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Knockdown of NAPA using short-hairpin RNA sensitizes cancer cells to cisplatin: Implications to overcome chemoresistance

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ABSTRACT

Cisplatin is a widely used anti-cancer drug which targets DNA in replicating cells. In the present study, we found that NAPA—a protein found in the endoplasmic reticulum (ER) and implicated in protein trafficking—protects cells against cisplatin. Accordingly, knockdown of NAPA using lentivirus-encoded shRNA (shNAPA) induced ER stress similar to cisplatin treatment in HEK293 cells. A low dose of cisplatin also elicited a mild ER stress response associated with the accumulation of the protective proteins BiP and NAPA. Remarkably, knockdown of NAPA induced apoptosis and enhanced cisplatin-induced cytotoxicity/apoptosis, thereby sensitizing cancer cells to cisplatin. On the other hand, overexpression of NAPA increased resistance to cisplatin by reducing cisplatin-induced ER stress and apoptosis. The modulatory effects of shNAPA required the tumor suppressor p53 since the effects of NAPA knockdown were reduced by the p53 inhibitor PFT-α and in H1299 cells which are p53-null. A partial reversal of cisplatin resistance was also observed in cisplatin-resistant HeLa cells following knockdown of NAPA. Our results also indicated that calpain is required for ER-mediated apoptosis. Importantly, combined cisplatin/shNAPA treatment suppressed tumor growth *in vivo* in xenograph experiments performed in nude mice. Taken together, these observations suggest that NAPA represents a target of cisplatin, and that knockdown of NAPA may improve cisplatin-based cancer therapy.

1. Introduction

Cisplatin is an anti-cancer agent used for the treatment of various human cancers. This chemotherapeutic agent is known to target chromosomal DNA and to induce apoptosis in actively replicating cells [1,2]. However, the development of cisplatin resistance is a serious problem which limits the efficacy of this cancer treatment. The mechanisms responsible for chemoresistance have been investigated intensively in the past [3–5]. These mechanisms include inadequate drug exposure and genetic alterations in cancer cells [6]. Accordingly, several studies have reported alterations in chemoresistant cancer cells, including altered membrane functions (i.e. increased drug efflux and decreased drug influx), cytoplasmic activation of detoxification systems, and altered nuclear functions (i.e. alterations of the drug's targets, increased DNA repair, and resistance to apoptosis). Chemoresistance may also be associated with a dysregulation in the expression of oncogenes and tumor suppressors [7–11]. While several cellular targets of cisplatin have been identified in the past, less is known about the molecules implicated in cell death and acquired chemoresistance resulting from cisplatin treatment.

The membranous endoplasmic reticulum (ER) in eukaryotic cells consists of a reticular network of membrane tubules, sheet-like structures, and lamellae [12,13]. The ER participates in several cellular processes, including trafficking of secretory and membrane proteins, lipid synthesis, stress response, and Ca^{2+} storage. The anti-apoptotic proteins Bcl-2 and Bcl-xL, as well as the pro-apoptotic Bax and Bak, have been shown to be located in both the ER and in mitochondria [14–17]. These proteins appear to regulate apoptosis partially by affecting Ca^{2+} storage in the ER. For instance, Bcl-2 can reduce the amount of Ca^{2+} released by the ER [18,19], whereas Bax and Bak act in the opposite way [20,21]. The pro-apoptotic BH3-only proteins [22], such as BNIP1 [23] and spike [24], are also located in the ER. Although mitochondria are known to be central to the process of apoptosis, the ER has also emerged as

Abbreviations: ALLN, N-acetyl-leucyl-leucyl-norleucinal; BH3, BCL-2 homology domain 3; BiP, binding immunoglobulin protein; BNIP1, BCL2/adenovirus E1B 19-kDa-interacting protein 1; CDDP, cisplatin; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; ERAD, ER-associated degradation; GFP, green fluorescent protein; HPV, human papillomavirus; Luc, luciferase; MDM2, murine double minute 2; MF, modification factor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAPA, NSF attachment protein α; NSF, N-ethylmaleimide-sensitive factor; ORF, open-reading frame; PARP, poly-ADP ribose polymerase; PCR, polymerase chain reaction; PFT-α, pifithrin-α; PIRH2, p53-induced RING-H2 domain protein; RT, reverse transcription; shRNA, short-hairpin RNA; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor; VSVG, vesicular stomatitis virus protein G.

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an important organelle in this process [25–27]. As such, BNIP1—which is part of the syntaxin-18 complex, a soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein (SNAP) receptor (SNARE) located in the ER—was shown to be implicated in maintaining the integrity of the ER network. Consistent with the possibility that these proteins regulate apoptosis, forced expression of NAPA, also known as α-SNAP, markedly delayed staurosporine-induced apoptosis in HeLa cells [28]. Similarly, loss of either NSF or SNAP protein led to a blockage of membrane trafficking, which was associated with the accumulation of SNARE complexes, and ultimately with cell death [29–33]. These observations suggest that NAPA may prevent apoptosis, possibly by competing with anti-apoptotic proteins for the BH3 domain of BNIP1.

It has been demonstrated earlier that cisplatin induces ER stress, and that this process is associated with nucleus-independent apoptotic signaling [34]. Here, we show that NAPA protects the cell from undergoing apoptosis in response to cisplatin. Accordingly, knockdown of NAPA using shRNA was shown to effectively sensitize cells to cisplatin in a p53-dependent manner. In line with these results, a combination of cisplatin and shNAPA could be used to reverse acquired cisplatin resistance in cancer cells.

2. Materials and methods

2.1. Cell lines and reagents

The cells used in this study were maintained in Dulbecco's modified Eagle's medium (Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml, Gibco), and streptomycin (100 μg/ml, Gibco) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. The cell lines used included human embryonic kidney cells (HEK293) and tumorigenic cell lines (cervix HeLa, ovarian Sk-ov-3, lung H1255 and H1299 cells were obtained from the American Tissue Type Collection, Manassas, VA, USA; nasopharyngeal CG-1 cells were a gift from Prof. Y.S. Chang at Chang Gung University). HeLa cisplatin-resistant variants HR1 and HR3 were obtained as described previously [35]. In some experiments, p53-null H1299 cells were transfected with the wild-type p53 expression plasmid pcep4-p53 (a generous gift from Dr. Y. S. Lin from Academia Sinica), using 2 mg/well of plasmid along with 3 ml/well of Lipofectamine (Invitrogen, Carlsbad, CA, USA) for 48 h. The cells were cultured in 6-well plates at 1.5×10^4 cells/well. Transient p53 protein expression in H1299 cells was driven by the cytomegalovirus (CMV) promoter. The chemotherapeutic agents cisplatin, vincristine, and taxol (also known as paclitaxel) were purchased from Bristol-Myers Squibb (New York, NY, USA). PFT-α was obtained from Biomol Research Laboratories (Plymouth Meeting, PA, USA). Other chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA). All reagents were prepared according to the instructions provided by the supplier.

2.2. Quantitative real-time reverse transcription-PCR

Quantitative reverse transcription-polymerase chain reaction (RT-PCR or qPCR) was performed on total RNA extracted from cells with the Trizol reagent (Invitrogen) using 200 nM of primers as described [36]. Primers for NAPA (GenBank sequence number: NM_003827) and GAPDH (NM_000996) were designed using Primer Express 2.0.0 (Applied Biosystems, Foster City, CA, USA). The resulting primers were as follows: NAPA, forward, 5′-GCGGAGCGCAAAGTGAAG-3′; reverse, 5′-TCGGCTTTCTTGAATGCGTT-3′; and GAPDH, forward, 5′-TCCTGCACCACCAACTGCTT-3′; reverse, 5′-GAGGGGCCATCCACGTCTT-3′. All samples and controls were used in triplicate on the same plate. Relative quantification was calculated using the ΔΔCt method with normalization to GAPDH. Namely, the ΔCt for each candidate was represented as ΔCt (candidate) = [Ct (candidate) − Ct (GAPDH)]. The relative abundance of the candidate gene X was shown as $2^{\Delta Ct(X)-\Delta Ct(GAPDH)}$.

2.3. Plasmids, transfection, cell extracts, and immunoblot analysis

NAPA cDNA sequence (NM_003827) was isolated by PCR using the following NAPA primer pairs: forward, 5′-GCTTTGCTGAGTCCCTTTGT-3′; reverse, 5′-AAAGGAGGGAAGCTCTCCAG-3′. The resulting amplicon was cloned into the pGEM-T easy vector. NAPA expression plasmid was constructed by removing NAPA open-reading frame (ORF) sequence from pGEM-T easy vector, and by inserting the ORF into the pcDNA3 vector using the restriction enzymes EcoRI and XhoI, thus resulting in the formation of pcDNA3-NAPA. Cells were transfected with plasmid cDNA to express NAPA as described [36]. Total protein extract for immunoblotting was prepared as described [37]. Fifty-μg protein extracts were separated using 12% SDS-PAGE, followed by transfer onto PVDF membranes, and incubation with primary antibodies raised against the following proteins: NAPA (Abcam, Cambridge, MA, USA), cleaved caspase-3 (Cell Signaling Technology, Danvers, MA, USA), GAPDH (FL-335), β-actin, p53 (DO-1), Bax (N-20), caspase-12 (M-108), caspase-4, PARP (H-250), calpain (H-240), ATF-6, or GFP (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA). The BiP antibody (diluted 1:3000) was generated by immunizing New Zealand rabbits with the full-length BiP protein. The membranes were then incubated with secondary antibodies: goat anti-mouse or goat anti-rabbit-horseradish peroxidase (Amersham, Buckinghamshire, UK). The resulting signal was visualized by enhanced chemiluminescence according to specifications from the supplier (Pierce, Rockford, IL, USA). The intensity of the protein bands was determined by scanning X-ray films through a scanning densitometer (GS 300, Hoefer, Holliston, MA, USA).

2.4. Knockdown of NAPA gene using shRNA

pLKO.1 plasmids expressing shRNA to knockdown NAPA were purchased from the National RNAi Core Facility (Academia Sinica, Taipei, Taiwan). A recombinant plasmid expressing luciferase-shRNA (shLuc; TRCN0000072244) was used as a negative control. Five recombinant plasmid clones that expressed shNAPA were tested for knockdown efficiency in HEK293 cells, which can be readily transfected. The plasmid expressing shNAPA (TRCN0000029169), which was the most effective in inhibiting NAPA expression, was used in this study. Transient transfection of the shRNA plasmids was performed by adding 2 μg/well of the plasmids with 3 μl/well Lipofectamine into cells maintained in 6-well plates (1.5×10^4 cells/well) according to instructions from the manufacturer. Furthermore, the stable cell clones which were inefficient for plasmid transfection were infected with recombinant lentivirus expressing shNAPA. The recombinant lentivirus carried puromycin-resistance gene for selection as described by the supplier (National RNAi Core Facility).

2.5. Stable overexpression of NAPA

HEK293 cells were infected with recombinant lentivirus carrying NAPA cDNA and a puromycin-resistance gene. Following culture in puromycin-containing medium for 2 weeks, two independent cell populations (i.e. NAPA#1 and NAPA#2) were established. Puromycin-resistant NAPA#1 and NAPA#2 cells were considered as 2 independent cell clones.

2.6. Indirect fluorescence microscopy

To visualize ER-to-Golgi protein transport, we used the viral glycoprotein ts045 VSVG tagged with green fluorescent protein (VSVG-GFP), pCDM8.1/VSVGts045-GFP (see Ref. [38]; kindly provided by Dr. Jennifer Lippincott-Schwartz of the National Institutes of Health, USA). The VSVG expression plasmid was transfected into HEK293 cells with or without NAPA knockdown. The VSVG-GFP location in cells was then visualized by fluorescence microscopy.

2.7. Cell viability, apoptosis, and FACS analysis

Cells were treated with cisplatin, vincristine, or taxol in serum-free medium for 2 h, and were subsequently cultured in drug-containing and serum-containing medium for 3 days unless indicated otherwise. Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay as described [34]. The percentage of cell viability was calculated as the ratio of total cells divided by the total number of cells counted. Cells with apoptotic nuclear phenotype were counted as described [37]. Percentage of apoptotic cell death was calculated as the ratio of dead cells divided by the total number of cells counted. To analyze drug-induced apoptosis, we prepared cell extracts for Western blot with antibodies specific for apoptotic markers (cleaved caspase-3 and cleaved PARP; see Ref. [37]). To confirm apoptosis, sub-G1 cells were measured by flow cytometry as described [39]. Three independent experiments were performed unless indicated otherwise. The data were reported as mean values ± standard deviation (SD). Statistical significance (p value) was calculated with a two-tailed Student's t-test for single comparison. The symbol * denotes $p < 0.05$; ** denotes $p < 0.01$.

2.8. Animals

Six-week-old female nude mice were purchased from the National Laboratory Animal Center (NLAC, Taiwan). Tumor cells were obtained by subcutaneous injection of 5×10^6 of either HR3-shLuc or HR3-shNAPA cells into 12 nude mice each. Six mice of the HR3-shLuc group were mock-treated, while another group of 6 mice were treated intraperitoneally every 6 days with cisplatin (8 mg/kg). The HR3-shNAPA group was treated the same way. Tumor growth was monitored at a regular interval by measuring two tumor diameters using calipers. Tumor volume was calculated with the following formula: $(d^2 \times D)/2$, where d and D represent respectively the shortest and longest tumor diameters. When tumors with a size of approximately 150 mm^3 were detected, cisplatin injections were started for 30 days until the mark of 80 days was reached.

3. Results

3.1. Knockdown of NAPA induces ER stress and potentiates cisplatin-induced apoptosis in HEK293 cells

In order to address the possibility that NAPA plays a role in response to DNA-damaging agents, we verified the effects of NAPA knockdown on HEK293 cells treated with cisplatin. Knockdown of NAPA mRNA and NAPA protein reached 95% in these experiments (Fig. 1A and B, respectively). To verify whether protein trafficking was altered following knockdown of NAPA, the VSVG-GFP plasmid was transiently overexpressed in HEK293 cells along with shNAPA or shLuc control (data not shown). We found that trafficking of the VSVG-GFP protein to the cell membrane was reduced following knockdown of NAPA (data not shown), therefore confirming that this protein plays a role in protein trafficking. Next, we verified whether NAPA knockdown induces ER stress by monitoring the level of BiP isoforms and the cleavage of ATF-6 by western blotting. We first observed that BiP accumulated and that ATF-6 was cleaved in response to the Ca^{2+} ionophore A23187, a compound known to induce ER stress and used here as a positive control (Fig. 1C, lanes 1–3; see also Ref. [40]). Interestingly, NAPA accumulated to a low level following treatment with A23187. Treatment with a low dose of cisplatin (5 μM) also induced BiP and NAPA accumulation to a lesser degree (Fig. 1C, lanes 4–6). Expression of the control shLuc did not affect the level of BiP following cisplatin treatment (Fig. 1C, lanes 7–9). In contrast, BiP accumulated to a high level following knockdown of NAPA (Fig. 1D, compare lane 10 with lane 7). Low dose of cisplatin further increased the level of BiP in cells expressing shNAPA (Fig. 1C, compare lanes 11 and 12 with lane 10). When the density of the two bands corresponding to BiP was quantified, we observed that BiP increased in a dose-dependent manner with the dose of cisplatin (Fig. 1D). In addition, knockdown of NAPA induced a 2-fold BiP increase when compared to either control non-treated HEK293 cells or HEK293 cells expressing shLuc (Fig. 1D). These results indicate that knockdown of NAPA elicits ER stress in HEK293 cells. In addition, these data suggest that NAPA knockdown may also promote cisplatin-induced ER stress in these cells.

It had been shown earlier that low level of BiP protein protects both tumorigenic and non-tumorigenic cells against ER stress [41–43]. To verify whether the increase of BiP protein seen above following knockdown of NAPA protects HEK293 cells against cisplatin, we monitored the cell cycle of HEK293 cells expressing shNAPA following treatment with a high dose of cisplatin (5 μM). In this case, we observed that the cells expressing shNAPA showed a higher level of apoptosis when compared to cells expressing shLuc (Fig. 1E). Notably, a low dose of shNAPA (2 μg/μl; added along with 2 μg/μl of plasmid DNA coding for shLuc), which produced knockdown of NAPA near 50%, was found to produce around 2% more apoptotic cells than the control shLuc (Fig. 1E, compare open bars). When we doubled the dose of shNAPA (4 μg/μl; knockdown of NAPA of 85%), we observed that apoptotic cells were 7% higher than the control shLuc (Fig. 1E). With high doses of shNAPA and cisplatin, we observed that apoptotic cells represented around 50% of the total cell population compared to 11% and 3% for the low dose of shNAPA and the control shLuc, respectively (Fig. 1E, compare closed bars). Therefore, knockdown of NAPA was shown to induce apoptosis, even without exposure to cisplatin (Fig. 1E). In addition, NAPA knockdown, even though it induced BiP accumulation as shown above, could sensitize HEK293 cells to cisplatin, suggesting that the protective role of BiP in stressed cells may require NAPA. Besides, cells expressing shNAPA also showed a larger population of sub-G1 cells when compared to cells expressing shLuc (Supplementary Fig. S1). The induction of apoptosis following knockdown of NAPA in HEK293 cells was further confirmed by the enhanced activation of caspase-3 as well as by the cleavage of PARP, a substrate of activated caspase-3 (Fig. 1F). Knockdown of NAPA alone using a high dose of plasmid DNA (4 μg/μl) not only induced PARP cleavage on its own but it also enhanced cisplatin-induced PARP cleavage (Fig. 1F). It has been shown earlier that cisplatin-induced apoptosis in human lung adenocarcinoma cells in a pathway that implicated both the ER and the calpain protein [44]. To verify whether a similar scenario applies here, we explored whether calpain plays a role in the cell system under study. We found that knockdown of NAPA induced calpain accumulation and enhanced cisplatin-induced calpain accumulation and caspase activation (Supplementary Fig. S2 [53,54]). Taken together, these results suggest that knockdown of NAPA not only has a pro-apoptotic effect on HEK293 cells, but it also sensitizes cells to cisplatin by enhancing apoptosis.

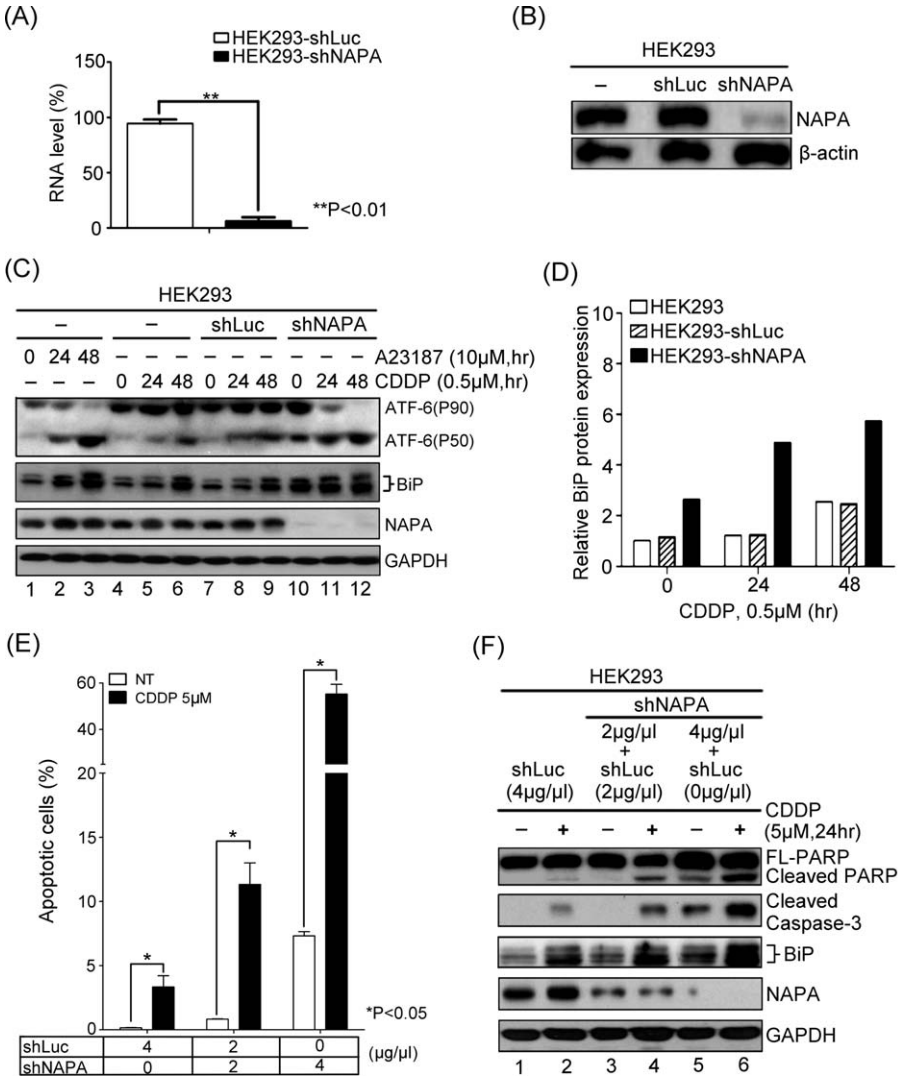

Fig. 1. Knockdown of NAPA induces ER stress and potentiates cisplatin-induced apoptosis in HEK293 cells. (A) Relative NAPA mRNA level determined by qPCR. shLuc was used as a negative control. (B) Knockdown of NAPA using shNAPA as determined by Western blotting. (C) BiP accumulation by shNAPA and cisplatin. A23187 was used as a positive control for the induction of ER stress markers. (D) Quantification of BiP from panel C (lanes 4–12). BiP protein level in untreated HEK293 cells represents a value of one. (E) Enhancement of cisplatin-induced apoptotic cell population following knockdown of NAPA. NT, no (CDDP) treatment. (F) Enhancement of cisplatin-induced caspase-3 activation and PARP cleavage following knockdown of NAPA. Note that shNAPA alone also induced caspase-3 activation. *p* values are indicated.

3.2. Knockdown of NAPA induces apoptosis and sensitizes cells to cisplatin in a p53-dependent manner

p53 is known to play a critical role in response to cisplatin. A recent study has shown that the ER-resident ubiquitin ligase "synoviolin" promotes the cytoplasmic degradation of p53 independently of other E3 ubiquitin ligases (i.e. MDM2, Pirh2, and Cop1; see Ref. [45]). To assess whether ER stress induced by the knockdown of NAPA could impair ER-mediated p53 degradation, we monitored the level of p53 protein in HEK293 cells following knockdown of NAPA. We first observed that p53 accumulated following knockdown of NAPA (Fig. 2A, compare lanes 1 and 4). As expected, we also observed that cisplatin induced the accumulation of p53 (Fig. 2A, lanes 2–4). Notably, the level of p53 in cisplatin-treated cells was considerably higher following knockdown of NAPA (Fig. 2A, compare lanes 2–4 with lanes 6–8). Similarly, cisplatin elicited Bax accumulation in a dose-dependent manner in cells expressing shLuc (Fig. 2A, lanes 1–4), and the level of Bax further increased following knockdown of NAPA (Fig. 2A, lanes 5–8).

To assess whether shNAPA-induced Bax in a p53-dependent manner, we treated cells expressing either shNAPA or control shLuc with PFT-α, a reversible inhibitor of p53, prior to cisplatin treatment. In control cells expressing shLuc, the accumulation of p53 and Bax following cisplatin treatment could be reduced by PFT-α (Fig. 2B, lane 2 vs. lane 3). Notably, the accumulation of p53 and Bax in shNAPA-expressing cells was also inhibited by PFT-α (Fig. 2B, lane 5 vs. lane 6). We also monitored the viability of the cells expressing shNAPA using the MTT assay (Fig. 2C). Sensitization to cisplatin was expressed as a sensitization factor (SF) which represents the IC_{50} of shLuc-expressing cells divided by the IC_{50} of shNAPA-expressing cells. Knockdown of NAPA sensitized cells to cisplatin 5-fold compared to shLuc control (SF = 5.33). On the other hand, this effect was partially reversed by treatment with PFT-α (SF = 2.67). Notably, PFT-α treatment appeared to improve the viability of shLuc-expressing cells treated with cisplatin (Fig. 2C). We also monitored the level of apoptosis of the various cells treated with cisplatin for either 24 or 48 h (Fig. 2D). For both time periods, apoptotic cells were shown to accumulate after knockdown of NAPA compared to control shLuc (Fig. 2D, $p < 0.05$). Cisplatin treatment further enhanced the accumulation of apoptotic cells, and this accumulation was prevented by PFT-α in cells expressing either shLuc or shNAPA (Fig. 2D). The decrease of sub-G1 cells by PFT-α was more pronounced in cells expressing

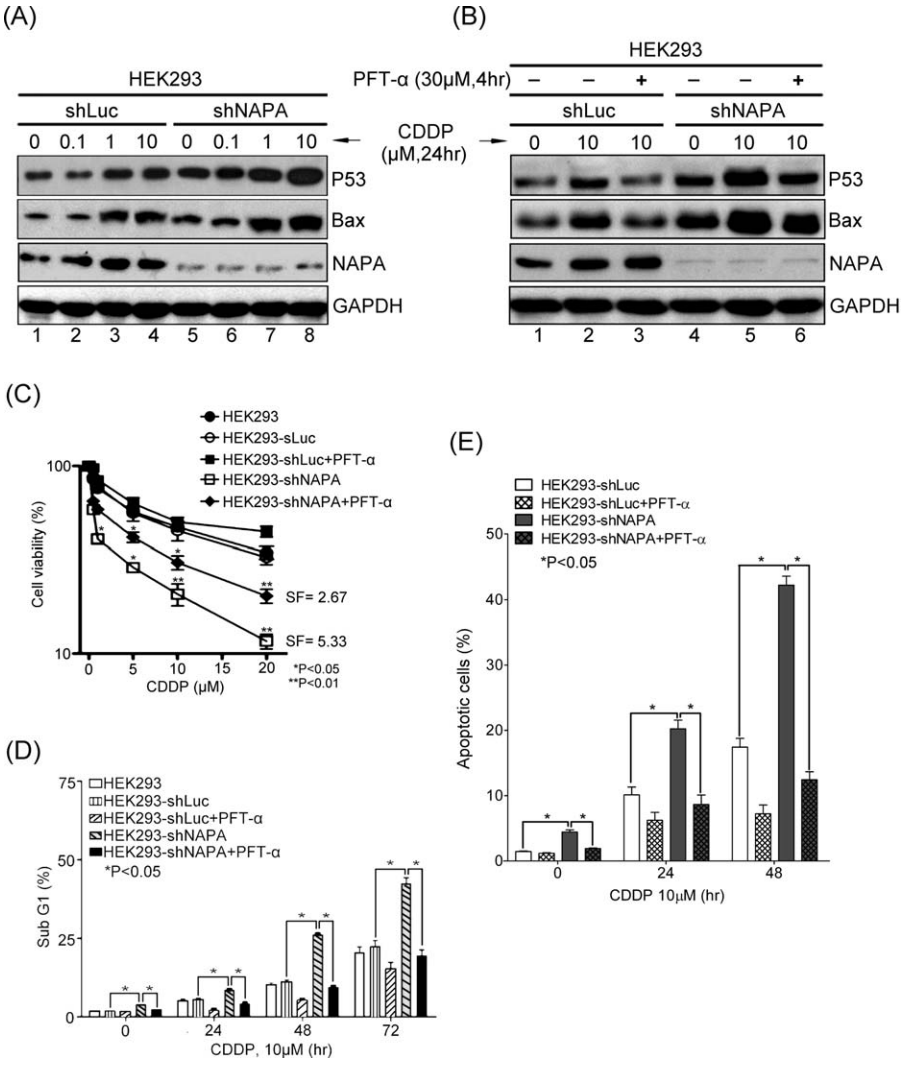

Fig. 2. Knockdown of NAPA sensitizes cells to cisplatin in a p53-dependent manner. (A) Accumulation of p53 and Bax by shNAPA/cisplatin treatments. HEK293 cells were treated with the indicated concentration of cisplatin for 24 h. (B) Reduction of shNAPA-induced Bax expression by PFT-α. (C) Enhancement of cisplatin-induced cytotoxic effect by shNAPA and reversal of this cellular response by PFT-α. Cells were treated as for panel B, and assayed by the MTT assay (see Section 2 for details). (D) Enhancement of cisplatin-induced sub-G1 cell population by shNAPA, and reversal of this response by PFT-α. The cells were treated as in C but were assayed by flow cytometry. (E) Enhancement of cisplatin-induced apoptotic cells by shNAPA, and reversal of this response by PFT-α. The cells were treated as in C but were assayed by counting apoptotic cells. *p* values are indicated.

shNAPA (Fig. 2D, only the *p* values for this group are shown). In addition, the cell cycle of the various treated cells was monitored using flow cytometry. In this case, apoptotic sub-G1 cells were shown to accumulate after knockdown of NAPA compared to control shLuc (Fig. 2E, $p < 0.05$). Cisplatin treatment further induced the accumulation of sub-G1 cells, and this phenomenon could be reduced by PFT-α (Fig. 2E). The decrease of sub-G1 cells by PFT-α was more pronounced in cells expressing shNAPA (Fig. 2E). These results indicate that knockdown of NAPA induces apoptosis and sensitizes cells to cisplatin in a p53-dependent manner.

3.3. Reduced sensitization to cisplatin following knockdown of NAPA in p53-null cells

To confirm the role of p53 in sensitizing cells to cisplatin following knockdown of NAPA, we performed further experiments with p53-null H1299 cells. Surprisingly, we observed that, following treatment with cisplatin, Bax accumulated to similar levels in H1299 cells expressing either shLuc or shNAPA (Fig. 3A, lanes 2 and 4). These results suggest that Bax may be regulated in a p53-independent manner. HEK293 cells were also treated in the same way as additional controls (Fig. 3A, lanes 5–8). In these cells, p53 and Bax protein level increased following either cisplatin treatment or knockdown of NAPA (Fig. 3A, compare lanes 6 and 7 with lane 5). The level of p53 and Bax in shNAPA-expressing cells further increased following treatment with cisplatin (Fig. 3A, compare lane 8 with lane 7). Furthermore, we also assessed the effects of restoring p53 expression in H1299 cells (see Section 2 for details; Fig. 3B). NAPA knockdown was shown to enhance the level of both cleaved caspase-3 and cleaved PARP in H1299 cells expressing exogenous p53 compared to controls (Fig. 3B, compare lanes 1 and 4). Both p53 and its transactivation target Bax accumulated following cisplatin treatment in H1299 cells over-expressing shLuc (Fig. 3B, lanes 1–3). This observation is probably due to cisplatin-induced post-translational modifications of p53, such as Ser46 phosphorylation, which leads to transactivation of Bax gene expression [46]. p53 and Bax accumulation was further enhanced in cells overexpressing shNAPA (Fig. 3B, lanes 4–6). When we monitored the viability of H1299 cells in response to cisplatin, we observed that knockdown of NAPA sensitized these cells to cisplatin when compared to shLuc (Fig. 3C). The exogenous expression of p53 in H1299 cells further increased this sensitiza-

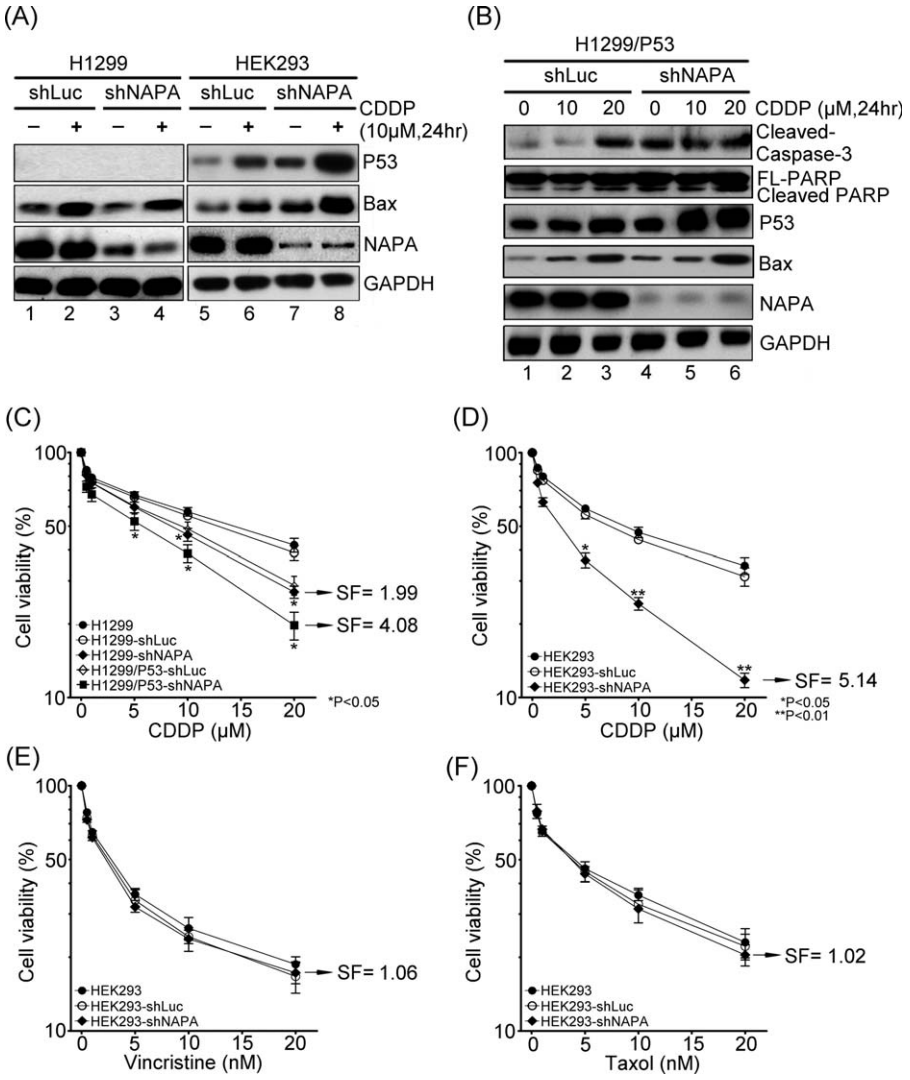

Fig. 3. Reduced sensitization to cisplatin by shNAPA in p53-null H1299 cells. (A) Lack of Bax accumulation following knockdown of NAPA. Bax was found to accumulate after a 10-μM-cisplatin treatment for 24 h in these cells but no increase was noticed following NAPA knockdown (lanes 1–4). Experiments were performed in parallel in HEK293 cells as control (lanes 5–8). (B) Effect of NAPA knockdown on H1299 cells that overexpress exogenous p53. H1299 cells which overexpress either shLuc (lanes 1–3) or shNAPA (lanes 4–6), were transfected with exogenous p53 for 48 h, prior to treatment with the indicated concentrations of cisplatin for 24 h. Fifty μg of total protein extracts were used for immunoblotting experiments to detect cleaved caspase-3 and PARP as well as protein levels of p53, Bax, NAPA, and GAPDH. (C) Low sensitization to cisplatin in H1299 cells that express shNAPA. (D) Sensitization to cisplatin by shNAPA in HEK293 cells. (E) Lack of sensitization effect to vincristine in H1299 cells that express shNAPA. (F) Lack of sensitization effect to taxol in shNAPA-expressing H1299 cells. Data in panels B–D were calculated from MTT assays. The extent of sensitization effects produced by shNAPA vs. shLuc was indicated for each panel. Knockdown (K.D.) efficiency of cells in panel C was the same as panel B, and efficiency for panels D–F was the same as panel A. *p* values are indicated.

tion effect to cisplatin (Fig. 3C, H1299/p53-shNAPA, SF = 4.08). The cytotoxic effect of cisplatin was also enhanced by exogenous p53 expression in shLuc-expressing H1299 cells (H1299/p53-shLuc) (SF = 1.99) compared to the shLuc cell group. Sensitization to cisplatin in HEK293 cells following knockdown of NAPA was also observed (Fig. 3D, SF = 5.14). The efficacy of NAPA knockdown in these experiments reached respectively 90% for H1299 and 90% for HEK293 cells, indicating that the reduced effect of shNAPA in H1299 was not due to low gene knockdown. Besides, knockdown of NAPA did not influence the viability of HEK293 cells in response to the mitotic damaging agents vincristine or taxol as assayed by both cell viability assay (Fig. 3E and F) and activation of caspase-3 (data not shown). These results indicate that the pro-apoptotic effects of NAPA knockdown are dependent on the level of p53.

3.4. Overexpression of NAPA protects cells against cisplatin

Since we observed that NAPA knockdown sensitized HEK293 cells to cisplatin, we hypothesized that overexpression of NAPA would on the other hand reduce sensitivity to cisplatin. To verify this possibility, we established two stable cell lines that overexpressed NAPA (NAPA#1 and NAPA#2). Profound accumulation of NAPA was detected in these cells following cisplatin treatment compared to either non-transfected cells or cells transfected with GFP (Fig. 4A). All cells treated with cisplatin showed accumulation of BiP, calpain, p53, and Bax. Caspase-7 and caspase-3 were also cleaved following cisplatin treatment. We observed that calpain also accumulated in following knockdown of NAPA (Supplementary Fig. S3A and B). On the other hand, the extent of protein increase (i.e. BiP, calpain, p53, and Bax) and the cleavage of caspase-7 and caspase-3 appeared to be reduced following overexpression of NAPA. Accordingly, quantification of band density showed a reduction of BiP (Fig. 4B) as well as p53 and Bax (data not shown) in NAPA-overexpressing HEK293 cells. The cytotoxic effect of cisplatin was profoundly reduced in cells overexpressing NAPA, with the resistance factor (RF) being calculated as the IC_{50} of NAPA-overexpressing cells divided by the IC_{50} of GFP-overexpressing cells (Fig. 4C, RF = 2.50 and 2.51 for

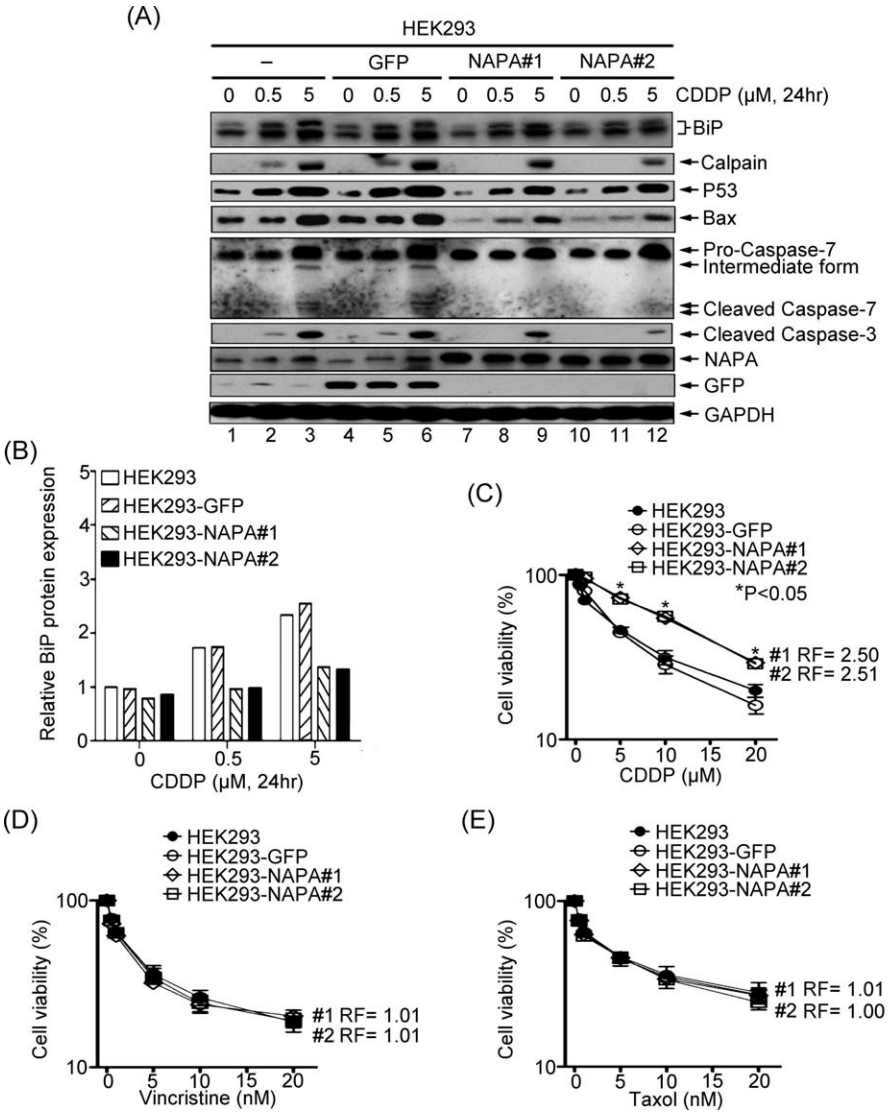

Fig. 4. Protection against cisplatin by forced expression of NAPA in HEK293 cells. (A) Reduction of ER stress by overexpression of NAPA. Two stable cell clones (NAPA#1 and NAPA#2) were treated with cisplatin, and cell extracts were immunoblotted with antibodies for the indicated proteins. HEK293 cells without transfection (lanes 1–3) and a stable cell clone overexpressing GFP (lanes 4–6) were used as controls. Cisplatin-induced p53 and Bax levels were profoundly reduced in cells overexpressing NAPA. (B) Decrease of BiP level by NAPA overexpression. The level of BiP protein was quantified from the experiments shown in panel A. (C) Increased resistance to cisplatin in cells that overexpress NAPA. (D) Lack of resistance to vincristine in cells that overexpress NAPA. (E) Lack of resistance to taxol in cells that overexpress NAPA. *p* values are indicated.

NAPA#1 and NAPA#2, respectively). Notably, overexpression of NAPA did not influence the sensitivity of the cells to either vincristine or taxol (Fig. 4D and E). These results suggest that forced expression of NAPA may rescue both cisplatin-induced ER stress and p53-mediated cisplatin cytotoxicity.

3.5. Reversal of cisplatin resistance following knockdown of NAPA in tumorigenic cells

Next, we verified whether knockdown of NAPA could reverse resistance to cisplatin in tumorigenic cells. We first used HeLa cells HR1 and HR3 which acquired resistance to cisplatin following repeated cycles of exposure to the drug [35]. We observed that the mRNA and protein levels of NAPA were up-regulated in HR1 and HR3 cells compared to parental HeLa cells (Fig. 5A and B). PARP cleavage was enhanced by knockdown of NAPA in HR3 cells (Fig. 5C, compare lanes 4–6 with lanes 7–9). We observed that shNAPA-expressing cells were profoundly sensitized to cisplatin in growth inhibition assays, with the HR3-cisplatin-resistant cells being sensitized to a much higher degree (Fig. 5D and E; note that HeLa and HR3 cells were treated with equitoxic doses in these experiments). As such, the shNAPA treatment sensitized HR3 cells more than 12-fold compared to 2.67-fold for the parental HeLa cells (Fig. 5D and E). It should be noted that, as seen in HEK293 cells, ER stress markers were also induced by shNAPA/cisplatin in these cancer cells (Supplementary Fig. S3).

Furthermore, the apoptotic cell population increased following prolonged treatment of cisplatin (1 μM or 5 μM for HeLa; 10 μM or 50 μM for HR3) in shNAPA-expressing cells as opposed to shLuc ones (Fig. 5F and G). NAPA protein levels following NAPA knockdown were shown for reference (Fig. 5F and G). Consistent with HEK293 cells, no sensitization effect was produced by shNAPA in cells treated with either vincristine or taxol (data not shown), suggesting that the sensitization effect may be specific to DNA-damaging agents. Interestingly, cells that were not treated with cisplatin showed more apoptotic cells in the shNAPA group compared to the shLuc one. The sub-G1 cell population was also increased following prolonged cisplatin treatment in shNAPA-

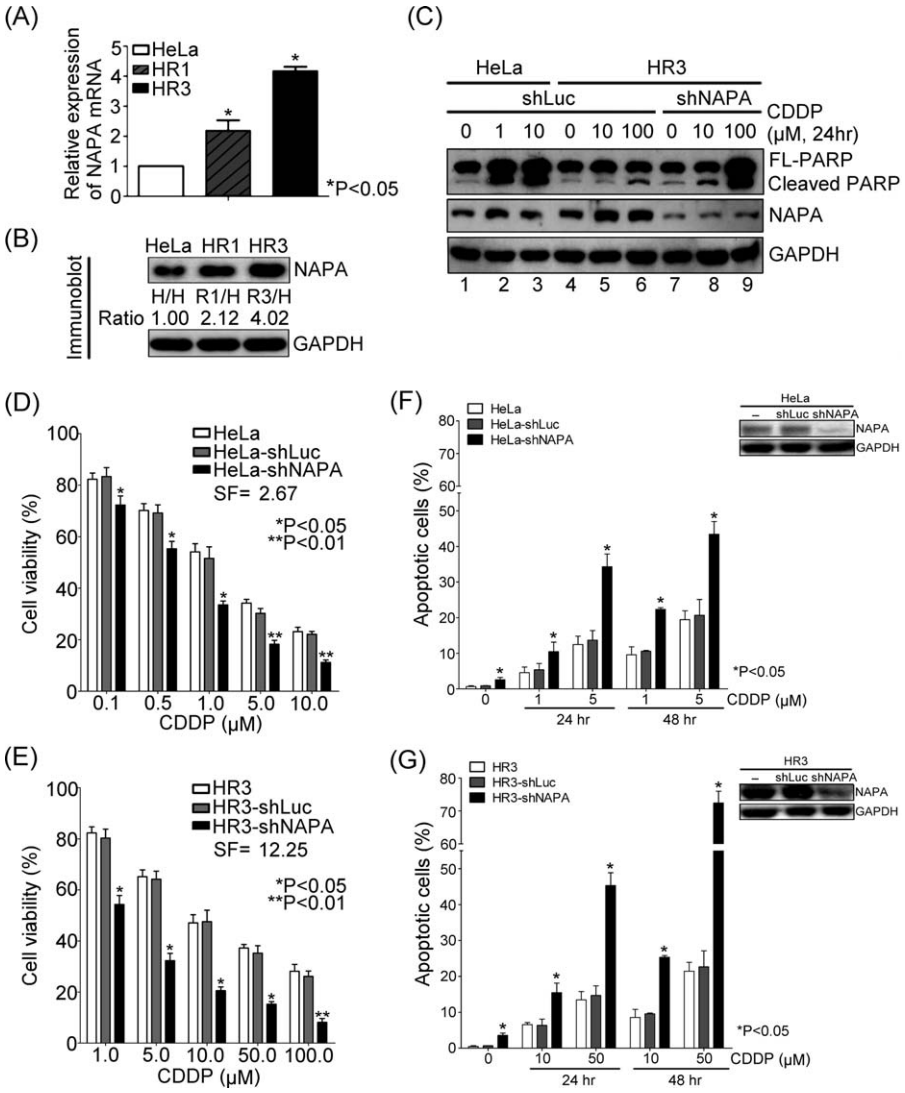

Fig. 5. Reversal of cisplatin resistance in HeLa cells that overexpress NAPA. (A) Up-regulation of NAPA mRNA in cisplatin-resistant HeLa cells. (B) Up-regulation of NAPA protein in cisplatin-resistant cells. The increase of NAPA protein level in resistant cells relative to HeLa was indicated. WB, Western blot. (C) Enhancement of caspase-3 activity following knockdown of NAPA in HR3 cells. HeLa and HR3 cells which overexpress either shNAPA or shLuc control were treated with the indicated concentrations of cisplatin for 24 h. Fifty μg of total protein extracts were used for immunoblotting experiments to detect cleaved caspase-3 and its substrate, full-length PARP (FL PARP) as well as cleaved PARP. (D) Sensitization to cisplatin following knockdown of NAPA in HeLa cells. Cisplatin-induced cytotoxicity was determined by MTT assays. (E) Sensitization to cisplatin following knockdown of NAPA in HR3 cells. The sensitization factor (SF) shown represents the IC_{50} for shLuc cells divided by the IC_{50} for shNAPA cells. (F) Enhancement of cisplatin-induced apoptotic cell accumulation following NAPA knockdown in HeLa cells. (G) Enhancement of cisplatin-induced apoptotic cell accumulation by shNAPA in HR3 cells. *p* values are indicated.

expressing cells, but not in shLuc ones (Supplementary Fig. S4). Notably, sensitization to cisplatin following knockdown of NAPA was also observed in the tumorigenic cell lines CG-1 and Sk-ov-3 (Supplementary Fig. S5). These results indicate that NAPA knockdown not only increases sensitivity to cisplatin but also reverses cisplatin resistance in cancer cells.

3.6. Knockdown of NAPA enhances sensitivity to cisplatin and suppresses tumor growth in nude mice xenografts

To assess the relevance of combining NAPA knockdown with cisplatin treatment *in vivo*, HeLa-R3 cells expressing either shNAPA (HR3-shNAPA) or control shLuc (HR3-shLuc) were inoculated subcutaneously into nude mice. We found that tumors formed after a 30-day lag-time, and they increased in size to similar rates until 50 days post-inoculation. At 50 days, tumors of approximately 150 mm^3 were detected in both groups (Fig. 6A). Eighty days post-inoculation, the sizes of tumors produced by HR3-shNAPA cells in untreated animals were considerably smaller than those produced by HR3-shLuc cells (Fig. 6A). As shown in Fig. 6B and C, tumor size was smaller in mice inoculated with HR3-shNAPA cells compared to those inoculated with HR3-shLuc cells (Fig. 6B and C, "untreated," 80 days post-inoculation). At 50 days post-inoculation, tumor-harboring mice from both groups were treated with cisplatin, and tumor size was measured every 3 days for the next 30 days. While tumor sizes were profoundly reduced in both mouse groups by repeated intraperitoneal injections of cisplatin, the tumors seen in the HR3-shNAPA group were much more responsive than those in the HR3-shLuc group (Fig. 6B–D, "CDDP"). That is, tumor growth was inhibited to a larger extent in the HR3-shNAPA/cisplatin group compared with the control HR3-shLuc/cisplatin group (Fig. 6D). These results indicate that shRNA which target NAPA induce potent anti-tumor effects *in vivo*. Based on the results presented here, we propose a mechanism describing the role of NAPA in modulating sensitivity to cisplatin (Fig. 6E, see Section 4).

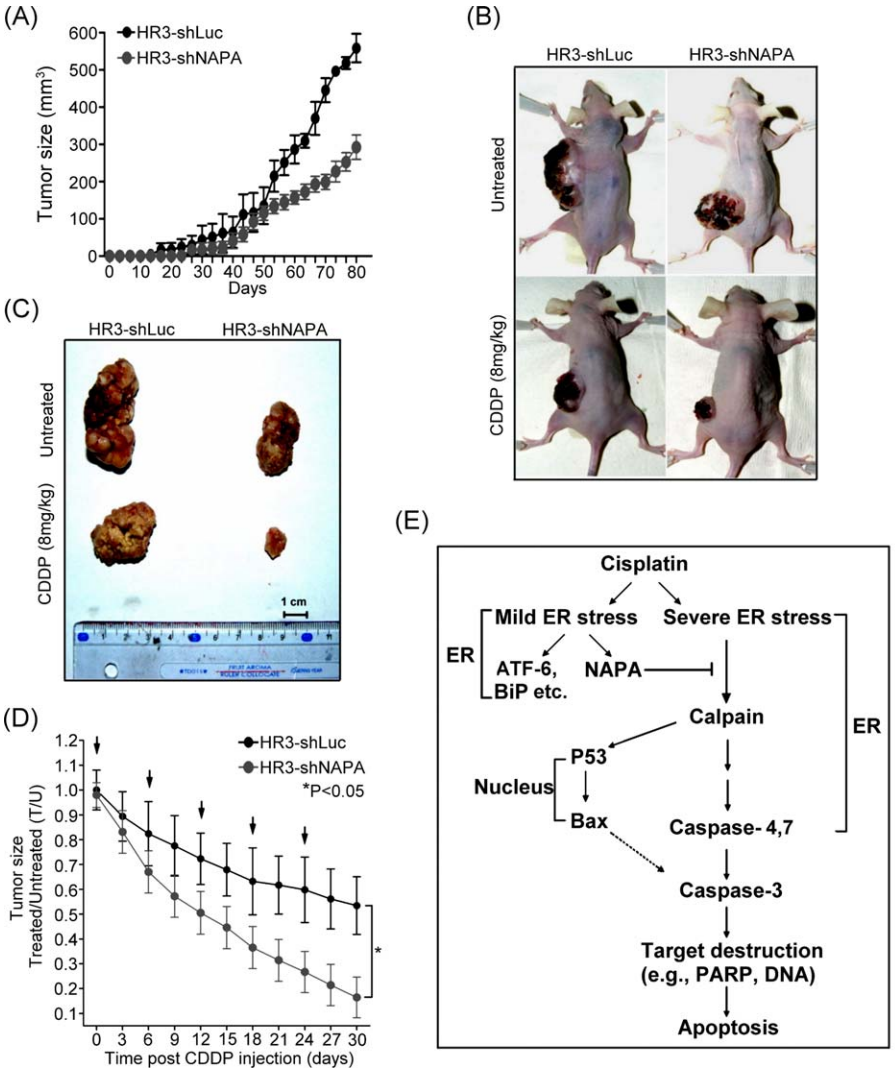

Fig. 6. Sensitization to cisplatin and suppression of tumor growth by combined shNAPA/cisplatin treatment in nude mice xenografts. (A) Reduced growth rate of HR3 cells expressing shNAPA. Compared to the tumorigenic cells expressing shLuc, the tumorigenic HR3 cells that expressed shNAPA grew slower starting 50 days post-inoculation in nude mice. (B) Representative nude mice with tumors 80 days post-inoculation. Nude mice 50 days post-inoculation were left untreated or were repeatedly treated with cisplatin for 30 days. (C) Representative tumor size excised 30 days after cisplatin treatment. The size reference (1 cm) was indicated below the figure. (D) Kinetic changes in tumor size of nude mice xenografts following repeated cisplatin injections. The average tumor size was calculated from tumors of six mice for each time point. (E) Working model of ER damage and related signal pathways implicated in cisplatin-induced apoptosis. While mild ER stress induced by low concentration of cisplatin activates the protective NAPA protein, severe ER stress induced by high concentration of cisplatin induces the pro-apoptotic protein calpain. Calpain further transmits signals to activate caspase-4 and probably also caspase-7 in the ER, leading to activation of caspase-3 in the cytoplasm. Calpain may also activate caspase-3 via p53 and Bax in the nucleus. Both pathways eventually lead to apoptosis. We also provide evidence that NAPA knockdown may mimic severe ER stress and enhance cisplatin-induced apoptosis. In contrast, NAPA overexpression may block cisplatin-induced calpain activation and prevent apoptosis. The known effect of Bax on caspase activation which was not performed in the present study is presented as a dashed line. *p* values are indicated.

4. Discussion

In the present study, we observed that the chemotherapeutic drug cisplatin-induced ER stress, and that the level of stress was dependent on the concentration of cisplatin applied as well as the length of treatment used. When mild ER stress was induced by low concentrations of cisplatin, cellular response included induction of BiP and NAPA which appeared to protect against the cytotoxic effects of cisplatin. This possibility was illustrated by the observation that NAPA knockdown induced apoptosis and sensitized cells to cisplatin.

Calpain appeared to be an important factor in mediating cisplatin-induced ER stress. This possibility was illustrated by the observation that activation of both caspase-12 and caspase-4 in the ER and subsequent cleavage of PARP by caspase-3 was blocked by calpain inhibitors. There is accumulating evidence indicating that calpain regulates both caspase-dependent and caspase-independent apoptosis induced by different apoptotic stimuli in various cells [47,48]. Recent studies suggest that the calpain pathway is an early event during cisplatin-induced apoptosis in human lung adenocarcinoma cells [44]. However, elucidation of the role of calpain in cisplatin-induced apoptotic cell death will require further studies.

Based on the mentioned studies and the results presented here, we propose a mechanism to explain the role of NAPA in modulating sensitivity to cisplatin (Fig. 6E). In this model, cisplatin-induced calpain activity in response to severe ER stress is suppressed by NAPA, which prevents the activation of downstream caspases, including caspase-4 and caspase-3. Interestingly, it was reported earlier that overexpression of BiP may suppress the ER-protein caspase-7 and may protect against drugs that target topoisomerases [41]. It will be interesting to investigate whether NAPA and BiP regulate distinct caspase-dependent apoptosis pathways. μ-Calpain is also involved in the regulation of apoptosis-inducing

factor (AIF)-mediated caspase-independent apoptosis by cisplatin [49]. It should be noted that the activation of caspases is more complex than depicted in our model, and this process is known to depend on the genetic context of the cell. p53, for instance, transcriptionally activates the expression of the caspase-6 and caspase-7 genes, but not the caspase-3 gene, in cisplatin-induced nephrotoxicity [50]. In addition to caspase-3, other caspases may also be regulated by calpain, and may therefore participate in the ER-mediated apoptosis induced by cisplatin.

Like cisplatin-induced severe ER stress, NAPA knockdown can lead to the accumulation of p53, probably via transactivation of its target Bax which mediates apoptosis via its action on mitochondria. Mouse studies strongly suggest that p53 is required for efficient execution of the apoptosis in tumor cells. Clinical studies also implicate p53 mutations in pleiotropic resistance to chemotherapy, suggesting that p53 is a potential drug target [51]. Consequently, the data obtained from p53 studies reinforce the notion that this protein is involved in a network of cellular response to anti-cancer drugs in tumors which can acquire cross-resistance to anti-cancer agents. It has been demonstrated that the oncogene adenovirus *E1A* gene can sensitize mouse fibroblasts to apoptosis induced by DNA-damaging agents such as ionizing radiation, 5-fluorouracil, etoposide, and adriamycin [2]. Likewise, the anti-cancer effects of cisplatin in most cases were considered to be mediated by nuclear damage. In our study, ER damage induced by cisplatin also appears to play an important role in p53-dependent inhibition of cell growth and apoptosis in non-tumorigenic cells like HEK293. The p53-dependence of the regulatory role of NAPA in cisplatin-sensitivity was profoundly hurdled by suppressing p53 activity. Surprisingly, however, ER-mediated cisplatin-sensitivity is also found in p53-null H1299 tumor cells. The p53-independent pathway of the regulatory role of NAPA may explain the significant reversal of acquired cisplatin resistance by NAPA knockdown in HeLa cells whose p53 activity is impeded by the E6 protein of HPV. Taken together, the regulatory role of NAPA in cisplatin-sensitivity appears to depend on the level of p53. It will be interesting to further investigate the p53-independent mechanism of ER-mediated drug sensitization for the development of potential therapy in p53-defective cancers.

The severe ER stress induced by cisplatin may cause disintegration of the ER network. The observations that knockdown of NAPA generated severe ER stress and resulted in p53 accumulation can possibly be explained by the ER-associated degradation (ERAD) process [52]. It is likely that NAPA knockdown led to disintegration of the ERAD activity, previously described to process and degrade misfolded ER proteins and also p53 [45], subsequently resulting in p53 accumulation. Alternatively, NAPA may physically interact with proteins that regulate apoptosis. Previous studies had revealed that NAPA interacts with several proteins including three membrane proteins (e.g. syntaxin 18, p31, and BNIP1; see Ref. [28]). Importantly, the BH3 domain of BNIP1, a domain responsible for the induction of apoptosis, provides a binding-site for NAPA, raising the possibility that BNIP1 plays a pivotal role in the cross-talk between apoptosis and membrane fusion. This hypothesis predicts that NAPA may suppress apoptosis by competing with anti-apoptotic proteins for the BH3 domain of BNIP1. In addition, this hypothesis may explain the findings that overexpression of NAPA suppressed cisplatin-induced apoptosis (as seen in this study) and markedly delayed staurosporine-induced apoptosis [28]. Finally, the relevance of our findings was illustrated by the observation that combining cisplatin and NAPA knockdown considerably decreased tumor growth *in vivo*, thereby providing a basis for the development of a new chemotherapeutic strategy against cancer.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2010.05.026.

References

[1] Kaufmanns SH. Induction of endonucleolytic DNA cleavage in human acute myelogenous leukemia cells by etopside, camptothecin, and other cytotoxic anticancer drugs: a cautionary note. Cancer Res 1989;49:5870–8.
[2] Lowe SW, Ruley HE, Jacks T, Housman DE. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell 1993;74:957–67.
[3] Andrews PA, Howell SB. Cellular pharmacology of cisplatin: perspectives on mechanisms of acquired resistance. Cancer Cells 1990;2:35–43.
[4] Chu G. Cellular responses to cisplatin. The role of DNA-binding proteins and DNA repair. J Biol Chem 1994;269:787–90.
[5] Chao CC. Molecular basis of cis-diamminedichloroplatinum(II) resistance: a review. J Formos Med Assoc 1996;95:893–900.
[6] Baird RD, Kaye SB. Drug resistance reversal—are we getting closer? Eur J Cancer 2003;39:2450–61.
[7] Siddik ZH. Cisplatin: mode of cytotoxic action and molecular basis of resistance. Oncogene 2003;22:7265–79.
[8] Dempke W, Voigt W, Grothey A, Hill BT, Schmoll HJ. Cisplatin resistance and oncogenes—a review. Anticancer Drugs 2000;11:225–36.
[9] Niedner H, Christen R, Lin X, Kondo A, Howell SB. Identification of genes that mediate sensitivity to cisplatin. Mol Pharmacol 2001;60:1153–60.
[10] Wang D, Lippard SJ. Cellular processing of platinum anticancer drugs. Nat Rev Drug Discov 2005;4:307–20.
[11] Stewart DJ. Mechanisms of resistance to cisplatin and carboplatin. Crit Rev Oncol Hematol 2007;63:12–31.
[12] Baumann O, Walz B. Endoplasmic reticulum of animal cells and its organization into structural and functional domains. Int Rev Cytol 2001;205:149–214.
[13] Voeltz GK, Rolls MM, Rapoport TA. Structural organization of the endoplasmic reticulum. EMBO Rep 2002;3:944–50.
[14] Krajewski S, Tanaka S, Takayama S, Schibler MJ, Fenton W, Reed JC. Investigation of the subcellular distribution of the bcl-2 oncoprotein: residence in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membranes. Cancer Res 1993;53:4701–14.
[15] Ng FW, Shore GC. Bcl-XL cooperatively associates with the Bap31 complex in the endoplasmic reticulum, dependent on procaspase-8 and Ced-4 adaptor. J Biol Chem 1998;273:3140–3.
[16] Hacki J, Egger L, Monney L, Conus S, Rossé T, Fellay I, et al. Apoptotic crosstalk between the endoplasmic reticulum and mitochondria controlled by Bcl-2. Oncogene 2000;19:2286–95.
[17] Zong WX, Li C, Hatzivassiliou G, Lindsten T, Yu QC, Yuan J, et al. Bax and Bak can localize to the endoplasmic reticulum to initiate apoptosis. J Cell Biol 2003;162:59–69.
[18] Foyouzi-Youssefi R, Arnaudeau S, Borner C, Kelley WL, Tschopp J, Lew DP, et al. Bcl-2 decreases the free Ca^{2+} concentration within the endoplasmic reticulum. Proc Natl Acad Sci USA 2000;97:5723–8.
[19] Pinton P, Ferrari D, Magalhães P, Schulze-Osthoff K, Di-Virgilio F, Pozzan T, et al. Reduced loading of intracellular Ca^{2+} stores and downregulation of capacitative Ca^{2+} influx in Bcl-2-overexpressing cells. J Cell Biol 2000;148:857–62.
[20] Nutt LK, Pataer A, Pahler J, Fang B, Roth J, McConkey DJ, et al. Bax and Bak promote apoptosis by modulating endoplasmic reticular and mitochondrial Ca^{2+} stores. J Biol Chem 2002;277:9219–25.
[21] Scorrano L, Oakes SA, Opferman JT, Cheng EH, Sorcinelli MD, Pozzan T, et al. BAX and BAK regulation of endoplasmic reticulum Ca^{2+}: a control point for apoptosis. Science 2003;300:135–9.
[22] Bouillet P, Strasser A. BH3-only proteins—evolutionarily conserved proapoptotic Bcl-2 family members essential for initiating programmed cell death. J Cell Sci 2002;115:1567–74.
[23] Boyd JM, Malstrom S, Subramanian T, Venkatesh LK, Schaeper U, Elangovan B, et al. Adenovirus E1B 19 kDa and Bcl-2 proteins interact with a common set of cellular proteins. Cell 1994;79:341–51.

[24] Mund T, Gewies A, Schoenfeld N, Bauer MK, Grimm S. Spike, a novel BH3-only protein, regulates apoptosis at the endoplasmic reticulum. FASEB J 2003;17:696–8.
[25] Breckenridge DG, Germain M, Mathai JP, Nguyen M, Shore GC. Regulation of apoptosis by endoplasmic reticulum pathways. Oncogene 2003;22:8608–18.
[26] Kuwana T, Newmeyer DD. Bcl-2-family proteins and the role of mitochondria in apoptosis. Curr Opin Cell Biol 2003;15:691–9.
[27] Thomenius MJ, Distelhorst CW. Bcl-2 on the endoplasmic reticulum: protecting the mitochondria from a distance. J Cell Sci 2003;116:4493–9.
[28] Nakajima K, Hirose H, Taniguchi M, Kurashina H, Arasaki K, Nagahama M, et al. Involvement of BNIP1 in apoptosis and endoplasmic reticulum membrane fusion. EMBO J 2004;23:3216–26.
[29] Novick P, Field C, Schekman R. Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. Cell 1980;21:205–15.
[30] Barnard RJ, Morgan A, Burgoyne RD. Stimulation of NSF ATPase activity by alpha-SNAP is required for SNARE complex disassembly and exocytosis. J Cell Biol 1997;139:875–83.
[31] Ungermann C, Nichols BJ, Pelham HR, Wickner W. A vacuolar v-t-SNARE complex, the predominant form in vivo and on isolated vacuoles, is disassembled and activated for docking and fusion. J Cell Biol 1998;140:61–9.
[32] Littleton JT, Barnard RJ, Titus SA, Slind J, Chapman ER, Ganetzky B. SNARE-complex disassembly by NSF follows synaptic-vesicle fusion. Proc Natl Acad Sci USA 2001;98:12233–8.
[33] Hanley JG, Khatri L, Hanson PI, Ziff EB. NSF ATPase and alpha-/beta-SNAPs disassemble the AMPA receptor-PICK1 complex. Neuron 2002;34:53–67.
[34] Mandic A, Hansson J, Linder S, Shoshan MC. Cisplatin induces endoplasmic reticulum stress and nucleus-independent apoptotic signaling. J Biol Chem 2003;278:9100–6.
[35] Chao CC, Huang SL, Huang HM, Lin-Chao S. Cross-resistance to UV radiation of a cisplatin-resistant human cell line: overexpression of cellular factors that recognize UV-modified DNA. Mol Cell Biol 1991;11:2075–80.
[36] Sun CL, Chao CC. Cross-resistance to death ligand-induced apoptosis in cisplatin-selected HeLa cells associated with overexpression of DDB2 and subsequent induction of cFLIP. Mol Pharmacol 2005;67:1307–14.
[37] Kamarajan P, Sun NK, Sun CL, Chao CC. Apaf1 overexpression partially overcomes apoptotic resistance in a cisplatin-selected HeLa cell line. FEBS Lett 2001;505:206–12.
[38] Presley JF, Cole NB, Schroer TA, Hirschberg K, Zaal KJ, Lippincott-Schwartz J, et al. ER-to-Golgi transport visualized in living cells. Nature 1997;389:81–5.
[39] Tsai SY, Sun NK, Lu HP, Cheng ML, Chao CC. Involvement of reactive oxygen species in multidrug resistance of a vincristine-selected lymphoblastoma. Cancer Sci 2007;98:1106–14.
[40] Li XA, Lee AS. Competitive inhibition of a set of endoplasmic reticulum protein genes (GRP78, GRP94, and ERp72) retards cell growth and lowers viability after ionophore treatment. Mol Cell Biol 1991;11:3446–53.
[41] Reddy RK, Mao C, Baumeister P, Austin RC, Kaufman RJ, Lee AS. Endoplasmic reticulum chaperone protein GRP78 protects cells from apoptosis induced by topoisomerase inhibitors: role of ATP binding site in suppression of caspase-7 activation. J Biol Chem 2003;278:20915–24.
[42] Gray MD, Mann M, Nitiss JL, Hendershot LM. Activation of the unfolded protein response is necessary and sufficient for reducing topoisomerase IIalpha protein levels and decreasing sensitivity to topoisomerase-targeted drugs. Mol Pharmacol 2005;68:1699–707.
[43] Ranganathan AC, Zhang L, Adam AP, Aguirre-Ghiso JA. Functional coupling of p38-induced up-regulation of BiP and activation of RNA-dependent protein kinase-like endoplasmic reticulum kinase to drug resistance of dormant carcinoma cells. Cancer Res 2006;66:1702–11.
[44] Liu L, Xing D, Chen WR, Chen T, Pei Y, Gao X. Calpain-mediated pathway dominates cisplatin-induced apoptosis in human lung adenocarcinoma cells as determined by real-time single cell analysis. Int J Cancer 2008;122:2210–22.
[45] Yamasaki S, Yagishita N, Sasaki T, Nakazawa M, Kato Y, Yamadera T, et al. Cytoplasmic destruction of p53 by the endoplasmic reticulum-resident ubiquitin ligase 'Synoviolin'. EMBO J 2007;26:113–22.
[46] Aylon Y, Oren M. Living with p53, dying of p53. Cell 2007;130:597–600.
[47] Goll DE, Thompson VF, Li H, Wei W, Cong J. The calpain system. Physiol Rev 2003;83:731–801.
[48] Suzuki K, Hata S, Kawabata Y, Sorimachi H. Structure, activation, and biology of calpain. Diabetes 2004;53:12–8.
[49] Liu L, Xing D, Chen WR. μ-Calpain regulates caspase-dependent and apoptosis inducing factor-mediated caspase-independent apoptotic pathways in cisplatin-induced apoptosis. Int J Cancer 2009;125:2757–66.
[50] Yang C, Kaushal V, Haun RS, Seth R, Shah SV, Kaushal GP. Transcriptional activation of caspase-6 and -7 genes by cisplatin-induced p53 and its functional significance in cisplatin nephrotoxicity. Cell Death Differ 2008;15:530–44.
[51] Wallace-Brodeur RR, Lowe SW. Clinical implications of p53 mutations. Cell Mol Life Sci 1999;54:64–75.
[52] Vembar SS, Brodsky JL. One step at a time: endoplasmic reticulum-associated degradation. Nat Rev Mol Cell Biol 2008;9:944–58.
[53] Chan SL, Mattson MP. Caspase and calpain substrates: roles in synaptic plasticity and cell death. J Neurosci Res 1999;58:167–90.
[54] Hitomi J, Katayama T, Eguchi Y, Kudo T, Taniguchi M, Koyama Y, et al. Involvement of caspase-4 in endoplasmic reticulum stress-induced apoptosis and Aβ-induced cell death. J Cell Biol 2004;165:347–56.