Supplementary information

Materials and Methods

Site-directed mutagenesis-

Table S1. Oligonucleotide primers used for site-directed mutagenesis.

| Mutation | Orientation | Primer sequence |
|----------|-------------|-----------------|
| Flag-B23 | Forward | 5’-GATCGGATCCACCATGGACTACAAGACGATGA-3’ |
| | Reverse | 5’-TAAAGAGACTCTCCATCCATGGCC-3’ |
| S125A | Reverse | 5’-CCTCCTCTTCATCTTCTGCTCTGCATCTTCTCCACA-3’ |
| T199A | Forward | 5’-GAAGAAATCTATACGAGATGCCTCCAGCAAAAATGCACAA-3’ |
| | Reverse | | |
| T199D | Forward | 5’-GAAGAAATCTATACGAGATGATCCAGCAAAAATGCACAA-3’ |
| T234/237A | Forward | 5’-CCTTCAAGAAACAGGAAAAAGCTCCTAAAGCAACAAAAGACCTAGTTC-3’ |
| T234/237D | Forward | 5’-CCTTCAAGAAACAGGAAAAAGATCCTAAAGATCCTCCAAAAGACCTAGTTC-3’ |

aAltered sequences are underlined.

Quantitative phosphopeptide profiling by mass spectrometry-

1. Preparation of protein digests

Endogenous NPM was isolated from control or PP1β RNAi knockdown HeLa cells by immunoprecipitation. The proteins bound to the magnetic beads were eluted by 2% formic acid, 30% acetonitrile, and dried by a speed vacuum. The protein samples were re-dissolved in 50mM ammonium bicarbonate. After reduction/alkylation, the protein solutions were diluted 10-fold with 25 mM ammonium bicarbonate and digested with trypsin at 37°C overnight. The digests were desalted by in-house reverse phase (RP) micro-columns packed with SOURCE-15RPC (GE Healthcare) and dried by speed vacuum.

2. Dimethyl stable isotope labeling

The protocol was modified from a previous publication (Hsu et al., 2003). The protein digests were dissolved in 40 μL 50 mM HEPES buffer, pH 7.5. Each peptide solution was mixed with freshly prepared sodium cyanoborohydride solution (0.5 M, 2 μL). Samples from the control and knockdown cells were then mixed respectively with d(0), 12C-formaldehyde and d(2), 13C-formaldehyde (4% in water, 2 μL). The reactions were allowed to proceed at 37°C for 1.5 h and then stopped with Tris-base (1 M, 5 μL). Preliminary LC-MS/MS analysis for each labeled sample was performed to ensure that the labeling efficiency was greater than 95%. Several mixing ratios of the heavy and light isotope-labeled samples were also analyzed to obtain the relative concentration of NPM in each sample. Finally, the mixture containing equal amounts of heavy and light isotope-labeled NPM was prepared for subsequent phosphopeptide analysis.

3. The TiO2/RP-2DLC system

The online TiO2/RP system was constructed on an ultimate (Dionex/ LCpackings) nano-LC system that composed of a gradient pump, an isocratic pump and two ten-port valves. One of the ten-port valves controlled whether the TiO2 (Titansphere, 5 μm, GL Sciences) column perform the loading/wash steps, via the auto-sampler, or the elution step. And the other ten-port valve determined the choice of the RP-trapping column for trapping or for separating. When the TiO2 elution steps were on, the loading pump delivered the elution buffer (150 mM sodium carbonate, 30% acetonitrile, 15 μL) at a flow rate of 0.5 μL/min to the TiO2 column. The effluents were immediately diluted 100-fold with 0.1% formic acid that was split from the same loading pump. After passing through a mixing tubing, the eluted peptides were trapped in one of the two RP-trapping columns (ZORBAX SB300-C18, 0.3 x 5 mm, Agilent). The TiO2 elution/RP trapping process was allowed to continue for 60 min while the other RP-trapping column was connected to the RP-separating column (0.1 x 80 mm, packed with ACQUITY UPLC BEH C18 particle, 1.7 μm, from Waters) for LC-MS/MS analysis. Typically, six TiO2 purification steps, i.e. regeneration, equilibration, loading and wash, were included in a 30-min run, while the RP separation
system went through regeneration/equilibration procedures. The TiO$_2$ elution and the RP separation processes took place simultaneously and took 60 min to complete.

4. Mass spectrometry
The effluent of RP chromatography was analyzed by an LTQ-Orbitrap hybrid mass spectrometer (Thermo Electron, Bremen, Germany). The mass spectrometer was operated in the information-dependent acquisition (IDA) mode. Survey full scan MS spectra (from m/z 350 to 2000) are acquired in the Orbitrap with resolution 60,000 at m/z 400 and the lock mass function enabled. In the IDA mode, eight most intense ions in each full scan spectrum are selected for isolation and fragmentation in the linear ion trap (MS/MS). Each precursor ion was allowed to be analyzed twice and then excluded in the subsequent 1 min. The precursor isolation width was set to 2 Da, normalized collision energy was at 35% and the maximum accumulation time for MS/MS was set to 150 msec. Neutral lost-directed MS$^3$ spectrum acquisition were enabled to aim at analyzing peptides with lost phosphoric acid, i.e. 98, 49, and 32.7 for ions with various charge status.

5. Mass spectrometric data analysis
The raw data were processed with DTAsupercharge software (MSquant software package) (Boersema et al., 2009; Mortensen et al., 2010). The generated MGF files were then used to search against SWISS-PROT human protein database by using Mascot Daemon (Matrix Science, London) search engine with the following parameters: 5 ppm MS tolerance, 0.5 Da MS/MS tolerance, carbamidomethylation of cysteine and dimethylation (light isotope) at peptide N-termini and lysine residues as fixed modification, and oxidation of methionine and dimethylation (heavy isotope) at peptide N-termini and lysine residues as variable modification. The protein identifications followed the criteria defined by MASCOT. The protein false-positive rates were always less than 1%. Since the protein N-terminus of NPM is usually acetylated, another search considering this modification was performed and the phosphopeptide hits were quantified independently.

Quantification was done with the MSquant software. MSQuant 1.5 was customized for dimethyl stable isotope labeling. Briefly, a new quantification mode was added by modifying the “new_MSQ_quantitationModes.xml” file in MSquant. After saving the MASCOT search results as MSquant-compatible html format, the search results and corresponding raw data were associated in MSquant. The final quantification result was exported as an Excel file and subjected to further calculation. All the parameter settings were based on the instruction of the developer.

We did not detect in this approach the sites under study (Thr199, Thr234, and Thr237). This is likely due to their location in the lysine-rich carboxyl-terminus of NPM, which hypothetically could be fragmented by trypsin into small peptides. Such small size is not suitable for MS/MS detection and analysis and/or for proper separation during the liquid chromatography due to low hydrophobicity. Consequently, this limitation may preclude successful identification by mass spectrometry of peptides that harbor these sites. Use of another proteaseV8 did not improve the experimental outcome in the MS analysis (data not shown).

Results

| Sequence of identified peptide$^a$ | Phospho-sites | Ratio$^b$ | StdDev | n$^c$ |
|-----------------------------------|---------------|-----------|--------|------|
| 104-CGSGPVHISGQHLVAEEDEAEpSEDEEEEDVK-134 | S125 | 0.6115 | 0.07931 | 4 |
| 55-DELIHEAEAMNYEGpSPIK-73 | S70 | 1.087 | 0.1509 | 7 |
| 1-MEDpSMDMDMSPLRPQNYLFGCELK-24 | S4 | 1.326 | 0.2351 | 3 |

$^a$identified phosphorylated residues are preceded by "p"

$^b$relative abundance of phosphopeptides between PP1[β] knockdown vs. control cells

$^c$number of quantified ratios
References
Boersema, P.J., Raijmakers, R., Lemeer, S., Mohammed, S., and Heck, A.J. (2009). Multiplex peptide stable isotope dimethyl labeling for quantitative proteomics. Nat Protoc 4, 484-494.
Hsu, J.L., Huang, S.Y., Chow, N.H., and Chen, S.H. (2003). Stable-isotope dimethyl labeling for quantitative proteomics. Anal Chem 75, 6843-6852.
Mortensen, P., Gouw, J.W., Olsen, J.V., Ong, S.E., Rigbolt, K.T., Bunkenborg, J., Cox, J., Foster, L.J., Heck, A.J., Blagoev, B., Andersen, J.S., and Mann, M. (2010). MSQuant, an open source platform for mass spectrometry-based quantitative proteomics. J Proteome Res 9, 393-403.

Legends to supplementary figures

**Figure S1.** Human 293T (left) or H1299 (p53-null, right) cells were exposed to 50 J/m² of UV irradiation. Lysates were prepared from cells collected at the indicated time points post-irradiation. Western blotting was performed using the indicated antibodies.

**Figure S2.** Phosphorylation of NPM is counterbalanced by phosphatase. (A) HeLa cells were treated with or without 50 nM OA for 3 h. Lysates were subsequently collected and subjected to immunoblotting using the indicated antibodies. T199-P, phospho-Thr199 of NPM. (B) HeLa cells were mock-treated (lane 1), UV-irradiated (50 J/m², lane 2), or treated with 5 nM calyculin A for 3 h (lane 3). Lysates were collected and subjected to immunoblotting using the indicated antibodies. (C) Lysates were prepared from HeLa cells (previously treated with 50 nM okadaic acid; lane 4) and subjected to immunoprecipitation using anti-NPM antibody (lanes 1-3). In vitro solid phase phosphatase assay was performed on the immunoprecipitates as described in the Experimental Procedures (with recombinant phosphatase, rPP1; lanes 2 & 3). In lane 3, an additional inhibition reaction in which 2 nM of calyculin A (“CA”) was added during incubation. The resultant products were probed with the indicated antibodies. (HC, heavy chain.)

**Figure S3.** Phosphorylation of NPM at Thr199, Thr234, and Thr237 was regulated by PP1β. (A) Lysates from the treated cells as described in Fig. 2B were harvested for immunoblotting with antibodies against different PP1 isoforms and β-actin. The blots are shown as an assessment for level of RNAi knockdown (PP1X: PP1α, top two blots; PP1β, middle two blots; PP1γ, bottom two blots). (B) Shorter exposure of selected blots (anti-T199P and anti-T234/237P of NPM) for the experiments described in Figure 2A. (C) 293T cells transiently harboring control- or PP1β-targeting shRNA (54 h) were exposed to UV irradiation (50 J/m²). Equal loadings of whole-cell extracts derived at 0 h, 3 h, and 6 h post-treatment were immunoblotted with the indicated antibodies. (D) To assess the effect of PP1β on NPM phosphorylation, we transiently transfected HeLa cells with increasing doses of plasmid that encode EGFP-PP1β and subsequently analyzed changes in the phosphorylation levels of NPM on Thr199 and Thr234/237. Whole-cell lysates were collected at 16 h post-transfection and analyzed by Western blot analysis using the indicated antibodies. (E) Representative fragmentation spectra and extracted ion chromatograms of precursor ions corresponding to the identified phosphopeptides: top, phosphopeptide MEDpSMDMDMSPLRPQNYLFGCELK²⁴, m/z 1532.1517, 2⁺; middle, phosphopeptide DELHlVEAEAMYEGpSPIK⁷³, m/z 761.0223, 3⁺; bottom, phosphopeptide CGSGPVIDHISQHLVAVEEDAEpSEDEEEEDVK¹³⁴, m/z 1172.8398, 3⁺. The modifications and peptide scores are shown below the peptide sequences. The fragmentation spectra and matched ions are shown in the left panels, whereas right panels represent extracted ion chromatograms of corresponding precursor ions. The integrated peak areas under the red trace (heavy isotope; PP1β-RNAi cells) and blue trace (light isotope; control cells) were used for peptide ratio calculation (see Supplementary Table S2 for quantified data and summary).

**Figure S4.** Expression of E2F1. (A and B) HeLa cells were exposed to 50 J/m² of UV irradiation. Cells were collected at the indicated time points post-irradiation. RT-PCR (A) and Western blotting (B) was performed to respectively monitor the mRNA and protein levels of E2F1. Relative levels of E2F1 protein induction (“rel. fold induction”) were determined by calculating the ratios of intensity of E2F1 bands.
induction ("rel. fold induction") were determined by calculating the ratios of intensity of E2F1 bands (normalized by β-actin) at various UV time points vs. time zero. (C) Promoter reporter assay was performed to characterize the effect of the S125A variant of NPM on E2F1 promoter. HeLa cells were co-transfected with E2F1-Luc reporter gene and the indicated constructs. Bars, means of triplicates ± standard deviations. (D) HeLa cells transiently harboring control shRNA or two different PP1β-targeting shRNA (PP1β and PP1β-2) were exposed to UV irradiation (50 J/m²). Cells were lysed at 6 h post-irradiation and subjected to E2F1-Luc reporter gene assay as in (C). For both (C) and (D), the blots on the bottom are shown as an assessment for expression levels of target proteins (as indicated on the right). (E) HeLa cells were treated with 5 nM calyculin A (caly. A) for 3 h before being mock or UV irradiated (50 J/m²). After 6 h, lysates were collected and subjected to immunoblotting using the indicated antibodies.

Figure S5. Cells were treated with UV irradiation (50 J/m²). At 4 h post-treatment, cell lysates were prepared and immunoprecipitated with control (IgG), α-NPM and α-pRB antibody. Immunoprecipitates and lysate input (Input, 1/60 of IP, right panel) were probed for pRB and NPM. This experiment was intended as a repeat for Figure 5B.

Figure S6. As in Figure 6A, except with another E2F1-targeting RNAi construct (E2F1-2). Capacity of these cells to remove thymine dimers upon UV treatment was measured as in Figure 6A. 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 vs. the vector/siE2F1 cells; **P<0.01, the 3A cells vs. the 3A/siE2F1 cells. Expression levels of the E2F1 protein in the indicated transfection groups were monitored by anti-E2F1 and anti-β-actin immunoblotting analysis, as shown on the right.

Figure S7. Roles of dephosphorylated NPM on p53-dependent transcription and apoptosis. (A) Promoter reporter assay was performed on HeLa cells transfected with p53-TA-Luc reporter gene. Cells were also co-transfected with the wild-type or 3A variant of NPM or subjected to UV irradiation (50 J/m²). Bars, means of triplicates ± standard deviations. (B) Cell cycle profiles of HeLa cells transfected with plasmids encoding FLAG alone, FLAG-NPM-wt, or FLAG-NPM-3A, as revealed by FACS measurement of DNA content. For each culture, proportions of cells in the indicated phases are shown on the right.
$^{15}$MEDp$^{55}$SMDMDMSPLRPQNYLFGCELK$^{24}$ m/z 1532.1517, 2$^+$
Modifications: C21: Carbamidomethyl (C), N-term: Acetyl (Protein N-term), S4: Phospho (ST),
K24: Dimethyl:2H(4)13C(2) (K)
Ions Score: 75

$^{55}$DELHIVEAEMNYEGpSPIK$^{73}$ m/z 761.0223, 3$^+$
Modifications: K19: Dimethyl (K), N-term: Dimethyl (N-term), S16: Phospho (ST)
Ions Score: 59

$^{104}$CGSGPVIHGQHLVAVEEADEpSEDEEEEDVK$^{134}$ m/z 1172.8398, 3$^+$
Modifications: C1: Carbamidomethyl (C), K31: Dimethyl (K), N-term: Dimethyl (N-term), S22: Phospho (ST)
Ions Score: 117
A

![Bar graph showing relative fold stimulation for p53-TA-Luc](image)

| UV  | FLAG-NPM | wt | 3A |
|-----|----------|----|----|
| -   | -        | 1  | 1  |
| +   | 24.5     | 1  | 1  |

B

![Histograms for different conditions](image)

| Condition       | sub-G1 | G1   | S    | G2/M  |
|-----------------|--------|------|------|-------|
| FLAG-vector     | 1.67%  | 39.38% | 33.81% | 25.13% |
| FLAG-NPM-wt     | 1.33%  | 42.50% | 36.38% | 19.78% |
| FLAG-NPM-3A     | 2.06%  | 37.70% | 40.10% | 20.13% |