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# Dephosphorylation of Nucleophosmin by PP1β Facilitates pRB Binding and Consequent E2F1-dependent DNA Repair

**Chiao Yun Lin,*†‡ Bertrand Chin-Ming Tan,§‖† Hsuan Liu,* Chii-Jiun Shih,§ Kun-Yi Chien,‖ Chih-Li Lin,§ and Benjamin Yat-Ming Yung*¶**

*Cancer Biochemistry Laboratory, Department of Pharmacology, §Department of Biomedical Sciences, and ‖Graduate Institute of Biomedical Sciences, College of Medicine, Chang Gung University, Tao-Yuan 333, Taiwan; and ¶Department of Health Technology and Informatics, The Hong Kong Polytechnic University, Kowloon, Hong Kong

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**Nucleophosmin (NPM) is an important phosphoprotein with pleiotropic functions in various cellular processes. Although phosphorylation has been postulated as an important functional determinant, possible regulatory roles of this modification on NPM are not fully characterized. Here, we find that NPM is dephosphorylated on various threonine residues (Thr199 and Thr234/237) in response to UV-induced DNA damage. Further experiments indicate that the serine/threonine protein phosphatase PP1β is a physiological NPM phosphatase under both the genotoxic stress and growth conditions. As a consequence, NPM in its hypophosphorylated state facilitates DNA repair. Finally, our results suggest that one possible mechanism of this protective response lies in enhanced NPM-retinoblastoma tumor suppressor protein (pRB) interaction, leading to the relief of the repressive pRB–E2F1 circuitry and the consequent transcriptional activation of *E2F1* and several downstream DNA repair genes. Thus, this study unveils a key phosphatase of NPM and highlights a novel mechanism by which the PP1β–NPM pathway contributes to cellular DNA damage response.**

## INTRODUCTION

Nucleophosmin (NPM) is an abundant and ubiquitously expressed nucleolar phosphoprotein indispensable for various cellular processes, such as ribosome biogenesis, cell cycle progression, apoptosis, and cell differentiation (Lim and Wang, 2006; Okuwaki, 2008). Its activities on these processes might be closely relevant to its role in human cancer, in which NPM is often found overexpressed or mutated (Grisendi *et al*., 2006). However, the biological connection of NPM to tumorigenesis and the underlying molecular mechanisms remain undetermined. One aspect of NPM-associated cell growth control lies in the acute response of mammalian cells to environmental stresses induced by DNA-damaging agents (Gjerset, 2006). Previously, we and others have demonstrated the significance of NPM in cellular DNA repair capacity, survival after DNA damage, and the maintenance of genomic stability (Wu *et al*., 2002a,b; Colombo *et al*., 2005; Li *et al*., 2006). Its role in this regard is partly mediated by its contribution to the stabilization and activation of the tumor suppressor p53 (Colombo *et al*., 2002; Maiguel *et al*., 2004). Underlying molecular mechanisms have not been characterized in detail.

Consistent with its pleiotropic functions in the cells, NPM is tightly regulated through multiple mechanisms. Its expression and subnuclear localization alter under various cellular contexts (Yung *et al*., 1985; Borer *et al*., 1989; Chan, 1992; Wu and Yung, 2002; Brady *et al*., 2004; Liu *et al*., 2007b). NPM also has been shown to undergo various types of posttranslational modification (Okuwaki, 2008). Several kinases, including casein kinase 2, nuclear kinase II, Polo-like kinase, ATR, and cyclin-dependent kinases (CDK1/cyclin B, CDK2/cyclin E, and CDK2/cyclin A) have been known to modify NPM, which is generally regarded as a phosphoprotein (Chan *et al*., 1990; Peter *et al*., 1990; Jiang *et al*., 2000; Okuda *et al*., 2000; Tokuyama *et al*., 2001; Okuwaki *et al*., 2002; Szebeni *et al*., 2003; Maiguel *et al*., 2004; Zhang *et al*., 2004; Negi and Olson, 2006). Consequently, certain functional attributes of NPM, such as RNA binding activity and centrosome duplication regulation, are tightly coordinated with its phosphorylation at various residues. Although these signaling pathways have been well documented, knowledge of NPM-targeting phosphatase, as well as the regulatory and functional significance of NPM dephosphorylation, has remained elusive.

In the present study, we discovered that NPM is targeted by the protein phosphatase PP1β. Depletion of PP1β by RNA interference (RNAi) in cycling or irradiated cells resulted in increased steady-state levels of phospho-NPM,

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† These authors contributed equally to this work.

‡ Present address: Department of Obstetrics and Gynecology, Chang Gung Memorial Hospital, Kwei-Shan, Tao-Yuan, Taiwan.

Address correspondence to: Bertrand Chin-Ming Tan (btan@mail.cgu.edu.tw) or Benjamin Yat-Ming Yung (htbyung@inet.polyu.edu.hk).

indicating that PP1$\beta$ is a physiological NPM phosphatase. We further demonstrated that the PP1$\beta$–NPM pathway is activated by DNA damage signal. Crucially, dephosphorylation of NPM enhances its activity in facilitating DNA repair. Finally, our results indicated that one possible mechanism of this protective response lies in the transcriptional upregulation of E2F1.

## MATERIALS AND METHODS

### *Antibodies and Reagents*

Anti-NPM monoclonal antibody (mAb) was kindly provided by Dr. P. K. Chan (Balyor College of Medicine, Houston, TX). Mouse monoclonal antibodies against E2F1, $\beta$-actin, retinoblastoma tumor suppressor protein (pRB), and rabbit polyclonal antisera against PP1$\alpha$ and PP1$\gamma$ were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-PP1$\beta/\delta$ rabbit polyclonal antibodies were purchased from Millipore (Billerica, MA). Rabbit antisera against phospho-NPM (Thr199), phospho-NPM (Thr234/237), and phospho-PP1$\alpha$ (Thr320) were from Cell Signaling Technology (Danvers, MA). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), except where otherwise indicated.

### *Cell Culture and Transfection*

HeLa, 293T, and H1299 cells were grown as a monolayer in DMEM supplemented with 10% fetal bovine serum and 100 U/ml penicillin and streptomycin, in a 5% $CO_2$-humidified incubator at 37°C. Cells were transfected using Lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Transient transfection was done for 24–54 h before cell harvest, unless otherwise noted.

### *Reporter Gene Assay*

For the E2F1 (Lin *et al*., 2006) and p53 (Chan *et al*., 2005) promoter reporter assay, the cells extracts were obtained with 1× Reporter lysis buffer (Promega, Madison, WI), and the reporter/luciferase activity was measured using Luciferase assay reagent (Promega) following the manufacturer's recommendations. Luciferase activity was measured with Luminometer AutoLunmat LB953 (Berthold, Norwalk, CT) and normalized to the corresponding $\beta$-galactosidase activity.

### *UV and Phosphatase Inhibitor Treatments*

Cells were grown to 80% confluence in 6-cm dishes. DNA damage was achieved by exposing the cells to UV irradiation (50 J/m$^2$). Cells were harvested at indicated times. In some experiments, they were treated with okadaic acid (OA; 50 nM) or calyculin A (5 nM) for 3 h before UV treatment.

### *Western Blot Analysis*

Cells were harvested and washed twice in phosphate-buffered saline (PBS) and then lysed in ice-cold Gold lysis buffer (1% Triton X-100, 10% glycerol, 1 mM sodium orthovanadate, 1 mM EGTA, 10 mM NaF, 1 mM sodium pyrophosphate, 10 $\mu$M $\beta$-glycerophosphate, 20 mM Tris-HCl, 137 mM NaCl, 5 mM EDTA, and cocktail protease inhibitor (Sigma-Aldrich), pH 7.9) for 30 min. Lysates were boiled in 2× urea sample buffer dye (100 mM Tris-HCl, pH 6.8, 4% SDS, 0.2% bromphenol blue, 20% glycerol, 200 mM $\beta$-mercaptoethanol, and 8 M urea), and then fractionated by SDS-polyacrylamide gel electrophoresis (PAGE).

Western blot analysis was performed after electrophoretic separation of polypeptides by 8 or 10% SDS-PAGE and transfer to Hybond-polyvinylidene difluoride membrane (GE Healthcare, Piscataway, NJ). Blots were probed with the indicated primary and appropriate secondary antibodies. Immunobands were subsequently detected by the enhanced chemiluminescence reaction (GE Healthcare).

### *Generation of Small Interfering RNAs (siRNAs)*

To establish a plasmid-based double-stranded RNAi system targeting endogenous PP1$\alpha$, PP1$\beta$, or PP1$\gamma$, annealed oligonucleotides corresponding to partial sequence were designed and ligated to pSuper.neo+GFP (Oligoengine, Seattle, WA) according to manufacturer's instruction. The cDNA sequence of the targeted mRNA region for different genes is as follows: PP1$\alpha$, 5′-GAGACGCTACAACATCAAA-3′; PP1$\beta$, 5′-GTTCGAGGCTTATGTATCA-3′; PP1$\beta$-2, 5′-TGTGCAGATGACTGAAGCA-3′, PP1$\gamma$, 5′-AGAGGCAGTTGGTCACTCT-3′; E2F1, 5′-CGCTATGAGACCTCACTGA-3′; and E2F1-2, 5′-GTGGACTCTTCGGAGAACT-3′.

### *Site-directed Mutagenesis*

To generate phosphorylation-site mutant constructs of NPM for expression in the cells, an appropriate set of oligonucleotide primers were used for site-directed amino acid substitutions (serine or threonine to alanine). We used full-length NPM cDNA in plasmid pCR3.1-FLAG-NPM as a template, and the primer sequences for cloning S125A, T199A, T199D, T234/237A, and T234/237D variants were listed in Supplemental Table S1.

### *Immunoprecipitation*

The cells were harvested, washed twice in ice-cold PBS, and the cell pellets were resuspended in hypotonic RSB buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, and 1 mM phenylmethylsulfonyl fluoride) at 4°C for 10 min. An equal volume of an RSB + NP40 (20%) buffer was added next and incubated at 4°C for 10 min. Nuclei were collected by centrifuged at 3000 rpm at 4°C for 5 min and resuspended in ice-cold Gold lysis buffer (20 mM Tris-HCl, pH 7.5, 10% glycerol, 1% Triton X-100, 1 mM sodium pyrophosphate, 100 mM $\beta$-glycorophosphate, 1 mM sodium orthovanadate, 137 mM sodium chloride, 5 mM EDTA, 1 mM EGTA, and protease inhibitor cocktail) for 30 min and subsequently centrifuged at 12,000 rpm at 4°C for 30 min. Equal amounts of nuclei extract protein (1 mg) were incubated with the indicated antibodies (2 $\mu$g) at 4°C for 2 h with rotation. The immunocomplexes were captured with protein G-Sepharose (30 $\mu$l) for 2 h at 4°C with rotation. The protein G–antigen–antibody complexes were washed four times with the Gold lysis buffer and boiled in 2× urea sample buffer dye (100 mM Tris-HCl, pH 6.8, 4% SDS, 0.2% bromphenol blue, 20% glycerol, 200 mM $\beta$-mercaptoethanol, and 8 M urea) for subsequent PAGE and immunoblotting analysis as described above.

### *Chromatin Immunoprecipitation (ChIP)*

ChIP experiments were done essentially as described previously (Liu *et al*., 2007a). Cross-linked, sonicated chromatin was precleared before being incubated with 2 $\mu$g of mouse mAb (pRB or E2F1) and rotated at 4°C overnight. Normal mouse immunoglobulin (Ig)G was used for the mock immunoprecipitation. After extensive washes, immunocomplexes were treated with proteinase K and decross-linked. Bound DNA in the pRB ChIP was extracted, purified, and subjected to polymerase chain reaction (PCR) analysis by using primers corresponding to the *E2F1* gene promoter sequence (Lin *et al*., 2006). After 35 cycles of amplification, PCR products were run on a 2% agarose gel and analyzed by ethidium bromide staining. For anti-E2F1 ChIP, bound DNA was subjected to quantitative real-time PCR analysis.

### *RNA Isolation and Reverse Transcription (RT)-PCR*

Total cellular RNAs were isolated from cells using the Trizol Reagent (Invitrogen) according to the manufacturer's instructions. Total RNA (2–5 $\mu$g) was reverse transcribed with reverse transcriptase (Moloney murine leukemia virus) (Invitrogen) at 37°C for 52 min. cDNA synthesis was primed with random hexamer. The *E2F1* and internal control *β-actin* genes were amplified with the following specific primers: *E2F1*, 5′-CCGAGGTGCTGAAGGTGCAGA-3′ (sense), 5′-TCTTCCCAGGGCTGATCCCAC-3′ (antisense); and *β-actin*, 5′-AGAAAATCTGGCACCACACC-3′ (sense), 5′-CCATCTCTTGCTCGAAGTCC-3′ (antisense). The PCR products were resolved on 1.5% agarose gels and stained with ethidium bromide.

### *Real-Time PCR*

For quantitative determination by real-time PCR, target transcripts or ChIP products were analyzed using the LightCycler system and Power SYBR Green PCR Master Mix (Roche Diagnostics, Indianapolis, IN). Transcript levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) values of the respective sample, and represent mean ± SD of at least three independent experiments. Sequences for the primers used in this experiment are as follows: *E2F1*, 5′-AGATGGTTATGGTGATCAAAGCC-3′ (sense), 5′-ATCTGAAAGTTCTCCGAAGAGTCC-3′ (antisense); *GAPDH*, 5′-GGTATCGTGGAAGGACTCATGAC-3′ (sense), 5′-ATGCCAGTGAGCTTCCCGT-3′ (antisense); *XPC*, 5′-CATCATCCCAGCCCGCTTTAC-3′ (sense), 5′-CCACTTCACCAGGTTTGAGAGG-3′ (antisense); *DDB2*, 5′-GCACCTCACACCTATCAAG-3′ (sense), 5′-GAGCTGACACATCATCTTCC-3′ (antisense); and *RPA3*, 5′-CGTCACTAAGCAGCCAATC-3′ (sense), 5′-GCACCAATCAGCGAAGAC-3′ (antisense). For quantitative analysis of anti-E2F1 ChIP experiments, primers corresponding to gene promoters were used: *XPC*, 5′-GAAATAGAGAGAAACCTGTTGT-3′ (sense), 5′-CTAGTCACGCCCCTAAAG-3′ (antisense); and *DDB2*, 5′-ATGTTTGGCGGGAAGTTG-3′ (sense), 5′-TCTGGGGAGAAACAAGGC-3′ (antisense). The results are given as a percentage of input and represent mean ± SD of at least three independent experiments. Triplicate PCRs were performed for all experiments.

### *In Vitro Phosphatase Assay*

The cells were okadaic acid treated (50 nM) for 8 h before harvest. Nuclei extracts were prepared and subjected to immunoprecipitation by using the anti-NPM antibody. The immunocomplexes were washed with reaction buffer (1 mM $MnCl_2$ and 5 mM caffeine). One unit of PP1 (New England Biolabs, Ipswich, MA) was added to the immunocomplexes for 1 h at 30°C. For inhibition studies, the immunocomplexes and PP1 were incubated in the presence of 1 $\mu$M OA. Degree of dephosphorylation was analyzed by SDS-PAGE and subsequently immunoblotting.

### *Measurement of Thymine Dimers*

To estimate the DNA repair capacity of cells, the amounts of thymine dimers in UV-irradiated cells were measured by enzyme-linked immunosorbent assays (ELISAs) as described previously (Zhai *et al.*, 2005) using an anti-thymine dimer antibody (Abcam, Cambridge, MA). DNA was extracted from the cells at the indicated times postirradiation. For statistical significance of quantitative comparisons, calculations were done by the SigmaPlot software (Systat Software, San Jose, CA).

### *DNA Repair Capacity/Host Cell Reactivation (HCR) Assay*

This method for measuring nucleotide excision repair (NER) in response to UV irradiation was performed based on a previous report (Athas *et al.*, 1991). The pCAT control vector (Promega) is a nonreplicated plasmid, under control of simian virus 40 promoter and enhancer sequences. The plasmid was treated with or without UV (254 nm) at the dose of 500 J/m$^2$ and then cotransfected with unirradiated pGL3luc (as an internal control) as well as plasmids encoding various phosphorylation-site mutants of NPM into HeLa cells. The cells were lysed in 1× Reporter lysis buffer 24 h after transfection, and chloramphenicol acetyltransferase (CAT) activity was detected as described previously (Wu *et al.*, 2002a,b). After normalization, relative CAT activity was expressed as the percent of activity in cells transfected with the UV-irradiated plasmid over those harboring the undamaged plasmid. For statistical significance of quantitative comparisons, calculations were done by the SigmaPlot software.

## RESULTS

### *NPM Is Subject to Dephosphorylation at Multiple Sites*

To characterize the involvement of NPM in cellular response to gentoxic stress, we first tested whether DNA damage affects the phosphorylation of several sites on NPM. HeLa cells were treated with UV radiation, and cell lysates collected at different time points posttreatment were probed with phosphospecific NPM antibodies. Interestingly, UV enhanced dephosphorylation of NPM at Threonine 199, 234, and 237 residues (Figure 1A). Dephosphorylation was detected at 6 h after UV treatment and sustained at 9 h. Similar patterns of modification changes were also evident in the 293T and p53-null H1299 cell lines (Supplemental Figure S1).

It was reported previously that PP1 is a candidate phosphatase for nucleolar proteins such as nucleolin and NPM (Kotani *et al.*, 1998). In line with this observation, we found that the phosphorylation levels of NPM on Thr199 and Thr234/237 were significantly enhanced in cells treated with PP1/PP2A phosphatase inhibitor OA or calyculin A (Supplemental Figure S2, A and B), thus providing further support to the notion that phosphorylation of this protein is counterbalanced by the action of phosphatase under normal growth condition. Based on these results, we next sought to determine whether PP1 is the specific phosphatase that is responsible for UV-induced NPM dephosphorylation. To this end, we first assessed the changes in the PP1 activity upon irradiation by performing immunoblotting with an antibody that specifically recognizes phospho-Thr320 of PP1. This modification was shown previously to be linked to the inactivation of PP1 enzymatic activity (Kwon *et al.*, 1997; Liu *et al.*, 1999). As shown in Figure 1B, our results showed that the level of phospho-Thr320 was reduced after UV treatment, indicating an up-regulation of PP1 phosphatase activity that is responsive to DNA damage. Next, to further investigate whether PP1 can indeed dephosphorylate NPM, we carried out an in vitro phosphatase assay. By monitoring the phosphorylation of endogenous NPM immunoprecipitated from cells, we found that purified recombinant PP1 is capable of removing phosphate groups on the Thr199 and Thr234/237 residues of NPM (Figure 1C and Supplemental Figure S2C). Furthermore, such specific phosphatase activity was lost upon inhibitor treatment (Supplemental Figure S2C). Together, these results showed that UV treatment is capable of inducing dephosphorylation of NPM at several sites and that PP1 is likely to be involved in this removal of phosphorylation after DNA damage.

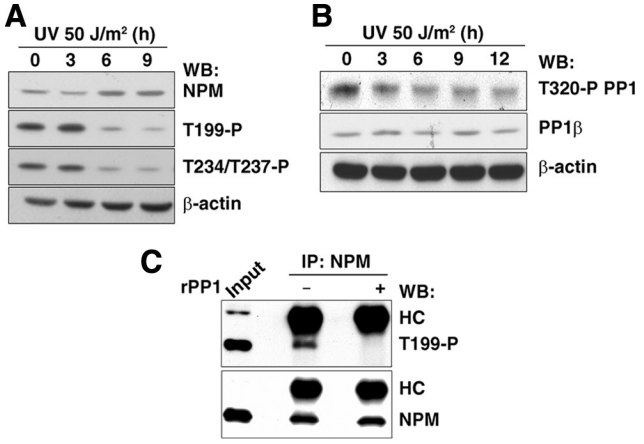


**Figure 1.** NPM undergoes dephosphorylation at multiple sites in response to UV-induced DNA damage. (A) HeLa cells were exposed to 50 J/m$^2$ UV irradiation. Lysates were prepared from cells collected at the indicated times postirradiation. Western blotting was performed using the indicated antibodies. (B) Unirradiated and irradiated HeLa cells were harvested at the indicated times after UV (50 J/m$^2$) treatment. Equal amounts of lysate proteins were separated by PAGE and subjected to immunoblotting with antibodies for phospho-PP1 (Thr320), PP1β, and β-actin. (C) Lysates were prepared from HeLa cells (previously treated with 50 nM okadaic acid; Input) and subjected to immunoprecipitation using anti-NPM antibody. In vitro solid phase phosphatase assay was performed on the immunoprecipitates as described in *Materials and Methods* (with recombinant phosphatase, rPP1). The resultant products were probed with the indicated antibodies. HC, heavy chain.

### *Identification of Specific Isoform of PP1 That Targets NPM after DNA Damage*

We next aimed to pinpoint the specific isoform of PP1 responsible for mediating UV-induced dephosphorylation of NPM. Because the mammalian genomes encode three closely related PP1 genes—α, β, and γ—specific ablation of individual PP1 isoform expression was achieved by expressing isoform-specific short hairpin RNA(shRNA) (Figure 2A). HeLa cells depleted of either PP1 isoform as well as control were UV irradiated (Supplemental Figure S3A), and the level of phosphorylation of NPM on Thr199 or Thr234/237 was measured at 0, 3, and 6 h after treatment. Among the three isoforms, depletion of PP1β caused dramatic NPM hyperphosphorylation, whereas PP1α and -γ knockdown had little effect on the basal phosphorylation level (Figure 2B and its shorter exposed version in Supplemental Figure S3B). Furthermore, knockdown of PP1β, but not of PP1α or PP1γ, blocked the UV-induced dephosphorylation of NPM on Thr199 and Thr234/237 (Figure 2B and Supplemental Figure S3B). Similar outcomes of PP1β knockdown also were observed in 293T cells (Supplemental Figure S3C). Consistently, when PP1β was overexpressed in cells, phosphorylation at these threonine residues underwent considerable reduction (Supplemental Figure S3D). An association of transiently overexpressed (Figure 2C) or endogenous (Figure 2D) PP1β with NPM also was found in coimmunoprecipitation experiments. Specificity of such interaction was reinforced by the observations that PP1α and PP1γ did not coprecipitate NPM (Figure 2C). Collectively, these results strongly indicate that PP1β plays an important role in

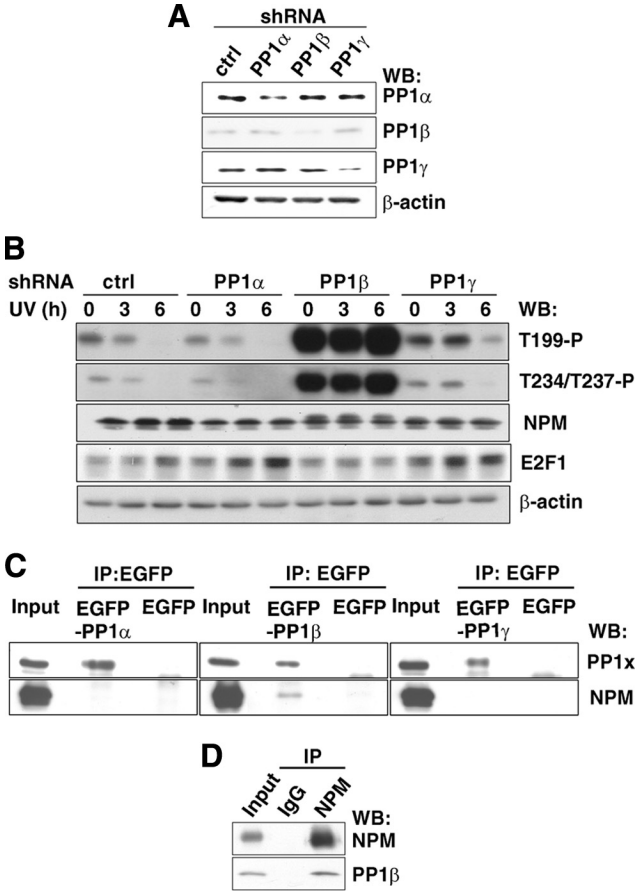


**Figure 2.** PP1$\beta$ is the physiological NPM phosphatase in response to UV. (A) Ablation of endogenous expression of the three PP1 isoforms was achieved by RNAi. HeLa cells transiently harboring control-, PP1$\alpha$-, PP1$\beta$-, or PP1$\gamma$-targeting shRNA (54 h) were harvested. Equal loadings of whole-cell extracts were immunoblotted with antibodies against specific PP1 isoforms, and $\beta$-actin. (B) HeLa cells transiently harboring control-, PP1$\alpha$-, PP1$\beta$-, or PP1$\gamma$-targeting shRNA (54 h) were exposed to UV irradiation (50 J/m$^2$). Equal loadings of whole-cell extracts derived at 0, 3, and 6 h posttreatment were immunoblotted with the indicated antibodies. (C and D) Coprecipitation of NPM with PP1$\beta$. HeLa cells were transfected with constructs encoding enhanced green fluorescent protein (EGFP) or EGFP-tagged PP1$\alpha$ (left), PP1$\beta$ (middle), or PP1$\gamma$ (right) (C). Immunoprecipitation (IP) of the cell lysates was done using EGFP antibodies and subsequently analyzed by immunoblotting with antibodies against corresponding PP1 isoforms (PP1x, top) and NPM (bottom). (D) Anti-NPM immunoprecipitates (IP) from HeLa cell extracts (Input) were probed with antibodies against NPM (top) and PP1$\beta$ (bottom).

dephosphorylating NPM on Thr199 and Thr234/237 upon UV irradiation.

We also characterized the dephosphorylation of NPM in a more comprehensive manner, by conducting a mass spectrometry-based comparative phosphoproteome analysis on NPM isolated from control and PP1$\beta$ knockdown cells (see Supplemental Material for detailed methods). Based on phosphopeptide profiling, we identified three additional phosphorylation sites on NPM (Ser4, Ser70, and Ser125; Supplemental Table S2 and Supplemental Figure S3E). However, quantitative comparison of phosphopeptides harboring Ser4 and Ser70 did not indicate significant changes in their abundance between the control and knockdown samples but revealed a slightly lower level of peptides with Ser125 phosphorylation in the PP1$\beta$-depleted cells (Supplemental Table S2 and Supplemental Figure S3E). We thus concluded that the phosphorylation levels of these residues are not subject to PP1$\beta$-mediated dephosphorylation.

### *Phosphorylation of NPM Impairs Cellular Response to DNA Damage by UV*

We demonstrated previously an ability of NPM in endowing the cells with a greater NER activity under UV damage (Wu *et al*., 2002a,b). It is therefore possible that the observed UV-induced dephosphorylation of NPM may be a determinant for this protective function of NPM. To address this issue, we next undertook a reporter plasmid-based assay to assess the effect of NPM phosphorylation on cellular DNA repair capacity. Consistent with the previous studies, overexpression of wild-type NPM rescued the expression of reporter from UV-damaged plasmid, indicating an increased host NER activity (Figure 3A). Interestingly, expression of a variant of NPM in which Thr199/234/237 were changed to Ala (3A) led to an even further elevated reporter activity, whereas the stimulatory effect was abolished in the T199/234/237D (3D) mutant.

To further examine the role of NPM dephosphorylation in cellular response to UV damage in vivo, we measured by ELISA the cellular capacity of removing UV-induced thymine dimers. HeLa cells were transiently transfected with various phosphorylation-site mutants of NPM and subse-

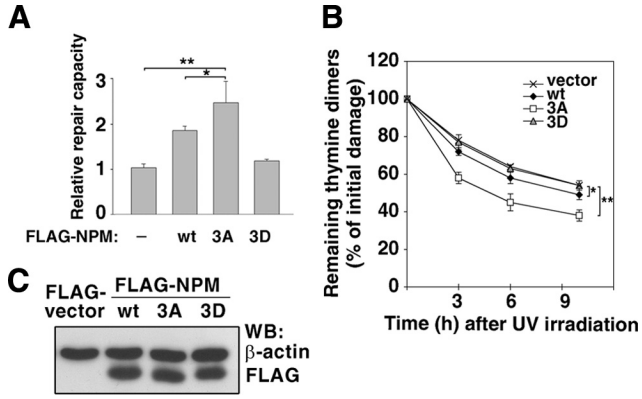


**Figure 3.** NPM phosphorylation status modulates cellular response to UV-induced DNA damage. (A) Assay for DNA repair capacity was performed as described in *Materials and Methods*. HeLa cells were transfected with control vector (−) or the indicated FLAG-tagged NPM (FLAG-NPM) constructs (3A and 3D represent site-directed mutants in which the Thr199, Thr234, and Thr237 residues were respectively altered to alanine and aspartic acid), along with a CAT reporter plasmid damaged with UV. Same experiments were done with an unirradiated CAT plasmid. DNA repair capacity was measured based on the normalized CAT activity and was expressed as percentage of activity in cells transfected with the UV-irradiated plasmid over those with unirradiated plasmid. *$p < 0.05$; **$p < 0.01$. (B) Phosphorylation-defective mutant of NPM enhances the capacity of cells to remove thymine dimers. Cells were transfected with the indicated constructs as described in A. These cells were subsequently irradiated with UV and lysed immediately (at time 0) or at the times indicated. The thymine dimers were analyzed by ELISA (see *Materials and Methods*) and presented as the percentage to those at time 0. Data presented are means ± SD of four independent experiments. *$p < 0.05$, the wild-type (wt) cells versus the vector; **$p < 0.01$, the 3A cells versus the 3D cells. (C) Protein expression of the overexpressed constructs in B (at time 0 of UV treatment) was monitored by Western blotting analysis, by using anti-FLAG and anti-$\beta$-actin antibodies.

quently irradiated (Figure 3C). In cells that expressed control vector and the phosphomimetic mutant (3D) of NPM, ~30% of thymine dimers detected immediately after UV irradiation (at time 0) were removed within 3 h after irradiation, and the remaining damage was slightly decreased up to 9 h (Figure 3B). In contrast, phosphorylation-defective version of NPM (3A) substantially enhanced the ability of the cells to repair thymine dimers, especially within the first 6 h of UV treatment. Together, these data clearly show that the dephosphorylated species of NPM promotes repair of DNA damage induced by UV.

### *PP1β-mediated Dephosphorylation of NPM Promotes E2F1 Expression*

Next, we sought to further delineate the mechanism underlying NPM's involvement in DNA repair. Recent evidence has supported a role of NPM in the transcriptional regulation of *E2F1* (Lin *et al*., 2006; Liu *et al*., 2007b). Coincidentally, E2F1 protein was reported to undergo accumulation after DNA damage (Lin *et al*., 2001; Ianari *et al*., 2004). As we uncovered the UV-induced NPM dephosphorylation, we also discovered a concomitant rise in E2F1 mRNA and protein levels in response to DNA damage (Supplemental Figure S4, A and B). Berton *et al*. (2005) further demonstrated in an in vivo context that such up-regulation promotes DNA repair. Based on these observations, we therefore set out to examine whether the role of dephosphorylated NPM in DNA repair is linked to *E2F1* regulation.

First, quantitative RT-PCR was carried out to examine the expression profile of *E2F1* gene in cells that harbor the wild-type, 3A, or 3D variant of NPM (Figure 4A). As shown previously (Lin *et al*., 2006), overexpression of the wild-type construct led to an increase in the *E2F1* transcript level compared with the control. Interestingly, the hypophosphorylated form of NPM (3A) augmented this expression even further. Consistently, this NPM mutant could highly activate the *E2F1* promoter activity, as shown by a reporter assay (Figure 4B). In contrast, expression of the phosphomimetic variant (3D) did not result in any significant alteration of the E2F1 mRNA level (Figure 4A). Another phosphodefective mutant that was altered on one of the MS-identified phosphorylation sites (S125A) also did not further activate E2F1 promoter activity compared with wild-type NPM (Supplemental Figure S4C), suggesting a lack of involvement of this residue in this functional aspect.

Our above-mentioned finding of PP1β's role in NPM dephosphorylation (Figure 2) also suggests its potential involvement in modulating E2F1 expression under DNA damage condition. To resolve this issue, we next assessed the effect of PP1β knockdown in a promoter reporter assay. Indeed, we found that reduction of PP1β by shRNA but not that of PP1α or PP1γ, abolished the UV-induced activation of *E2F1* promoter (Figure 4C and Supplemental Figure S4D). Moreover, the increase in E2F1 protein levels in response to UV irradiation was lost upon depletion of PP1β (Figure 2B). In line with these observations, inactivation of phosphatase activity by calyculin A treatment also down-regulated the UV-induced E2F1 protein expression (Supplemental Figure S4E). Collectively, our data imply that the UV-responsive transcriptional activation of *E2F1* may be mediated through the action of PP1β on NPM.

### *Dephosphorylated NPM Alleviates pRB-mediated Transcriptional Repression of E2F1*

pRB is known to, via complex formation with E2F1, counterbalance the activation potential of E2F1 at the transcriptional level, and its occupancy of the *E2F1* gene promoter

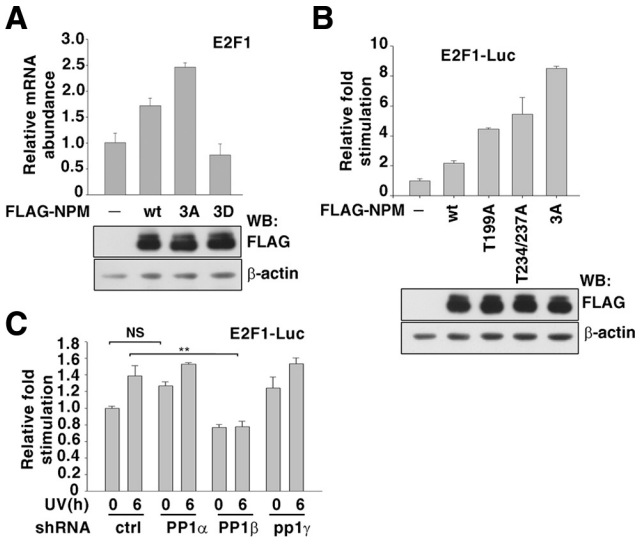


**Figure 4.** PP1β-mediated NPM hypophosphorylation triggers *E2F1* expression. (A) Total RNA was extracted from HeLa cells ectopically expressing empty vector (−), wild-type (wt), T199/234/237A (3A), or T199/234/237D (3D) variant of NPM. The mRNA level of *E2F1* gene in these cells was measured by quantitative RT-PCR (see *Materials and Methods*). Western blotting results shown on the bottom illustrate the expression levels of the indicated constructs. (B) Promoter reporter assay was performed on HeLa cells cotransfected with E2F1-Luc reporter gene and the indicated variant of NPM. Bars are means of triplicates ± SDs. Expression levels of the indicated constructs were monitored by anti-FLAG and anti-β-actin immunoblotting analysis, as shown on the bottom. (C) HeLa cells transiently harboring control-, PP1α-, PP1β-, or PP1γ-targeting shRNA (54 h) were exposed to UV irradiation (50 J/m$^2$). Cells were lysed at 6 h postirradiation and subjected to reporter gene assay as described in B. NS, $p > 0.05$; **$p < 0.01$.

has been linked to its gene regulation (Polager and Ginsberg, 2008). Recent studies have pointed to an involvement of NPM in the regulation of such transcriptional network (Lin *et al*., 2006; Liu *et al*., 2007b). Therefore, to elucidate how dephosphorylated NPM could activate *E2F1* gene expression, we next assessed the binding of pRB to the *E2F1* promoter in the context of DNA damage by using ChIP. After UV irradiation, we found a loss of pRB binding to the E2F1 binding site within the *E2F1* gene promoter (Figure 5Aa, lanes 1 and 2). Interestingly, compared with the wild-type protein, expression of the nonphosphorylatable variant of NPM (3A) also led to a decrease of pRB promoter binding (Figure 5Ab, lanes 3 and 4). On the contrary, knockdown of PP1β expression augmented promoter recruitment (Figure 5Ac, lanes 5 and 6). Collectively, these findings are in close agreement with the above observations that linked PP1β-mediated dephosphorylation of NPM to *E2F1* gene activation (Figure 4).

Consistent with an activation of E2F1, there was a disruption of the E2F1–pRB complex after UV treatment (Figure 5B). Intriguingly, coimmunoprecipitation experiments also showed that there was a coprecipitation of pRB with NPM upon UV irradiation, which was not detectable under mock treatment (Figure 5C and Supplemental Figure S5). Such association of NPM with pRB was further evident when Thr199/234/237 were substituted with Ala, compared with wild-type and the 3D mutant of NPM (Figure 5D), strongly suggesting that the NPM–pRB interaction depends on dephosphorylation of these residues. Consistently, upon siRNA-mediated reduction of intracellular PP1β, which was

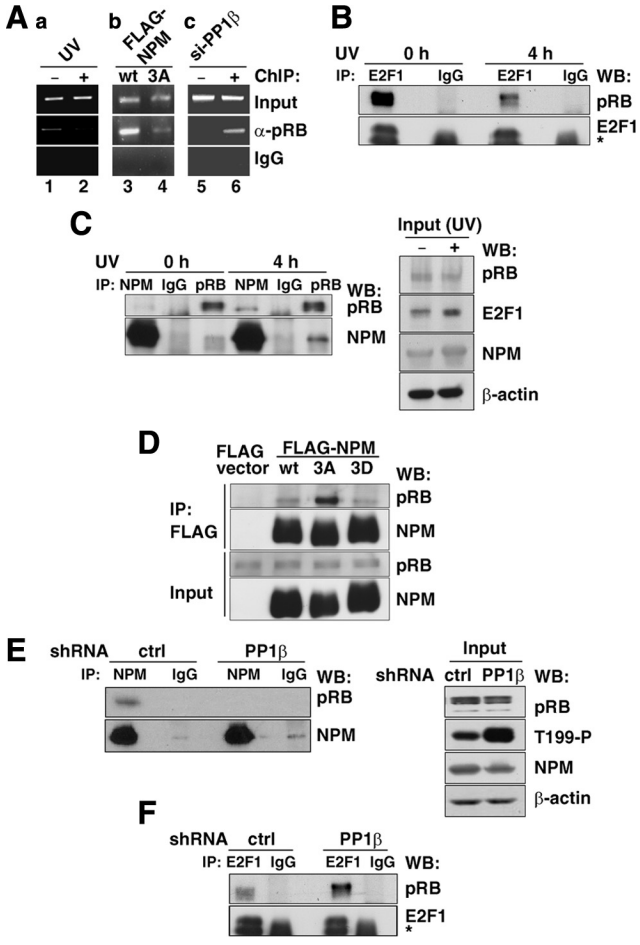


**Figure 5.** Dephosphorylated NPM prefers to associate with pRB and disrupts the pRB-E2F1 interaction. (A) ChIP was performed as described in *Materials and Methods*. Chromatin fragments were prepared from HeLa cells subjected to the following treatments: (a) mock (lane 1) or UV irradiation (50 J/m$^2$, lane 2); (b) ectopic expression of wild type (lane 3) or the 3A mutant (lane 4) of NPM; or (c) expression of control (lane 5) or PP1$\beta$-targeting (lane 6) shRNA. Immunoprecipitation was done with either control antibody (immunoglobulin G [IgG], bottom) or an antibody against pRB ($\alpha$-pRB, middle). Products (~200 base pairs) from final PCR analysis by using primers specific to *E2F1* promoter region were resolved by 1.5% agarose gel. DNA input (1/50 of immunoprecipitation) is shown in the top panels. (B) Cells were treated with mock or UV irradiation (50 J/m$^2$). At 4 h posttreatment, cell lysates (panels on the right) were prepared and immunoprecipitated with control (IgG) and anti-E2F1 antibody. Immunoprecipitates and lysate input (Input, 1/60 of IP, right) were probed for the specified proteins. (C) Immunoprecipitation was done as described in B, except with the use of control (IgG), $\alpha$-NPM, and $\alpha$-pRB antibody. (D) HeLa ells were transfected with empty vector (vector), FLAG-tagged wild-type NPM (NPM-wt), or phosphorylation site mutants T199/234/237A (NPM-3A) and T199/234/237D (NPM-3D) for 24 h. Lysates (60× input) were immunoprecipitated with anti-FLAG antibody (IP) and subsequently immunoblotted using anti-pRB (top) and anti-FLAG (bottom) antibodies, as indicated. Lysate input (Input) is shown on the bottom. (E and F) Extracts were prepared from HeLa cells ectopically expressing control or PP1$\beta$-specific shRNA and subsequently subjected to immunoprecipitation using anti-NPM (E) or anti-E2F1 (F) antibody. Control antibody (IgG) was used for mock immunoprecipitation. Immunocomplexes were probed with specified antibodies. Immunoblotting of the input proteins (1/50 of IP) is shown on the right of E. *, heavy chain of immunoglobulin G.

shown to increase the phosphorylation of NPM (Figure 2B), a loss of interaction between NPM and pRB was observed (Figure 5E). Conversely, lack of PP1$\beta$ led to an up-regulated complex formation between E2F1 and pRB (Figure 5F). Together, these results demonstrate that the interaction of pRB with hypophosphorylated NPM, in response to UV damage and mediated by PP1$\beta$, relieves its transcriptional repression activity on E2F1.

### *Dephosphorylated NPM Promotes DNA Damage Repair through an E2F1-dependent Mechanism*

Finally, to clarify whether E2F1 underlies the protective effect of NPM dephosphorylation in DNA damage response, we again performed the in vivo DNA repair assay in the context of E2F1 knockdown. Compared with control, cells lacking E2F1 exhibited diminished ability to repair UV-induced DNA damage (Figure 6A and Supplemental Figure S6). Moreover, although overexpression of NPM-3A enhanced the DNA repair activity under UV treatment, down-regulation of E2F1 expression effectively abolished such augmentation of DNA repair. Consistent with a repressive role on E2F1, overexpression of pRB also decreased the cellular capacity of removing thymine dimers (Figure 6B), mimicking the effect of E2F1 knockdown as well as the phospho-mimetic mutant 3D (Figure 3B). Furthermore, when expressed along with the NPM-3A mutant, pRB antagonized the repair-promoting activity of the hypophosphorylated NPM. Collectively, these results demonstrate the connection between NPM-mediated regulation of pRB-E2F1 pathway and the consequent activation of DNA repair.

To provide further mechanistic basis for the promotion of DNA repair in the context of NPM-mediated E2F1 up-regulation, we next evaluated possible expression of E2F1 target genes that encode DNA repair proteins. To this end, quantitative RT-PCR experiments demonstrated that the dephosphorylated form of NPM promotes the expression of several NER genes, such as *RPA3*, *XPC*, and *DDB2*, that were previously linked to E2F1 (Polager *et al*., 2002; Nichols *et al*., 2003; Prost *et al*., 2007; Lin *et al*., 2009; Figure 6C). Concomitantly, there was an increase in E2F1 occupancy of the *XPC* and *DDB2* promoters in the presence of NPM-3A (Figure 6D), in line with the notion that these genes are regulated by E2F1 in a UV-dependent manner (Prost *et al*., 2007; Lin *et al*., 2009). Conversely, no significant changes in these E2F-dependent attributes were evident in the context of NPM–3D overexpression. Finally, we performed additional experiments (Supplemental Figure S7) to determine whether dephosphorylated NPM also could functionally interact with p53, another critical regulator of DNA damage response. As shown by the reporter assay, whereas UV treatment caused a dramatic up-regulation of p53 activity, overexpression of wild-type NPM exerted a negative regulation, consistent with previous observations (Maiguel *et al*., 2004). However, overexpression of NPM-3A, compared with the wild-type form, did not lead to discernible alteration in the p53 transactivation activity (Supplemental Figure S7A). Results of the flow cytometry-based cell cycle profiling also revealed that overexpression of NPM-3A failed to affect the progression of cell cycle and extent of apoptosis (Supplemental Figure S7B). These data therefore suggest that up-regulation of E2F1 and its downstream DNA repair genes may be a major mechanism through which dephosphorylated NPM favors repair of UV-induced DNA damage. Considered together, our findings provide strong evidence that, rather than acting as a repair protein per se, NPM serves as an integral component of the DNA damage response pathway that regulates expression of DNA repair genes.

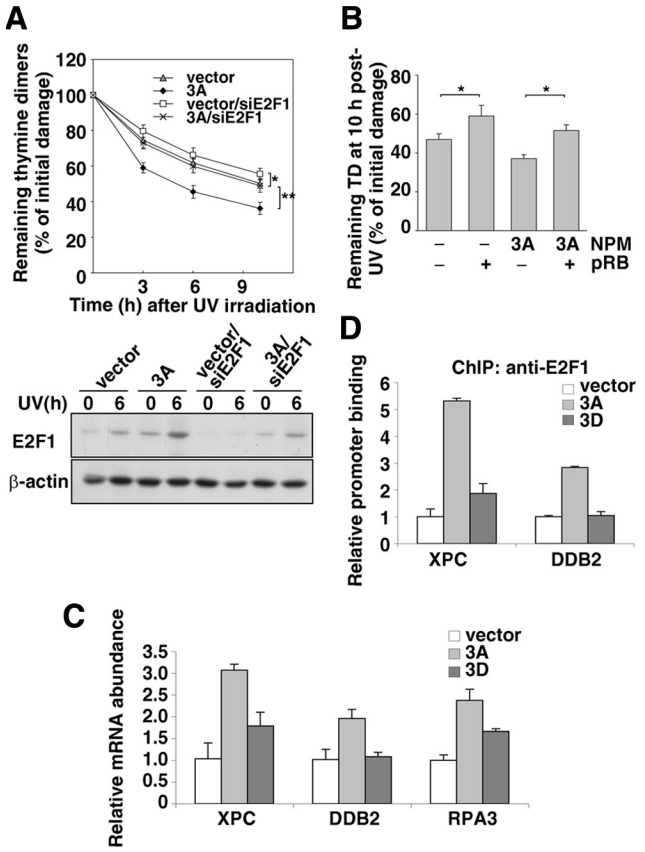


**Figure 6.** Protective role of dephosphorylated NPM in DNA damage response is mediated through E2F1 and downstream NER genes. (A) HeLa cells were transfected with control vector or the FLAG-tagged NPM-3A construct, in combination with plasmids encoding control or E2F1-targeting shRNA. Capacity of these cells to remove thymine dimers upon UV treatment was measured as in Figure 3B. The levels of thymine dimers were presented as the percentage to those at time 0. Data presented are means ± SD of four independent experiments. *$p < 0.05$, the vector cells versus the vector/siE2F1 cells; **$p < 0.01$, the 3A cells versus the 3A/siE2F1 cells. Western blotting results shown on the bottom illustrate the expression levels of E2F1 in the indicated transfectants (β-actin as a control). (B) HeLa cells were transfected with control vector or the FLAG-tagged NPM-3A construct, in combination with control or plasmids encoding pRB. DNA repair assay was done as described in A. Normalized data, at 10 h post-UV treatment, from the indicated groups were plotted in the bar graph. TD, thymine dimers. *$p < 0.05$. (C and D) HeLa cells were transiently transfected with empty vector (−), T199/234/237A (3A), or T199/234/237D (3D) variant of NPM and subsequently subjected to gene expression (C) and ChIP (D) analyses. (C) Total RNA was extracted from these cells. The relative mRNA level of E2F1 downstream genes *XPC*, *DDB2*, and *RPA3* was measured by quantitative RT-PCR (see Materials and Methods). (D) ChIP was performed as described in *Materials and Methods*. Immunoprecipitation was done with either control antibody or an antibody against E2F1. Bound DNA was subjected to quantitative real-time PCR analysis to monitor E2F1 binding to the *XPC* and *DDB2* promoters. Relative promoter binding, expressed as normalized ratio of IP to input, is shown (the values of the empty vector group are represented as 1).

## DISCUSSION

Here, we report a new pathway through which PP1β signals to NPM in response to DNA damage and consequently promotes cellular DNA repair. DNA damage induces dephosphorylation of NPM at multiple sites, leading to enhancement of complex formation between NPM and pRB and the subsequent upregulation of E2F1. Consequently, such signaling pathway potentiates the cellular DNA repair capacity.

As a multifunctional protein, dephosphorylated NPM may exert its functions in DNA damage signaling through modulating other critical regulators of cell growth and stress in addition to E2F1. For example, disruption of the NPM–ARF complex is known to contribute to p53-dependent apoptosis in the context of UV-induced DNA damage (Lee *et al*., 2005; Gjerset, 2006). However, our attempt to characterize the role of NPM dephosphorylation in this functional aspect did not reveal any effect of NPM–3A overexpression on the p53 transactivation activity (Supplemental Figure S7A) or on the cell cycle profile and extent of apoptosis (Supplemental Figure S7B), further implying that the PP1β–NPM pathway may be functionally independent from the ARF–NPM interaction and the p53-associated network. Furthermore, the above-mentioned findings also potentially excluded the possibility that pRB-specific functions, such as regulation of Skp2 and expression of apoptotic genes (Young and Longmore, 2004; Assoian and Yung, 2008), are altered by NPM binding in this stress condition. Finally, although our previous report implicated nuclear factor (NF)-κB in the negative regulation of *E2F1* during quiescence (Lin *et al*., 2006), we did not detect association of NF-κB with the *E2F1* promoter under normal growth or DNA damage condition (data not shown). Together, our findings strongly suggest that derepression of the pRB–E2F1 circuitry is a predominant functional consequence of the PP1β-mediated NPM dephosphorylation during UV-induced DNA damage repair.

Although it is widely assumed that phosphorylation of NPM is intimately linked to its cellular roles in various processes, we demonstrated that its dephosphorylation also represents an activating event. Compared with the extensive knowledge of the NPM phosphorylation and the kinases involved, much less is understood about NPM dephosphorylation and the enzymes that target this process. Our present work attributed this phosphatase activity to PP1β, which is responsible for mediating the dephosphorylation of at least three residues: Thr199, Thr234, and Thr237. Interestingly, PP1β was shown previously to be predominantly in the nucleolus and exhibit phosphatase activity toward nucleolar phosphoproteins such as NPM (Kotani *et al*., 1998). Such subcellular localization and putative substrate preference, together with our findings, are consistent with the notion that it is an authentic NPM phosphatase. Although our current results excluded the other two PP1 isoforms (α and γ), it is a formal possibility that other types of protein phosphatase also may target NPM, either at overlapping or distinct sites. Identity of such enzyme, as well as the functional context under which it dephosphorylates NPM, remain to be investigated. Dissecting such signaling pathways can provide further insight into how the multifunctional NPM is regulated.

Although kinases play important roles in every signaling pathway, removal of phospho-moiety from proteins by phosphatases represents another key determinant in the functional outcome of the signal transmission. In recent years, it has become increasingly clear that phosphatases, particularly those with serine/threonine specificity, are intimately involved in the cellular response to genotoxic stress. Among the numerous members of this phosphatase family, PP2A and PP5 were implicated previously in regulating ATM autophosphorylation after DNA damage (Ali *et al*., 2004; Goodarzi *et al*., 2004; Zhang *et al*., 2005). A recent

report also pinpointed PP4 in the recovery from DNA damage checkpoint by acting as a γH2AX phosphatase (Nakada *et al*., 2008). Interestingly, PP1 is activated in an ataxia telangiectasia mutated-dependent manner after ionizing irradiation-induced DNA damage (Guo *et al*., 2002). This is in line with our observations of the DNA damage-induced activation of the PP1β–NPM pathway. Despite these putative roles, more detailed investigations, particularly on identifying cellular substrates, are necessary to fully delineate the functional link of these phosphatases to DNA damage response.

Hyperphosphorylation of the pRB protein has been known as an inactivating signal that promotes dissociation of the pRB–E2F1 complex and reversion of pRB-mediated transcriptional repression. Evidence from recent studies supports a role of NPM in mediating the transcriptional regulation of *E2F1* (Lin *et al*., 2006; Liu *et al*., 2007b). Our present work presents another mechanism for the negative regulation of pRB: the dephosphorylated form of NPM preferentially associates with pRB and sufficiently induces the coincident dissociation of pRB from the *E2F1* promoter region (Figure 5). This sequestration may be mediated by a direct interaction between these two proteins, which was demonstrated previously (Takemura *et al*., 1999; Liu *et al*., 2007b). In addition, change in the subcellular localization also may alter the functional attributes of pRB after DNA damage. In support of this notion, recent reports have illustrated that NPM triggers a nucleolar migration of pRB after DNA replication is completed (Takemura *et al*., 2002) and that nucleolar residence of pRB critically depends on the sumoylation of NPM (Liu *et al*., 2007b). It is therefore a formal possibility that dephosphorylation of NPM contributes to or effects a spatiotemporal modulation of pRB in response to DNA damage that ultimately leads to E2F1 up-regulation.

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