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# Cell cycle-dependent phosphorylation of nucleophosmin and its potential regulation by peptidyl-prolyl cis/trans isomerase

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## Abstract

Nucleophosmin (NPM) is a ubiquitously expressed phosphoprotein involved in many cellular processes. Phosphorylation is considered the major regulatory mechanism of the NPM protein, associated with diverse cellular events. In this study, we characterized the phosphorylation status of several physiological phosphorylation sites of NPM, especially the newly confirmed *in vivo* site threonine 95 (Thr95). NPM-Thr95 exhibits a transient and cell cycle-dependent phosphorylation state compared to several other *in vivo* phosphorylation sites examined, including Ser4, Thr199 and Thr234/Thr237. In addition, we characterized a functional interaction between NPM and the peptidyl-prolyl isomerase Pin1, which specifically bind to each other during mitosis. The demonstration of this binding represents a novel post-phosphorylation regulatory mechanism for NPM that has not been investigated before. Mutated Pin1 putative binding sites result in defected cell division and reduced number of mitotic cells, suggesting that post-phosphorylation is important for NPM in regulating cell cycle progression.

## Introduction

Nucleophosmin (NPM) is an abundant phosphoprotein predominantly localized in nucleoli, involved in many distinct biological processes including ribosome biogenesis, preribosomal RNA processing, chromatin remodeling and centrosome duplication (Herrera *et al.* 1995, Lindstrom 2011, Okuda *et al.* 2000). NPM undergoes nucleocytoplasmic trafficking by the Ran/CRM1 nucleocytoplasmic complex, to regulate centrosome duplication (Budhu & Wang 2005, Wang *et al.* 2005). Cytoplasmic NPM associates with unduplicated centrosomes and, by suppressing their duplication, maintains a strict number of centrosomes. However, the phosphorylation on Thr199 by cdk2/cyclin E could dissociate NPM from centrosomes and allow their duplication (Okuda *et al.* 2000). Therefore, this process must be tightly controlled in coordination with cell cycle progression. Aberrant transportation or inappropriate phosphorylation of NPM could result in cell cycle defects, genome instability

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and malignancy. This is supported by the fact that approximately one-third of acute myeloid leukemia (AML) cases heterozygously express a mutant form of NPM that is delocalized to the cytoplasm, which results in G2/M phase arrest (Chan & Meng 2015). Therefore, fully understanding the translocation mechanism and characterizing the phosphorylation events of NPM are critical to decipher its roles in cancer cell signaling that may help reveal therapeutic targets.

Wang *et al.* (2005) have previously identified a nuclear export signal (NES) of NPM, recognized by the Ran/CRM1 complex, that is responsible for its cytoplasmic translocation and enrichment on the centrosome. A putative Thr95 phosphorylation site within this NES region has been further identified. Mutation of Thr95 to alanine (T95A) inhibits centrosome duplication while the change to aspartic acid (T95D) that mimics phosphorylation results in centrosome duplication. Since phosphorylation plays a vital role in regulating NPM biological functions, a number of phosphorylation sites and their associated kinases have been identified both *in vitro* and *in vivo* (Okuwaki 2008). In the present study, we aimed to further examine the physiological phosphorylation sites of NPM. By using mass spectrometry analysis of cultured human cells, several such sites were identified, including a newly confirmed Thr95 that has not been reported before.

Notably, many discovered phosphorylation sites possess a Ser/Thr-Pro motif consensus and are potential substrates of certain kinases, such as cyclin-dependent kinases (CDKs), Jun-N-terminal protein kinases (JNKs), polo-like kinases (PLK) and glycogen synthase kinases (GSK3). In addition, a phosphorylated Ser/Thr followed by a proline (pSer/Thr-Pro) represents potential substrates of the peptidyl-prolyl *cis/trans* isomerase Pin1. The latter catalyzes the conformational change of the peptide bond between *cis* and *trans* conformations (Lu *et al.* 1996). An N-terminal WW binding domain targets Pin1 to its substrates and a C-terminal catalytic domain PPIase isomerizes the peptide bond of the specific motifs (pSer/Thr -Pro) (Ranganathan *et al.* 1997). Over the last decade, more than 40 proteins have been identified as Pin1 targets. Most of these are well known cell-cycle regulators, such as cyclin D1, Rb, p27, cyclin E and p53 (Liou *et al.* 2002, Rizzolio *et al.* 2012, Yeh *et al.* 2006, Zheng *et al.* 2002, Zhou *et al.* 2009), indicating an important role for Pin1 in cell cycle regulation. Also, Pin1 overexpression has been shown to correlate with centrosome amplification. In line with this, its ablation in murine embryonic fibroblasts (MEFs) delays centrosome duplication, suggesting its potential function in the process (Suizu *et al.* 2006). Here we report a functional interaction between NPM and Pin1 during mitosis. Mutation of potential Pin1 binding sites results in impaired cell cycle progression. Taken together, these results indicate a new post-phosphorylation regulation of NPM by Pin1.

## Materials and Methods

### Mass spectrometry

EGFP-NPM was immunoprecipitated from mitotic HeLa cells and then subjected to mass spectrometry analysis. The resulting immunoprecipitates were separated by 10% SDS-PAGE. After staining, the protein was excised from the gel and then in-gel digested with trypsin (Promega, Madison, WI). The resulting peptides were analyzed using nano LC

tandem mass spectrometry as described previously (Maher *et al.* 1990, Yu *et al.* 2007). Briefly, nano flow reversed-phase liquid chromatographic separation was coupled online to an LTQ linear ion trap mass spectrometer (Thermo Electron, San Jose, CA) for MS/MS and MS/MS/MS analysis (nanoLC-$MS^2$-$MS^3$). The peptides were separated at a flow rate of ~200 nL/min using a step gradient of 2%–42% solvent B (0.1% formic acid in acetonitrile) for 40 min, 42%–98% solvent B for 10 min and 98%–98% solvent B for 5 min, while mobile phase A was 0.1% formic acid in water. The mass spectrometer was operated in a data-dependent mode to sequentially acquire MS, $MS^2$, and neutral phosphate loss-dependent $MS^3$ spectra with dynamic exclusion. Normalized collision energy was 35% for both $MS^2$ and $MS^3$. The raw $MS^2$ and $MS^3$ data were searched using TurboSEQUEST (Thermo Electron) against a protein database including EGFP-NPM to identify phosphopeptides. The identified tryptic phosphopeptides were further subjected to manual validation of the peptide sequence and phosphorylation sites by examining the corresponding $MS^2$ and/or $MS^3$ spectra.

## Development of a rabbit monoclonal p-Thr95 antibody

A phospho-Thr95 monoclonal antibody was successfully developed in collaboration with Epitomics Inc (project EPNCIR117; antibody commercial name ab133453). Peptide cSLGGFEIpTPPVVLR (NCI-117P) was used for immunization and antibody screening, and peptide cSLGGFEITPPVVLR (NCI-117NP) was used for counter-screening of the antibodies. A total of 3 animal immunizations were performed. The pre-, 2$^{nd}$ and 3$^{rd}$ bleed sera were tested by ELISA and western blot for candidate polyclonal antibody identification. NHF-hTERT cell lysates expressing either EGFP-NPM or EGFP-T95A NPM were used for western blot screening. Candidates were then subjected to monoclonal antibody cloning. After recombinant cloning and transient expression of candidate antibodies in 293T cells, supernatants negative against NCI-117NP and positive against NCI-117P peptide were chosen for bulk production.

## NHF-hTERT cell cycle synchronization

The doubling time of NHF-hTERT cells was found to be 39 hours. Asynchronous cells were split when 100% confluent, at a ratio of 1:4. They were harvested 36 hr later for FACS and WB analysis. For G1/G0 phase arrest, cells were split when 100% confluent at a ratio of 1:4. Five days later and without changing medium, cells were harvested for FACS and WB analysis. For G1/S phase arrest, exponentially growing NHF-hTERT cells were plated at 30–50% confluence in DMEM with 2 mM thymidine and incubated for 15 hours at 37ºC. The thymidine-containing medium was removed and the cells were rinsed twice with pre-warmed DMEM. They were then trypsinized using E-PET and re-plated at 30–35% confluency, with pre-warmed DMEM and incubated at 37ºC. Twenty-four hours later, the medium was changed to pre-warmed DMEM containing 2 mM thymidine and incubated for another 24 hours at 37ºC before harvesting. For G2/M phase arrest, 100% confluent cells were split at a ratio of 1:4. Thirty-six hours later, they were treated with 0.4-mg/μl of nocodazole for 19 hours before collection. For enrichment of mitotic cells, exponentially growing cells were shaken off every 4 hours. Collected cells were subjected to FACS and western blot analysis.

### Immunofluorescence staining

NHF-hTERT cells were plated on chamber slides with appropriate medium one day before immunostaining. The media was then removed and cells were fixed using 4% paraformaldehyde for 10 minutes at room temperature, pre-chilled methanol for 10 minutes at room temperature and pre-chilled methanol for 10 minutes at −20ºC. Cells were then permeabilized with 1% NP40 in PBS for 5 minutes at room temperature. Blocking was performed using 10% Normal Donkey Serum (Jackson Immuno Research 017-000-121) in PBS for 1 hour in room temperature. Cells were then incubated with rabbit anti-γ-tubulin (Sigma-Aldrich, T-3559, 1:1000 diluted in PBS) and rabbit monoclonal anti-phospho-NPM (Thr95) antibodies (Epitomics Inc., #5188-1, 1:1000 diluted in PBS) for 1 hour at 37 º C. They were washed 4 times with PBS and incubated with secondary antibodies (Alexa Fluor 568/488: 1:1000 diluted in PBS) for 1 hour in room temperature. They were washed with PBS again and slides were mounted using Vecta Shield containing 0.5 μg/ml DAPI.

### Pull down assay

Pull down of GST-Pin1 and its mutants was performed as previously described (Lu *et al.* 1999). Lysates of NHF-hTERT were obtained using lysis buffer supplemented with 50 mM Tris-HCl (pH 8.0), 200 mM NaCl, 10% glycerol, 1% Triton X-100 and 1 mM DTT. Phosphatase inhibitor, 1 mM sodium orthovanadate ($Na_3VO_4$), 5 mM NaF, and protease inhibitors were added right before use. 1 μg GST or GST-Pin1 or its mutant recombinant protein was used for each pull down assay.

### Time lapse imaging

$5\times10^5$ NHF-hTERT cells were cotransfected with 300 nM NPM siRNA and 5 μg GFP-NPM or GFP-NPM $_{\Delta Pin1}$ plasmid using the Nucleofector™ Kit for Human Dermal Fibroblast (NHDF) (Lonza, Basel, Switzerland), program U23. Twenty-four hours post nucleofection, each sample was spread into 2 × Ø35mm glass bottom dishes for imaging. Another 24 hours later, time-lapse imaging was performed using an LCV110 incubator fluorescence microscope system (Olympus Corp, Tokyo, Japan). Three random positions of each dish were chosen for time-lapse observations with DIC and GFP frame acquisition, with a 15-minute interval.

## Results

### Identification of physiological NPM phosphorylation sites

Although many phosphorylation sites of NPM have been described, many of them are putative or identified by *in vitro* kinase assays. To better understand the phosphorylation function at a physiological level, a mass spectrometry analysis of HeLa cells expressing GFP-NPM was carried out. A total of ten physiological NPM phosphorylation sites were successfully identified (highlighted in red, Figure 1A; data not shown), including some previously described sites, such as Ser70, Thr199, and Thr237. Interestingly, among these *in vivo* phosphorylation sites, Thr95, which is localized within the nuclear export signal (NES) region previously described as a putative phosphorylation site implicated in centrosome duplication (Wang *et al.* 2005), was confirmed to be phosphorylated *in vivo*. Furthermore,

sequence alignment of NPM orthologues across 5 different species indicates that Thr95 is conserved from *Xenopus laevis* to human, suggesting a critical role of this residue for NPM protein function. Figure 1B shows a MS signal of the phosphorylation of threonine 95.

## Characteristics of NPM phosphorylation during the cell cycle

We next sought to determine whether phosphorylation of NPM is cell cycle dependent. Three phosphor-specific commercial antibodies against phospho-Ser4, phospho-Thr199 and phospho-Thr234/Thr237 are available (Figure 2A). Among these, an anti-phospho-MKK1/MKK2 antibody is used for phospho-Thr234/Thr237 detection due to cross-activity of this antibody (Cha *et al.* 2004). In addition, we developed a phosphor-specific antibody against Thr95 in collaboration with Epitomics Inc (see Materials and Methods). We synchronized an hTERT-immortalized normal human fibroblast line (NHF-hTERT) into different phases, i.e. G0/G1, S, G2/M and M phases, which was confirmed by FACS analysis (Figure 2B). A "shaking-off of the rounded up mitotic cells" method has been used to collect cells in metaphase without introducing any drug. As shown in Figure 2C, we found that the phosphorylation of Ser4, Thr95, Thr199, and Thr234/Thr237 all occur during G2/M transition to M phase, with cyclin B1 and MPM2 (a marker of mitosis) (Davis *et al.* 1983) as controls. However, the phosphorylation of Thr95, interestingly, was found to occur dominantly at the onset of mitosis but was rapidly dephosphorylated in M phase, which is different from other sites examined. This very rapid phosphorylation turnover suggests a rapid conformational or/and subcellular localization change, and that the phosphorylation of Thr95 may be an essential early signal in initiating mitosis. To gain a better idea, we performed immunofluorescence using the anti-phospho-Thr95 antibody to determine the subcellular localization of phosphor-Thr95 in NHF-hTERT cells. Shown in Figure 2D, phosphorylation of Thr95 was specifically detected in mitotic spindle poles. This subcellular localization strongly indicates an important role of phosphor-Thr95 in mitosis entry, mitotic bipolar spindle organization or cell division.

## Functional interaction between NPM and Pin1

Although kinase-mediated phosphorylation is considered a key regulatory mechanism for the roles of NPM in several cellular processes, other mechanisms in regulating NPM functions await discovery. One possible mechanism is post-phosphorylation regulation, mediated by Pin1 since several phosphorylation sites contain a Pin1-binding consensus motif (p-Thr/Ser-Pro). To determine if Pin1 regulates NPM in mitosis, a pull down assay was carried out using recombinant GST-Pin1 and cell lysates of NHF-hTERT. A specific binding between GST-Pin1 and endogenous NPM of NHF-hTERT was observed (Figure 3A). Furthermore, using cell lysates prepared from synchronized cells in various cell cycles, we found that mitosis-derived, but not G0 or S phase-derived, NPM preferentially bound GST-Pin1 (Figure 3B). Functionally, Pin1 is composed of two domains, a type IV WW domain and the catalytic PPIase domain. Both domains recognize p-Thr/Ser-Pro substrates; however the WW domain exhibits higher binding affinity (Innes *et al.* 2013). The PPIase domain isomerizes substrates at peptidylprolyl bonds of the consensus motifs (Ranganathan *et al.* 1997). In order to investigate whether the interaction between GST-Pin1 and endogenous NPM is catalytic or non-catalytic, we introduced two mutants of Pin1, i.e. W34A and K63A within the Pin1 WW domain and PPIase domain, respectively. Pull down

assays showed that only wild type GST-Pin1 and neither W34A within the WW domain nor the catalytically inactive K63A is able to bind NPM (Figure 3C), suggesting that both domains are necessary for NPM binding.

We speculated that all seven putative Pin1 sites of NPM are functionally important in contributing to Pin1-mediated NPM activities (Figure 3D, upper panel). To investigate this hypothesis specifically in the context of cell cycle progression, we constructed a mutant of NPM named $NPM_{\Delta Pin1}$, in which all seven phosphorylation sites were mutated to alanine. To rule out the effects of endogenous NPM, we cotransfected NHF-hTERT cells with siRNA, which targets the UTR region of NPM mRNA, along with either GFP-NPM or GFP-$NPM_{\Delta Pin1}$ that does not contain a native NPM UTR (Figure 3D, lower panel). While the expression of endogenous NPM was knocked-down to a very low level, a significant decrease of mitotic cell number was observed in cells that were transfected with GFP-$NPM_{\Delta Pin1,}$ compared to GFP-NPM (Figure 3E). Using a real time live cell imaging system, as shown in Figure 3F, cells expressing GFP-NPM exhibited normal cell division process. In contrast, cells expressing GFP -$NPM_{\Delta Pin1}$ exhibited defected cell division, as evident by nucleolar fusion. Another parameter in evaluating cell cycle progression is the dynamic change of the nucleoli numbers (Figure 3G) (Hernandez-Verdun 2011). By counting about 500 cells in each experimental group, we found a greater number of cells containing multiple nucleoli in GFP-$NPM_{\Delta Pin1}$ expressing cells compared to GFP-NPM cells (Figure 3H). All these result showed that mutation of the potential Pin1 binding sites of NPM results in the blockage of mitosis initiation, suggesting a potential post-phosphorylation regulation by Pin1.

## Discussion

Centrosomes are the major microtubule-organizing center, and their life cycle is coordinated by the cell cycle in animal cells (Hinchcliffe & Sluder 2001, Nigg & Stearns 2011). Aberrant centrosome numbers are believed to contribute to the dysregulation of the cell cycle and the development of cancer (Nigg *et al.* 2014). Correct centrosome numbers ensure spindle biopolarity in proliferating cells. However, an excessive number of centrosomes, either caused by overduplication or by cell division failure, frequently result in the formation of multipolar spindles. Although studies have shown that cells with multipolar spindles do not inevitably lead to multipolar division, and cells apply a centrosome-independent spindle assembly mechanism to cluster extra centrosomes into biopolar spindles, excessive numbers of centrosomes constitute a common cause of chromosome segregation errors (Ganem *et al.* 2009, Silkworth *et al.* 2009). Cancer has been proposed to develop as a consequence of chromosomal imbalances, and centrosome aberrations constitute one prominent cause of such imbalances. Many human tumors carry extensive centrosome aberrations and there is a strong correlation between the extent of these aberrations and the clinical outcomes (Nigg 2002, Zyss & Gergely 2009). Therefore, there is a high demand to investigate the numerical centrosome aberrations as a potential cause of chromosome instability in human tumors.

We have previously demonstrated a NPM putative phosphorylation site at Thr95 within the nuclear export sequence motif (NES) that is critical for centrosome duplication. In this study, we explored the regulating machinery controlling NPM phosphorylation and

mechanically linking NPM phosphorylation, centrosome duplication, and cell cycle progression. Mass spectrometry studies in cultured human cells confirmed that Thr95, Thr199 and Thr237 along with several other sites were indeed NPM phosphorylation sites *in vivo*. Noticeably, Thr95, along with the three other sites Ser70, Thr199 and Thr237, is followed by a proline (T/S-P), indicating that they could be substrates of proline-directed kinases, such as MAPKs, cyclin-dependent kinase (CDKs), JNKs, PLK or GSK3 (Ubersax & Ferrell 2007). Using phospho-specific antibodies, we demonstrated that Ser4, Thr199 and Thr234/Thr237 were all phosphorylated during mitosis, while Thr95 was phosphorylated more specifically on the G2/M boundary, earlier than the other sites. All of these sites with the exception of Ser4 are potential targets of proline-dependent protein kinases and when phosphorylated, form potential binding sites (pS/T-P) for Pin1, an isomerase that regulates protein function by switching peptidylprolyl bond between *cis* and *trans* conformations.

Pin localizes to centrosomes during the inter-phase of cell cycle. Functionally, Pin1 ablation dramatically delays centrosome duplication for several rounds in MEFs. Overexpression of Pin1 is able to drive multiple rounds of centrosome duplication under S phase arrest, which can be fully abolished by the Pin1 inhibitor ATRA, in NIH 3T3 cells, in a dose-dependent manner (Wei *et al.* 2015). Moreover, Pin1 overexpression induces cell transformation *in vitro* and tumor development *in vivo*, together with the presence of overduplicated centrosomes, indicating that the latter is the cause of cell transformation (Lu *et al.* 1996). Despite a strong implication in centrosome duplication and carcinogenesis regulation by Pin1, substrates of Pin1 remain to be identified.

In this study, we demonstrate a physical interaction between NPM and Pin1 during mitosis in NHF-hTERT cells. Cells expressing a mutant NPM that lacks seven potential Pin1 binding sites (GFP-NPM$_{\Delta Pin1}$) had prolonged interphase and centrosome amplification (data not shown) compared to those that express wild-type NPM, indicating that cell-cycle dependent phosphorylation of NPM is highly correlated with cell cycle progression and Pin1 plays a critical role in this post-phosphorylation regulation. Comprehensive studies await to be performed to determine the detailed interaction between Pin1 and NPM, and their effect on centrosome duplication.

### Conclusions

Taken together, our results indicate that phosphorylation of Ser4, Thr95, Thr199, and Thr234/Thr237 of NPM is G2/M cell cycle-dependent, and that Pin1 may regulate NPM through its binding.

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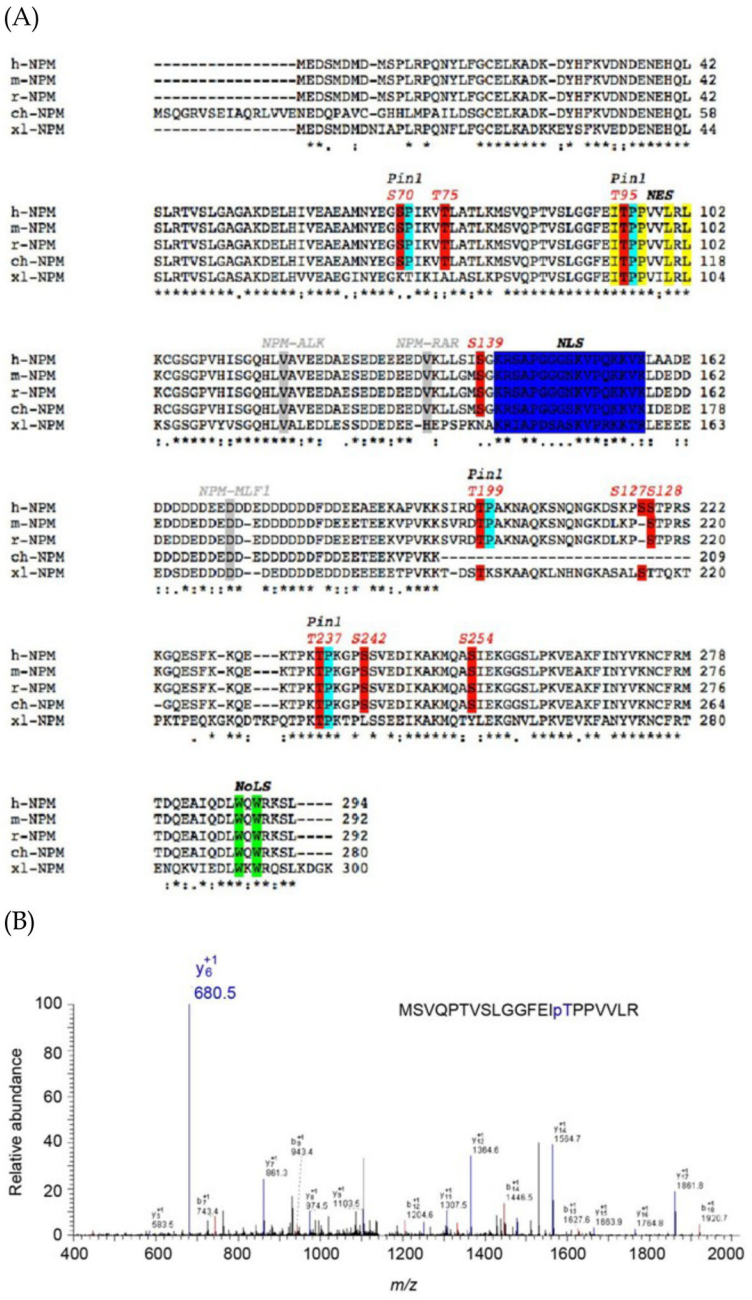


**Figure 1.**

*In vivo* phosphorylation site(s) of NPM. **(A)**. Alignment of primary amino acid sequences of NPMs from five different species was performed by CLUSTAL 2.1. The NES (yellow), NLS (blue) and NoLS (green) motifs are highlighted. *In vivo* phosphorylation sites (red) were determined by mass spectrometry. Potential Pin1 binding sites (light blue) were determined by motif consensus [p-Thr/Ser-Pro]. NPM fusion protein sites (grey) that result from translocation of the N-terminal NPM to ALK, RAR, or MLF1 in lymphoma and leukemia are also indicated. h, human; m, mouse; r, rat; ch, Chinese hamster; xl, *Xenopus laevis*. (**B)**.

*In vivo* phosphorylation site T95 was determined by mass spectrometry analysis as described in Materials and Methods.

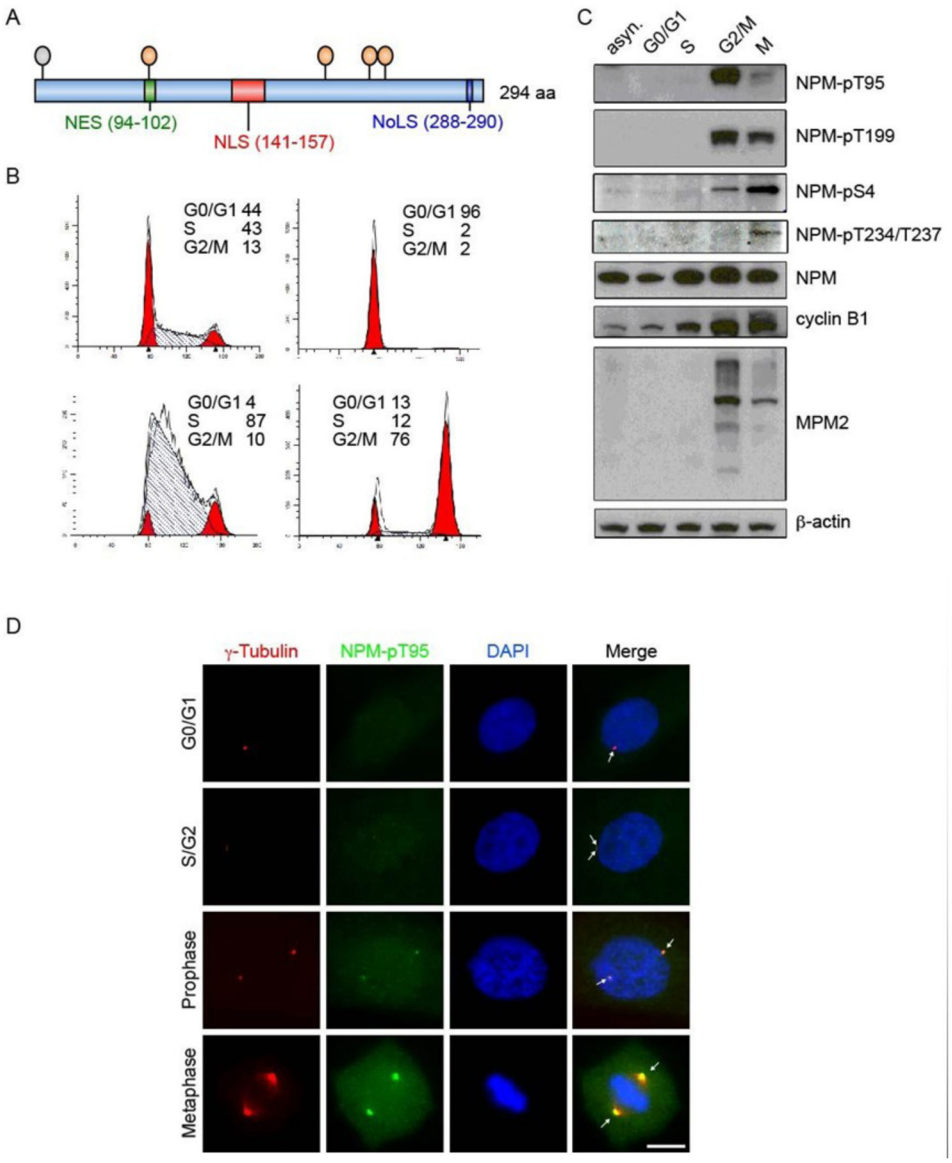


**Figure 2.**

Cell phase characterization of different NPM phosphorylation sites. **(A).** Schematic depiction of NPM and its phosphorylation sites examined in this study. Except S4 (grey), T95, T199, and T234/T237 (orange) are putative Pin1 binding sites followed by a proline. **(B).** Flow cytometry of cultured NHF-hTERT cells that were either untreated (asynchronously cycling), or arrested in G0/G1-phase (serum starvation), S-phase (double thymidine treatment) or G2/M phase (nocodazole treatment). Red peaks indicate 2N and 4N DNA content. **(C).** Five different phosphorylation sites at different cell phases were examined by western blot using phospho-specific antibodies in NHF-hTERT cells treated as (B). The mitotic (M) cells were collected by the “shaking-off” method. **(D).** Subcellular localization of NPM-pT95. Coimmunofluorescence was performed using anti-NPM-pT95 (green) and anti-γ-tubulin (red) antibodies. Scale bar, 10 μm.

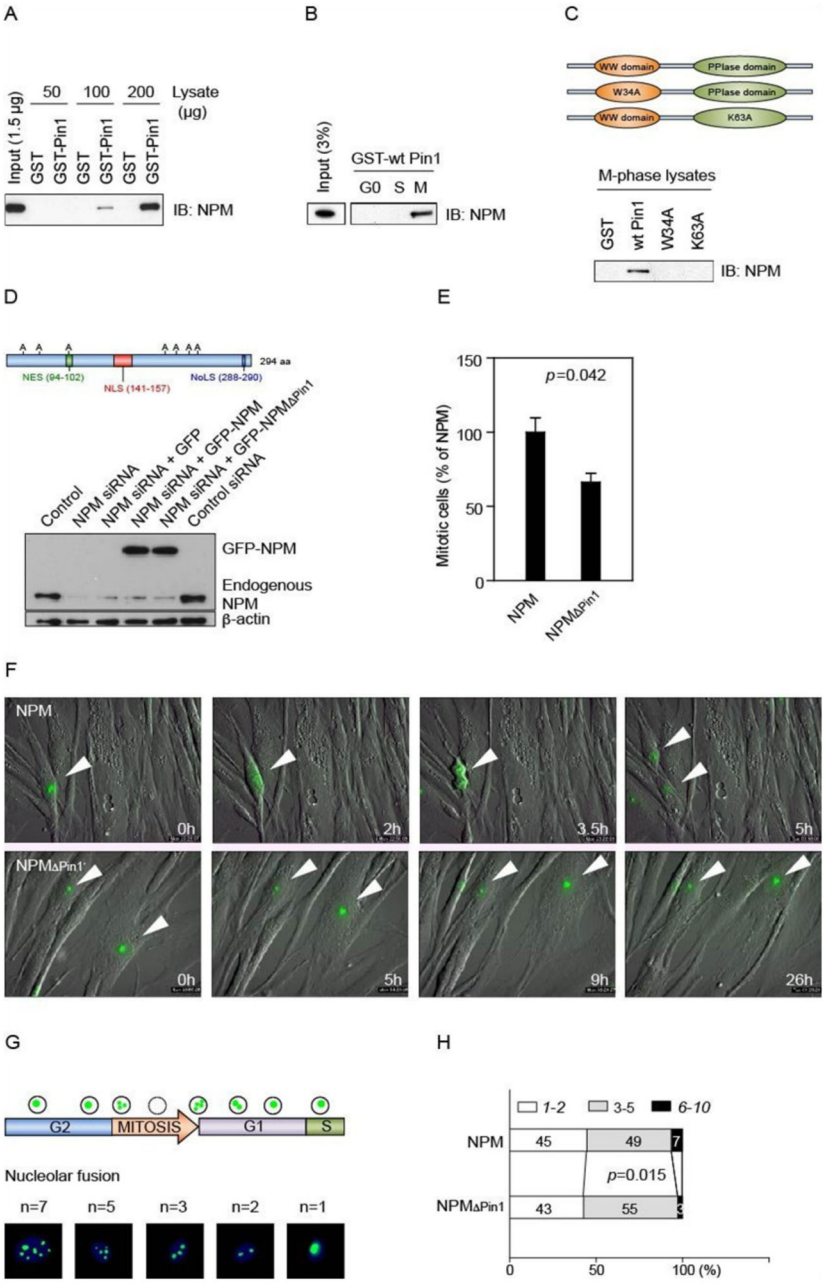


**Figure 3.**

NPM interaction with Pin1. **(A).** Pull down analysis using the whole cell lysate from NHF-hTERT cells showing specific interaction between endogenous NPM and GST-Pin1 expressed in E.coli, GST was used as negative control. **(B).** Pull down analysis using the whole cell lysate from different cell phase of NHF-hTERT cells showing a preferable binding of GST-Pin1 with endogenous NPM at mitotic phase. **(C).** Using M-phase lysates from NHF-hTERT cells, pull down analysis showed that only wild type GST-Pin1, but not W34A or K63A, binds to endogenous NPM. W34A, a Pin1 mutant at the WW binding domain; K63A, a Pin1 mutant at the PPIase catalytic domain. **(D).** Schematic depiction of

seven putative Pin1-binding-sites mutant ($NPM_{\Delta Pin1}$) and the examination of the expression of exogenous NPM (GFP-NPM or GFP-$NPM_{\Delta Pin1}$) and depletion of endogenous NPM using siRNA targeting 5′-UTR of NPM mRNA. **(E).** Mitotic cells were quantified in NHF-hTERT cells that express EGFP-NPM or GFP- $NPM_{\Delta Pin1}$ with endogenous NPM knockdown. ~500 cells were counted. Shown as *mean ± S.E.M.*, unpaired *t-test* was performed. Three independent experiments were analyzed. **(F).** Time lapse of the live cell division treated as (E). Representing images of different time points are shown. **(G and H).** Nucleolus number was also quantified in NHF-hTERT cells that express EGFP-NPM or GFP- $NPM_{\Delta Pin1}$ with endogenous NPM knockdown. ~500 cells were counted.