NPM aptamers sensitized MCF-7 and SGC7901 cells to apoptosis induced by cisplatin and etoposide. MCF-7 (**e**, **f**) SGC7901 (**g**, **h**) cells stably expressing NPM aptamers were incubated with cisplatin or etoposide at indicated concentrations for 48 h. Cell viability was determined as above.

(Korgaonkar *et al*., 2005). In HeLa cells expressing NPM aptamers, p14ARF was found to localize mainly in the nucleoplasm, which may antagonize the degrading activity of Mdm2 so as to promote p53 stabilization. As a result, the increased p53 level could contribute to the elevated apoptosis observed in cells stably expressing NPM aptamers.

Our findings that disruption of NPM oligomerization and its nucleolar localization induced apoptosis of cancer cells suggest a feasible approach to identify small-molecule compounds for apoptosis-based cancer therapy. Similar to our findings, Qi *et al*. (2008) have identified a small molecule, NSC348884, as an NPM inhibitor. Although NSC348884 possibly binds to the N terminus of NPM, which is different from where NPM aptamers bind to, it can disrupt NPM oligomerization and cause an upregulation of p53 as do NPM aptamers. Nevertheless, it is still not known how this small compound increases p53 level to induce apoptosis. In addition, several other cytotoxic compounds have been found to cause mislocalization of NPM in nucleoplasm, including actinomycin D, daunomycin,

camptothecin, toyocamycin and aloe-emodin (Chan *et al*., 1999; Lee *et al*., 2005), but how they exert such effect is less understood. It will be interesting to determine whether they can bind to NPM and affect its function. Alternatively, it will be interesting to test whether these compounds can compete with the NPM-specific aptamers obtained in our study for binding to NPM, which will provide important mechanistic insight for understanding the cytotoxic effect of these compounds. As NPM-specific aptamers obtained in this research can act on their own or synergize with other cancer therapeutic drugs to induce apoptosis of cancer cells, they may serve as potential lead compounds for developing better strategies for cancer treatment in the future.

Materials and methods

Plasmids and recombinant proteins

Bacterial and mammalian expression vectors were constructed according to standard procedures (Sambrook and Russell, 2001). Mammalian expression vectors for aptamers were constructed by cloning aptamers into the RNAi vector pSIREN-DNR-DsRed-Express (Clonetech, Mountain View, CA, USA) with some modifications. Briefly, the original promoter of the U6 snRNA was replaced with a longer promoter region of the U6 snRNA (nucleotides −265 to +27). Aptamer cDNA followed by the U6 snRNA transcription terminator and a poly(U) sequence were cloned between the *Bam*HI and *Eco*RI sites. Recombinant NPM peptides were expressed in bacteria BL21(DE3) cells and purified with glutathione-Sepharose beads (Amersham, Pittsburgh, PA, USA) or Ni-NTA agarose beads (Qiagen, Hilden, Germany) according to the instructions provided by suppliers. GST-NPM/NPMHis$_6$ and GST-p53/NPMHis$_6$ complexes were achieved by co-expressing NPMHis$_6$ with GST-NPM or GST-p53, which were further purified and immobilized on glutathione-Sepharose beads (Amersham).

SELEX procedures

An initial RNA library of approximately 10^{11} RNA molecules was generated by *in vitro* transcription of DNA templates containing 49 randomized nucleotides flanked by fixed linker regions and a T7 promoter. SELEX was performed by using EMSA for the first five rounds and dot-blot assay for the last six rounds. The EMSA steps of SELEX were essentially performed as previously described (Yang *et al*., 2006). For dot-blot assay, different amount of NPMHis$_6$ was dotted on nitrocellulose membrane and incubated with ^{32}P-labeled RNA. After extensive wash, RNA bound to NPM on the membrane was detected with autoradiography and further recovered. At each round, the selected RNA was reverse-transcribed into single-stranded cDNA and further amplified into double-stranded DNA with standard procedures (Sambrook and Russell, 2001). The amplified DNA was used for next round of transcription and selection. After 11 rounds, cDNAs encoding the selected RNA aptamers were cloned into a vector and sequenced. The binding of individual aptamer to NPM was further determined by dot-blot assay as described above. The secondary structures of aptamers were predicted using the Mfold program (Zuker, 2003).

GST pulldown of RNA aptamers

^{32}P-labeled aptamer was prepared by *in vitro* transcription and incubated with 5 μg of glutathione *S*-transferase (GST), GST-NPM, GST-NPM$_{1-113}$, GST-NPM$_{114-186}$ and GST-NPM$_{187-294}$ proteins immobilized on glutathione-Sepharose beads in RNA-binding buffer for 30 min at 30 °C, the beads were then extensively washed and bound RNA was extracted with phenol/chloroform and resolved on 7.5% denature gel. Bound RNA was detected by autoradiography.

Cell culture and transfections

Human breast cancer MCF-7 cells, cervical cancer HeLa cells and gastric cancer SGC7901 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (HyClone, Novato, CA, USA). Transfections were performed using the calcium phosphate method or Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. To generate cell lines stably expressing aptamers, we co-transfected mammalian vectors expressing aptamers with pcDNA3.1hygro(+) (Invitrogen) and cells were selected in DMEM containing 10% FBS and 400 μg/ml hygromycin B for 2 weeks. The surviving colonies were picked and propagated to examine the expression of aptamers by reverse transcription (RT)–PCR.

Assays for interfering NPM oligomerization

Two assays were used to detect the effect of aptamers on NPM oligomerization *in vitro*. In the first assay, NPMHis$_6$ at a concentration of 5 μM was preincubated with *in vitro* transcribed aptamers at indicated concentrations (0, 1.0, 2.5, 5.0 μM, respectively) in a reaction mixture of 20 μl containing the same RNA binding buffer as above for 1 h at room temperature. The reaction mixture was further incubated with GST or GST-NPM (5 μg for each) immobilized on glutathione-Sepharose beads in 300 μl reaction mixture for another 2 h. The beads were washed extensively and bound NPMHis$_6$ was detected by western blotting using anti-His$_6$ antibody. In the second assay, purified GST-NPM/NPMHis$_6$ complexes (5 μg) immobilized on glutathione-Sepharose beads were incubated with aptamers (1.0 μM) in a reaction mixture of 100 μl for 1 h at room temperature. After spinning down, the released NPMHis$_6$ in half the supernatant (50 μl) was detected with anti-His$_6$ antibody.

To examine the *in vivo* effect of aptamers on NPM oligomerization, we prepared nuclear lysates from MCF-7 and HeLa cells stably expressing aptamers and resolved on native polyacrylamide gel. Samples were further blotted to nitrocellulose membrane for western blotting using anti-NPM antibody.

RNA aptamer immunoprecipitation

MCF-7 cells were transiently transfected with aptamer expression vectors. After 48 h, cells were harvested and treated with 1% formaldehyde and subsequently sonicated in a buffer containing 50 mM Tris-HCl (pH 7.5), 1% NP-40, 0.5% sodium deoxycholate, 0.5% SDS, 1 mM EDTA, 150 mM NaCl and 1 mM phenylmethylsulfonyl fluoride. Cell lysate was then incubated with mouse anti-NPM antibody for 1 h followed by incubation with protein A/G Sepharose beads for another 2 h. The beads were extensively washed and heated at 70 °C for 1 h to reverse the cross-link. Half the beads were used for western blot analysis and the other half was used to extract RNA by using the TRIzol reagent (Invitrogen). Extracted RNA was further reverse transcribed and amplified by PCR using aptamer-specific oligos.

Immunostaining

Cells were fixed in 4% paraformaldehyde and further permeabilized in 0.5% Triton X-100 and followed by sequential incubation with mouse anti-NPM or anti-Flag antibodies (1:100) and fluorescein isothiocyanate (FITC)-conjugated

anti-mouse IgG for 1 h at room temperature. Extensive wash was carried out between each step. Nuclei were stained by 4′,6-diamidino-2-phenylindole (DAPI). Fluorescent images were visualized using confocal laser scanning microscope (Nikon, Tokyo, Japan).

Apoptosis assays

Colonogenic survival assay was performed by seeding 800 cells stably expressing aptamers in DMEM supplemented with 10% FBS and 200 μg/ml hygromycin B in 10 cm dishes. After 3 weeks, colonies were fixed with 4% formaldehyde and stained with 0.2% methylene blue for visualization. TUNEL assay was performed by using TUNEL label mix (Roche, Nutley, NJ, USA) according to the protocol provided by the supplier. Synergistic cell killing of NPM aptamers and cisplatin or etoposide was determined by Trypan blue staining.

Conflict of interest

The authors declare no conflict of interest.

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