Biochemical Pharmacology 82 (2011) 1630–1640

Contents lists available at SciVerse ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Silencing of the SNARE protein NAPA sensitizes cancer cells to cisplatin by inducing ERK1/2 signaling, synoviolin ubiquitination and p53 accumulation

Zchong-Zcho Wu [a,b,1], Nian-Kang Sun [a,c,2], Kun-Yi Chien [a,b,3], Chuck C.-K. Chao [a,b,*]

[a] Department of Biochemistry and Molecular Biology, Chang Gung University, Gueishan, Taoyuan 333, Taiwan
[b] Graduate Institute of Biomedical Sciences, Chang Gung University, Gueishan, Taoyuan 333, Taiwan
[c] Division of Biomedical Sciences, Chang Gung University of Science and Technology, Gueishan, Taoyuan 333, Taiwan

ARTICLE INFO

Article history:
Received 6 July 2011
Accepted 23 August 2011
Available online 31 August 2011

Keywords:
Apoptosis
Chemotherapy
Cisplatin resistance
NAPA
Synoviolin

ABSTRACT

We found earlier that NAPA represents an anti-apoptotic protein that promotes resistance to cisplatin in cancer cells by inducing the degradation of the tumor suppressor p53. In the present study, we investigated the cellular mechanism underlying the degradation of p53 by NAPA. Knockdown of NAPA using short-hairpin RNA was shown to induce p53 accumulation and to sensitize HEK293 cells to cisplatin. On the other hand, this sensitization effect was not found in H1299 lung carcinoma cells which lack p53. Expression of exogenous p53 in H1299 cells was increased following knockdown of NAPA and these cells showed increased sensitivity to cisplatin-induced apoptosis. Notably, knockdown of NAPA induced the ubiquitination and degradation of the E3 ubiquitin ligase synoviolin and the accumulation of p53 in unstressed HEK293 cells. Conversely, NAPA overexpression decreased the ubiquitination and degradation of synoviolin, and reduced p53 protein level. Knockdown of NAPA disrupted the interaction between synoviolin and proteins that form the endoplasmic reticulum-associated degradation (ERAD) complex and in turn decreased the ability of this complex to ubiquitinate p53. In addition, knockdown of NAPA induced the activation of the MAPK kinases ERK, JNK and p38, but only inhibition of ERK reduced synoviolin ubiquitination and p53 accumulation. These results indicate that NAPA promotes resistance to cisplatin through synoviolin and the ERAD complex which together induce the degradation of p53 and thus prevent apoptosis. Based on these findings, we propose that the combination of cisplatin and knockdown of NAPA represents a novel and attractive strategy to eradicate p53-sensitive cancer cells.

1. Introduction

Platinum-containing drugs have been used for several years to treat human cancers. Despite the recent introduction of new platinum-based chemotherapeutic compounds, cisplatin remains widely used for the treatment of a wide range of human malignancies. Cisplatin exerts its anti-cancer activity by inducing DNA damage and apoptosis in replicating cells [1,2]. However, chemoresistance represents a major problem of cisplatin chemotherapy. The mechanisms underlying cisplatin chemoresistance have been intensively investigated in the past [3–6]. These mechanisms include increased efflux and decreased influx of the drug, alterations of cellular targets and detoxification systems, increased DNA repair, and resistance to apoptosis. Cisplatin

Abbreviations: BiP, binding immunoglobulin protein; CHX, cycloheximide; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated degradation; ERK, extracellular signal-regulated kinases; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IP, immunoprecipitation; JNK, c-Jun N-terminal protein kinase; Luc, luciferase; MAPK, mitogen-activated protein kinase; NAPA, NSF attachment protein α; ORF, open-reading frame; PARP, poly-ADP ribose polymerase; PCR, polymerase chain reaction; PVDF, polyvinylidene fluoride; RT, reverse transcription; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; shRNA, short-hairpin RNA; SYVN1, synoviolin/Der3p/Hrd1p; UPR, unfolding protein response.

* Corresponding author at: Tumor Biology Laboratory, Department of Biochemistry and Molecular Biology, Chang Gung University, 259 Wen-Hua 1st Road, Gueishan, Taoyuan 333, Taiwan. Tel.: +886 3211 8136; fax: +886 3211 8700.
E-mail addresses: D9601101@stmail.cgu.edu.tw (Z.-Z. Wu), sunn@mail.cgu.edu.tw (N.-K. Sun), kychien@mail.cgu.edu.tw (K.-Y. Chien), cckchao@mail.cgu.edu.tw (Chuck C.-K. Chao).
[1] Tumor Biology Laboratory, Department of Biochemistry and Molecular Biology, Chang Gung University, 259 Wen-Hua 1st Road, Gueishan, Taoyuan 333, Taiwan. Tel.: +886 3211 8800x5151.
[2] Division of Biomedical Sciences, Chang Gung Institute of Technology, 261 Wen-Hua 1st Road, Gueishan, Taoyuan 333, Taiwan. Tel.: +886 3211 8999x5421.
[3] Tumor Biology Laboratory, Department of Biochemistry and Molecular Biology, Chang Gung University, 259 Wen-Hua 1st Road, Gueishan, Taoyuan 333, Taiwan. Tel.: +886 3211 8800x5971.

0006-2952/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2011.08.018

chemoresistance is also associated with dysregulated expression of cellular oncogenes and tumor suppressors [7–11]. While several cellular targets of cisplatin have been identified in the past, the cellular mechanisms underlying cisplatin-induced cell death and chemoresistance remain less clear. The sensitivity of cancer cells to cisplatin has been shown to rely on the activation of p53 and apoptosis [2,7]. Several E3 ubiquitin ligases such as MDM2, Pirh2 and COP1 have also been shown to regulate the degradation of p53 [12,13]. Notably, compounds that block the interaction between MDM2 and p53 can stabilize p53 and sensitize cancer cells to cisplatin [14,15]. Therefore, blocking the interaction between p53 and its E3 ubiquitin ligases represents a powerful strategy to reduce the degradation of p53 and prevent resistance to cisplatin.

The endoplasmic reticulum (ER) consists of a reticular network of sheet-like structures, membrane tubules, and transport vesicles [16,17]. Recently, the ER has been shown to be implicated in regulating apoptosis [18–20]. Accordingly, the pro-apoptotic BH3-only proteins BNIP1 and spike have been shown to be located in the ER [21–23]. Consistent with the possibility that some ER proteins regulate apoptosis, a recent report has shown that forced expression of NAPA – an ER SNARE protein also known as α-SNAP and which interacts with BNIP1 – markedly delayed staurosporine-induced apoptosis in HeLa cells [24]. In addition, the E3 ubiquitin ligase synoviolin (SYVN1), which represents a mammalian homolog of the yeast Der3p/Hrd1p protein, is also located in the ER and is involved in degrading misfolded ER and cell membrane proteins [25]. As such, SYVN1 participates in the unfolding protein response (UPR) which represents a cellular system that leads to the disposal of unfolded proteins [26–28]. As a component of the ER-associated degradation (ERAD) complex, SYVN1 also targets p53 for ubiquitination and proteasomal degradation [12,29], suggesting a possible crosstalk between ERAD and p53 in regulating apoptosis and ER stress.

Previous work has shown that cisplatin induces ER stress, and that this process is associated with nucleus-independent apoptosis [30]. Our previous studies also revealed that the newly identified cisplatin-resistance gene NAPA protects cancer cells against cisplatin-induced apoptosis and ER stress [31,32]. Accordingly, knockdown of NAPA was shown to induce the accumulation of p53 and to sensitize cancer cells to cisplatin [31]. In the present study, we show that knockdown of NAPA enhances cisplatin-induced apoptosis by inducing the degradation of SYVN1 which in turn stabilizes p53.

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. Human embryonic kidney cells (HEK293) and p53-null lung cancer cells (H1299) were obtained from the American Tissue Type Collection (Manassas, VA, USA). In some experiments, H1299 cells were transfected with the p53 expression plasmid pcep4-p53 (a generous gift from Dr. Y.S. Lin, Academia Sinica, Taipei, Taiwan) in the presence of Lipofectamine (Invitrogen, Carlsbad, CA, USA) for 48 h as described before (Wu and Chao, 2010). Cisplatin was purchased from Bristol-Myers Squibb (New York, NY, USA). Other chemicals were purchased from Sigma (St. Louis, MO, USA). All reagents were used according to the instructions provided by the supplier.

2.2. Gene knockdown using short-hairpin RNA

pLKO.1 plasmids expressing shRNA to knockdown either NAPA or SYVN1 were purchased from the National RNAi Core Facility (Academia Sinica). The most effective shNAPA (TRCN0000029169) and shSYVN1 (TRCN000034007) plasmids were used in the present study. A plasmid expressing shRNA to downregulate luciferase (TRCN0000072244) was used as a negative control. Transient transfection of shRNA plasmids was performed by adding 2 μg/well of plasmid and 5 μl/well of Lipofectamine in cells cultured in 6-well plates (1.5×10^4 cells/well). Three days following plasmid transfection, NAPA or SYVN1 mRNA level was determined by qRT-PCR as described below. Recombinant lentivirus constructs were incubated with the cells for two weeks in puromycin-containing selection medium according to the procedure provided by the supplier (National RNAi Core Facility).

2.3. Quantitative real-time PCR

Quantitative real time-polymerase chain reaction (qPCR in short) was performed on total RNA extracted with Trizol (Invitrogen) and 200 nM of primers as before [33]. Primers for NAPA (GenBank sequence number NM_003827), p53 (NM_001126112.1), SYVN1 (NM_032431.2) and GAPDH (NM_000996) were designed using Primer Express 3.0 (Applied Biosystems, Foster City, CA, USA). The resulting primers consisted of NAPA, forward, 5′-GCGGAGCG-CAAAGTGAAG-3′, reverse, 5′-TCGGCTTTCTTGAATGCGTT-3′; p53, forward, 5′-TCAACAAGATGTTTTGCCAACTG-3′, reverse, 5′-ATGTGCTGTGACTGCTTGTAGATG-3′; SYVN1, forward, 5′-GTTTA-CAGGCTTCATCAAGG-3′, reverse, 5′-CATGATGGCATCTGTCACAG-3′; and GAPDH, forward, 5′-TCCTGCACCACCAACTGCTT-3′, reverse, 5′-GAGGGGCCATCCACGTCTT-3′. All samples and controls were prepared in triplicate on the same plate. Relative quantification was calculated using the ΔΔCt method with normalization against GAPDH [31].

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

NAPA (NM_003827) or SYVN1 (NM_032431.2) cDNA sequences were isolated by PCR from HEK293 cell mRNA using the following primers: NAPA, forward, 5′-GAATTCGCTTTGCTGAGTCCCTTTGT-3′, reverse, 5′-CTCGAGAAAGGAGGGAAGCTCTCCAG-3′; and SYVN1, forward, 5′-GAATTCGGCCAGGGCAATGTTCCGC-3′, reverse, 5′-CTCGAGGGGCTGCTCAAAAGAGCAGAGGC-3′. The resulting amplicons containing restriction enzyme sites (underlined) were cloned into the pGEM-T easy vector and confirmed by DNA sequencing. The expression plasmid was constructed by removing the open-reading frame (ORF) sequences from pGEM-T easy vector, and by inserting the ORF into the pcDNA3 vector as described earlier [31]. The restriction enzymes *EcoRI* and *XhoI* were used to produce pcDNA3-NAPA and pcDNA3-SYVN1, respectively. Cells were transfected with plasmid cDNA to express NAPA and SYVN1. Fifty micrograms of total protein extract was separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transfer to polyvinylidene fluoride (PVDF) membrane, 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), PARP (H-250), (DO-1), SYVN1 (A-21), SEL1L (N-15), and ubiquitin (P4D1) (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 the following secondary antibodies: goat anti-mouse, goat anti-rabbit, or donkey anti-goat IgG HRP (Santa Cruz Biotechnology). The resulting signal was visualized by enhanced chemiluminescence according to

specifications from the supplier (Pierce, Rockford, IL, USA). Protein band intensity was determined by scanning X-ray films with a densitometer (Personal Densitometer SI; Amersham Biosciences, Sunnyvale, CA, UK). Western blotting experiments were performed in triplicate.

2.5. Apoptotic cell analysis

Apoptotic cells following drug treatment for 24 h were determined by nuclear phenotype [34]. To evaluate drug-induced apoptosis, we also used cell extracts for immunoblotting experiments with antibodies specific for the apoptotic markers cleaved caspase-3 and PARP. To confirm apoptosis, sub-G1 cells were measured by flow cytometry as described [35]. Three independent experiments were performed.

2.6. Analysis of SYVN protein stability

Cells (5×10^5) plated on 60-mm tissue culture dishes were grown for 24 h, and cycloheximide was then added at 50 μg/ml. At various times after the addition of cycloheximide, the cells were harvested and lysed in lysis buffer (1× PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail; BD Biosciences, San Jose, CA, USA), and detected by western blot with SYVN1 antibody.

2.7. Co-immunoprecipitation (co-IP) assay

Cells were grown in 10-cm culture dishes (5×10^6 cells/dish). The cells were transfected with expression plasmids, and were treated or not with cisplatin, prior to harvesting in lysis buffer. IP experiments were performed with antibodies against either SYVN1 or p53. Immunoprecipitated complexes were separated by 10% SDS-PAGE, and subjected to Western blot with the indicated antibody.

2.8. Ubiquitination assay

Cells were transfected with expression plasmids encoding either shRNA (shLuc control or shNAPA) or proteins (pcDNA3 vector control or pcDNA3-NAPA) in the presence of MG132 (20 μM) for 8 h. Three days following transfection, the cells were harvested and lysed. Total protein lysates (500 μg) were subjected to immunoprecipitation with either anti-SYVN1 or anti-p53 antibodies. The immunoprecipitation products were analyzed by Western blotting using anti-ubiquitin antibodies.

2.9. Statistical analysis

The data were reported as means ± standard deviation (SD). Statistical significance (p value) was assessed by a two-tailed Student's t test for single comparison. The symbol * denotes $p < 0.05$; ** denotes $p < 0.01$.

3. Results

3.1. Knockdown of NAPA induces p53 accumulation and enhances cisplatin-induced apoptosis

We found earlier that knockdown of NAPA using shRNA sensitized various cancer cell lines to cisplatin [31]. Knockdown of NAPA caused UPR-like stress and p53 accumulation. Notably, the sensitizing effects of NAPA knockdown were shown to be dependent on p53 [32]. In order to determine the mechanism underlying the sensitizing effect of NAPA knockdown, we verified the effects of NAPA knockdown on HEK293 cells that were treated or not with cisplatin. We first observed that NAPA knockdown enhanced the cleavage of caspase-3 and PARP (Fig. 1A). Activation of caspase-3 and PARP was further enhanced following cisplatin treatment (Fig. 1A, lane 4 vs. lane 2). Notably, the protein level of p53 increased following either NAPA knockdown or cisplatin treatment (Fig. 1A). We quantified the protein level of p53 and observed that NAPA knockdown produced an increase of p53 either with or without cisplatin treatment (Fig. 1B). As expected, knockdown of NAPA considerably increased cisplatin-induced apoptosis (Fig. 1C) and sub-G1 cells (Fig. S1A). We also examined the effect of NAPA knockdown on p53-null H1299 cells. NAPA knockdown slightly enhanced the cleavage of caspase-3 and PARP in untreated H1299 cells (Fig. 1D, lane 3 vs. lane 1). Ectopic expression of p53 in H1299 cells enhanced the cleavage of caspase-3 and PARP induced by NAPA knockdown (Fig. 1D). Next, we also monitored apoptosis in H1299 cells that expressed exogenous p53. NAPA knockdown alone induced H1299 cell apoptosis both with and without p53 overexpression. In the presence of cisplatin, NAPA knockdown further enhanced apoptosis (Fig. 1E) and sub-G1 cells (Fig. S1B). Taken together, these results indicate that NAPA knockdown may sensitize cells to cisplatin in both p53-dependent and p53-independent manners. However, these results also suggest that the p53-dependent pathway may play a more prominent role in this sensitization effect.

3.2. Knockdown of NAPA enhances the ubiquitination and degradation of synoviolin

A recent study has shown that the E3 ubiquitin ligase SYVN1 promotes the degradation of p53 independently of other E3 ubiquitin ligases such as MDM2 [12]. To assess whether knockdown of NAPA increases the transcription of p53, we monitored the mRNA level of SYVN1 and p53 in HEK293 cells following knockdown of NAPA. We observed that the mRNA level of SYVN1 was increased following knockdown of NAPA; however, the mRNA level of NAPA was only slightly increased following knockdown of SYVN1 (Fig. 2A), suggesting that NAPA may be an upstream regulator of SYVN1 gene expression. While the mRNA level of p53 was not affected by knockdown of NAPA (Fig. 2B), the mRNA level of SYVN1 was increased by more than 3.5-fold in this case (Fig. 2C). Furthermore, we found that the protein level of SYVN1 was decreased by the shNAPA treatment and this process was associated with accumulation of p53 (Fig. 2D, lane 2 vs. lane 1). While p53 also accumulated in cells expressing shSYVN1, the protein level of NAPA appeared to remain constant protein level in this case (Fig. 2D, lane 3 vs. lane 1). Densitometry analysis indicated that the cells expressing shNAPA indeed produced more p53 compared to shLuc control cells ($p < 0.05$) (Fig. 2E). Notably, the protein level of SYVN1 was considerably reduced in cells expressing shNAPA ($p < 0.01$) (Fig. 2F). Following treatment with the inhibitor of protein synthesis cycloheximide (CHX), we observed that the degradation rate of SYVN1 in shNAPA-expressing cells was faster than that in control shLuc-expressing cells (Fig. 2G and Fig. S2A), indicating that knockdown of NAPA induced the degradation of SYVN1. To determine whether the degradation of SYVN1 was due to ubiquitin-associated proteasomal degradation, we examined the ubiquitination of SYVN1. We found that immunoprecipitated SYVN1 displayed increased ubiquitination in shLuc control cells treated with the proteasome inhibitor MG132 (Fig. 2H, lane 2 vs. lane 1). Notably, the ubiquitination of SYVN1 was greatly increased following knockdown of NAPA (Fig. 2H, compare lanes 4 and 2). These results suggest that knockdown of NAPA induces the degradation of SYVN1, and in turn causes p53 accumulation.

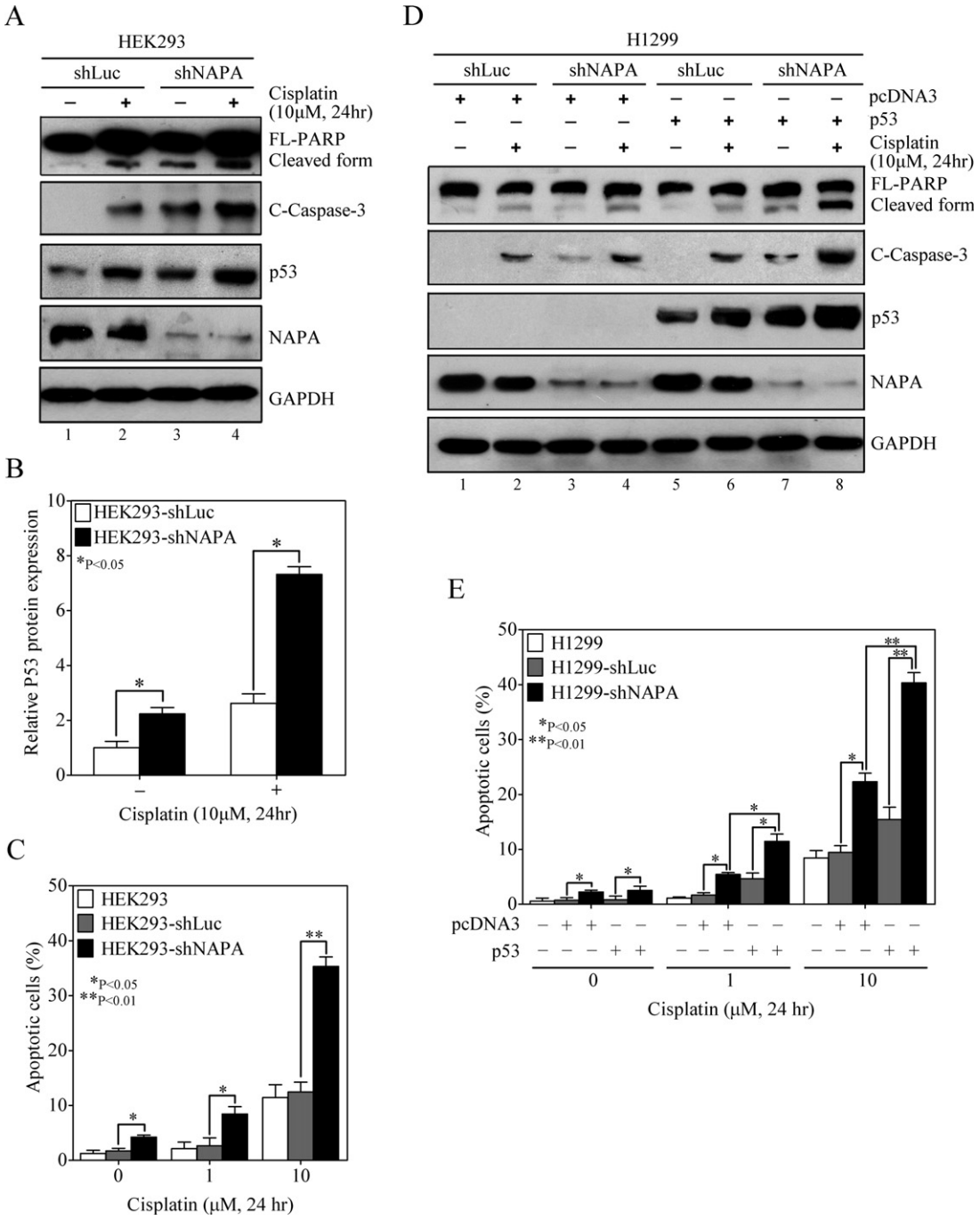

Fig. 1. Knockdown of NAPA induces p53 protein accumulation and enhances cisplatin-induced apoptosis. (A) Enhancement of cisplatin-induced caspase-3 activation, PARP cleavage and accumulation of p53 following NAPA knockdown in HEK293 cells. Total protein extracts (50 μg) were used for immunoblotting experiments. (B) Quantification of p53 protein from the experiments presented in panel A and performed in triplicate. (C) Enhancement of cisplatin-induced apoptosis following NAPA knockdown in HEK293 cells. (D) Enhancement of cisplatin-induced caspase-3 activation following NAPA knockdown in H1299 cells. pcDNA3 plasmid control (lanes 1–4); wide-type p53 overexpression (lanes 5–8). (E) Enhancement of cisplatin-induced apoptosis following NAPA knockdown in H1299 cells. Transient expression of pcDNA3 control or p53 is indicated. The results B, C and E are expressed as mean values ± SD for experiments performed in triplicate. *p*-Values are indicated.

3.3. Overexpression of NAPA reduces the ubiquitination and degradation of synoviolin

To confirm that SYVN1 regulates the protein level of p53 following knockdown of NAPA, we overexpressed NAPA in HEK293 cells. We observed that the mRNA level of SYVN1 was not significantly affected by overexpression of NAPA (Fig. 3A). Furthermore, the mRNA level of p53 and SYVN1 was not significantly affected by overexpression of NAPA compared to the control pcDNA3 vector (Fig. 3B and C). NAPA overexpression did not affect the transcription of SYVN1 and p53; however, reduced p53 and accumulation of SYVN1 was detected in cells overexpressing NAPA (Fig. 3D). Densitometry analysis confirmed that p53 protein level significantly decreased whereas that of SYVN1 increased in this case (Fig. 3E and F). Furthermore, the degradation rate of SYVN1 was reduced in cells overexpressing NAPA compared to control (Fig. 3G and Fig. S2B). In addition, ubiquitination of SYVN1 was dramatically reduced in cells overexpressing NAPA (Fig. 3H, compare lanes 4 and 2). These results indicate that NAPA overexpression stabilizes the SYVN1

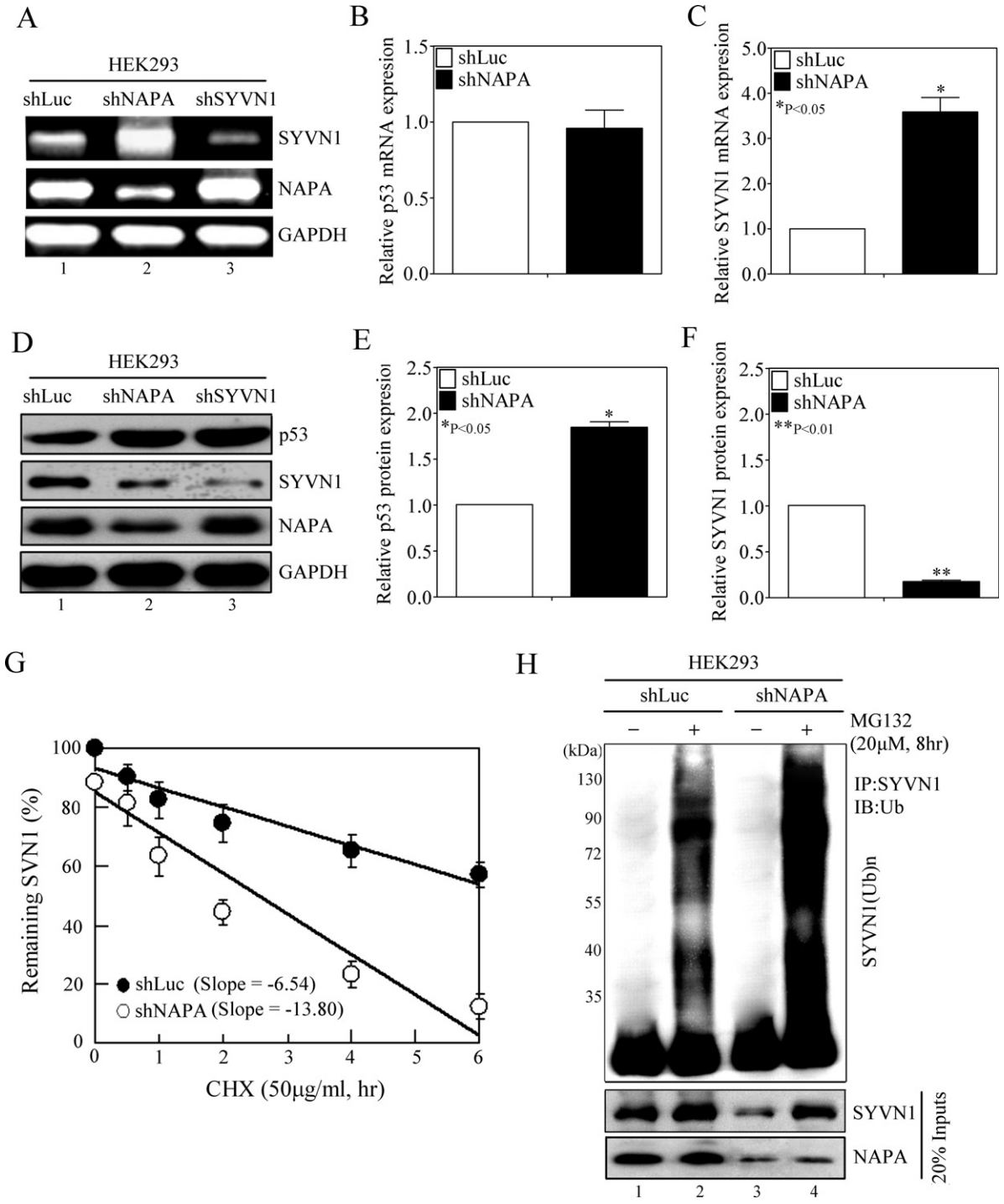

Fig. 2. Enhanced ubiquitination and degradation of SYVN1 following NAPA knockdown in HEK293 cells. (A) Increased mRNA level of SYVN1 following NAPA knockdown. The RT-PCR products were compared with shLuc control and shSYVN1. (B) Lack of change in the level of p53 mRNA following NAPA knockdown as measured by qPCR. (C) Increase in the mRNA level of SYVN1 by NAPA knockdown as measured by qPCR. (D) Increase in the protein level of p53 and reduction in the protein level of SYVN1 by NAPA knockdown. Data of shLuc and shSYVN1 were also indicated as control. Total protein extracts (50 μg) were used for immunoblotting. (E) Quantification of p53 protein level from the data of panel D. (F) Quantification of SYVN1 protein level from the data of panel D. (G) Increase in the degradation rate of SYVN1 protein by NAPA knockdown (shNAPA). The slope of the linear regression representing the SYVN1 protein level of shNAPA group and shLuc control was indicated. (H) Increased ubiquitination of SYVN1 protein following NAPA knockdown. SYVN1 protein was immunoprecipitated followed by immunoblotting with anti-ubiquitin antibody. Results of panels B, C, E, F and G are expressed as mean values ± SD for experiments performed in triplicate. *p*-Values are indicated.

protein by reducing its ubiquitination, thereby leading to reduced p53 protein level. Taken together, these findings support the idea that NAPA regulates p53 protein level by affecting the stability of SYVN1. In the presence of cisplatin, NAPA overexpression further reduced apoptosis (Fig. S3A) and sub-G1 cells (Fig. S3B).

3.4. NAPA regulates p53 protein level through the ERAD complex

The ERAD complex consists of SYVN1/Hrd1, p97/VCP, BiP/Grp78, SEL1L, and other factors [36]. Among these ERAD components, SYVN1 plays a critical role by ubiquitinating p53 in the cytoplasm [12]. We have shown above that knockdown of

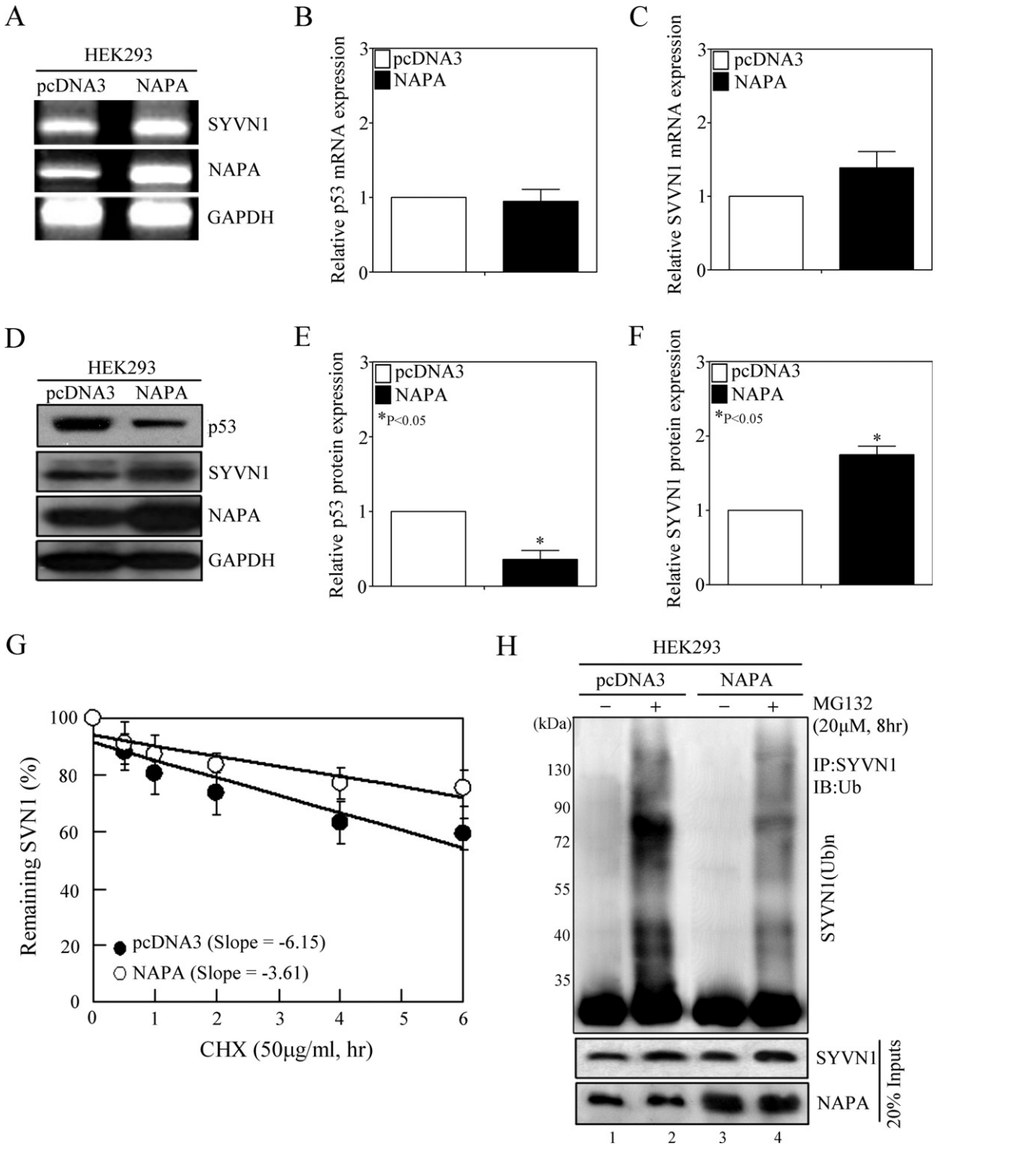

Fig. 3. Reduction of p53 protein and ubiquitination of SYVN1 protein by NAPA overexpression in HEK293 cells. (A) Slight increase of SYVN1 mRNA by NAPA overexpression. pcDNA3 transfection was used as control. The RT-PCR product was indicated. (B) Lack of change in the level of p53 mRNA following NAPA overexpression as measured by qPCR. (C) Slight increase in the mRNA level of SYVN1 by NAPA overexpression as measured by qPCR. (D) Decrease in the protein level of p53 and increase in the protein level of SYVN1 following NAPA overexpression. Data of pcDNA3 transfection was also indicated as control. Total protein extracts (50 μg) were used for immunoblotting. (E) Quantification of p53 protein level from the data of panel D. (F) Quantification of SYVN1 protein level from the data of panel D. (G) Decrease in the degradation rate of SYVN1 protein by NAPA overexpression. The slope of the linear regression corresponding to SYVN1 protein level for the NAPA overexpression and pcDNA3 control groups was indicated. (H) Decreased ubiquitination of SYVN1 protein following NAPA overexpression. SYVN1 protein was immunoprecipitated followed by immunoblotting with anti-ubiquitin antibody. Direct immunoblot for SYVN1 and NAPA from 20% input protein was indicated at the bottom. Results of panels B, C, E, F and G are expressed as mean values ± SD for experiments performed in triplicate. *p*-Values are indicated. Ub, ubiquitin.

NAPA led to p53 accumulation by increasing SYVN1 ubiquitination and degradation. Therefore, we investigated whether knockdown of NAPA impairs the formation of the ERAD complex which would in turn lead to p53 accumulation. While knockdown of NAPA decreased SYVN1 protein level, the two representative components of the ERAD complex BiP and SEL1L were not affected (Fig. 4A, lane 2 vs. lane 1). Notably, co-IP experiments using SYVN1 as bait indicated that the interaction of SYVN1 with BiP, SEL1L and

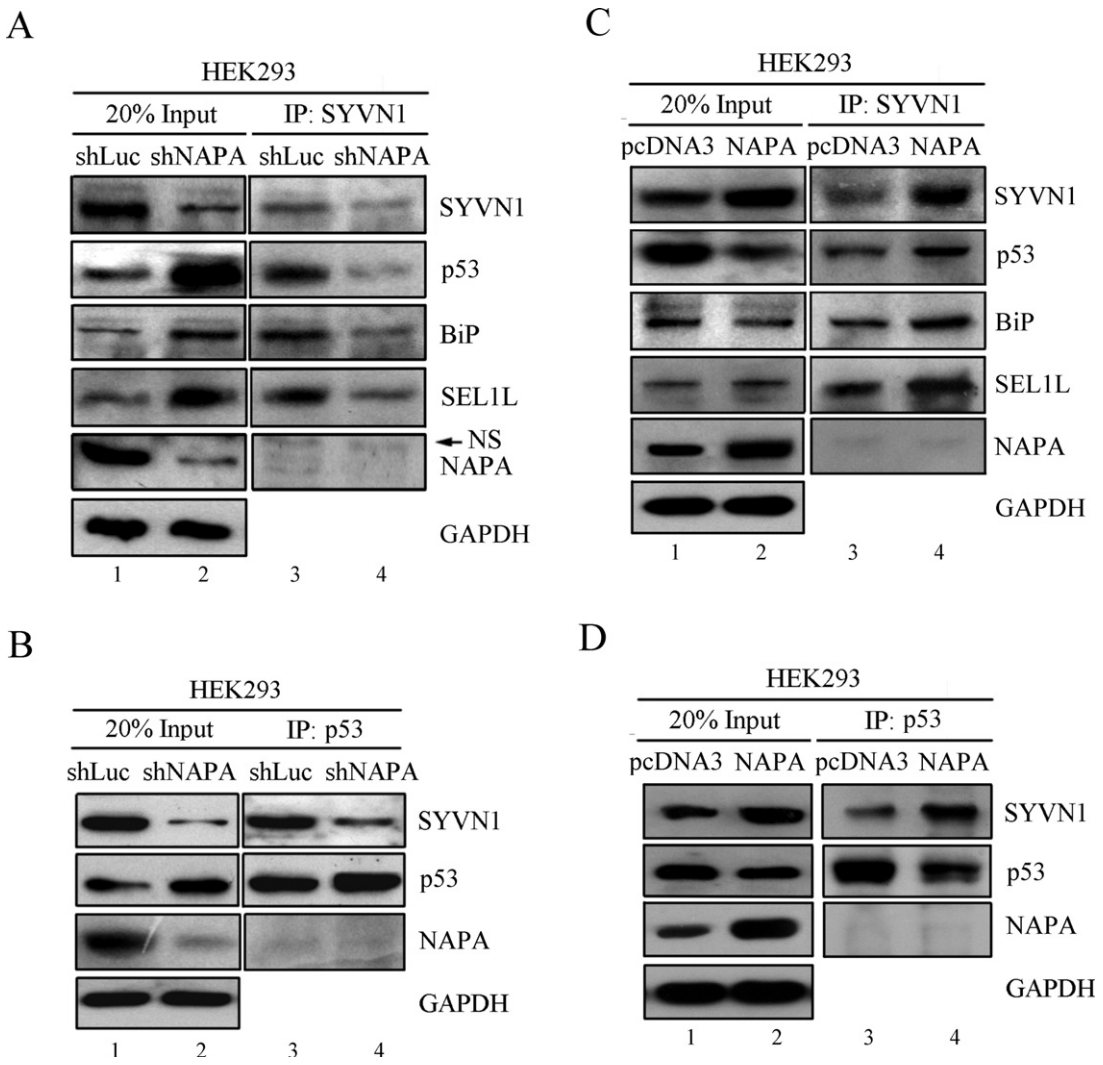

Fig. 4. Disruption of the ERAD complex following NAPA knockdown and enhancement of ERAD complex formation following NAPA overexpression in HEK293 cells. (A) Reduced interaction between SYVN1 and p53 or other ERAD components (BiP and SEL1L) by NAPA knockdown (shNAPA). SYVN1 protein from cell extracts of shNAPA or shLuc control group was immunoprecipitated and subjected to immunoblotting with the indicated antibodies. NS indicates non-specific bands. (B) Reduced interaction between p53 and SYVN1 following NAPA knockdown. p53 protein was immunoprecipitated and subjected to immunoblotting with the indicated antibodies. (C) Slight increase in the interaction between SYVN1 and p53 or other ERAD components (BiP and SEL1L) after NAPA overexpression. Sample preparation and symbols were the same as for panel A. (D) Slight increase in the interaction between p53 and SYVN1 following NAPA overexpression. Sample preparation and symbols were similar to those of panel B.

p53 was considerably reduced by shNAPA (Fig. 4A, lane 4 vs. lane 3). Notably, NAPA was not detected in these IP experiments. Consistent with these results, co-IP experiments using p53 yielded a reduced level of SYVN1 following knockdown of NAPA (Fig. 4B, lane 4 vs. lane 3). Conversely, overexpression of NAPA reduced p53 and led to an increase of SYVN1; however, there was no significant change in the level of BiP or SEL1L in cells overexpressing NAPA (Fig. 4C, lane 2 vs. lane 1). Moreover, co-IP experiments indicated that SYVN1 brought down p53, BiP, and SEL1L, but not NAPA, in cells overexpressing NAPA (Fig. 4C, lane 4 vs. lane 3). Similarly, p53 brought down abundant amounts of SYVN1, but not NAPA (Fig. 4D, lane 4 vs. lane 3). These results suggest that overexpression of NAPA may enhance the formation of the ERAD complex. To confirm these results, we transfected SYVN1-RFP and BiP-GFP fusion plasmids in HEK293 cells and examined the formation of the ERAD complex following knockdown of NAPA. These results indicate that knockdown of NAPA may impair the formation of the ERAD complex and in turn decrease its ability to ubiquitinate p53. Accordingly, accumulation of p53 plays a major role in sensitizing cells to cisplatin-induced apoptosis.

3.5. Inhibition of ERK signaling reduces the ubiquitination and degradation of synoviolin induced by knockdown of NAPA

To investigate whether cellular kinases provide the upstream signals that regulate the ubiquitination of SYVN1 following knockdown of NAPA, we examined the phosphorylation and activation of MAPK. The phosphorylation of JNK and ERK, but not p38, was significantly induced by knockdown of NAPA in HEK293 cells (Fig. 5A, compare lanes 5 and 1). In the presence of cisplatin, all MAPK were activated in these cells (Fig. 5A). To verify the possibility that knockdown of NAPA enhances the activation of JNK and ERK, we examined the phosphorylation of these kinases following overexpression of NAPA. Accordingly, cisplatin-induced phosphorylation of JNK and ERK was reduced in cells overexpressing NAPA (Fig. S4). Furthermore, we found that the suppression of SYVN1 protein following knockdown of NAPA was reversed by the ERK inhibitor PD98059 (Fig. 5B, compare lanes 4 and 1 as well as lanes 6 and 4). On the other hand, the suppression of SYVN1 was not affected by the JNK inhibitor JNK II (Fig. 5B, compare lanes 5 and 4). Notably, the increase of SYVN1 protein

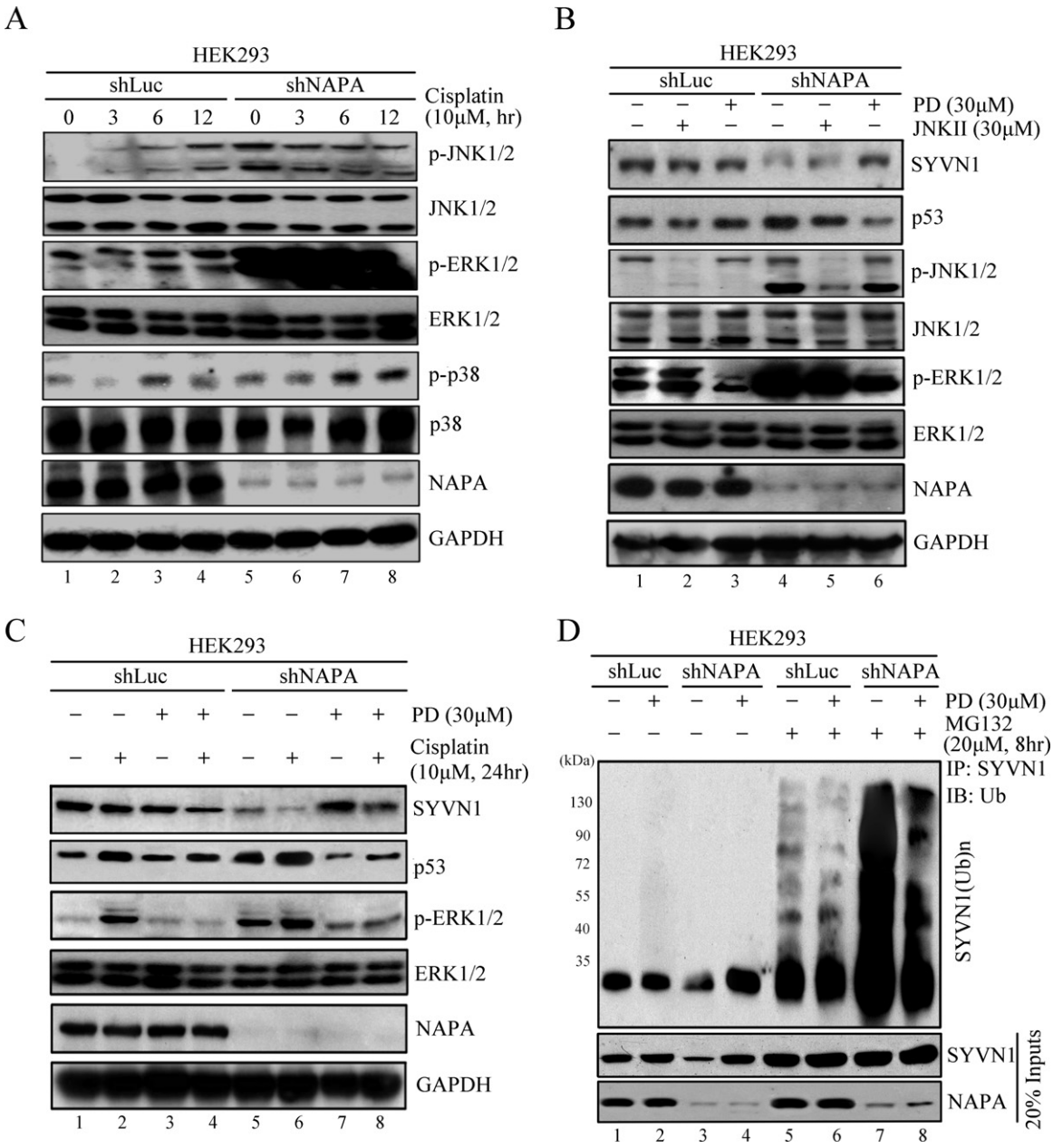

Fig. 5. NAPA knockdown-induced ubiquitination and degradation of SYVN1 depends on the activation of ERK in HEK293 cells. (A) Enhancement of cisplatin-induced phosphorylation of MAPK following NAPA knockdown. JNK1/2, ERK1/2 and p38 were investigated for their phosphorylation (p-JNK1/2, p-ERK1/2 and p-p38) in shLuc (lanes 1–4) and shNAPA (lanes 5–8) cells. (B) Recovery of protein level of SYVN1 from NAPA suppression and reduction in the protein level of p53 by ERK inhibitor. Cells were exposed to pre-treatment with either PD98059 or JNK II for 4 h, followed by NAPA knockdown. (C) Minimal effect of cisplatin on the recovery of protein level of SYVN1 from NAPA suppression and reduction of p53 by ERK inhibitor. (D) Reduction of NAPA knockdown-induced ubiquitination of SYVN1 protein by ERK inhibitor. Ubiquitinated SYVN1 was revealed by treatment of cells with the proteasome inhibitor MG132 (lanes 5–8), compared with shLuc control (lanes 1–4). SYVN1 was immunoprecipitated and subjected to immunoblotting with anti-ubiquitin antibody. Symbols are same as for Fig. 3.

level caused by the ERK inhibitor was associated with a decrease of p53 (Fig. 5B, compare lanes 6 and 4). Furthermore, cisplatin-induced p53 accumulation was dramatically reduced following overexpression of NAPA (Fig. S1). In line with these results, shNAPA induced ERK activation and p53 accumulation, whereas the level of SYVN1 was suppressed in this case (Fig. 5C, compare lanes 5 and 1). This regulatory effect was enhanced by cisplatin (Fig. 5C, compare lanes 6 and 5). Notably, inhibition of ERK phosphorylation/activation reduced the level of p53, whereas this treatment increased the level of SYVN1 (Fig. 5C). To confirm these observations, we examined whether the reduction of SYVN1 protein was due to ubiquitin-associated proteasomal degradation. Indeed, we found that SYVN1 ubiquitination was considerably enhanced by the shNAPA treatment compare to the shLuc control (Fig. 5D, compare lanes 5 and 7). In addition, the enhanced ubiquitination of SYVN1 was reduced by the ERK inhibitor (Fig. 5D, compare lanes 8 and 7). It should be noted that, in the absence of MG132, the changes of SYVN1 protein level in shNAPA-expressing cells were eliminated in parallel experiments performed in the presence of MG132 (Fig. 5D, compare lanes 1–4 with lanes 5–8). Collectively, these observations indicate that the ubiquitination and degradation of SYVN1 protein that is induced by knockdown of NAPA may occur through the activation of ERK. Namely, phosphorylation of SYVN1 may be required and occur prior to the ubiquitination and degradation of this protein. On the other hand, we analyzed the proteins that immunoprecipitated with

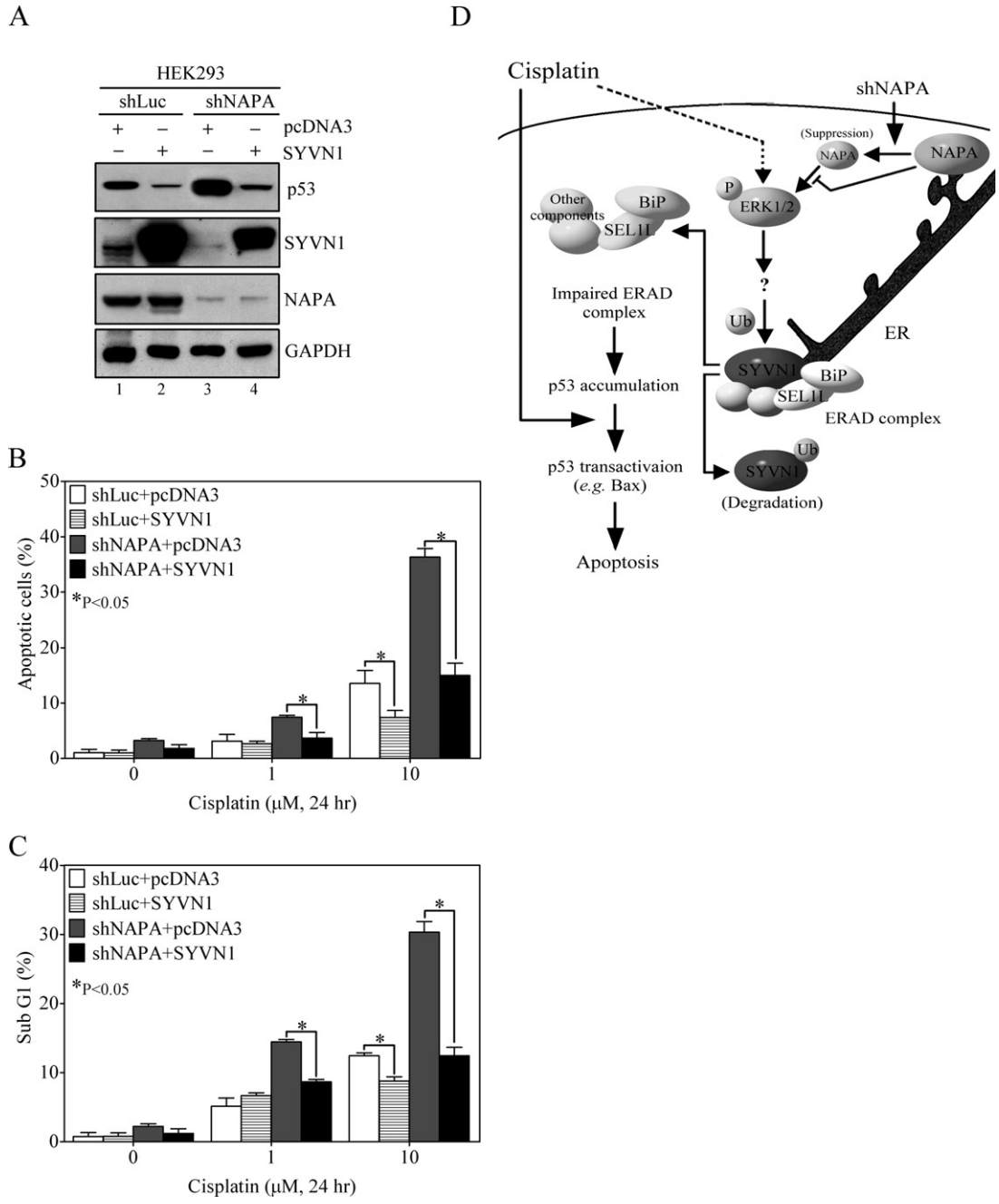

Fig. 6. Reversal of NAPA knockdown-induced SYVN1 degradation, p53 accumulation and apoptosis following overexpression of SYVN1 in HEK293 cells. (A) Reversal of NAPA knockdown-induced SYVN1 degradation and p53 accumulation following overexpression of SYVN1. Overexpression of pcDNA3 vector was used as control. (B) Reduction of shNAPA or cisplatin-induced apoptotic cell following SYVN1 overexpression. (C) Reduction of shNAPA or cisplatin-induced sub-G1 cell following SYVN1 overexpression. (D) Working model of cisplatin stress and related signal pathways implicated in the regulation of SYVN1 degradation, impaired ERAD complex formation, accumulation of p53, and enhancement of cisplatin-induced apoptosis following NAPA knockdown. Results are expressed as mean values ± SD for experiments performed in triplicate. *p*-Values are indicated.

SYVN1 by mass spectrometry analysis and failed to detect any difference in the phosphorylation of SYVN1 in shNAPA-expressing cells that were treated or not with the ERK inhibitor (data not shown). These results suggest that the ubiquitination of SYVN1 may involve a yet-to-be-defined protein that is regulated by ERK-dependent phosphorylation.

3.6. Overexpression of SYVN1 reverses NAPA knockdown-induced p53 accumulation and apoptosis

The results presented above suggest that knockdown of NAPA impairs the formation of the ERAD complex, which in turn fails to perform SYVN1-dependent ubiquitination and degradation of p53 in the cytoplasm. To confirm this possibility, we verified whether overexpression of SYVN1 could reverse this effect and decrease sensitivity to cisplatin. SYVN1 expression plasmid and control vector (pcDNA3) were independently transfected in HEK293 cells that stably expressed either shNAPA or shLuc. While the protein level of SYVN1 was reduced by the shNAPA treatment, the level of p53 was increased compared to the shLuc control (Fig. 6A, lane 3 vs. lane 1). Notably, overexpression of SYVN1 greatly suppressed p53 in both shNAPA and shLuc-expressing cells (Fig. 6A). Furthermore, we found that NAPA knockdown-induced p53 accumulation was reversed by overexpression of SYVN1 (Fig. 6A, lane 4 vs. lane 3). While shNAPA-expressing cells showed a higher level of apoptosis with or without cisplatin, the level of

apoptosis and sub-G1 cells in these cells was reduced to that of control shLuc cells following overexpression of SYVN1 (Fig. 6B and C, respectively). These results indicate that the accumulation of p53 induced by knockdown of NAPA can be reversed by overexpression of SYVN1. Based on the results presented here, we propose a mechanism to describe how NAPA and SYVN1 regulate the level of p53 and sensitivity to cisplatin (Fig. 6D).

4. Discussion

In this study, we found a novel mechanism to explain cisplatin resistance based on the activity of the ER protein NAPA. Collectively, our results suggest a model as summarized in Fig. 6D. Depletion of NAPA leads to ubiquitination and degradation of SYVN1 which in turn impairs the formation of the ERAD complex, and leads to accumulation of p53 in the cytoplasm. Involvement of the E3 ubiquitin ligase SYVN1 in ubiquitinating and degradading p53 has been described previously [12]. In the presence of cytotoxic concentrations of cisplatin, p53 is modified post-translationally through phosphorylation at Ser46 by the DYRK2 [37], for instance, a process that selectively transactivates the *Bax* gene. The pro-apoptotic Bax protein then enhances apoptosis (Fig. 6D). Our results support several reports which showed that p53 expression can enhance cisplatin-induced apoptosis [38,39]. These findings may also partly explain the observation that cisplatin treatment is more efficient against cancers that express p53 [2]. Our findings in HEK293 cells indicate that the regulation loop of NAPA-SYVN1-p53 may be important for cisplatin resistance. Notably, NAPA knockdown also reduced SYVN1 level and led to the accumulation of p53 in several cancer cell lines (CG-1, HepG2, and SK-Hep1) (Fig. S5A, B, and C). Furthermore, cancer (HepG2 and SK-Hep1) or transformed (VA13) cell lines displayed higher NAPA protein levels than non-cancer cell lines (HEK293 and Chang liver) and non-transformed WI-38 cell line (Fig. S5D), confirming that the signaling network described in this study is of significance in cancer cells. Other E3 ligases such as MDM2 are unlikely to be involved in this process since knockdown or overexpression of NAPA did not affect the level of MDM2 mRNA or protein (Fig. S6A, B, C, and D). However, cisplatin resistance may still occur in cancer cells that express p53 since there are many factors that can degrade or inactivate p53, including E3 ubiquitin ligases [12].

In previous studies, we found that NAPA knockdown could sensitize p53-null H1299 cells to cisplatin and that these cells could be re-sensitized to cisplatin if exogenous p53 was reintroduced [32]. Previous studies by others have revealed that NAPA interacts with several proteins including the three membrane proteins syntaxin 18, p31, and BNIP1 [24]. Importantly, BNIP1 contains a BH3 domain, which can induce apoptosis and may provide a binding site for NAPA. In this case, NAPA may suppress apoptosis by competing with anti-apoptotic proteins for the BH3 domain of BNIP1. Accordingly, NAPA knockdown may cause the release of BNIP1 and its translocation to the mitochondria to induce apoptosis. In line with this possibility, overexpression of NAPA may suppress cisplatin-induced apoptosis (as seen in the present study) and delay staurosporine-induced apoptosis [24]. It is important to note that knockdown of NAPA sensitizes cells to cisplatin in both p53-dependent and, but to a lesser extent, p53-independent manners. Notably, NAPA knockdown also sensitized HEK293 cells to other chemotherapeutic agents such as adriamycin and etoposide, which causes DNA damage; however, the degree of sensitization in this case was lower than for cisplatin (Fig. S6E and F).

Our findings provide a link between apoptosis and membrane fusion. The major function of the ER membrane-resident NAPA is to transport proteins involved in the ER network [40–43]. We initially found that knockdown of NAPA activates the unfolded protein response (UPR), and is associated with p53 accumulation and hypersensitivity to cisplatin [32]. In the present study, we observed that SYVN1, which represents an important component in the ERAD process, bridges a gap between NAPA and p53. As such, NAPA knockdown induced the ubiquitination and degradation of SYVN1, thereby disrupting the interaction between this protein and other components of the ERAD complex such as BiP and SEL1L. The impaired ERAD process in turn resulted in accumulation of the pro-apoptotic p53 protein. Our findings provide a framework to explain the role of NAPA and protein transport (i.e. membrane fusion) in regulating the sensitivity of cancer cells to cisplatin-induced apoptosis.

In this study, we also described the involvement of ERK signaling following cisplatin treatment and NAPA knockdown. Notably, inhibition of the ERK pathway blocked the degradation of SYVN1 induced by NAPA knockdown and led to the recovery of the ERAD complex that functionally degrades p53 in the cytoplasm (Fig. 6D). Unlike NAPA knockdown, however, SYVN1 is not degraded following cisplatin treatment in Luc-knockdown cells (Fig. 5C). The ubiquitination and degradation of SYVN1 induced by knockdown of NAPA may occur via activation of ERK. Namely, phosphorylation of SYVN1 may be required and may occur prior to the ubiquitination and degradation of the protein. Therefore, we subjected SYVN1 to mass spectrometry analysis to determine its phosphorylation sites. An online TiO2/RP-2DLC (titanium oxide/reverse phase two-dimensional liquid chromatography) system described previously [44] was used to enrich phosphopeptides from immunoprecipitated, SDS-PAGE-resolved, and trypsin-digested SYNV1 preparations. Coupled to a highly sensitive LTQ-Orbitrap mass spectrometer, numerous phosphopeptides originating from other proteins co-purified with SYNV1 and were identified by this way (data not shown). However, we failed to detect any differences in the phosphorylation sites of SYNV1 proteins between NAPA-knockdown cells treated or not with the ERK inhibitor. Although it is possible that the level of existing phosphopeptides of SYNV1 are below the limits of the technology used, this result suggests that ubiquitination of SYVN1 may involve a yet-to-be-defined protein that is regulated by ERK-dependent phosphorylation. Collectively, we observed that NAPA knockdown leads to the disruption of the ERAD and to the accumulation of p53 which, in the presence of cytotoxic concentrations of cisplatin, transactivates various pro-apoptotic genes like *Bax* and activates apoptosis. Combination of cisplatin and agents that disrupt the ERAD complex, like knockdown of NAPA, considerably increases both p53-dependent and p53-independent apoptosis, thereby providing a novel and attractive strategy to improve cisplatin-based chemotherapy against human cancers.

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgements

The authors would like to thank Dr. S.-H. Chen for reagents and Jan Martel for help during preparation of the manuscript. This study was supported by the National Science Council, Taiwan (Contracts No. NSC96-2320-B-182-034 and NSC97-2320-B-182-024-MY3) and by Chang Gung University (Contracts No. CMRPD150291 and CMRPD150292). RNAi reagents were obtained from the National RNAi Core Facility which is located at the Institute of Molecular Biology/Genomic Research Center (Academia Sinica), and is supported by the National Research Program for Genomic Medicine Grants of the National Science Council (NSC 97-3112-B-001-016).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2011.08.018.

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-diamminedicholoroplatinum(II) resistance: a review. J Formosan 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] Song K, Li Z, Seth P, Cowan KH, Sinha BK. Sensitization of cis-platinum by a recombinant adenovirus vector expressing wild-type p53 gene in human ovarian carcinomas. Oncol Res 1997;9:603–9.
[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] Siddik ZH. Cisplatin: mode of cytotoxic action and molecular basis of resistance. Oncogene 2003;22:7265–79.
[11] Wang D, Lippard SJ. Cellular processing of platinum anticancer drugs. Nat Rev Drug Discov 2005;4:307–20.
[12] 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.
[13] Wang L, He G, Zhang P, Wang X, Jiang M, Yu L. Interplay between MDM2, MDMX Pirh2 and COP1: the negative regulators of p53. Mol Biol Rep 2011;38:229–36.
[14] Heminger K, Markey M, Mpagi M, Berberich SJ. Alterations in gene expression and sensitivity to genotoxic stress following HdmX or Hdm2 knockdown in human tumor cells harboring wild-type p53. Aging (Albany NY) 2009;1:89–108.
[15] Koster R, Timmer-Bosscha H, Bischoff R, Gietema JA, de Jong S. Disruption of the MDM2-p53 interaction strongly potentiates p53-dependent apoptosis in cisplatin-resistant human testicular carcinoma cells via the Fas/FasL pathway. Cell Death Dis 2011;2:e148.
[16] Baumann O, Walz B. Endoplasmic reticulum of animal cells and its organization into structural and functional domains. Int Rev Cytol 2001;205:149–214.
[17] Voeltz GK, Rolls MM, Rapoport TA. Structural organization of the endoplasmic reticulum. EMBO Rep 2002;3:944–50.
[18] Breckenridge DG, Germain M, Mathai JP, Nguyen M, Shore GC. Regulation of apoptosis by endoplasmic reticulum pathways. Oncogene 2003;22:8608–18.
[19] Kuwana T, Newmeyer DD. Bcl-2-family proteins and the role of mitochondria in apoptosis. Curr Opin Cell Biol 2003;15:691–9.
[20] Thomenius MJ, Distelhorst CW. Bcl-2 on the endoplasmic reticulum: protecting the mitochondria from a distance. J Cell Sci 2003;116:4493–9.
[21] 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.
[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] 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.
[24] Nakajima K, Hirose H, TaniguchiM, Kurashina H, Arasaki K, Nagahama M, et al. Involvement of BNIP1 in apoptosis and endoplasmic reticulum membrane fusion. EMBO J 2004;23:3216–26.
[25] Bordallo J, Plemper RK, Finger A, Wolf DH. Der3p/Hrd1p is required for endoplasmic reticulum-associated degradation of misfolded lumenal and integral membrane proteins. Mol Biol Cell 1998;9:209–22.
[26] Friedlander R, Jarosch E, Urban J, Volkwein C, Sommer T. A regulatory link between ER–associated protein degradation and the unfolded–protein response. Nat Cell Biol 2000;2:379–84.
[27] Travers KJ, Patil CK, Wodicka L, Lockhart DJ, Weissman JS, Walter P. Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER–associated degradation. Cell 2000;101:249–58.
[28] Meusser B, Hirsch C, Jarosch E, Sommer T. ERAD: the long road to destruction. Nat Cell Biol 2005;7:766–72.
[29] Yamasaki S, Yagishita N, Nishioka K, Nakajima T. The roles of synoviolin in crosstalk between endoplasmic reticulum stress-induced apoptosis and p53 pathway. Cell Cycle 2007;6:1319–23.
[30] 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.
[31] Wu ZZ, Chao CC. Knockdown of NAPA using short-hairpin RNA sensitizes cancer cells to cisplatin: implications to overcome chemoresistance. Biochem Pharmacol 2010;80:827–37.
[32] Wu ZZ, Lu HP, Chao CC. Identification and functional analysis of genes which confer resistance to cisplatin in tumor cells. Biochem Pharmacol 2010;80:262–76.
[33] 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.
[34] 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.
[35] 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.
[36] McCracken AA, Brodsky JL. Evolving questions and paradigm shifts in endoplasmic-reticulum-associated degradation (ERAD). Bioessays 2003;25:868–77.
[37] Aylon Y, Oren M. Living with p53, dying of p53. Cell 2007;130:597–600.
[38] Tsuruya K, Yotsueda H, Ikeda H, Taniguchi M, Masutani K, Hayashida H, et al. Involvement of p53-transactivated Puma in cisplatin-induced renal tubular cell death. Life Sci 2008;83:550–6.
[39] Guntur VP, Waldrep JC, Guo JJ, Selting K, Dhand R. Increasing p53 protein sensitizes non-small cell lung cancer to paclitaxel and cisplatin in vitro. Anticancer Res 2010;30:3557–64.
[40] 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.
[41] 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.
[42] 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.
[43] 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.
[44] Lin CY, Tan BC, Liu H, Shih CJ, Chien KY, Lin CL, et al. Dephosphorylation of nucleophosmin by PP1β facilitates pRB binding and consequent E2F1-dependent DNA repair. Mol Biol Cell 2010;21:4409–17.