The Hong Kong Polytechnic University

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The Involvement of NPM1 in Transcriptional Activation of PCNA

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DECLARATION

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it reproduces no materials previously published or written, nor material that has been accepted for the award of any other degree or diploma, except where due acknowledgment has been made in the text.

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Abstract

Nucleophosmin (NPM/B23, NPM1) is an important nucleolar phosphoprotein with pleiotropic functions involving in a board range of cellular processes such as ribosome biogenesis, transcription regulation and cell cycle regulation. Our previous results have shown that NPM/B23 plays an important role in resistance to UV-induced cell death by up-regulating *PCNA* through YY1 and thereby enhancing nucleotide excision repair (NER) activity (Weng and Yung, 2005; Wu and Yung, 2002). However, the underlying mechanism of this process is still unclear. Here we show that NPM/B23 drives *PCNA* promoter activity depending on its acetylation state. NPM/B23 cooperates with the transcription factor YY1 at an upstream YY1 binding site and regulates the *PCNA* promoter. The treatment by UV irradiation increased expression level and acetylation state of NPM/B23, and this alteration was accompanied by an increase in PCNA. Additionally, the p300 was bound to *PCNA* promoter and enhanced the recruitment of NPM/B23 and YY1 to promoter upon UV irradiation. p300 is a transcription co-activator with intrinsic histone acetyltransferase (HAT) activity. Therefore, the *PCNA* promoter activity in p300 knockdown condition and in NPM/B23 deacetylation-mimicking mutants was examined as compared to wild-type. At 48 h after the addition of p300 shRNA, *PCNA* promoter activity was significantly decreased ($P<0.05$). NPM/B23 deacetylation-mimicking mutants which defects in p300 HAT activity, the *PCNA* promoter activity and subsequently, DNA repair capacity was decreased after UV treatment. These results demonstrate that p300 is importantly participated in PCNA up-regulation by

triggering NPM/B23 acetylation. Taken together, we anticipate our findings may be a starting point for anti-cancer treatment. A combination therapy with NPM/B23 antisense and HAT inhibitor to impair the expression level and the acetylation state of NPM/B23 could be a novel therapeutic strategy for preventing recurrence and drug resistance.

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“Always ask myself a simple but important question in your research aspect”

Golden words spoken by my Chief Supervisor,

Prof. Benjamin Yung, without his supports and advices

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(2) The postgraduate scholarship recipient in 2010 of Hong Kong Association of University Women

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List of Abbreviations

A/a

ALCL	Anaplastic large-cell lymphoma
AKT	a serine/threonine-specific protein kinase, also known as protein kinase B
ALK	Anaplastic lymphoma kinase
AML	Acute myeloid leukemia
AMP	Adenosine monophosphate
AOS1 (SAE1)	SUMO1 activating enzyme subunit 1
AP2α	Activating enhancer binding protein 2α
ARF	Alternate-reading-frame protein
ATP	Adenosine triplephosphate
ATF5	Activating transcription factor 5

B/b

b-Myb	v-myb myeloblastosis viral oncogene homolog (avian) - like 2
BARD1	BRCA1-associated RING domain 1
BRCA1	Breast cancer 1 (early onset), a human caretaker gene
BRCA1-BARD1	Heterodimeric complex of BRCA1 and BARD1
BuONa	Sodium butyrate

C/c

CAD	Caspase-activated DNase
CD30	Cell membrane protein, also known as TNFRSF8
CDKs	Cyclin-dependant kinases
c-fos	Cellular proto-oncogene
ChIP	Chromatin immunoprecipitation assay
c-Jun	a protein interacting with c-fos, forms AP-1 early response transcription factor
cICAT	Cleavable isotope-coded affinity tag
CID	Collision-induced dissociation
CK2	Casein kinase 2
COPD	Chronic obstructive pulmonary disease

List of Abbreviations

E/e	
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E2F1	E2F transcription factor 1
E3	Ubiquitin-protein ligase
ELISA	Enzyme-liked immunosorbent assay
EMSA	Electrophoresis gel mobility shift assay
ETD	Electron-transfer dissociation
F/f	
Fen1	Flag structure-specific endonuclease 1
Fbw7γ	F-box and WD repeat domain contain 7
G/g	
GADD45α	Growth arrest and DNA-damage-inducible, alpha
H/h	
HMDM2 (Mdm2)	p53 E3 ubiquitin protein ligase homolog, negative regulator of p53
HIF-1α	Hypoxia inducible factor 1α
HIV-1	Human immunodeficiency virus 1
HSP60	Heat shock protein 60
I/i	
IRF-1	Interferon regulatory factor 1
ITC	Isothermal titration calorimetry
J/j	
JAK	Janus Kinase, a non-receptor tyrosine kinase

List of Abbreviations

M/m

MALDI-TOF	Matrix-assisted laser desorption inoization-time of flight mass apectrometry
MDS	Myelodysplastic syndrome
MnSOD	Superoxide dismutase 2

N/n

NAD^+	Nicotinamide adenine dinucleotide, a coenzyme
NDF	Nucleolus-derived foci
NER	Nucleotide excision repair, a type of DNA repair pathways
NES	Nuclear export signal
NF-κB	Nuclear factor κ-light chain enhancer of active B cells
NLS	Nuclear localization signal
NoLS	Nucleolus localization signal
NPM1	Nucleophosmin
$NPMc^+$	Cytoplasmic NPM1 protein

P/p

p120	Catenin (cadherin-associated protein), delta 1
p21	Cyclin-dependant kinase inhibitor 1A
p300	E1A binding protein p300
PAR	Poly-ADP-ribose
PARG	Poly(ADP-ribose) glycohydrolase
PARP	Ploy(ADP-ribose) polymerase
PCNA	Proliferating Cellular Nuclear Antigen
PI3K	Phosphoinositide 3-kinase
$PI(3,4,5)P_3$	Phosphatidylinositol (3,4,5) – trisphophate
PLK1	Polo-like kinase 1
PP1	Protein phosphatase 1
pRB	Retinoblastoma 1

List of Abbreviations

R/r	
Rad51	RAD51 homolog (S. cerevisiae)
Rad52	RAD52 homolog (S. cerevisiae)
recA	an essential E. coli core protein with 38 kilodalton be involved in repair and maintenance of DNA
RNAPII	RNA polymerase II
S/s	
SDS-PAGE	Sodium dodecyl polyacrylamide electrophoresis
SIM/SBM	SUMO-interaction/binding motif
STAT	a protein with a full-name: Signal Transducer and Activator of Transcription
SUMO	Small ubiquitin-like modifier
T/t	
TNFα	Tumor necrosis factor α
U/u	
Ub	Ubiquitin
UBA2	Ubiquitin-like modifier activating enzyme 2
Ubc9	Ubiquitin-conjugating enzyme E21
ULP	Ubiquitin-like protein
UV	Ultraviolet radiation
Y/y	
YY1	Ying Yang 1

Chapter One:

Literature review of nucleophosmin and cellular responses to ultraviolet irradiation

1.1 Introduction

Nucleophosmin (NPM1) is a multifunctional protein initially characterized as a nucleoprotein that functions in the processing and transport of ribosomal RNA. NPM1 was found to be more abundant in proliferating and cancer cells than in normal quiescent cells (Chan et al., 1989). Evidence has also shown a positive relationship between the expression level of NPM1 and the proliferative state of the cell. Various cellular functions have been ascribed to NPM1, including, involvement in cell proliferation, cytoplasmic-nuclear shuttle transportation, acting as a ribonuclease, and molecular chaperone (Hingorani et al., 2000). It has also been implicated in several distinct physiological functions such as control of centrosome (spindle pole) duplication, cell cycle regulation, and regulation of the ARF/p53 tumor suppressor pathway.

In this review of the literature, a brief overview of NPM1, physiological functions of NPM1, and the alteration of NPM1 in human cancer are presented.

In particular, the physiological functions of NPM1 are discussed in detail, including molecular chaperoning activities, ribosome synthesis, transcriptional regulation, modulation of tumor suppressors, cell cycle regulation, and post-translational modifications in NPM1.

1.2 A brief overview of NPM1

NPM1 has other aliases such NPM, B23 and NPM1/B23.1, also been designated numatrin in mammals, or NO38 in amphibians. It was originally identified as a major phosphoprotein localized in granular regions of the nucleolus and has been shown to be associated with pre-ribosomal particles. (Feuerstein and Mond, 1987; Orrick et al., 1973; Schmidt-Zachmann et al., 1987; Spector et al., 1984). The NPM1 protein belongs to the family of nulceophosmin/nucleoplasmin, and consists of NPM1, NPM2, NPM3 and other invertebrate NPM-like proteins. Moreover, NPM1, NPM2 and NPM3 have all been identified in mammals and have shown highly homology in sequence (MacArthur and Shackleford, 1997). The detailed structures and functional domains of NPM proteins (NPM1 to NPM3) are shown in Figure 1.1. In this

Introduction section of thesis, the biological functions of NPM1 protein as well as the alternation of NPM1 in cancer will be discussed.

Comparing sequences of NPM proteins have shown an interesting characteristic in sequence homology, however, they have distinct effects in biological functions (Frehlick et al., 2007). NPM1 protein is also referred to as B23 because it was the 23rd protein in the B region of the gel slab when the protein spots were numbered in decreasing mobility in both electrophoretic dimensions. However, the calculated molecular mass of the cDNA-derived NPM1 is 32 kDa which is smaller than the recognized molecular size (37 kDa) estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). It is possible that unusual localization of charged amino acids and the tertiary structure retard the mobility of NPM1 in SDS-PAGE, resulting in an apparent larger molecular mass (Chan et al., 1989). The NPM1 protein contains several functional domains, including an N-terminal region for oligomerization, molecular chaperone, acidic domains for histone binding and a C-terminal region for DNA/RNA binding. The overlapping region consists of the acidic domain and partial C-terminal region containing ribonuclease activity. The

detailed structural functions of NPM1 will be listed and described in *1.2.1 structural characteristics of the NPM1 protein*. However, various functional domains of NPM1 have been identified within protein in different, but slightly overlapping segments (Grisendi et al., 2006). Basically, NPM1 contains two important, specific sequence motifs required for protein transportation. One is the nuclear localization signal (NLS) sequence (Hingorani et al., 2000), and the other is the nuclear export signal (NES) sequence (Wang et al., 2005) which are required for protein translocation between the nucleus and the nucleolus. In addition, the *NPM1* gene has shown that alternative splicing results in multiple transcript variants, termed B23.1 and B23.2 (Chan et al., 1990). In 2002, the National Institutes of Health Mammalian Gene Collection (MGC) tried to sequence and verify the full open reading frame (ORF) for a non-redundant set of human and mouse genes. Therefore, full-ORF clones for an additional 7,800 human and 3,500 mouse genes have been identified (Strausberg et al., 2002). Due to sequencing results, there are three NPM1 transcript variants: B23.1 (also known as NPM1 isoform1), NPM1 isoform2 and B23.2 (isoform3). The well-known NPM1 protein also termed B23.1 which contains 294 amino acids in sequence and shares 255 identical N-terminal residues with B23.2, where the

last 35 C-terminal amino acids of B23.1 are absent in B23.2. Taken together, the C-terminal domain (bases from 243–294) being only present in B23.1 is essential for its nucleolar localization. Since the immunohistochemistry staining also reveals that B23.1 is nearly exclusively in the nucleolus, and a minor fraction of B23.1 is found to localize at the centrosome. B23.2 (also known as NPM1 isoform3) utilizes an alternative 3′ terminal exon of the *NPM1* gene to translate a shorter protein containing 259 amino acids in sequence and with a distinct C-terminus compared to B23.1. However, the sequencing lost in the C-terminus affects the localization of B23.2, which is found in both nucleoplasm and nucleoli. The last *NPM1* transcript variant encoded the isoform2 protein, which lacks an alternative in-frame exon and results in a protein containing 265 amino acids in sequence and without an internal segment in structure comparing to B23.1. Interestingly, although NPM1 proteins have several isoforms, NPM1/B23.1 is the most studied because its expression level in tumors is often altered when mutated or translocated in hematological malignancies (Grisendi et al., 2006).

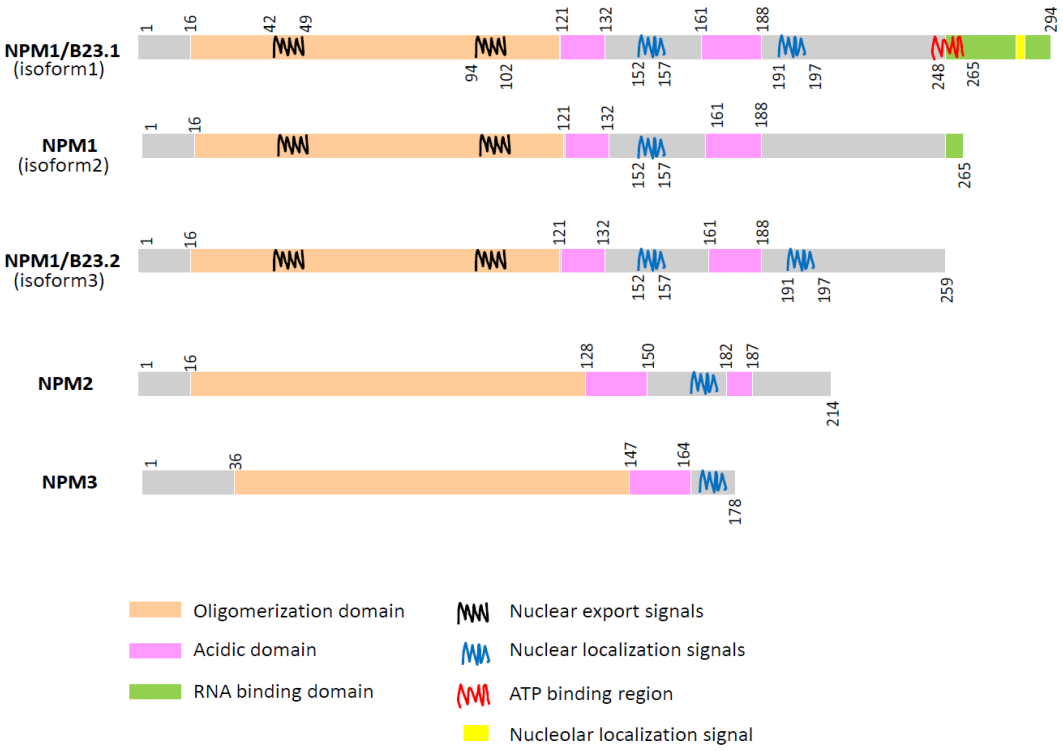


Figure 1.1: Schematic representations of the NPM family proteins.

The NPM family proteins share high homology in their N-terminal domains, which is required for protein oligomerization. The human *NPM1* gene with alternative splicing variants has been identified and termed B23.1 (isoform 1), B23.2 (isoform 3) and isoform2. B23.1 contains 294 amino acids in sequence and shares 255 identical N-terminal residues with B23.2. However, the last 35 C-terminal amino acids of B23.1 are absent in B23.2 resulting in a shorter protein with 259 amino acids. The other NPM1 transcript variant, isoform2, lacks an alternative in-frame exon resulting in a protein that contains 265 amino acids and lacks an internal segment in protein structure; other members of the NPM family include NPM2 and NPM3 proteins. NPM2 mainly expresses in ovarian tissues and contains 214 amino acid sequences including two acidic domains and one nuclear localization signal motif. NPM3 is the shortest member in the NPM family; it contains one acidic domain in the central portion and ubiquitously expresses in cell as well as NPM1. [This figure is adapted from (Okuwaki, 2008)].

1.2.1 Structural characteristics of the NPM1 protein

The human NPM1 (B23.1) protein contains 294 amino acids and can be divided into several domains with distinct properties. The brief structural map for functional domains of NPM1 is shown in Figure. 1.2.

Starting from the N terminus, residues 1–120 represents the core region containing eight-stranded β-sheets (Akey and Luger, 2003). Moreover, this region is the most conserved among the NPM family of proteins. Further, analysis and comparison of the crystal structures of NPM proteins N-terminus from different species, such as *Drosophila* nucleoplasmin-like protein (dNLP) (Namboodiri et al., 2003), *Xenopus* NO38/NPM1 (Namboodiri et al., 2004), human NPM1 (Dutta et al., 2001) and NPM2 (Platonova et al., 2011), demonstrated that the N-terminal core region forms a pentameric ring, then two pantamers form a decamer structure (Lee et al., 2007). That means the core region is required for protein oligomerization (Okuwaki, 2008). Furthermore, two acidic stretches, A1 (residues 121–132) and A2 (residues 160–188) extended from the N-terminus containing many acidic aspartate and glutamate residues. The distinct electrostatic property of this region suggests that it can be

engaged in an interaction with basic proteins such as histones to facilitate assembly of nucleosomes and chromatin remodeling (Hingorani et al., 2000). All this information indicates that acidic stretches are important for binding to histones and influence histone chaperone activity (Frehlick et al., 2007). Due to this characteristic, NPM1 can suppress the misfolding and aggregation of target proteins in the crowded environment of the nucleolus (Szebeni and Olson, 1999). The term “chaperone” was first used to describe the biological function of NPM1. Chaperones refer to molecules that are associated with their target proteins to prevent misfolding and aggregation, but are not found in the final products comprising the target proteins (Okuwaki, 2008).

Further, there are two well-known nuclear export signal (NES) motifs in the NPM1 N-terminal core region, both of which are responsible for nucleo-cytoplasmic shuttling of NPM1 (Maggi et al., 2008; Wang et al., 2005). *Maggi* et al. (2008) reported that the mutation of two conserved leucine residues (Leu42Ala and Leu44Ala) in the first NES motif (residues 42–47) leads to a defective NPM1 that cannot be shuttled out of the nucleus. The second NES motif (residues 94–102) is responsible for nuclear export of NPM1 by Ran-

Crm1 complex, and enables the association of NPM1 with the centrosome during mitosis to prevent centrosome re-duplication (Wang et al., 2005). *Wang* et al. (2005) has demonstrated that removal of this NES motif by deleting residues 94–102 or mutating two conserved leucine residues (Leu100Ala and Leu102Ala) leads to nuclear retention of NPM1 and a supernumerary centrosome.

The next region, termed the central portion, is located in two acidic stretch domains of NPM1 and is required for ribonuclease activity. Moreover, the central portion and the C-terminal domain of NPM1 contain basic amino acids in sequence resulting in a weak alkaline charge for nucleic-acid binding (Hingorani et al., 2000).

Following the acidic stretches is an unstructured segment (residues 189–243) that is enriched in the basic residues lysine and arginine. This basic domain is important for the nucleic acid binding activity of NPM1, likely due to its electrostatic properties interacting with acidic DNA/RNA molecules (Hingorani et al., 2000). Unlike other proteins of the NPM family, the

furthermost C-terminal region of NPM1 protein is an aromatic stretch (residues 244–294) required for RNA-binding. Moreover, this region contains two tryptophan residues (288 and 290) that are required for efficient nucleolar localization of NPM1 (Nishimura et al., 2002; Wang et al., 1993).

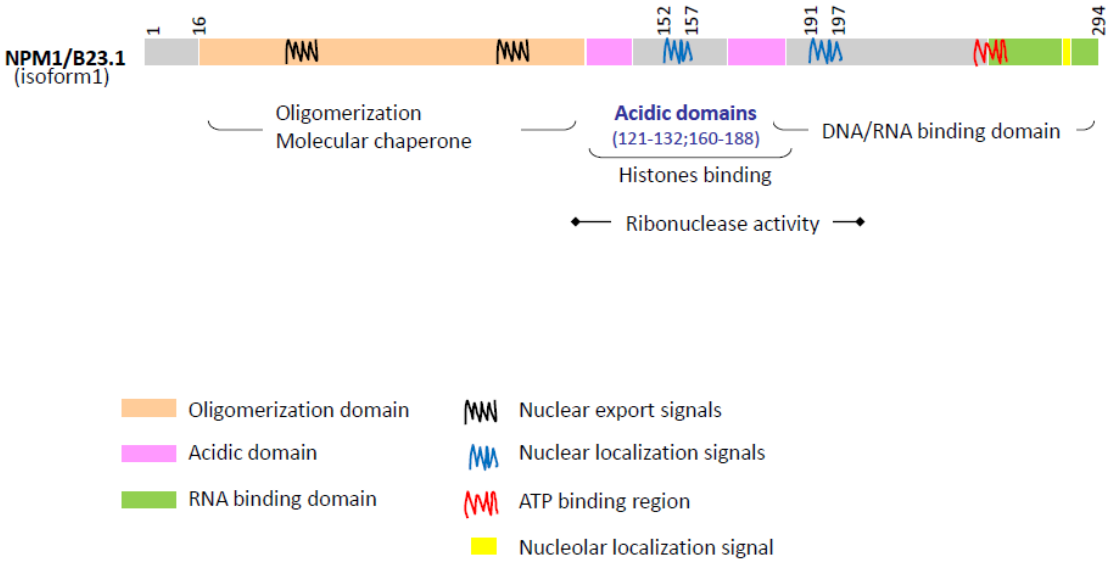


Figure1.2: The structural map for function domains of human NPM1.

The human NPM1 protein contains 294 amino acids and can be divided into several domains. Starting from the N-terminus is a hydrophobic segment, which is required for protein oligomerization as well as chaperone activity. The two acidic domains extending from the N-terminus are important for histone binding. The central portion between the two acidic domains is required for ribonuclease activity while the C-terminus is involved in nucleic acid binding. The NPM1 protein also contains several sequence motifs including: nuclear localization signal (NLS in base from 152–157 and 191–197), nuclear export signal (NES) and the nucleolus localization signal (NoLS) that are important for the localization of NPM1 in the nucleolus as well as their shuttling between the nucleus and cytoplasm. [Figure 1.2 is modified from (Grisendi et al., 2006; Okuwaki, 2008)].

1.3 Physiological functions of NPM1 protein

Research into the physiological functions of NPM1 protein has become increasingly important, particularly in the field of cancer research. Studies show that NPM1 is a multi-functional protein involved in many important biological processes such as cell proliferation and anti-apoptotic pathway. This section will focus on the physiological functions of NPM1 such as molecular chaperone, ribosome biogenesis, transcription regulation, inhibition of apoptosis, post-translation modification of NPM1 and tumor suppressor gene modulation.

1.3.1 Role as a molecular chaperone

In the last decade, several research groups have shown that NPM1 can function as a molecular chaperone for protein and nucleic acids (Okuwaki et al., 2001; Szebeni and Olson, 1999). According to Szebeni and Olson (1999), the observed molecular chaperone activity of NPM1 may serve to prevent protein aggregation and misfolding. Indeed, it has been confirmed that NPM1 suppresses the aggregation and misfolding of target proteins through the structural properties of its N-terminus (Hingorani et al., 2000). Moreover, NPM1 works as a histone chaperone to control the assembly and disassembly of

chromatin formation during the cell cycle (Okuwaki et al., 2001). To act as a molecular chaperone, NPM1 has to shuttle between the nucleus and the cytoplasm. This ability of NPM1 is attributed to the presence of nuclear export signal (NES) and nuclear localization signal (NLS) in NPM1 (Dingwall et al., 1987; Hingorani et al., 2000).

1.3.2 Involvement in ribosome biogenesis

NPM1 is abundantly expressed in the nucleolus. Its nucleo-cytoplasmic shuttling activities are due to its C-terminal end portion that carries the specific nucleolus localization signal (NoLS) and is involved in the function of RNA-binding. NPM1 also has the ability to bind nucleic acids and to transport pre-ribosomal particles. All of these are important and necessary machinery for the processing and assembly of ribosomes (Borer et al., 1989; Dumbar et al., 1989; Wang et al., 1994; Yun et al., 2003). In addition, the RNA binding region of NPM1 is closely related to its nucleolus localization signal. It has been suggested that NPM1 may be essential for ribosome biogenesis (Huang et al., 2005; Itahana et al., 2003). Furthermore, two tryptophan residues (W288 and W290) have been identified within the RNA-binding domain of NPM1 and are required

for nucleolar localization of NPM1 (Nishimura et al., 2002). This RNA-binding domain is unique to NPM1 and is not found in other proteins. Indeed, NPM1 has been found to have intrinsic ribonuclease activity which was verified using an RNA-embedded gel and perchloric acid assay. Additionally, NPM1 also processes pre-ribosomal RNA in the nucleolus (Herrera et al., 1995; Savkur and Olson, 1998). The molecular chaperone property of NPM1 can facilitate this pre-ribosomal RNA processing by preventing the aggregation of proteins in the nucleolus during the process of RNA assembly. *Grisendi* et al. (2005) have also reported that knockdown of NPM1 expression changes the ribosome profile and inhibits the processing of pre-ribosomal RNA (Grisendi et al., 2005). Furthermore, a few enlarged nucleoli are frequently observed in cancer cells. This phenomenon is conceivably linked to the proposed oncogenic role of NPM1, as the aberrantly high expression of NPM1 in rapidly proliferating cancer cells is generally consistent with rapid ribosome biogenesis in maintaining the proliferative potential of cancer cells (Ruggero and Pandolfi, 2003).

1.3.3 Participation in transcriptional regulation

NPM1 has also been found to be involved in transcriptional regulation and contributes to cell growth control. The function of transcriptional regulation of NPM1 is related to the ability of NPM1 to interact with various transcription factors, such as NF-κB (nuclear factor κ-light chain enhancer of active B cells), Yin Yang 1 (YY1), alternate-reading-frame protein (ARF), interferon regulatory factor 1 (IRF-1) and activating transcription factor 5 (ATF5) (Colombo et al., 2002; Inouye and Seto, 1994; Kondo et al., 1997; Korgaonkar et al., 2005; Lix et al., 2012). NPM1 has been shown to act as an NF-κB co-activator in regulating the expression of the antioxidant enzyme MnSOD (Dhar et al., 2004). NPM1 can also alter the transcriptional activity of IRF1 and p53 (Colombo et al., 2002; Kondo et al., 1997). *Kondo* et al. (1997) have reported that NPM1 inhibited the DNA-binding and transcriptional activity of IRF-1 by interacting with IRF-1. Moreover, *Colombo* et al. (2002) have found that NPM1 interacts directly with p53 and regulates the stability and transcriptional activation of p53 subsequent to different types of stress. Furthermore, NPM1 has been found to form a stable complex with YY1, and the transcriptional repressive function of YY1 can be relieved by interacting with NPM1 (Inouye and Seto, 1994). This YY1-NPM1

complex can also up-regulate proliferating cell nuclear antigen (PCNA) promoter activity (Weng and Yung, 2005). PCNA is a nuclear protein whose appearance correlates with the proliferative state of the cell. The p300 protein may have a role in DNA repair synthesis through its direct interaction with PCNA (Hasan et al., 2001a). Additionally, NPM1 also has been reported to modulate the binding of NF-κB, E2F1 and pRB for the activation of E2F1 promoter (Lin et al., 2006).

During the retinoic acid-induced cell differentiation in human leukemia HL-60 cells, the protein expression level of c-myc and NPM1 and their promoter activities are reduced (Yung, 2004). However, the underlying mechanism of the down-regulation of NPM1 during the retinoic acid-induced cell differentiation has been revealed in recent years. NPM1 is involved in dynamic changes in the promoter occupancy of different transcriptional factors, which include NPM1 and AP2α (Yung, 2004). It has been found that NPM1 and AP2α can interact with each other. NPM1 can also be recruited by AP2α to the promoters of retinoic acid-responsive genes such as b-Myb, HSP60 and p120. These findings demonstrate that NPM1 potentially acts as a negative co-regulator during

retinoic acid signaling-induced gene expression. All these data indicate the potential function of NPM1 as a molecular regulator of gene expression at the transcriptional level.

1.3.4 Apoptosis inhibition

Overexpression of NPM1 in cells can promote cell survival, partly due to inhibition of apoptosis (Ye, 2005). Several lines of evidence, as further discussed, support this anti-apoptotic role of NPM1.

First, the expression of NPM1 is strongly induced in response to hypoxia (Li et al., 2004). Since NPM1 is a transcriptional target of hypoxia inducible factor 1α (HIF-1α), the NPM1 promoter region containing a functional HIF-1-responsive element can be activated by hypoxia or forced expression of HIF-1α. During hypoxia, NPM1 can inhibit hypoxia-induced phosphorylation of the tumor suppressor p53 at Ser-15 and interact with p53 in hypoxic cells. This might result in inhibition of the activation of p53 and hence dampen p53-mediated activation of apoptosis during hypoxia-driven tumor progression (Li et al., 2004). Secondly, NPM1 overexpression has been shown to induce

resistance to ultraviolet (UV) irradiation-induced apoptosis which is mediated by the tumor suppressor IRF-1 in NIH3T3 cells (Kondo et al., 1997; Wu et al., 2002b). Third, being highly expressed in hematopoietic cells, NPM1 has been shown to be related to the suppression of RNA-dependent protein kinase (PKR) activation, which normally induces apoptosis (Jagus et al., 1999; Pang et al., 2003). This observation has been associated with the aberrant proliferation of tumor cells. Fourth, NPM1 can function as a nuclear receptor of phosphatidylinositol (3,4,5)-trisphophate [PI(3,4,5)P_3], which is an essential second messenger implicated in various cellular processes. The NPM1-[PI(3,4,5)P_3] complex is a downstream effector of phosphatidylinositol 3-kinase. NPM1 inhibits apoptotic pathway via directly interaction with PI(3,4,5)P_3 to inhibit DNA fragmentation activity of caspase-activated DNase (CAD) resulting in an anti-apoptotic effect in NGF-treated PC12 cells (Ahn et al., 2005). Fifth, NPM1 has been shown to have an inhibitory effect on tumor suppressor p53. NPM1 can interact with p53 in hypoxic cells to block the hypoxia-induced activation of p53 phosphorylation (Li et al., 2004). *Li* et al. (2005) found that NPM1 inhibits the phosphorylation of p53 at serine 15 and thus abrogates the induction of downstream genes such as p21. Finally, *Gao* et al. (2005) have

reported that NPM1 can interact with pro-apoptotic protein GADD45α, which is responsive to genotoxic stress. While GADD45α lacks a nuclear localization signal, NPM1 interacts with GADD45α directly to serve as the molecular chaperone in controlling the subcellular distribution of GADD45α. However, the disruption of NPM1-GADD45α complex has been shown to impair cell cycle arrest and apoptotic function of GADD45α (Gao et al., 2005).

Lui and *Yung* (1998) have reported that NPM1 is an important factor in negatively regulating the nucleolar function for cellular apoptosis. This group used sodium butyrate (BuONa; a cell growth inhibitor) and vandate (a tyrosine phosphate inhibitor) to induce cell death in human promyelocytic leukemia HL-60 cells (Liu and Yung, 1998). The results showed that the transcription of NPM1 was decreased during the BuONa/vandate-induced apoptotic pathway. However, there were no decreases in NPM1 mRNA level and the telomerase activities during the serum starvation-induced cell growth arrest. The decreases in NPM1 mRNA expression and the telomerase activity in HL-60 cells subsequent to BuONa/vandate treatment is, therefore, suggested to be due to cellular apoptosis rather than the growth arrest (Liu and Yung, 1998). More

intriguingly, NPM1 may also serves as an important factor in the down regulation of nucleolar function for cellular differentiation. The role of NPM1 in cellular differentiation has been illustrated in the granulocytic differentiation of HL-60 cells induced by retinoic acid treatment (Hsu and Yung, 1998). The mRMA expression level of NPM1 is reduced during the retinoic acid-induced differentiation. On the contrary, there is no decline in NPM1 mRNA level during the cellular growth arrest induced by serum starvation. These data suggest that the decrease of NPM1 mRNA expression level in HL-60 cells subsequent to retinoic acid treatment is attributed to cellular differentiation rather than the growth arrest induced by retinoic acid (Hsu and Yung, 1998).

1.3.5 Modulation of tumor suppressors

NPM1 has also been involved in regulating the activity and stability of some tumor suppressors such as ARF and p53. ARF is a tumor suppressor ($p19^{Arf}$ in the mouse and $p14^{ARF}$ in humans) that localizes within the nucleolus. ARF activates p53-dependent cell cycle checkpoints by binding the ubiquitin ligase Mdm2, which promotes p53 degradation and leads to p53-dependent cell cycle arrest (Bertwistle et al., 2004; Brady et al., 2004; Sugimoto et al., 2003). It has

been shown that NPM1 stabilizes p53 through direct interaction with Mdm2 (Kurki et al., 2004b). ARF prevents the proliferation of cells lacking Mdm2 and p53, albeit less efficiently. In addition, ARF is a relatively stable protein whose degradation depends upon its N-terminus polyubiquitination and is mediated through the ubiquitin-proteasome pathway. Moreover, NPM1, ARF and other proteins can form high-molecular-weight complexes in the nucleolus (Kuo et al., 2004). This interaction has been associated with the stabilization of ARF by retarding the turnover of ARF. Overall, this stabilization is essential to maintain the biological function of ARF (Kuo et al., 2004; Kuo et al., 2008).

Furthermore, the protective effect of NPM1 on ARF turnover is involved in the influence of the proteasome-dependent and –independent degradation. While ARF is recognized for suppressing cell proliferation through p53-dependant and p53–independent pathways (Bertwistle et al., 2004; Itahana et al., 2003; Korgaonkar et al., 2005), the deficiency of NPM1 has been shown to result in accelerated tumorgenesis and this is probably attributable to the destabilization of ARF (Sherr, 2006). ARF has been reported to suppress cell proliferation by inhibiting the biogenesis of ribosomes through retarding the

processing of 47/45S and 32S precursors of ribosomal RNA (Sugimoto et al., 2003). Thus, the interaction of NPM1 and ARF in the nucleolus is another way of controlling cell proliferating processes.

The tumor suppressor p53 is another protein that is proposed to be modulated by NPM1. p53 is an important protein, serving as a cellular gatekeeper for cell growth and division. Generally, in the cell, the protein level of p53 is kept at a minimal concentration by its relatively short half-life (about 20 min). However, several different types of DNA damage can activate p53, such as DNA double-strand breaks or severely damaged DNA integrity (Levine, 1997). Moreover, the integrity of the nucleolus and p53 stability is intimately linked. The nucleolus is a cellular stress sensor responsible for maintaining low levels of p53, which are automatically elevated as soon as nucleolar function is disrupted in response to cellular stresses (Rubbi and Milner, 2003). NPM1 has been reported to promote p53 stability when undergoing nucleoplasmic relocalization (Horn and Vousden, 2004). *Kurki* et al. (2004a & 2004b) reported that NPM1 in the nucleolus can increase the stability of p53 by suppressing the physical binding interaction between Mdm2 and p53 in response to UV

irradiation. In response to several cellular stresses, the impaired nucleolar integrity induces the translocation of nucleolar NPM1 between subcellular compartments. The re-localized NPM1 subsequently participates in the p53-mediated reaction (Horn and Vousden, 2004; Rubbi and Milner, 2003). Indeed, overexpression of NPM1 induces cell cycle arrest in normal fibroblasts, whereas this promotes the S-phase entry of the cell cycle in cells lacking p53 (Itahana et al., 2003).

1.3.6 Maintenance of genomic stability

NPM1 also binds to chromatin in a DNA-damage-dependent manner and is implicated in the maintenance of genomic stability through participation in the DNA repair process (Lee et al., 2005). The early to immediate response of NPM1 to DNA damage involves rapid transcriptional up-regulation of NPM1 following UV irradiation (Wu et al., 2002a; Wu and Yung, 2002). Moreover, many DNA repair-associated genes have shown to be associated with elevated expression of NPM1 (Wu et al., 2002b). NPM1 has also been implicated in maintaining genomic stability during the process of cell cycle through the regulation of centrosome duplication (Grisendi et al., 2005).

1.3.7 Cell cycle regulation

The nucleolus undergoes reversible disassembly when the cell enters mitosis. During mitosis, NPM1 is observed to translocate from nucleolar remnants to cytoplasm (Hernandez-Verdun and Gautier, 1994). NPM1 has been found to move to chromosome periphery and the cytoplasmic entities, known as nucleolus-derived foci (NDF) (Dundr et al., 2000). NDF can redistribute to the poles of the mitotic spindle and interact with NuMA (a nuclear matrix protein) subsequently to control the formation of centrosomes in pro-metaphase and mitotic poles in metaphase (Compton and Cleveland, 1994; Zatsepina et al., 1999). The interesting observation that NPM1 is localized in mitotic spindle poles suggests the protective role of NPM1 in preventing hyper-amplification of the centrosome to ensure a successful progression through G2-M phases (Tokuyama et al., 2001; Zatsepina et al., 1999; Zhang et al., 2004). Although NPM1 is not a classical centrosomal protein, it is associated with unduplicated centrosomes and is a direct substrate of cyclin-dependent kinase 2-cyclin E (CDK2-cyclin E) complex in centrosome duplication (Andersen et al., 2003; Okuda, 2002; Tokuyama et al., 2001).

Indeed, one of the major functions of NPM1 in promoting cell proliferation is linked to its positive role in cell cycle regulation. One mechanism by which NPM1 promotes cell cycle progression is its influence on two important negative cell cycle regulators, ARF and p53. Another role of NPM1 in promoting cell cycle progression is its function as an effector for several positive cell cycle regulators such as protein kinase C (Beckmann et al., 1992), mitotic cdc2 kinase (Peter et al., 1990), and nuclear casein kinase 2 (Pfaff and Anderer, 1988). These protein kinases can phosphorylate NPM1 and hence, regulate its physiological functions. For example, NPM1 has been identified as a substrate of CDK2-cyclin E complex in late G1 phase to regulate centrosome duplication in dividing cells (Okuda et al., 2000; Tokuyama et al., 2001). NPM1 has also been reported to be phosphorylated by cdc2 kinase during mitosis (Peter et al., 1990) and by nuclear casein kinase 2 during the interphase (Chan et al., 1990). Furthermore, NPM1 has been suggested to be a candidate substrate for BRCA1-associated RING domain1 (BRCA1-BARD1) ubiquitin ligase. Sato et al. (2004) has reported that NPM1 forms a complex with BRCA1-BARD1 ubiquitin ligase and presents at the centrosome during mitosis. It is also suggested that the

interaction of NPM1 with BRCA1-BARD1 ubiquitin ligase might lead to ubiquitination of NPM1 and facilitate in maintaining the integrity of spindle poles and genomic integrity (Grisendi et al., 2006).

1.3.8 Post-translational modification of NPM1 protein

To date, NPM1 has been reported to undergo various post-translational modifications involving downstream or/and further gene expressions. There are five types of post-translational modifications of NPM1 including phosphorylation, acetylation, poly(ADP-ribosyl)ation, ubiquitination and sumoylation. Table 1.1 summarizes the cellular physiological functions that are regulated by post-translational modifications of NPM1 and alterations of NPM1 in human cancers.

Table 1.1: Summary of physiological functions regulated by post-translational modifications of NPM1 and alterations of NPM1 in human cancers

Post-translational modifications of NPM1:	***Physiological functions:***
Phosphorylation/ Dephosphorylation	▪ Cell cycle regulation, (Chan et al., 1986; Descombes and Nigg, 1998; Szebeni et al., 2003; Tokuyama et al., 2001; Wianny et al., 1998) ▪ Initiation of centrosome duplication (Okuda et al., 2000) ▪ Molecular chaperone (Szebeni et al., 2003)
Acetylation	▪ Enhanced histone chaperone activity (Swaminathan et al., 2005) ▪ Transcriptional activation of genes involved proliferation during carcinogenesis (Shandilya et al., 2009) ▪ Involved in viral gene transactivation (Gadad et al., 2011) ▪ Facilitation of DNA repair * *studying in progress*
Poly(ADP-ribosyl)ation	▪ Mitosis regulation (Leitinger and Wesierska-Gadek, 1993; Meder et al., 2005)
Ubiquitination	▪ Mitosis regulation (Hayami et al., 2005), ▪ Induced by ARF to regulate the stability of NPM1 (Itahana et al., 2003; Sato et al., 2004)
SUMOylation	▪ Regulation of stability and localization of NPM1 (Okada et al., 2007; Okuwaki, 2008; Tago et al., 2005)
Alterations of NPM1 in human cancers:	
▪ Over-expression in tumors of different origins, such as thyroid tumors (Pianta et al., 2010), gastric (Tanaka et al., 1992), colon (Nozawa et al., 1996) and breast (Wang et al., 2010a) cancers ▪ Causing NPMc+ acute myeloid leukemia ▪ Balanced translocations in lymphomas and leukemias (Rau and Brown, 2009; Shiseki et al., 2007; Zhang et al., 2007)	

1.3.8.1 Phosphorylation of NPM1

NPM1 has been identified as a phosphoprotein, many of its amino acid residues are found to be phosphorylated in cells. It has been reported that the phosphorylation of NPM1 is catalyzed by various protein kinases, including nuclear casein kinase 2 (CK2) (Chan et al., 1986), protein kinase C (Beckmann et al., 1992), polo-like kinase 1 (PLK1), and cyclin-dependent kinases such as CDK1/cyclin B (also called cdc2 kinase), CDK2/cyclin E, and CDK2/cyclin A (Tokuyama et al., 2001). Reported phosphorylation sites on NPM1 and the responsible kinases are summarized in Figure 1.3 and Table 1.2, respectively.

CK2 is a cell cycle-related serine/threonine protein kinase which is highly accumulated in nucleoli rather than the nucleoplasm or the cytoplasm (Pfaff and Anderer, 1988) and is required for cell viability as well as cell cycle progression (Pinna and Meggio, 1997). The serine 125 (S125) residue of NPM1 can be phosphorylated by CK2 during the interphase (Szebeni et al., 2003). However, overexpression of a S125A NPM1 mutant with serine replaced by alanine at position 125, results in the segregation of the nucleolar structure and inhibition of ribosome biogenesis (Louvet et al., 2006). That means the NPM1

phosphorylation at S125 by CK2 serves to regulate NPM1 function during ribosome biosynthesis, and also enables NPM1 to perform its function as a molecular chaperone (Szebeni et al., 2003).

Plk1 is another enzyme that is reportedly associated with NPM1 phosphorylation (Zhang et al., 2004). In general, Plks are conserved protein-serine/threonine kinases that act as pivotal components of cell cycle regulation (Descombes and Nigg, 1998; Wianny et al., 1998). Scientists have found that ectopic over-expression of an NPM1 mutant in which S4 residue is replaced by alanine induces multiple mitotic defects. S4 residue of NPM1 might play a role in mitosis via Plk1-mediated phosphorylation (Zhang et al., 2004). Not only Plk1 is involved in NPM1 phosphorylation, another Polo-like kinase 2 (Plk2) has been found as a novel interacting partner of NPM1. The major role of Plk2 is involved in centrosome reproduction as it is localized to centrosomes and its kinase activity is activated near the G1/S phase transition (Cizmecioglu et al., 2008; Warnke et al., 2004). Interestingly, Plk2 can interact with, and phosphorylate NPM1 on serine 4 residue in S-phase and mitosis. Notably, overexpression of NPM1 S4 mutant in which replaced serine residue by alanine

interrupts the progress of centriole reduplication in S-phase and normal centrosome reproduction. These results demonstrated that Plk2 can trigger centriole duplication by inducing phosphorylation of NPM1 on S4 residue (Krause and Hoffmann, 2010).

Furthermore, the cell cycle regulation is tightly controlled by several regulatory checkpoints in normal cells. Specifically, coordinated initiation of centrosomes and DNA duplication are two major regulatory checkpoints for the centrosome duplication cycle. Cyclin-dependent serine/threonine kinases (CDKs) are necessary for initiation of centriole duplication (Hinchcliffe and Sluder, 2002) and control the onset of cell cycle events such as DNA synthesis and mitosis (Nurse, 1994). NPM1 has been reported to associate with, and disassociate from, centrosomes in a cell cycle stage-specific manner (Dyson, 1998; Okuda et al., 2000). This cell cycle dependent association and disassociation of NPM1 with centrosomes is perhaps controlled by differential phosphorylation of NPM1 by CDK-cyclin complexes. For instance, during early to middle G_1 phase, NPM1 is associated with unduplicated centrosomes. CDK2-cyclin E is highly activated in the late G_1 phase leading to phosphorylation of

NPM1. This triggers the disassociation between NPM1 and centrosomes (Koff et al., 1992; Lees et al., 1992). CDK2-cyclin E-induced NPM1 phosphorylation leads to the dissociation between NPM1 and centrosomes, which is a prerequisite step for centrosomes to initiate duplication. The typical cyclin-dependent kinase consensus sites are distributed throughout the NPM1 sequence, including S10, S70, T199, T219, T234 and T237. Four threonine residues T199, T219, T234 and T237 have been reported to be phosphorylated by CDK2-cyclin E or CDK1-cyclin B (cdc2 kinase) in vivo and in vitro (Okuda et al., 2000; Okuwaki et al., 2002; Tokuyama et al., 2001). Phosphorylation of NPM1 at T199 by CDK2-cyclin E permits NPM1 disassociation from the centrosome during the G_1 phase. As mentioned before, it has been suggested that NPM1 can link to the paired centriole (Shinmura et al., 2005), and the disassociation of NPM1 is essential to the initiation of centrosome duplication. However, T199 residue of NPM1 is also a major phosphorylation site for CDK2-cyclin A, which has a similar efficiency to CDK2-cyclin E. It is possible that the continual presence of active CDK-cyclin A is responsible for preventing the re-association of NPM1 to centrosomes during the S and G_2 phases (Tokuyama et al., 2001). Recently, investigators have reported that T199 phosphorylation of NPM1 is localized at

the nuclear speckles that contain splicing machineries such as SC35 (Tarapore et al., 2006). These observations suggest that T199 phosphorylation of NPM1 plays a role in the localization of NPM1 at the speckles and in repressing pre-mRNA processing. Moreover, the phosphorylation of T199 has also been implicated in inhibiting GCN5-mediated histone acetylation (Zou et al., 2008).

In addition to the T199 site, NPM1 is also phosphorylated on T219, T234 and T237 during mitosis. Furthermore, the RNA-binding activity of NPM1 was found to be abolished by CDK1-cyclin B-mediated phosphorylation of these four threonine sites in vitro (Okuwaki et al., 2002). The T199 residue of NPM1 is also highly phosphorylated by CDK1-cyclin B during mitosis. The mitosis-specific phosphorylated form of NPM1 may play a role in maintaining chromosomes in their condensed state during mitosis (Lu et al., 1996) or nucleolar disassembly. This might correlate with NPM1 re-association with the centrosome during mitosis (Wang et al., 2005). Recent studies have suggested that the phosphorylation and dephosphorylation cycle of NPM1 during mitosis depends upon the balance between the CDK1 and the protein phosphatase 1 (PP1) activities (Negi and Olson, 2006). Moreover, the proteomic analysis of the

mitotic spindle indicated that NPM1 are phosphorylated at S70, S125 and S254 residues (Nousiainen et al., 2006). However, it remains to be determined which kinases are responsible for phosphorylation of NPM1 at S70, S125 and S254 residues. The biological functions of phosphorylation at these sites remain unknown.

PKC is a serine/threoine protein kinase required for ubiquitous signal transduction systems in mammalian cells and participates in cell differentiation, tumor promotion and oncogenesis (Nishizuka, 1986). Analysis of nuclear preparations by 2-D SDS/polyacrylamide gel and subsequent microsequencing has shown that NPM1 is a substrate for nuclear PKC and is possibly involved in the transport of ribosomal proteins (Beckmann et al., 1992; Borer et al., 1989).

Additionally, most of cellular processes are regulated by proteins with post-translational modifications. For some post-translational modifications (e.g., acetylation and tyrosine phosphorylation), identifying the modified amino acid is relatively straightforward because they are quite stable, even in the presence of the high energy required for collision-induced dissociation (CID) experiments.

However, for other post-translational modifications (e.g., O-linked N-acetylglucosamine (O-GlcNac) and phosphorylated serine and threonine residues), localization is substantially more difficult because the peptides lose the modification either in a charge separation process (O-GluNac), or by a β-elimination event with a neutral loss of phosphoric acid (e.g., phosphoserine into dehydroalanine). *Molina* et al. (2007) performed a global proteomics profiling of phosphopeptides by means of electron transfer dissociation (ETD) in an ion trap mass spectrometer. They also identified a total of 1435 phosphorylation sites from human embryonic kidney 293T cells treated with phosphatase inhibitors finding that NPM1 is phosphorylated at S70, S125, S137, T199, Thr234/237 and S260.

Dephosphorylation is another mechanism in regulating the function of NPM1. As previously mentioned, a protein phosphatase 1 (PP1) is required for regulating phosphorylation and dephosphorylation of NPM1 during mitosis. Recently, a specific protein phosphatase called PP1β has been reported to de-phosphorylate NPM1 in response to DNA damage during ultraviolet irradiation. This process is believed to facilitate the DNA repair pathway (Lin et al., 2010).

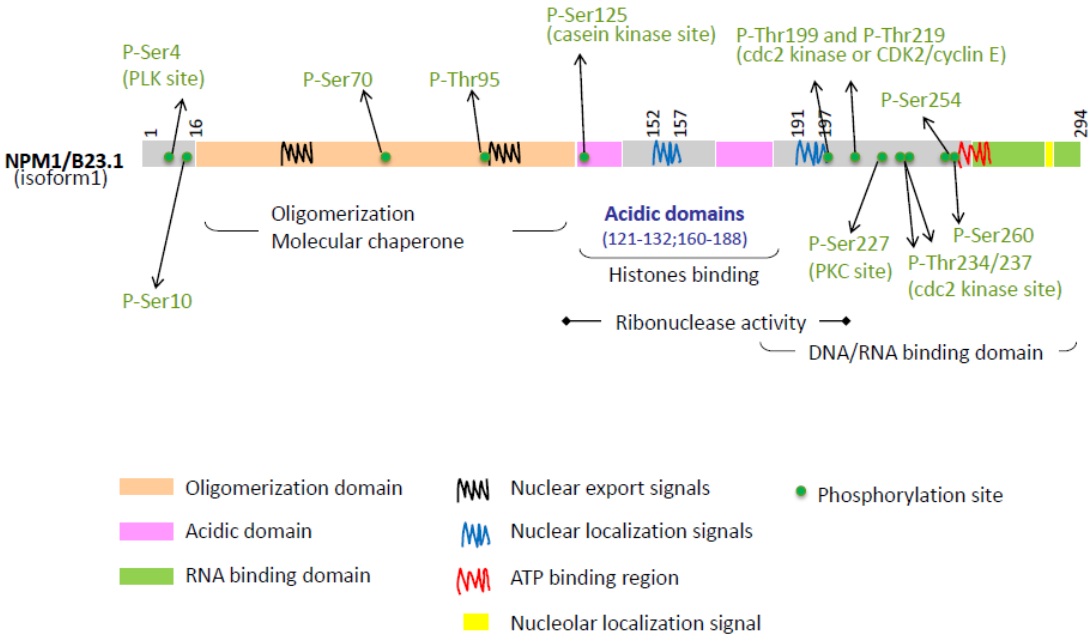


Figure 1.3: The phosphorylation sites of human NPM1 protein.

The green dots presented in Figure 1.3 demonstrate the phosphorylation sites of NPM1. These serine and/or threonine sites could be phosphorylated by casein kinase2, cyclin-dependent serine/threonine kinases (CDKs) and protein kinase C (PKC), respectively. NPM1 phosphorylation is involved in a variety of gene regulations; the detailed functions of NPM1 phosphorylation is described in *1.3.8.1 phosphorylation of NPM1* [This figure is modified from (Grisendi et al., 2006; Okuwaki, 2008)].

Table1.2. Summary of phosphorylation sites, phosphorylation kinases and physiological functions of NPM1 phosphorylation

Phosphorylation Sites	**Kinases**	**Physiological functions**
Serine 4	Polo-like kinase 1 (Plk1) Polo-like kinase 2 (Plk2) G protein-coupled receptor kinase 5 (GRK5)	▪ Cell cycle regulation (Zhang et al., 2004) ▪ Trigger centriole duplication (Krause and Hoffmann, 2010) ▪ Regulate cellular sensitivity to PLK1 inhibition (So et al., 2012)
Serine 10 Serine 70	CDK2/cyclin E* or cdc 2kinase*	▪ Regulation of centrosome duplication and ribosomal biogenesis (Okuda et al., 2000; Okuwaki et al., 2002)
Serine 125	Casein kinase	▪ Ribosome biogenesis (Louvet et al., 2006) ▪ Molecular chaperone (Szebeni et al., 2003)
Threonine 199 Threonine 219 Threonine 234/237	cdc 2 kinase or CDK2/cyclin E	▪ The localization of NPM1 (Tarapore et al., 2006) and the regulation of centrosome duplication (Tokuyama et al., 2001), ▪ Inhibiting GCN5-mediated histone acetylation (Zou et al., 2008), ▪ Cell cycle regulation (Lu et al., 1996; Okuwaki et al., 2002)
Seine 227	Protein kinase C	▪ Biological functions are unknown(Beckmann et al., 1992)
Serine 254 Serine 260	Unknown	▪ Biological functions are unknown (Molina et al., 2007)

" * ", the kinases are required for phosphorylation have not been verified.

1.3.8.2 Acetylation of NPM1

NPM1 interacts with core histone proteins H2A, H2B, H3 and H4 and functions as a human histone chaperone in facilitating nucleosome assembly (Okuwaki et al., 2001). Acetylation of NPM1 is suggested to be involved in the chromatin regulation, as NPM1 acetylation increases its binding affinity to acetylated core histones. Notably, acetylation of NPM1 has also been suggested to be involved in the NPM1-mediated regulation of chromatin transcription by loosening nucleosomal structure in the regulatory region (Swaminathan et al., 2005). Moreover, several lysine residues at the C-terminal of NPM1 can acetylate by histone acetyltransferase p300 in vitro. While p300 activates transcription by acetylating the histones and "loosening" the tightly packed chromatin structure, it also acetylates NPM1 resulting in transcriptional activation from chromatin template (Swaminathan et al., 2005). The acetylation sites of NPM1 have been mapped by MALDI-TOF analysis. The result showed that most of the NPM1 C-terminal region (Lys212, Lys229, Lys230, Lys248 or Lys250, Lys257 and Lys292) are the acetylation sites; Lys292 only can be acetylated in vitro condition. However, the function of NPM1 acetylation is under investigation. Recently, the latest finding demonstrated that NPM1

acetylation influences its subcellular localization and increases its role in carcinogenesis. Because acetylated NPM1 was predominantly distributed in the nucleoplasm and significantly colocalized with RNAP II (RNA polymerase II) at the active transcription foci (Shandilya et al., 2009). Furthermore, the acetylation level of NPM1 also increases in later stages of oral carcinoma as compared with early stage. This suggests that augments of the NPM1 acetylation level are associated with the malignancy of oral cancer. This manifestation suggested that acetylated NPM1 protein might possibly increase its occupancy on gene promoters which relate with metastasis. Further experimental results also showed that acetylated NPM1 associated with RNAP II and increased their occupancies on TNF-α promoter region resulting in activation of TNF-α (Shandilya et al., 2009). Notably, p300 is not the only factor mediating NPM1 acetylation, viral infection also induces NPM1 acetylation. Previously, NPM1 has been reported as directly interacting with the viral HIV-1 Tat protein (Li, 1997). Experimental data have demonstrated that HIV-1 infection not only induces NPM1 acetylation, but also synergistically further enhances Tat-mediated transactivation of HIV-1 promoter (Gadad et al., 2011). In this research project, attempts were made to elucidate the role of NPM1 and

its acetylation in the transactivation of the DNA repair-associated gene PCNA under UV irradiation.

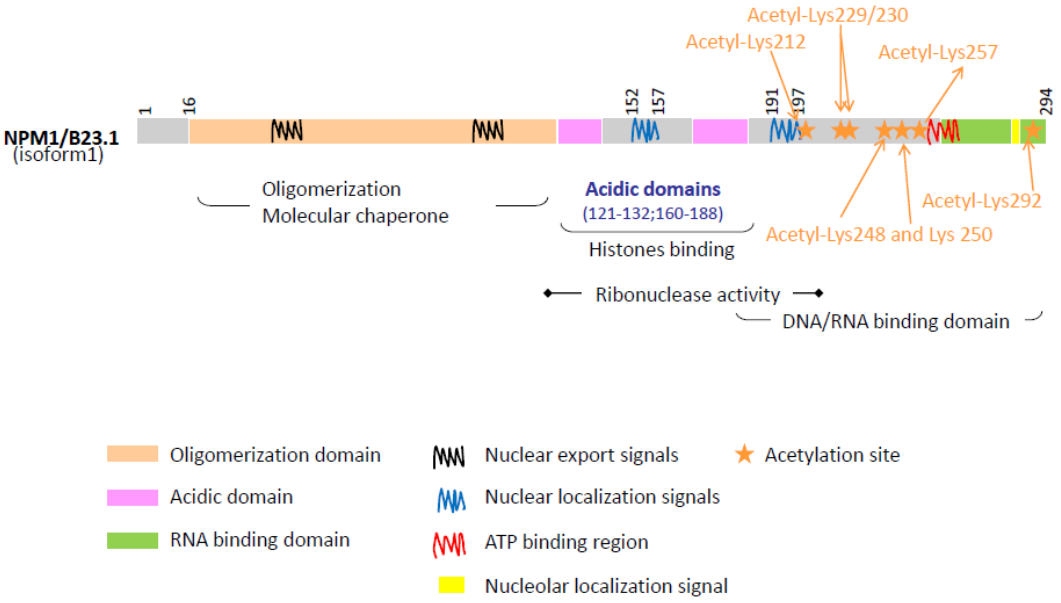


Figure 1.4: The acetylation sites of human NPM1 protein.

The star shapes shown in Figure 1.4 are the putative acetylation sites of NPM1. The majority of these lysine residues can be acetylated by p300. NPM1 acetylation might transactivate genes involved in cell proliferation and survival [This figure is modified from (Grisendi et al., 2006; Okuwaki, 2008)].

1.3.8.3 Poly(ADP-ribosyl)ation of NPM1

In addition to previously described post-translational modifications, NPM1 can also undergo a variety of modifications including poly(ADP-ribosyl)ation (Ramsamooj et al., 1995), unbiquitination (Itahana et al., 2003; Sato et al., 2004) and sumoylation (Tago et al., 2005). In this section, Poly(ADP-ribosyl)ation will be discussed. This kind of modification is an enzyme-mediated reversible post-translational modification of proteins (Lakadong et al., 2010). In this process, ADP-ribose groups are added to, or detached from, the acceptors or target proteins. The target proteins can become poly-ADP-ribosylated or deribosylated, respectively. Poly-ADP-ribose (PAR) is a large and branched negatively charged polymeric macromolecule. This kind of polymeric macromolecule has added to target proteins, a family of enzymes known as poly(ADP-ribose) polymerases (PARPs) (Chang et al., 2004; Smith, 2001). Most importantly, these poly-ADP-ribosylated proteins are involved in several physiological functions and are required for normal cell division. For instance, *Tulin* et al. (2002) reported that knockouts of PARP inhibited the process of poly(ADP-ribosyl)ation in Drosophila, leading to embryonic lethality. PARPs could catalyze the polymerization of ADP-ribose groups from the donor NAD^+ to

target proteins. This process created the linear or branched structure of polymers attached to target proteins (Kim et al., 2005). In addition, PAR groups-attached was bound to specific amino acid residues of target proteins, especially on the aspartate, glutamate or lysine residue surface of target proteins (Imamura et al., 2004).

As previously mentioned, poly(ADP-ribosyl)ation is an enzymatic reversible reaction. PARPs catalyze the polymerization of ADP-ribose groups on target proteins while the other enzyme, poly(ADP-ribose) glycohydrolase (PARG), breaks down the adducts on target proteins (Chang et al., 2004; Hatakeyama et al., 1986). *Leitinger* et al. (1993) used 1-dimenstional and 2-dimenstional polyacrylamide gel electrophoresis (PAGE) to analyze nucleolar proteins labeling with ^{32}P-NAD^{+} and detected the modified proteins by autoradiography. These results indicated that, in addition to nuclear ADP-ribosyltransferases and histones, NPM1 and nucleolin/C23 also were modified by ADP-ribosylation (Leitinger and Wesierska-Gadek, 1993). Other groups have also reported that NPM1 is one of the nuclear proteins that are postulated to be poly-ADP-ribose-associated in vivo (Adolph, 1985; Song and Adolph, 1983). Two PARP enzymes,

PARP1 and PARP2, have been reported to be associated with NPM1 (Meder et al., 2005) and thus might be responsible for poly-ADP-ribosylation of NPM1. NPM1 poly-ADP-ribosylation might be involved in the formation of chromatin and preventing hyper-amplification of the centrosome during the G_2–M phase of the cell cycle. This is because most PARPs have been localized to the cellular spindle, and NPM1 is also present at mitotic centrosomes during the G2–M phase. For instance, both PARP1 and PARP2 have been found to localize in centromeres (Saxena et al., 2002) and PARP1 and PARP3 have been found to associate with centrosomes (Smith, 2001). In addition, PARP1 is also an important structural component of chromatin, similar to linker histones in modulating the chromatin structure (Kim et al., 2004). Experimental results also indicate that NPM1 could interact with PARP1 via its DNA-binding domain (Meder et al., 2005). Additionally, cellular PAR concentrations were shown to increase dramatically by PARP activity during metaphase and anaphase in mitosis (Bakondi et al., 2002). In summary, PARPs and PAR groups might play an important role in spindle function (Chang et al., 2004). The poly(ADP-ribosyl)ation of NPM1 are most likely involved in chromatin association and disassociation through an interaction with PARP1.

1.3.8.4 Ubiquitination of NPM1

Ubiquitination is a complex post-translational modification and mainly regulates the selective degradation of regulatory proteins by proteasomes. Many cellular functions are regulated by ubiquitin/proteosome-dependent proteolysis including, the cell cycle (Koepp et al., 1999), inflammatory response induction (Ghosh et al., 1998), and antigen presentation (York et al., 1999). The dysregulation of ubiquitin-dependent proteolysis has been reported to act as a causative factor in cancer and several inherited diseases, such as colorectal carcinoma (Loda et al., 1997), familial cylindromatosis (Bignell et al., 2000), and Angelman syndrome (Kishino et al., 1997). However, protein degradation is not the only fate for ubiquitin-tagged proteins. Ubiquitination can also regulate certain cellular processes such as ribosomal function, post-replicational DNA repair, the initiation of inflammatory response, and the functions of transcription factors (Pickart, 2001).

The entire process of ubiquitination is carried out by three enzymes, namely E1, E2, and E3. E1 enzyme is also known as ubiquitin-activating enzyme, which is shared among all ubiquitin ligases. The first process is ubiquitin activation,

which is a two-step reaction activated by E1 ubiquitin-activating enzyme. The E1 enzyme uses ATP as an energy source to produce an ubiquitin-adenylate intermediate. Following, is the transfer of ubiquitin to the E1 activating cysteine and the simultaneous release of AMP. In the initial process, the E1 enzyme creates a thiol ester linkage between the E1 cysteine sulfhydryl group and the C-terminal carboxyl group of ubiquitin (Ub), thereby activating the C-terminal of the conserved 76-residue ubiquitin. The second process is the transfer of the Ub from E1 enzyme to the ubiquitin-conjugating enzyme (E2). E2 will transiently carry the activated ubiquitin molecules by thiol ester and then transfer it to the target protein. In general, the E2 enzyme interacts with a specific E3 enzyme partner. The final process involves the ubiquitin-protein ligase (E3, also known as ubiquitin ligase), which is responsible for the combination with E2 and the transfer between the activated ubiquitin of the E2 and the lysine residue of the target protein (or other ubiquitins) through an isopeptide bond (Hershko, 1983; Pickart, 2001). E3 enzymes are involved in recognizing the substrate of the ubiquitination pathway and interacting with both E2 enzymes and substrates. E3 enzyme can receive the ubiquitin from the E2 enzyme and transfer itself to the target protein (also called substrate) in some cases. However, E3 enzyme

can also interact with the E2 enzyme and the substrate in other cases, but itself never receives the ubiquitin (Woelk et al., 2007). The schematic illustration of ubiquitination is shown in Figure 1.5.

These three processes initiate all known ubiquitination reactions. In an ubiquitination cascade, an E1 enzyme binds with many different kinds of E2 enzymes, which subsequently binds with hundreds of E3 enzymes in a hierarchical way. Furthermore, other ubiquitin-like proteins (ULPs) can also be modified via this E1-E2-E3 cascade. In addition, ubiquitin can be appended as a monomer or as isopeptide-linked polymers which are referred to as polyubiquitin chains. The binding of polyubiquitin chains label proteins for degradation by proteasomes. However, polyubiquitination may not end up in proteasomes-degradation. When polyubiquitination occurs by adding lysine 6-linked poly-Ub chain to substrates, the target proteins can be degraded by 26S proteasomes. It is a kind of post-translational modification of substrate proteins.

NPM1 has been reported to have two different types of polyubiquitination, one for gene regulation and the other for protein degradation. The functions of

NPM1 ubiquitination are briefly described in below. In the gene regulation aspect, NPM1 has been shown to be a possible ubiquitination substrate of BRCA1 E3 enzyme. BRCA1 formed a complex with BRDA1 to mediate the addition of lysine 6-linked polyubiquitination to NPM1. The experimental results demonstrated the co-localization of NPM1 and BRCA1-BARD1 complex at the mitotic spindle. Notably, the BRCA1-BARD1 complex-induced polyubiquitination is proposed to modulate the function of NPM1 during mitosis. Besides, BRCA1 inactivation has been reported to induce centrosome amplification and lead to genomic instability (Nishikawa et al., 2004), and the enzymatic activity of E3 ligase of BRCA1-BARD1 can be down-regulated by cyclin A/E-cdk2 (Hayami et al., 2005). This means that NPM1 might be the downstream substrate of BRCA1-mediated ubiquitination during mitosis. Another NPM1 of polyubiquitination is induced by tumor suppressor ARF. NPM1 is known to directly associate with ARF which can triggers ubiquitin-proteasome dependent degradation to further regulate the stability of NPM1 (Itahana et al., 2003; Sato et al., 2004). Recently, the precise ubiquitination site of NPM1 relating protein stability has been reported to lie in lysine 263 residue of NPM1. Replacement of lysine 263 to asparagines (K263N) curtails the protein

half-life approximately 10 hr comparing with wild-type NPM1. This K263N mutant is unstable because the mutagenesis of K263 residue abrogates ATP binding and redistributes NPM1 localization form the nucleolus to the nucleoplasm, and consistently contributes ubiquitin-proteasome dependent degradation mediated by caspase-3 cleavage (Choi et al., 2008b; Liu et al., 2007b). Taken together, K263 is a crucial site which is required for NPM1 stability and ATP binding, confining the localization of NPM1. K263 mutant further impedes the mitogenic effects and inhibits the anti-apoptotic function of NPM1 in PC12 cells (Choi et al., 2008a; Choi et al., 2008b).

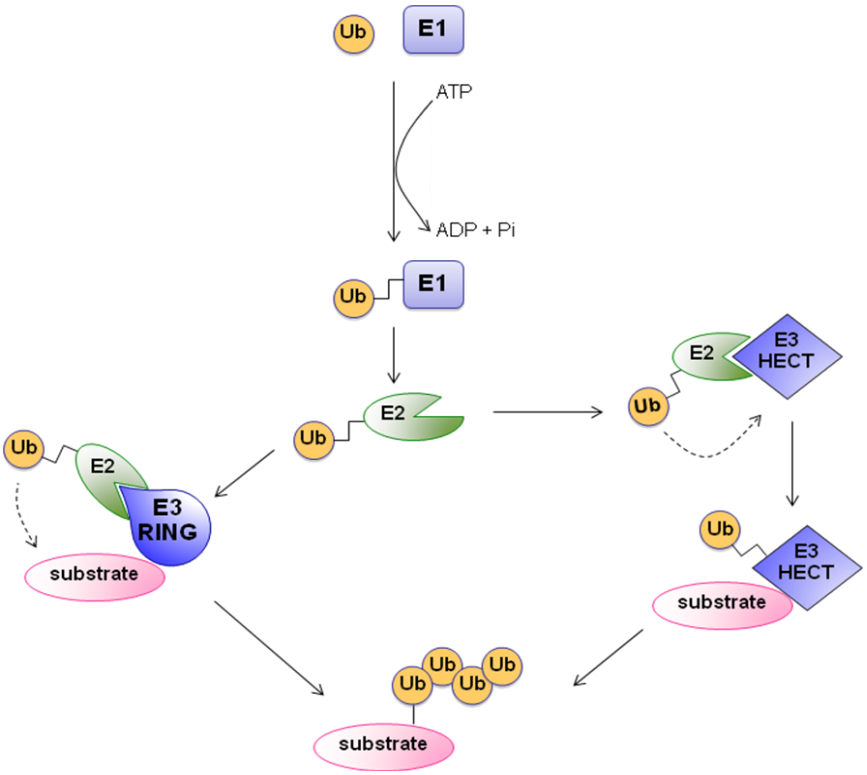


Figure 1.5: The ubiquitination pathway.

A schematic illustration of ubiquitination pathway. Three types of enzymes are required for protein ubiquitination: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin-protein ligase (E3). E1 enzyme uses ATP to activate ubiquitin and covalently conjugates ubiquitin to intracellular proteins (substrates). E2 enzyme transiently carries activated ubiquitin. E3 enzyme receives the activated ubiquitin from E2, and then transfers itself to substrate or interacts with E2 and substrate [This figure is adapted from (Woelk et al., 2007)].

1.3.8.5 SUMOylation of NPM1

SUMOylation is another post-translational modification which can reversibly modulate the protein activities of NPM1. *S*mall *u*biquitin-like *mo*difier or SUMO can be covalently bound to its target proteins. To date, many proteins have been identified to carry this modification. SUMO can be attached onto and detached from target proteins by covalently binding, and hence further modulates the functions of the substrate proteins. SUMOylated proteins are known to be involved in various cellular functions including DNA repair, transcription activation and nuclear body formation (Hay, 2005; Kerscher, 2007).

The sizes of SUMO proteins are approximately 10 KDa and very similar to ubiquitin in size, and three-dimensional structure even though they share less than 20% amino acid sequence identity. Moreover, the enzymatic cascade of SUMOylation is analogous to that of ubiquitination (Seeler and Dejean, 2003). However, in contrast to the ubiquitination pathway, SUMOylation is not used to label proteins for degradation. Instead, it influences the functional properties of proteins, such as their activation, localization, interactions and half-life (Geiss-Friedlander and Melchior, 2007). In addition, all SUMO proteins contain an unstructured stretch of up to 22 amino acid residues at their N-terminus that is not found in other ubiquitin-related proteins.

Like ubiquitination, SUMOylation makes an isopeptide bond between the C-terminal glycine residue of the modifier protein and the ε-amino group of the lysine residue in the acceptor protein. Both ubiquitination and SUMOylation are stepwise processes and are regulated by an enzymatic cascade including three

classes of enzymes. The first step of SUMOylation is activating a mature SUMO protein at its C-terminus by the SUMO-specific E1 activating enzyme. This E1 enzyme is a heterodimer formed by AOS1 (also named as SAE1, SUMO1 activating enzyme subunit 1) (Desterro et al., 1999; Johnson et al., 1997), and UBA2 (ubiquitin-like modifier activating enzyme 2), (Gong et al., 1999; Okuma et al., 1999). This ATP-dependent step serves to form a SUMO-adenylate conjugate, which acts as an intermediate in the formation of a thioester bond between the C-terminal carboxy group of SUMO and the catalytic cysteine residue of UBA2. In the second step, SUMO is transferred from UBA2 to the E2 conjugating enzyme, Ubc9. Ubc9 is the only E2 enzyme required for SUMOylation and can create a thioester linkage between its own catalytic cysteine residue and the C-terminal carboxy group of SUMO (Desterro et al., 1997; Johnson and Blobel, 1997; Lee et al., 1998; Saitoh et al., 1998). Finally, Ubc9 transfers SUMO to the accepter protein; a step facilitated by SUMO E3 ligases that form an isopeptide bond between the C-terminal glycine residue of SUMO and a lysine side chain of the acceptor protein (Geiss-Friedlander and Melchior, 2007). Importantly, Ubc9 can interact with a downstream substrate through its unique motif ΨKxE (also known as SUMO-accepter site), where Ψ is a bulky aliphatic branched amino acid and x is any amino acid (Johnson and Gupta, 2001; Melchior et al., 2003; Seeler and Dejean, 2003). SUMO is coupled to the lysine (K) residue of this motif in a target protein. Removal of SUMO from an acceptor protein requires SUMO proteases, which are ubiquitin-like protein/sentrin-specific proteases (Ulp/SENPs). Those protease are required for SUMO removal from their target proteins and correct SUMO conjugation by processing SUMO precursors and exposing their carboxyl terminal diglycine motif (Kerscher, 2007)

NPM1 is one of the nuclear proteins that can be modified by SUMOylation. The NPM1 SUMOylation seems not to be a spontaneous reaction even though NPM1 contains the ΨKxE consensus sequence at lysines 24, 32, and 54. Notably, NPM1 SUMOylation cannot be eliminated when these three NPM1 consensus sequences were mutated individually or in combination (Tago et al. 2005).

Moreover, ARF protein expression can induce the SUMOylation of nuclear proteins (e.g., NPM1) and some of the ARF-interacting proteins (e.g., Mdm2 and NPM1) (Tago et al., 2005). In particular, it has been proven that ARF induces SUMOylation of NPM1 at lysine residues (K230 and K263). Substitution of arginine for lysine 230 or 263 of NPM1 abolishes the localization of NPM1 in the nucleolus and at the centrosome, and further leads to premature centrosome duplication and NPM1 aggregation in outside of nucleolus (Liu et al., 2007b). Additionally, K263R mutation also has an effect on the stability of NPM1 by avoiding the caspase-3 mediated cleavage (Okuwaki, 2008) that is in opposition to NPM1 ubiquitination in K263. However, all above experimental results also proved that lysine 263 residue is a major SUMOylation site in NPM1. K263R SUMOylation can affect the stability and modulate the subcellular localization of NPM1 (Okada et al., 2007; Okuwaki, 2008). Interestingly, NPM1 can interact with SUMO proteases, SENP3 and SENP5, to control the SUMO-pathway. Knockdown, either of NPM1 or nucleolar SENPs, results in the accumulation of SUMOylated proteins in nucleoli. Depletion of SENPs reveals ribosome biogenesis defects similar to those in NPM1 depletion (Yun et al., 2008). In total, results conclude that NPM1 may regulate the SUMO-pathway by interacting with SUMO proteases.

Also, it can affect its stability and localization when NPM1 itself is SUMOylated (Hay, 2007; Yun et al., 2008).

1.4 Alteration of NPM1 in Human cancers

NPM1 is an abundant phosphoprotein with multiple functions. The *NPM1* gene is located on chromosome 5q35 and contains 12 exons (Chang and Olson, 1990). The NPM1 protein is dominantly located in the nucleolus, but shuttles between the nucleus and the cytoplasm, an activity attributed to its nuclear export signal sequence (Borer et al., 1989). NPM1 possesses both oncogenic and tumor suppressive functions (Grisendi et al., 2005). In addition, NPM1 overexpression increases cell growth rate and proliferative ability, particularly through enhancing ribosome biogenesis and apoptosis inhibition (see section 3.4) via several cellular pathways (Grisendi et al., 2005; Ye, 2005).

Indeed, NPM1 has been found to be overexpressed in many tumors of different histological origins including gastric (Tanaka et al., 1992), colon (Liu et al., 2012b; Nozawa et al., 1996), liver (Yun et al., 2007), breast (Skaar et al., 1998), ovarian (Shields et al., 1997), prostate (Subong et al., 1999), bladder (Tsui et al., 2004), thyroid (Pianta et al., 2010), brain (Gimenez et al., 2010) and multiple myeloma (Weinhold et al., 2010). Notably, NPM1 overexpression might be correlated with clinical features in some cases. For instance, the level of NPM1 overexpression in hepatocellular carcinoma was found to be closely correlated with clinical prognostic parameters such as serum alpha fetal protein level, tumor pathological grading and liver cirrhosis (Yun et al., 2007). Recent study examines the expression pattern of NPM1 in clinical colonic carcinoma samples,

adjacent normal tissues and metastatic lymph nodes from the same patients by using Immunohistochemistry staining. Highly expression of NPM1 statistically correlates with lymph node metastasis and poor survival rate of patients with colon cancer (Liu et al., 2012b). Therefore, expression level of NPM1 could act as a biomarker for hepatocellular carcinoma and colon cancer and may serve as a potential marker for the prognosis of patients with colonic carcinoma. Furthermore, another group also reported that NPM1 overexpression was associated with recurrence and progression of bladder cancer (Tsui et al., 2004). It is worth noting that genetic alteration of the *NPM1* gene was not found in common solid cancers including lung, hepatocellular, breast, colorectal and gastric carcinomas (Jeong et al., 2007).

Intriguingly, the *NPM1* gene is a common target for genetic alteration in hematological malignancies such as lymphomas and leukemias (Falini et al., 2007a; Naoe et al., 2006; Rau and Brown, 2009). The genetic alterations include frameshift mutations, translocations and deletions. For instance, when *NPM1* is disrupted in the t(2;5) chromosome translocation in anaplastic large-cell lymphoma (ALCL), the translocation creates a chimeric gene encoding a fusion protein NPM-ALK (Morris et al., 1994). Different fusion proteins are created in different chromosomal translocations. In myelodysplasia/acute myeloid leukemia (AML), t(3;5) creates the fusion protein NPM-MLF (Yoneda-Kato et al., 1996). In rare cases of acute promyelocytic leukemia, t(5;17) creates the NPM-RAR fusion protein (Redner et al., 1996).

In 2005, scientists further examined the subcellular localization of NPM1 in bone marrow biopsy specimens from patients with primary AML by immunohistochemical staining (Falini et al, 2005). They showed that the *NPM1* gene was mutated at exon 12 resulting in aberrant cytoplasmic (instead of nucleolar) localization of NPM1 in those specimens from adult AML patients. Furthermore, cytoplasmic NPM1 was detected in approximately 35% of patients with primary AML. It was concluded that NPM1 might be one of the most frequently mutated genes in AML. AML with aberrant cytoplasmic NMP1 localization is designation as NPMc+ AML. However, cytoplasmic NPM1 (NPMc+) is found in an exiguous proportion in chronic myeloproliferative disorders and myelodysplastic syndrome (Caudill et al., 2006; Oki et al., 2006; Rau and Brown, 2009; Shiseki et al., 2007; Zhang et al., 2007). *Rau & Brown* (2009) indicated that NPMc+ has an intimate relationship with de novo AML and other myeloproliferative disorders/myelodysplasia that have sporadic *NPM1* exon 12 mutations. This result concluded that *NPM1* mutations and relocalization in cytoplasm is an event restricted to AML.

1.4.1 NPM1 mutations in acute myeloid leukemia (AML)

In the clinical setting, immunohistochemical detection of NPM1 in AML patients has a predictive implication of *NPM1* mutations (Falini et al., 2006b). Surprisingly, *NPM1* gene mutations are heterozygous and lead to frameshift mutations in exon 12 (Falini et al., 2007b). Exon 12 mutations of *NPM1* in AML have similar alterations at the C-terminus of the mutant NPM1 proteins.

The wild-type NPM1 protein has two nuclear export signal (NES) motifs and one nucleolar localization signal (NLS) at its C-terminus (see section 1.2.1). The NPM1 protein can shuttle from the cytoplasm to the nucleoplasm and then into the nucleolus owing to these specific motifs. In normal cells, NPM1 is predominantly located in nucleus due to the prevalence of NoLS over the NES motifs (Bolli et al., 2007). However, exon 12 mutations of *NPM1* create an extra NES motif at the C-terminus of mutant NPM1 and at the same time disrupts the natural NoLS sequence due to mutations of both tryptophan residues 288 and 290 or 290 alone (Falini et al., 2006a; Falini et al., 2007a; Mariano et al., 2006). *Falini* et al. (2006a) transfected previous mutants of NPM1 containing 288/290 mutation or 290 residue mutations into cells to study the localization of NPM1 protein. They concluded that both alterations of NPM were crucial to aberrant relocalization of NPM1c+ mutants.

Falini et al. (2005) classified different mutation types in exon 12 of *NPM1* from 51 patients with AML. In Falini's report, the most frequent mutation is a 4-bp duplication in exon 12 of the *NPM1* gene (tandem duplication of TCTG in at position 956 in the DNA sequence). This duplication results in a shift in the reading frame and alters the C-terminal portion of the NPM1 protein by replacing the last seven amino acids with eleven different residues. This mutation is designated as mutation A (Falini et al., 2005). The next most frequent is a 4-bp insertion in exon 12 of *NPM1* (insertion of CATG at position 960 in the DNA sequence) that results in a shift in the reading frame and alters the C-terminal portion of the NPM1 protein. This mutant is called mutation B in Falini's report. Mutation C is a 4-bp insertion in exon 12 of *NPM1* (insertion of CGTG at

position 960 in the DNA sequence site) and mutation D is a 4-bp insertion in exon 12 of NPM1 (insertion of CCTG at position 960 in the DNA sequence site). In adult AML, mutation A accounts for about 80% of all NPMc+ cases. However, mutation A only accounts for 11.1–50% of all NPMc+ cases in childhood AML. It seems that this mutation tends to occur less frequently in children suggesting that different molecular mechanisms are operative in NPMc+AML in children and in adults (Rau and Brown, 2009).

1.4.2 NPM1 mutations and the pathogenesis of leukemia

The cytoplasmic localization of NPM1 is a pathognomonic feature of NPMc+AML, but the exact role of NPMc+ in the pathogenesis of leukemia is still unclear. One possible mechanism is that NPMc+ might be leukemogenic through its interaction with the tumor suppressor ARF. Both ARF and NPM1 are nuclear proteins and their interaction retains both proteins in the nucleolus. In response to stimuli of DNA damage such as UV light, NPM1 and ARF will redistribute to the nucleoplasm, where HMDM2 competes with NPM1 for binding to ARF. This leads to the formation of NPM1-HMDM2 and ARF-HMDM2 binary complexes. These binary complexes inhibit the ubiquitin ligase activities of HMDM2 and ARF-BP1, resulting in p53 stabilization and activation of p53-dependent pathway. Upon activation of p53-dependent pathway, the cell will undergo cell cycle arrest, cell death or senescence in response to DNA damage (Gallagher et al., 2006). However, NPMc+ can still bind to ARF and then translocate to the cytoplasm together with ARF in leukemic cells. Subsequently, NPMc+ abolishes ARF's binding affinity with HMDM2. Thus, the ubiquitin ligase activity of HMDM2 is increased and this in turn inhibits p53 initiation (Colombo et al., 2006; den

Besten et al., 2005). In addition to disrupting ARF-induced p53 activation, NPMc+ can also perturb ARF-induced p53-independent tumor suppressive pathways via destabilization of the ARF protein. In normal cells, wild-type NPM1 is localized in the nucleus. It can inhibit ARF turnover and radically increases the stability of ARF. In contrast, NPMc+ is localized in the cytoplasm and supposedly has lost its function to maintain ARF's stability (den Besten et al., 2005; Falini et al., 2006b).

However, perturbation of ARF function is not the only explanation for the leukemogenic property of NPMc+ (den Besten et al., 2005). Perturbation of other NPM1 functions is also possible. For instance, loss of NPM1 affects genomic instability and leads to increased susceptibility to oncogenic transformation (see section 3.6 for details of NPM1's function in maintaining genomic stability). Surprisingly, transgenic mouse study provides more interesting results about NPM1's function in hematopoietic disease. NPM1 heterozygous knock-out mice ($NPM1^{+/-}$) develop hematologic features similar to those seen in human patients with myelodysplastic syndrome (MDS). In fact, it is a common feature that these transgenic mice develop hematopoietic malignancy, especially myeloid malignancy (Grisendi et al., 2005; Sportoletti et al., 2008). While numerical and structural chromosomal abnormalities are always found in these mice, NPMc+ is seen predominantly in AML patients with normal karyotype and lacking recurrent chromosomal abnormalities (Sportoletti et al., 2008). Overall, these results suggest that NPMc+ is not simply a loss of genomic stability leading to oncogenic transformation (Rau and Brown, 2009).

In 2008, scientists revealed another possible mechanism to show the role of NPMc+ involved in the pathogenesis of leukemia. Wild-type NPM1 has an ability to regulate the turnover of the oncoprotein c-Myc by interacting with F-box protein Fbw7γ. Fbw7γ is a component of the E3 ligase complex involved in the ubiquitination and protease degradation of c-Myc. However, NPMc+ can still interact with and translocate Fbw7γ to cytoplasm. In the cytoplasm, Fbw7γ is degraded and this then increases the stability of c-Myc (Bonetti et al., 2008). Consequently, NPMc+ could facilitate the c-Myc-induced hyperproliferation. In the normal cellular condition, such hyperproliferation will activate ARF and p53-dependent cell cycle arrest or apoptotic pathway to turn on cell cycle checkpoint and prevent the abnormal cell growth (Di Micco et al., 2006). However, as discussed above, NPMc+ can disturb ARF and p53 activity and further attenuate cell cycle checkpoints. It seems that NPMc+ could play a role to trigger hyperproliferation and attenuate the ARF-dependent and p53-dependent checkpoints involved in leukemogenesis without the need for cooperating with genomic instability.

1.4.3 NPM1/ALK fusion protein in anaplastic large-cell lymphomas (ALCLs)

The *NPM1* gene, located at chromosome 5q35, is translocated in anaplastic large-cell lymphoma (ALCL) and in rare variants of AML (Mason et al., 1990; Morris et al., 1994; Orscheschek et al., 1995; Schlegelberger et al., 1994). To date, chromosomal translocation is among the most frequent genetic alterations identified in cancer (Futreal et al., 2004; Look, 1997). Chromosomal translocations may produce chimeric transcripts that encode oncogenic fusion

proteins (Futreal et al., 2004). In the context of NPM1, the fusion protein is constituted by the N-terminus of NPM1 and the C-terminus of the partner protein encoded by other gene involved in the chromosomal translocation. The fusion results in the constitutive activation of tyrosine kinases that are important to tumorgenesis (Lim et al., 2009). Anaplastic large-cell lymphoma (ALCL) is the most common type of T-cell lymphoma in children (Lim et al., 2009) and is characterized by CD30 expression (Drexler et al., 2000; Falini et al., 2007a). About 60% of ALCL patients express the *ALK* gene (anaplastic lymphoma kinase gene) and such patients are identified as ALK^+ALCL. Patients with ALK^+ALCL have been found to harbor the t(2;5)(p23;q35) chromosomal translocation, which fuse the *NPM1* gene and the *ALK* gene to create a chimeric transcript encoding an oncogenic fusion protein (NPM1-ALK). This NPM1-ALK fusion protein is made up of the N-terminal portion of the NPM1 protein and the entire cytoplasmic catalytic domain of the ALK protein. Due to the oligomerization domain of NPM1 protein, the NPM1-ALK fusion protein can form homodimers with itself and heterodimers with wild-type NPM1 protein (Bischof et al., 1997). Moreover, the homodimeric form of NPM1-ALK is localized in the cytoplasmic and the nuclear compartments of the cell. However, the heterodimers are translocated to the nucleus. This provides an explanation for the nuclear and nucleolar distribution of NPM1-ALK (Drexler et al., 2000). The NPM1-ALK fusion protein is a constitutively active tyrosine kinase. There are numerous in vivo and in vitro experimental data showing that NPM1-ALK plays an important role in the pathogenesis of the t(2;5) expressed-ALCLs (Morris et al., 1997; Shiota and Mori, 1997). Furthermore, NPM1-ALK can modulate several signaling pathways including PI3K/AKT (Nieborowska-Skorska et al., 2001) and JAK/STAT

pathways (Nieborowska-Skorska et al., 2001; Ruchatz et al., 2003; Zamo et al., 2002). However, the global view of the constitutive NPM1-ALK activation in cancer cells is still unclear. Recently, Lim's group performed quantitative proteomic analysis of lymphoid cells with or without NPM1-ALK expression by cleavable isotope-coded affinity tag (cICATs) and electrospray-ionization tandem mass spectrometry. Their data indicate that the NPM1-ALK fusion protein deregulates multiple cellular pathways, and also affects cell proliferation, cell survival, apoptosis evasion and tumor dissemination through PI3K/AKT, JAK/STAT and other novel signaling pathways (Lim et al., 2009).

1.5 The cellular response to ultraviolet irradiation

UV irradiation is frequently used as a tool to induce cellular stress responses in experimental studies. It can activate a variety of gene expressions by several cellular pathways, which are different depending on the UV wavelengths. For instance, ultraviolet C (UVC, 200–290 nm) irradiation is strongly absorbed by nucleic acids and thus leads to DNA damage. The longer wavelength of ultraviolet A (UVA, 320–380 nm) irradiation can produce active oxygen intermediates and is weakly absorbed by most biomolecules. Intermediate wavelength of ultraviolet B (UVB, 290–320 nm) irradiation produces effects similar to those of UVC. In addition, UVB is also directly absorbed by cellular macromolecules such as DNA, and can produce active oxygen intermediates and DNA damage. The cellular effect of UVB irradiation is very similar to those of UVC irradiation in their gene activating properties (Tyrrell, 1996). Actually, the major cellular effect of UVC irradiation on cells is DNA damage and the worse effect is cell death or

genomic mutation, which is regarded as an initial step of neoplastic transformation (Pourzand and Tyrrell, 1999; Tyrrell, 1996; Wu et al., 2002b).

However, UV irradiation not only results in the damage of cellular components, but also leads to the induction of several specific cellular responses. For instance, the well-documented UV-induced cellular response is the "SOS response" first characterized in *E. coli.* It is regulated by the recA locus. Genes in the SOS response are involved in enhanced DNA repair or cell division inhibition (Walker, 1985). Moreover, the cellular responses for UV irradiation are characterized by activation or repression of genes transcription. The proto-oncogenes c-fos and c-jun are considered as the earlier and immediate UV-induced response genes. UV irradiation can induce and activate c-fos and c-jun, which can regulate various genes containing AP-1 transcriptional factor binding sites in their promoters (Tyrrell, 1996). Furthermore, the induction of c-fos and c-jun is considered to help cells fight against the UV-induced apoptosis (Lallemand et al., 1998; Schreiber et al., 1995; Wisdom et al., 1999).

In addition, p53 is another immediate UV-induced response gene. UV irradiation can activate gene expression of p53 and increase the protein level of p53. The increase of p53 will block cell cycle progression to provide time for repairing DNA damage (Cox and Lane, 1995). Proliferating cellular nuclear antigen (PCNA) is one of the p53 downstream target genes and is detected simultaneously together with p53 expression following UV irradiation (Cox and Lane, 1995). PCNA is an important component involved in DNA repair and replication machinery (Moldovan et al., 2007). Similar to the PCNA and p53

genes, *NPM1* is another UV-inducible gene and is rapidly upregulated after UV irradiation (Wu et al., 2002b). Interestingly, *NPM1* mRNA expression is induced after UV irradiation in UV-resistant cells when compared with the UV-sensitive control cells (Higuchi et al., 1998) However, the blockage of *NPM1* gene expression by *NPM1* antisense cDNA makes UV-resistant cells become sensitive to UV irradiation. In another study, the NPM1 protein expression in UV-sensitive cells were lower than that in normal fibroblast cells (Hirano et al., 2000). Moreover, NPM1 inhibition via antisense transfection led to a decrease in PCNA protein expression level, inhibition in DNA repair capacity (nucleotide excision repair activity), and cell death (Wu et al., 2002b).

1.6 Proliferating cell nuclear antigen (PCNA)

In prokaryotes and eukaryotes, PCNA is essential for DNA replication and repair synthesis. It forms a ring-shaped homotrimeric structure, encircles DNA and slides freely on the DNA double helix. Due to this characteristic, PCNA acts as a scaffold protein interacting with DNA and many partners (e.g., regulatory proteins). PCNA is known to interact with many proteins related to DNA replication, DNA repair, translesion DNA synthesis, DNA methylation, cell cycle regulation, chromatin metabolism, sister chromatin cohesion and apoptosis (Hashiguchi et al., 2007; Maga and Hubscher, 2003; Prosperi, 2006; Warbrick, 2000).

PCNA can directly participate in DNA repair pathways. However, these repair systems are very complicated owing to different types of DNA lesions and damages that require different repair machinery. For instance, nucleotide

excision repair (NER) is the major repair pathway for removing DNA damages induced by UV irradiation, and bulky lesions induced by carcinogens (Tornaletti and Pfeifer, 1996; Wood and Shivji, 1997). PCNA directly plays a role in NER (Shivji et al., 1992) and is also induced in cells exposed to UV irradiation (Hall et al., 1993). Furthermore, PCNA can facilitate DNA polymerase δ and ε to remove UV-induced DNA damages (Kelman and Hurwitz, 1998; Zeng et al., 1994).

Notably, NPM1 and PCNA have an intimate relationship because both proteins are rapidly upregulated after UV irradiation (Cox and Lane, 1995; Wu et al., 2002b). Furthermore, the protein expression level and promoter activity of PCNA are elevated in NPM1 overexpressed cells. It seems that NPM1 can modulate PCNA upregulation and further increase DNA repair capacity to remove UV-induced DNA damages (Wu et al., 2002b). NPM1 antisense transfection causes an inhibitory effect on PCNA expression and NER activity, further leading to cell death induced by UV irradiation (Wu et al., 2002b). PCNA is overexpressed in chronic lymphocytic leukemia and this overexpression reflects the intrinsic DNA repair activity in leukemic cells and their resistance to UV-mimetic chemotherapeutic agents, such as cisplatin (Del Giglio et al., 1993; Olaussen et al., 2006).

1.7 Summary and focus of the study

NPM1, an ambiguous protein with multiple biological functions, has an intimate relationship with carcinogenesis and pathogenesis in various cancers such as leukemias and lymphomas. NPM1 modulates several cellular signaling pathways through transcriptional regulation to activate or repress expression of

downstream genes as the details are shown in section 1.3.3. Interestingly, NPM1 also directly participates in the DNA repair pathway and resistances to UV-induced cell death via upregulating DNA repair associated gene, *PCNA*. Recent studies show that PCNA promoter activity was increased while NPM1 was over-expressed in HeLa cells (Wu et al., 2002b). However, analysis of NPM1 amino acid sequence indicates that it does not have any characteristics of transcription factor. Nevertheless, there still are many proteins and transcriptional factors interacting with NPM1 directly, including NF-κB, p53, AP-2α, and Yin Yang 1 (YY1) (Inouye and Seto, 1994).

YY1 is a ubiquitous transcription factor containing four C_2H_2-type zinc finger motifs with two specific domains that characterize its function as a repressor or activator. YY1 reveals transcriptional repression via several particular sequence regions including YY1 C-terminus region (aa 298–397), sequence domains within zinc finger motifs and glycine-rich domain between amino acids 157 to 201. Moreover, the transcriptional activation domains of YY1 are laid in N-terminal region (aa 43–53), followed by glycine-rich domain and histidine residues from amino acids 70 to 80 (Gordon et al., 2006). These regions may have possible implications for genes activation. Notably, several lines of evidences also indicate that many genes contain YY1 binding sequence in promoter regions, thereby; YY1 could directly or indirectly participate in gene regulations via transcriptional repression or activation models.

Human *PCNA* gene is one of that found to contain YY1 binding site in its promoter region (Labrie et al., 1995). It has been demonstrated that YY1 might

be an important factor for PCNA regulation. Due to this reason and the transcriptional regulatory properties of YY1, further experimental results showed that transcriptional activation of *PCNA* promoter is potentially through YY1 and YY1 binding by using knockdown YY1 protein expression and mutagenesis of YY1 site on *PCNA* promoter with NPM1 overexpression condition (Weng and Yung, 2005). All of results can draw an inference that NPM1 and YY1 have a relationship for PCNA activation.

Moreover, both NPM1 and PCNA are UV-inducible proteins. Elevated protein and mRNA expression level of PCNA are followed by UV-induced NPM1 overexpression (Wu et al., 2002b). Notably, our preliminary results showed a novel finding that UV irradiation can induce post-translational modification of NPM1. The previously introduction sections also emphasized that NPM1 modifications play an important role in gene regulation. However, the physiological function of NPM1 modification induced by UV irradiation is still unknown. Does it affect or modulate *PCNA* promoter activity? Additionally, the underlying mechanism of PCNA activation regulated by NPM1 modification needs to be further investigated. These questions form an area of focus in this study and are addressed in Part A of Chapter Four.

Furthermore, the detailed interacting mechanism of NPM1 and YY1 in PCNA upregulation is another interesting issue to be investigated. How does NPM1 modulate PCNA activity via transcription factor YY1? Do NPM1 modification forms present different binding affinity with *PCNA* gene? In mechanism of NPM1-mediated *PCNA* activation whether NPM1 recruits on YY1 site directly or

interacts with YY1 firstly and thus binds to YY1 sites for PCNA activation. These questions also are important points in this study and are addressed in Part B of Chapter Four.

1.8 Organization of the thesis

In addition to the literature review (Chapter One) which provides the context and rationale for this study, and Chapter Two, which describes the study aims and specific questions to be addressed, this thesis is organized as follows:

Chapter Three contains the detailed description of the experimental procedures, materials and methods that are common to Parts A and B. The specific "materials and methods" of each experimental parts that are described in the subsequent chapter. Further, a description of the statistical analysis methods used is also included.

Chapter Four is the experimental chapter. This chapter has two major parts each containing several sections, including an introduction, a brief section on materials and methods, statistical analysis, experimental results, discussion and conclusion.

Chapter Five gives a summary of the important findings in relation to the study aims and specific questions addressed. This final chapter presents overall findings, discussion of the results, research implications and limitations, as well as conclusions and suggestions for further study.

Chapter Two:

Aims, Specific Questions of Thesis

2.1 Aims of Study

NPM1 is an important nuclear phosphoprotein with multiple functions including ribosome biogenesis, proliferation, cell division and other cellular processes through transcriptional regulation. Notably, NPM1 has been identified to interact with various transcription factors and proteins such as NF-κB (Lin et al., 2006), AP-2α (Liu et al., 2007a), ARF (Itahana et al., 2003), ATF5 (Liu et al., 2012a) and YY1 (Weng and Yung, 2005). Our previous study also demonstrated that NPM1 regulates PCNA promoter through YY1. However, the underlying mechanism of PCNA activation mediated by NPM1 and YY is still unclear.

This study had two main aims, and was organized in two parts:

Part A aimed to elucidate the underlying mechanism of PCNA activation mediated by NPM1 and YY1.

Part B aimed to further indentify the physiological function of PCNA activated by NPM1.

2.2 Specific questions to be addressed in Part A are as follows:

1. What is the expression pattern of NPM1 and PCNA in response to low dose UV-irradiated treatment at up to 3, 6, and 9 hours on
    a. protein expression level?
    b. mRNA expression level?
2. What type of posttranslational modification of NPM1 is induced by UV irradiation?

3. Can NPM1 overexpression mediated *PCNA* promoter activity?
4. Is there evidence that NPM1 recruitment depends on YY1 and via the YY1 recognition binding site on *PCNA* promoter?
5. Is there evidence that overexpression of ectopic NPM1 still has effect in PCNA activation with YY1 site mutation?
6. Is there evidence that PCNA activation is through a specific domain of NPM1?
    a. Generate different length of NPM1 deletion mutants to narrow down ds*PCNA*-NPM interaction domain for PCNA activation (*in vivo* assay)
    b. Physical interaction evidence for interacting reaction of human ds*PCNA* oligonucleotide and recombinant NPM1 protein required for PCNA activation (*in vitro* assay)
    c. Physical interaction evidence for interacting reaction of human ds*PCNA* oligonucleotide, recombinant YY1 zinc finger domain and NPM1 protein has synergic effect for PCNA activation (*in vitro* assay)

2.3 Specific questions to be addressed in Part B are as follows:

1. Is there evidence that the modification enzyme influences *PCNA* promoter activity?
2. What regulatory proteins are recruited to *PCNA* promoter region involved in UV-induced PCNA upregulation?
3. Is there evidence that the function of histone acetyltransferase p300 will lead to post-translational modification of NPM1 upon low dose UV-irradiated treatment?

4. Is there evidence that NPM1 mutants mimicking the deacetylated state inhibit the transcription of PCNA via reducing its binding affinity with *PCNA* promoter?
5. Is there evidence that NPM1 mutants mimicking the deacetylated state have inhibitory effect on DNA repair capacity?

Chapter Three:

Materials and methods

This section presents a general "materials and methods" which are used in Parts A and B of chapter four. Additionally, more specific details are stated in the subsequent chapter. The detailed procedures and protocols are given for "materials and methods" that were developed in, or modified by, our laboratory. Only brief descriptions are provided for the commercial kit methods.

3.1 Cell culture and transfection

Human HeLa cells were grown as a monolayer in DMEM/F12 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 LipofetAMINE 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Cells were harvested after 18–24 hr transient transfection. Mouse NIH3T3 fibroblasts were maintained in DMEM supplemented with 10% fetal calf serum (FCS) and 100 U/ml penicillin and streptomycin. The cell culture condition of NIH3T3 is similar with HeLa cells.

3.2 Reagents and antibodies

All reagents and anti-Flag mouse monoclonal antibody (mAb, F3165) were purchased from Sigma-Aldrich (St.Louis, MO), except where otherwise indicated. Anti-nucleophosmin/B23 (NPM1) mAb was kindly provided from Dr. P.K. Chan (Department of Pharmacology, Bayler College of Medicine, Houston, TX). Anti-

PCNA monoclonal antibody was obtained from BD Bioscience (San Diego, CA). Rabbit polyclonal antibodies against NPM1 and YY1 were from Santa Cruz Biotechnology (Santa Cruz, CA).

3.3 Reporter gene assay

The human wild-type *PCNA* promoter reporter gene, pGL3-wt-PCNA, contains human *PCNA* promoter region from −153 to +160 bp harboring transcription start site and one putative YY1-binding site (Labrie et al., 1995). Comparing with wild-type, the YY1 site-mutated *PCNA* reporter gene (pGL3-mtYY1-PCNA) was generated by site-directed mutagenesis. The detailed plasmid construction of both PCNA reporter genes were according to pervious description (Weng and Yung, 2005) and described in detail (See "materials and methods" in chapter four). For reporter gene assay, cells extracts were obtained with 1 × reporter lysis buffer (Promega, Madison, WI). The reporter/luciferase activity were performed with luciferase assay reagent (Promega) following the manufacturer's recommendations and subsequently measured by Luminometer Victor3 (Perkin-Elmer, Waltham, MA) and normalized to the corresponding β-galatosidase activity.

3.4 Western bolt analysis

Cells were harvested and washed twice in phosphate-buffered saline (PBS) and then lysed in ice-cold WCE buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 10% glycerol, 0.1% NP-40, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1X protease inhibitors [Roche Applied Science, Werk Penzberg, Germany]) for 30

min. The lysates were boiled in 2X urea sample buffer dye (100 mM Tris-HCl pH 6.8, 200 mM β-mercaptoethanol, 20% glycerol, 4% SDS, 0.2% bromophenol blue and 8 M urea), and then fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Western blot analysis was performed after eletrophoretic separation of polypeptides by 8 or 10% SDS-PAGE and transferred to Hybond-polyvinylidene difluoride (PVDF) membrane (PALL, Port Washington, NY). Blots were probed with the indicated primary antibodies and appropriate secondary antibodies. The immunobands were detected by the chemiluminescence reaction (GE Healthcare, Amersham place, England).

3.5 RNA isolation and reverse transcription-PCR (RT-PCR)

RNA was harvested from cells using TriZOL reagent using standard procedures. Total RNA (1–2 μg) was used to synthesize the complementary DNA (cDNA) using MMLV transcriptase (Invitrogen) and Oligo dT primers (Invitrogen) at 37°C for 1hr. cDNA was stored at −20°C or immediately used for PCR. RT-PCR was performed in cDNA samples using the following primers shown in Table 3.1. All the PCR products were resolved on 1.5% agarose gels and stained with STBR Green dye (Invitrogen).

Table 3.1 Oligonucleotide primers used for RT-PCR

Gene	Primer sequence
human *NPM*	5'-TTGTTGAAGCAGAGGCAATG-3'
	5'-ACTTCCTCCACTGCCAGAGA-3'
human *PCNA*	5'-TGGTCCAGGGCTCCATCCTCAA-3'
	5'-ATACTGGTGAGGTTCACGCC-3'
human *β-actin*	5'-AGAAAATCTGGCACCACACC-3'
	5'-CCATCTCTTGCTCGAAGTCC-3'
murine *NPM*	5'-TCGGCTGTGAACTAAAGGCT-3'
	5'-GAGCAGATCGCTTTCCAGAC-3'
murine *PCNA*	5'-ATGTTTGAGGCACGCCTGAT-3'
	5'-ACATGCTGGTGAGGTTCACG-3'
murine *β-actin*	5'-GGGAATGGGTCAGAAGGACT-3'
	5'-ATACAGGGACAGCACAGCCT-3'

3.6 Real-time quantitative PCR

For each treatment point, at least two ChIP assays were performed on each of at least three separate formaldehyde cross-linked cell extracts. DNA samples from ChIP preparations were quantitated by real-time PCR using the ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA) and Maxima™ SYBR Green qPCR Master Mix (Fermentas of Fisher Scientific, UK). The sequences for the primers and the probes are shown in Table 3.2. Duplicate PCRs for each sample were carried out. The results are given as percentages of inputs and represent the mean ± SD of at least three independent experiments. For statistical significance of quantitative comparisons, calculations were done by the SigmaPlot software (*$P < 0.05$, **$P < 0.001$, ***$P < 0.001$).

Table 3.2 Oligonucleotide primers used for ChIP assay

Specificity	**Primer sequence**
human *PCNA* promoter	5'-CGCTTCCTCCAATGTATGCT-3'
	5'-GCGGGAAGGAGGAAAGTCTA-3'
human *NPM* promoter	5'-GGGGTAACGATTAACTGCGA-3'
	5'-GCGTGCTTTCCAGTCGTAAC-3'
ectopic human *PCNA* promoter (-153 to +160 bp)	5'-CTAGCAAAATAGGCTGTCCC-3'
	5'-GCGGGAAGGAGGAAAGTCTA-3'
human *Vimentin*	5'-CTGAAGTAACGGGACCATGC-3'
	5'-AGAAGAGGCGAACGAGGG-3'
murine *PCNA*	5'-CTCAAACCACGGGTACGATT-3'
	5'-CGGAGTTGTGGCGACTAGAT-3'

3.7 Chromatin immunoprecipitation (ChIP)

Cells were collected by trypsin-EDTA (Invitrogen) treatment, and 1×10^6 cells were used for each experimental groups. Subsequently, cells were cross-linked by formaldehyde (final concentration, 1%) for 10 min at RT and collected by centrifugation and washed twice with ice-cold PBS. Cell pellet were lysed in lysis buffer (with proteinase inhibitor) and incubated on ice for 30 min. Next, samples were sonicated on ice and centrifuged at 14,000 rpm for chromatin collection. Those cross-linked, sonicated chromatin samples were used for ChIP assay by standard procedures of MAGNIFY™ ChIP kit (Invitrogen). The magnetic beads from ChIP assay kit were incubated with 2 μg rabbit polyclonal antibodies (such as NPM1, YY1 or P300, respectively) and rotated at 4°C for 2 h. Normal rabbit immunoglobulin (Ig)G was used for the mock immunoprecipitation. The incubation time is similar to previous descriptions in interested primary antibodies. Thus, the supernatant from 1×10^6 cells were added into magnetic beads-antigen complexes and rotatory incubated at 4°C for 4 h. Immunoprecipitating, washing, and elution of immune complexes with elution buffer were carried out. The final samples were added to 100 μl ddH_2O for elution. Eluted samples were determined by quantitative real-time PCR with Maxima™ SYBR Green qPCR Master Mix or conventional-PCR with Hotstart *Taq* DNA polymerase reactions (Promega). PCR products were run on a 2.5% agarose gel and analyzed by SyBr Green (Invitrogen) staining.

3.8 Recombinant proteins cloning, expressions and purifications

Different lengths of NPM1 recombinant proteins and YY1 zinc finger domain involved in further experiments such as electrophoresis gel mobility shift assay (EMSA) and isothermal titration calorimetry (ITC) have been produced and highly purified as described in this section. In total, ten different lengths of NPM1 cDNA fragments were amplified by PCR reactions using pCR3.1-FLAG-B23 as the PCR template. Detailed oligonucleotide primers used for different NPM1 fragments generation are listed in Table 3.3 and the schematic presentations of each NPM1 recombinant protein is shown in Appendix 2. All PCR amplified NPM1 fragments were treated with BamHI and EcoRI, and then subcloned into the pET-32M-3C expression vector (Novagen of EMD Millipore, Darmstadt, Germany) to generate the NPM1-His_6-tagged constructs. This expression vector contains the selection of tag (His_6-tag), fusion partner (Thioredoxin [TRX]) and human rhinovirus (HRV) 3C protease cleavage site. The His_6-tag domain of pER-32M-3C vector can used to facilitate the protein purification; fusion partner can further used to improve the solubility of protein and apply in affinity chromatography for protein purification. Additionally, the protease cleavage site is designed for a specific purpose to remove the His_6-tag and/or fusion partner obtaining the purified target proteins. The recombinant YY1 zinc finger domain was amplified using 5′-ATCGGGATCCATAGCTTGCCCTCATAA-3′ and 5′CTGGAATTCTCAATGTGTTAAGATGTG-3′ primers and then cloned into the pET-49M BamH1 and EcoRI sites, as indicated by the underlined sequences respectively. Similarly, this pET-49M vector carries His_6-tag coding sequences in its C-terminus followed by a human rhinovirus (HRV) 3C protease cleavage site and a

glutathione-S-transferase (GST) fusion partner. All cloning steps were performed using the *E.coli* XL-1 strain.

For protein expressions, the plasmids were freshly transformed into the *E.coli* BL21 (DE3) strain and were grown on the LB plates with appropriate antibiotics. Ampicillin was used at 100 μg/ml for pET-32M constructs and kanamycin was used at 30 μg/ml for pET-49M construct). For the small-scale expression, 5 ml broth cultures were inoculated from the solid support into the LB broth with the appropriate antibiotics and grown overnight at 30°C; these cultures were used as inoculums for 50 ml cultures. Protein expression was induced at an O.D. 600 of 0.5–0.6 with 0.5mM IPTG for 5 h at 30°C. For large-scale expression, LB broth cultures were induced with 0.5 mM IPTG at an O.D. 600 of approximately 0.6. Bacteria were then growth for 5 h, pelleted and stored frozen at -20°C.

Protein extraction was performed on the bacterial pellets using a CelLytic ™ B Plus Kit (Sigma-Aldrich) according to the manufacturer's standard procedures. Recombinant NPM1 proteins and YY1 zinc finger domain were initially purified by affinity chromatography with HisTrap and GSTrap, respectively. The His-tag and GST-tag of recombinant proteins were removed by 3C protease cleavage and subsequently further purified by size-exclusion chromatography (Superdex 75, GE Healthcare).

Table 3.3: Oligonucleotide primers for NPM1 cDNA fragments generation

NPM1 constructs	Primer sequences	Region (amino acids)
Construct 1	5’-AGTCGGATCCATGAGCCCCCTGAG-3’	9-122
	5’-GACGAATCCTCAATCTTCCTCCACAGC-3’	
Construct 2	5’-ATCGGGATCCATGAGCCCCCTGAG-3’	9-134
	5’-TACGGAATTCTCATTTCACATC-3’	
Construct 3	5’-ATCGGGATCCATGAGCCCCCTGAG-3’	9-188
	5’-ATCGGAATTCTCATTCTTCAGCTT-3’	
Construct 4	5’-ATCGGGATCCAAGAAACAGG-3’	229-294
	5’-ACTGGAATTCTCAAAGAGACTTC-3’	
Construct 5	5’-ATCGGGATCCAAGTCAAATCAGAAT-3’	206-294
	5’-ACTGGAATTCTCAAAGAGACTTC-3’	
Construct 6	5’-ATCGGGATCCTCTGTAGAAGACAT-3’	243-294
	5’-ACTGGAATTCTCAAAGAGACTTC-3’	
Construct 7	5’-AGTCGGATCCATGAGCCCCCTGAG-3’	9-294
	5’-ACTGGAATTCTCAAAGAGACTTC-3’	
Construct 8	5’-CATGGATCCAGGAAGATGCAGAGTCAG-3’	120-188
	5’-GGCGAATTCCATTCTTCAGCTTCCTCATC-3’	
Construct 9	5’-AGCTGGATCCAGATGTGAAACTCTTAAG-3’	131-294
	5’-ACTGGAATTCTCAAAGAGACTTC-3’	
Construct 10	5’-CATGGATCCGAGGAAGATGCAGAGTCAG-3’	120-294
	5’- ACTGGAATTCTCAAAGAGACTTC-3’	

Note: Each pair of primers contains two artificial restriction enzymes cutting sites, BamH1 and EcoRI respectively, as indicated by the underlined sequences.

3.9 Electrophoresis Gel Mobility Shift Assay (EMSA)

The electrophoresis gel mobility shift assay (EMSA) is a classical DNA-binding assay using nondenaturing polyacrylamide gel electrophoresis (PAGE) to detect sequence-specific DNA-binding proteins. As proteins bind to the end-labeled DNA fragment they will retard the mobility of the fragment during electrophoresis, resulting in discrete bands corresponding to the individual protein-DNA complexes. Hence, we performed EMSA using two different lengths of ds*PCNA* oligonucleotide that both contain an YY1 recognition site in sequence and without radio-isotope labeling on the end of DNA. One with 400 bp in length and was cut from pGL3-wt-PCNA reporter gene by restriction enzymes (SmaI and NocI) and then isolated, purified from agarose gel; the other was synthesized by a commercial company and annealed to complementary oligonucleotides containing 30 bp. To the recombinant NPM1 C-terminus and YY1 zinc finger domain were interested proteins to detect and verify the targeted proteins-DNA interaction.

We performed EMSA in an agarose gel system by using ds*PCNA* oligonucleotides with 400 bp. The binding reactions contained a constant amount of the long ds*PCNA* probe at 0.25 pmol, and the protein concentration (NPM1 C-terminus or YY1 zinc finger domain) was varied from 0 pmol to 2 pmol. All reactions were prepared on ice in the EMSA buffer (20 mM HEPES pH 7.5, 40 mM KCl, 10 mM $MgCl_2$, 10 mM $CaCl_2$, 10 mM β-mercaptoethanol, 8% glycerol, 0.25 mg/ml bovine serum albumin), and the reaction mixture was gently mixed

and incubated on ice for 15 min. After incubation, the reaction mixtures were resolved by 1.5% agarose gel.

For short ds*PCNA* oligonucleotides, our EMSA used nondenaturing polyacrylamide gel electrophoresis (PAGE) to determine the minimal interacting domain of DNA and interested proteins. Firstly, the commercial synthesized *PCNA* oligonucleotide sequence is 5′-GCGGACGCGGCGGCATTAAACGGTTGCAGG-3′ annealing with complementary *PCNA* oligonucleotide by heating at 95°C for 10 min and then slow cooling to room temperature. Similar to previous EMSA experimental conditions, the binding reactions contained a constant amount of ds*PCNA* probe at 0.25 pmol, and the protein concentration (NPM1 C-terminus or YY1 zinc finger domain) was varied from 0 pmol to 2 pmol. All reactions were prepared on ice in the EMSA buffer, and the reaction mixture was gently mixed and incubated on ice for at least 15 min. Samples were then loaded into a 7.5% (36.5:1 acrylamide:bisacrylamide) polyacrylamide gel (PAGE gel pre-run at 85 V, 1 h) in 1x TBE (Tris-borate EDTA) buffer and run at 4°C for 4 h at 200 V. After electrophoresis separation, the resulting bands were visualized with the Kodak digital camera 4000 mm system.

Following, we added ethidium bromide (EtBr) into the nondenaturing PAGE gel for image capturing to instead of using radioactive labeling. As well, we did not add bulk carrier DNA such as poly (dI: dC) for the binding reaction. Since all protein samples had an aborative purification performed such as HPLC.

Chapter Four:

Experimental design and results of the study

Part A: NPM1 upregulates proliferating cellular nuclear antigen (PCNA) activation through transcription factor Yin Yang 1 (YY1)

A4.1 Introduction

Nucleophosmin (NPM1, B23 or NPM) is a nucleolar protein with multiple physiological functions. NPM1 is involved in various cellular processes including cell cycle regulation, ribosome biogenesis (Maggi et al., 2008), transcription regulation, cell proliferation, differentiation (Lim and Wang, 2006; Okuwaki, 2008) and acts as histone chaperone (Lindstrom, 2011). Histone chaperones are a set of proteins interacting with histones. The major function of histone chaperones is to facilitate the ordering of nucleosome assembly in the cellular replication process. As a chaperone, NPM1 is acetylated with functional consequence and turns on gene expression in an acetylation-dependent manner (Shandilya et al., 2009). Acetylation is one of the post-translational modifications of NPM1, which include phosphorylation, sumoylation, poly(ADP-ribosyl)ation and ubiquitination. The detailed biological functions of NPM1 post-translational modifications are described in Chapter One. NPM1 is also involved in cell proliferation and transformation. Its expression level and activity are intimately related to carcinogenesis because NPM1 is often found overexpressed or mutated in human cancer cells of diverse origins (Grisendi et al., 2006). Clinical experimental results have shown that NPM1 expression level is significantly higher in hepatocellular carcinoma (HCC) tissues than in non-malignant

hepatocytes. In parallel, the enhanced PCNA expression level is detected in HCC patients as well. Interestingly, statistical analysis of NPM1 and PCNA expression revealed a correlation in HCC tissues and non-malignant hepatocytes (Yun et al., 2007). A flurry of papers from 1997 to 2008 revealed intricate interactions among NPM1, resistance to apoptosis and the maintenance of genomic stability (Grisendi et al., 2005; Lim and Wang, 2006; Lindstrom and Zhang, 2008). NPM1 overexpression makes the cells resistant to UV irradiation-induced apoptosis, which is mediated through the tumor suppressor interferon regulatory factor-1 (IRF-1) in NIH3T3 cells (Kondo et al., 1997; Wu et al., 2002a; Wu et al., 2002b; Wu and Yung, 2002). Notably, the manifestation of UV-induced NPM1 overexpression is observed together with PCNA expression, and the subsequent result is elevated DNA repair capacity. Taken together, the upregulation of PCNA would lead cells to increase DNA repair capacity and consequently their resistance to UV irradiation-induced cell death. This may be one of the reasons why NPM1 makes cells resistant to UV-induced cell death (Wu et al., 2002b).

Proliferating cellular nuclear antigen (PNCA) is an indispensable protein for replication and DNA repair pathway, particularly in nucleotide excision repair (NER), which is responsible for removing bulky DNA induced by UV irradiation (Shivji et al., 1992). PCNA encircles DNA and acts as scaffold protein for interacting with DNA and other regulatory proteins, thereby facilitating DNA polymerase ε and δ to more easily remove DNA damage (Kelman and Hurwitz, 1998; Zeng et al., 1994). The latest finding reveals that PCNA expression is significantly correlated with pathologic stages of prostate cancer (Wang et al.,

2010b). Elevated expression of PCNA in prostate cancer and high-grade prostatic intraepithelial neoplasia (PIN) is a common manifestation; however, benign prostatic epithelium shows only negligible or negative reactivity to PCNA antibody (Wang et al., 2010b).

Collectively, NPM1 has an intimate relationship with PCNA. Based on aforementioned evidences, the relationship can be summarized as follows. First, NPM1 and PCNA both show elevated expressions in cancer cells when compared with non-malignant or benign tissues. Second, increased expression of PCNA is correlated with NPM1 overexpression and malignant stages of cancer. Third, UV-induced PCNA expression is followed by NPM1 overexpression. Finally, knockdown of NPM1 expression level by antisense consequently reduces the expression of PCNA. However, the role and the underlying mechanism of NPM1 in UV-induced PCNA upregulation still remain elusive. In part A of this project, attempts will be made to elucidate whether UV-induced PCNA upregulation is mediated by NPM1 independently or by the collaboration of NPM1 and other interacting proteins.

In the present study, we found that UV treatment evoked NPM1 acetylation, which was induced by p300, a histone acetyltransferase. ChIP assay results showed that p300 directly bound to the *PCNA* promoter region in a similar way as YY1 and NPM1 did after UV treatment. This physiological condition triggered NPM1 acetylation by p300, resulting in chromatin remodeling and consequently

PCNA up-regulation. Furthermore, we have also demonstrated that *PCNA* promoter region with YY1 site is important for NPM1 recruitment.

A4.2 Specific Questions to be addressed in Part A were as follows

First of all, whether NPM1 and PCNA are induced after UV irradiation, we will harvest cell lysates at different post-UV irradiated time point to measure protein and mRNA level.

1. What is the expression pattern of NPM1 and PCNA after low dose UV-irradiated treatment (30 J/m^2) at up to 3, 6, and 9 hours in

    a. protein expression level?

    b. mRNA expression level?

Several types of posttranslational modification of NPM1 have been identified and participated in various cellular processes. Biological functions of NPM1 posttranslational modification are elucidated in "*section 1.3.8 of Chapter One*" in detail. In here, the posttranslation modification of NPM1 should be considered. Further to identify and measure posttranslational modification of NPM1 after UV irradiation.

2. What type of posttranslational modification of NPM1 is induced by UV treatment?

Since the ability of transcriptional regulation of NPM1, the interacting partners and domains are important issues. According to transcription element sites search (TESS) and previously published paper in 2005, YY1 is an important regulatory factor involved in NPM1-mediated PCNA up-regulation. Following questions will answer which domain of NPM1 is required for PCNA activation and whether YY1 and YY1 recognition site is participated in PCNA up-regulation.

3. Is there evidence to show which domain of NPM1 is responsible for up-regulation of PCNA?

4. Is there evidence that NPM1 through YY1 and YY1 recognition site up-regulates *PCNA* promoter activity?

5. Is there evidence that ectopic NPM1 overexpression still has effect on YY1 site-mutated *PCNA* promoter?

A4.3 Materials and methods

The common "materials and methods" are listed and described in chapter three. Here presents the specific materials or methods that are performed in this Part A section.

A4.3.1 UV treatment

UV treatment (at 254 nm) was performed by using UVC 500 UV Crosslinker (Amersham, GE Healthcare). For UV treatment, mouse NIH3T3 cells were grown to 80% confluence in 10-cm dishes and the cells were exposed to low-dosage UV irradiation (30 J/m^2). NIH3T3 cells were harvested at specific time points as indicated in the respective figure legends.

A4.3.2 Plasmids construction

The pCR3.1-FLAG-B23 expression vector was amplified by PCR using full-length NPM1 cDNA in plasmid pET-T7 (a gift from Dr. Pui Kwong Chan) as a PCR template. The N-terminal primer 5′-ACC**ATG**GACTACAAAGACGATGACGACAAG CTTATGGAAGATTCGATGGAC-3′ encoded an AUG translation initiation codon (**boldface**) followed by the codons for the eight amino acids in the Flag epitope (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys; double-underlined) and six amino acids from NPM1. The C-terminal primer was 5′-CGCCGCGGATCCTTAAAGAGACTTCC TCCACT-3′ with a BamHI site (underlined). Amplified PCR products were separated and purified from 1% agarose gel electrophoresis by using QIAquick Gel extraction kit (QIAGEN). Next, the purified Flag-tagged full-length NPM1

cDNA was subcloned into the cloning sites of pCR3.1 vector supplied in the Eukaryotic TA cloning kit (Invitrogen). The orientation of Flag-NPM1 cDNA in pCR3.1 vector was verified by nucleotide sequencing. The pCR3.1-Flag-B23 plasmid with NPM1 cDNA in the sense orientation was driven by cytomegalovirus (CMV) immediate-early promoter and the expressed recombinant NPM1 protein was recognized by anti-Flag-mAb (Sigma-Aldrich).

The human *PCNA* promoter reporter gene (pGL3-PCNA) contains a human *PCNA* promoter region from −153 to +160 bp harboring the transcription start site. This human *PCNA* region was amplified using 5′-GAAGATCTCGCTTCCTCC AATGTATGCT-3′ and 5′-TCGACCATGGTGGCGGAGTGGCAACAA-3′ primers and cloned into the pGL3 BglII and NcoI sites; underlined sequences represent BglII and NcoI sites, respectively. Notably, this inserted human *PCNA* promoter region contains putative YY1-binding site (Labrie et al., 1995). In the following experiments, this plasmid was referred to as a wild-type PCNA promoter reporter gene (pGL3-wt-PCNA promoter). Therefore, when compared to wild-type reporter gene, the inserted region with YY1 site-mutation was generated by site-directed mutagenesis and subsequently subcloned into pGL3-control vector. The detailed reporter gene construction of pGL3-mtYY1-PCNA has been described in detail (Weng and Yung, 2005).

To verify the exclusive interacting domain of NPM1 required for *PCNA* binding, NPM1 expression vectors of different lengths were generated with the following procedures. First, an EGPF-tagged plasmid with full-length NPM1

sequence was generated and referred to as pEGFP-N3 full-length NPM1. This plasmid was amplified by PCR using the pCR3.1-FLAG-B23 as PCR template and the following pair of primers. Full-length NPM1 cDNA was amplified using 5′-CCGCCGCTCGAGATGGAAGATTCGATGGACATG-3′ and 5′-CCCCCCAAGCTTGAAG AGACTTCCTCCACTGCCA-3′ primers and cloned into the pEGFP-N3 XhoI and HindIII sites, where the underlined sequences are the artificial XhoI and HindIII recognition sites. For further generating other EGFP-tagged NPM1 C-terminal deletion mutant clones, the previously listed forward primer was used and paired with different C-terminal reverse primers as shown in the list below.

For pEGFP-N3-NPM1 (1-222):
5′-CCC CCC AAG CTT GTG ATC TTG GTG TTG ATG ATGG-3′

For pEGFP-N3-NPM1 (1-187):
5′-CCC CCC AAG CTT GTT CAG CTT CCT CAT CAT CAAA-3′

For pEGFP-N3-NPM1 (1-137):
5′-CCC CCC AAG CTT GAC TTA AGA GTT TCA CAT CCTC-3′

The artificial HindIII restriction enzyme cutting site is identified as underlined sequences. Similarly, the inserted fragments of EGPF-tagged NPM1 N-terminal deletion mutants were amplified by PCR with the previous full-length reverse primer and different N-terminal forward primers as shown in the list below.

For pEGFP-N3-NPM1 (58-294):
5′-CCG CCG CTC GAG ATG CAC ATT GTT GAA GCA GAG GCA-3′

For pEGFP-N3-NPM1 (131-294):
5′-CCG CCG CTC GAG ATG GAG GAT GTG AAA CTC TTA AGT-3′

For pEGFP-N3-NPM1 (216-294):

5′-CCG CCG CTC GAG ATG CCA TCA TCA ACA CCA AGA TCA-3′

The artificial XhoI restriction enzyme site is identified as underlined sequences.

All amplified PCR products were purified by gel purification, and subsequently inserted into pEGFP-N3 control vector. The orientation of each pEGFP-N3-NPM1 clone was analyzed by restricting mapping and full-length nucleotide sequencing. A schematic presentation of several NPM1 deletion mutants are shown in Appendix 1.

Before cell transfection, HeLa cells were seeded into a 6-well plate with 80–90% confluence by using culture medium without antibiotics. On the day of transfection, cells were first washed twice with PBS and then transiently co-transfected with 1 μg pGL3-PCNA reporter gene and 1 μg pCR3.1-FLAG-B23, or 1 μg pGL3-control vector and 1 μg pCR3.1 vector, 0.5 μg pCMV β-galatosidase plasmid by using LipofetAMINE reagent according to the manufacturer's protocol (Invitrogen). In total, 2.5 μg plasmid DNAs and 5 μl of LipofectAMINE reagent were each diluted into 100 μl of OPTI-MEM medium. Next, the diluted plasmid DNAs and LipofectAMINE reagent were gently mixed and incubated at room temperature for 30 min to allow the formation of DNA-liposome complexes. Cells were placed in OPTI-MEM medium (1.5 ml) and then overlaid with DNA-liposome complexes and incubated for 6 h at 37°C. Cells were changed to complete medium after 6 h incubation.

A4.3.3 Immunoprecipitation

The cell lysate (600 μg) was diluted to 600 μl with WCE buffer (see "3.3 Western Blot Analysis" in chapter three for the detailed procedure). The lysate was incubated with a desired antibody overnight at 4°C. For analysis of the acetylation status of NPM1, the cell lyaste was incubated with anti-NPM1 pAb overnight at 4°C. The immune complex was subsequently captured by protein G-sepharose (30 μl) for 2 hr at 4°C. The protein G-antigen-antibody complexes were washed four times with the WCE buffer and boiled in 2X urea sample buffer dye for subsequent SDS-PAGE and immunoblotting analysis as described in section "3.3 Western Blot Analysis" in chapter three.

A4.3.4 Biotin-streptavidin pull-down assay

Oligonucleotides with biotin-labeling on the 5′-end of the sense strand were used in pull-down assay. The sequences of these two oligonucleotides are as follows (The YY1 recognition site is underlined):

PCNA-wt-oligo: 5′-CGCGGCGGCATTAAACGGTTG-3′
PCNA-YY1 mut-oligo: 5′-CGCGGCGGGTTTAAACGGTTG-3′

These biotin-labeled oligonucleotides were annealed to their respective complementary oligonucleotides, and subsequently incubated with 50 μl of Streptavidin-MagneSphere® (Promega) in binding buffer (20 mM Tris-HCl pH 7.9, 10% glycerol, 1 mM sodium orthovanadate, 1 mM EGTA, 5 mM EDTA, 10 mM NaF, 1 mM sodium pyrophosphate, 0.5% Triton X-100, 200 mM NaCl, 0.2 μg/μl ssDNA) for at least 4 h at 4°C. The DNA-bound beads were washed three times

with binding buffer. Nuclear extracts were prepared from HeLa cells. Then, 1000 μg of pre-cleared nuclear extracts were added to the precipitated beads and incubated overnight at 4°C. After four washes with binding buffer, the precipitated proteins were separated by SDS-PAGE, and detection of YY1 and NPM1 was achieved by western blot analysis (see "*3.3 Western Blot Analysis*" in chapter three).

Some detailed descriptions about common or general "materials and methods" are reviewed in chapter three.

A4.4 Results

A4.4.1 NPM1 and PCNA are induced by UV irradiation

In 2002, NPM1 has been found to be an UV-inducible protein (Wu et al., 2002b) and immediately response to DNA damage lesions (Wu and Yung, 2002). To verify expression patterns of NPM1 and PCNA inducing by low dosage of UV irradiation (30 J/m^2), we measured the mRNA and protein levels of NPM1 and PCNA in various post-UV irradiated time points. In this UV irradiation experiments, we found previously research articles and used UV-sensitive mouse fibroblasts, NIH3T3 cells accordingly. Before we performed the UV irradiation, NIH3T3 cells with 80% confluence were changed to a culture medium without antibiotic supplement. After exposure to UV irradiation, cells and cell lysates were collected at different post-UV irradiated time points for RNA and protein extraction.

Consistent with previous findings, protein and mRNA expression levels of NPM1 and PCNA were induced by UV irradiation (Wu et al., 2002a; Wu and Yung, 2002). The increasing manifestation was detected at 3 h post-UV-irradiation treatment and sustained to 24 h. The similar patterns were also revealed for PCNA expression (Figure A4.1A & B). It seems that PCNA up-regulation was followed by NPM1. PCNA also is an UV-induced gene and directly involved in DNA repair pathways, particularly in nucleotide excision repair (Hashiguchi et al., 2007; Shivji et al., 1992; Weng and Yung, 2005; Wu et al., 2002b).

A4.4.2 Increased NPM1 acetylation is induced by UV irradiation

The posttranslational modifications of NPM1 have been reported to involve in various cellular processes. Recently, a research article demonstrated that high expression and acetylation status of NPM1 were detected in malignant oral cancer tissues. Interestingly, acetylated NPM1 colocalized with RNA polymerase II (RNAP II) in nucleoplasm and occupied promoter region of TNFα gene to regulate transcriptional activations of genes implicated in oral cancer (Shandilya et al., 2009). Whether UV irradiation could trigger posttranslational modification of NPM1, particularly the acetylation status of NPM1, we performed a series of experiments to detect the acetylation status of NPM1. To characterize the post-translational modification status of NPM1 after UV irradiation, we carried out immunoprecipitations of NPM1 from nuclear extracts of non-irradiated and irradiated NIH3T3 cells. Immunocomplexes were analyzed for acetyl-lysine amount by western blotting. The results showed that acetylation status of NPM1 was increased after UV irradiation (Figure A4.1.C). Importantly, we firstly identified that the elevated NPM1/B23 acetylation status was induced by UV irradiation. Increased acetylated NPM1 might play an important role in cellular response to UV-induced damage.

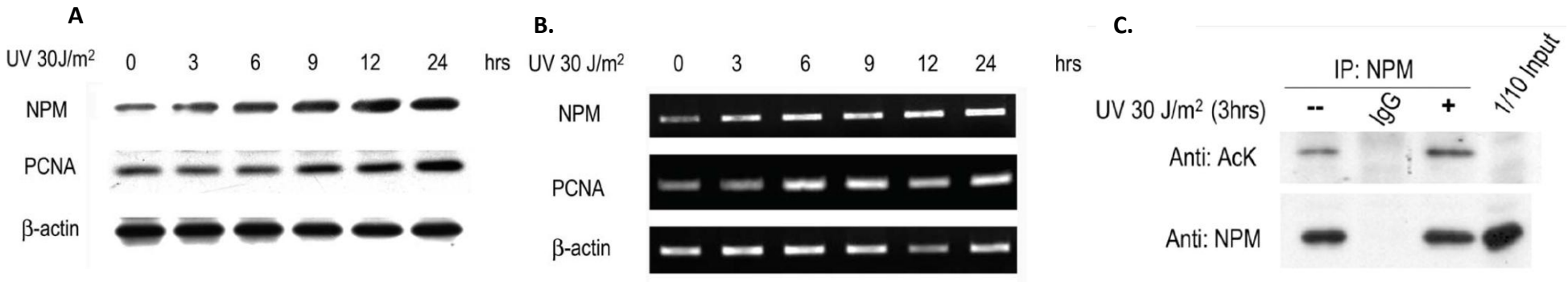


Figure A 4.1: Inductions of NPM1 and PCNA are triggered by UV irradiation.

(A) NIH3T3 cells were exposed to UV and harvested at indicated time points after UV radiation (30 J/m^2). Equal amount of lysate proteins were separated by 10% SDS-PAGE and subjected to western blotting with the indicated antibodies.

(B) Non-irradiated and irradiated NIH3T3 cells were harvested for RNA extraction. The mRNA level of endogenous NPM1 (NPM), PCNA and β-actin (internal control) were measured by RT-PCR with specific primers as listed in Table 3.1 of chapter three. The detailed procedures of RT-PCR are described in chapter three.

(C) NIH3T3 cells were exposed to UV and harvested at 3 h post-treatment time point. Lysates were immunoprecipitated with anti-NPM1 antibody (IP) and subsequently immunoblotted using anti-acetyl lysine (AcK, top) and anti-NPM1 (NPM, bottom) antibodies, as indicated. Acetyl lysine antibody is against all acetyl lysine residues of proteins and is used to recognize (measure) the acetyl group (status) of immunoprecipitated NPM1. The internal control of immunoprecipitation was immunoblotted by NPM1 to confirm equal amount of cell lysates.

A4.4.3 <u>The nuclear diffusion of NPM1 after UV irradiation</u>

The localization of NPM1 has been reported to localize in nucleolus. UV irradiation induces the expression and posttranslational modification of NPM1, whether the localization of NPM1 would be affected after UV treatment. To characterize the localization of NPM1 in cellular response UV, NIH3T3 cells were cultured on slide overnight and treated with UV irradiation (30 J/m^2). Cells were fixed at 3h post-irradiated time point and co-stained NPM1 antibody and DAPI for nucleic acids detection. The results observed from Figure A4.2 that NPM1 mainly expressed in the nucleoli of un-irradiated cells, whereas it diffused to the nucleoplasm of irradiated cells (Figure A4.2). These data suggested that the nuclear diffusion of NPM1 is important for regulation of PCNA-mediated DNA repair in UV-irradiated cells.

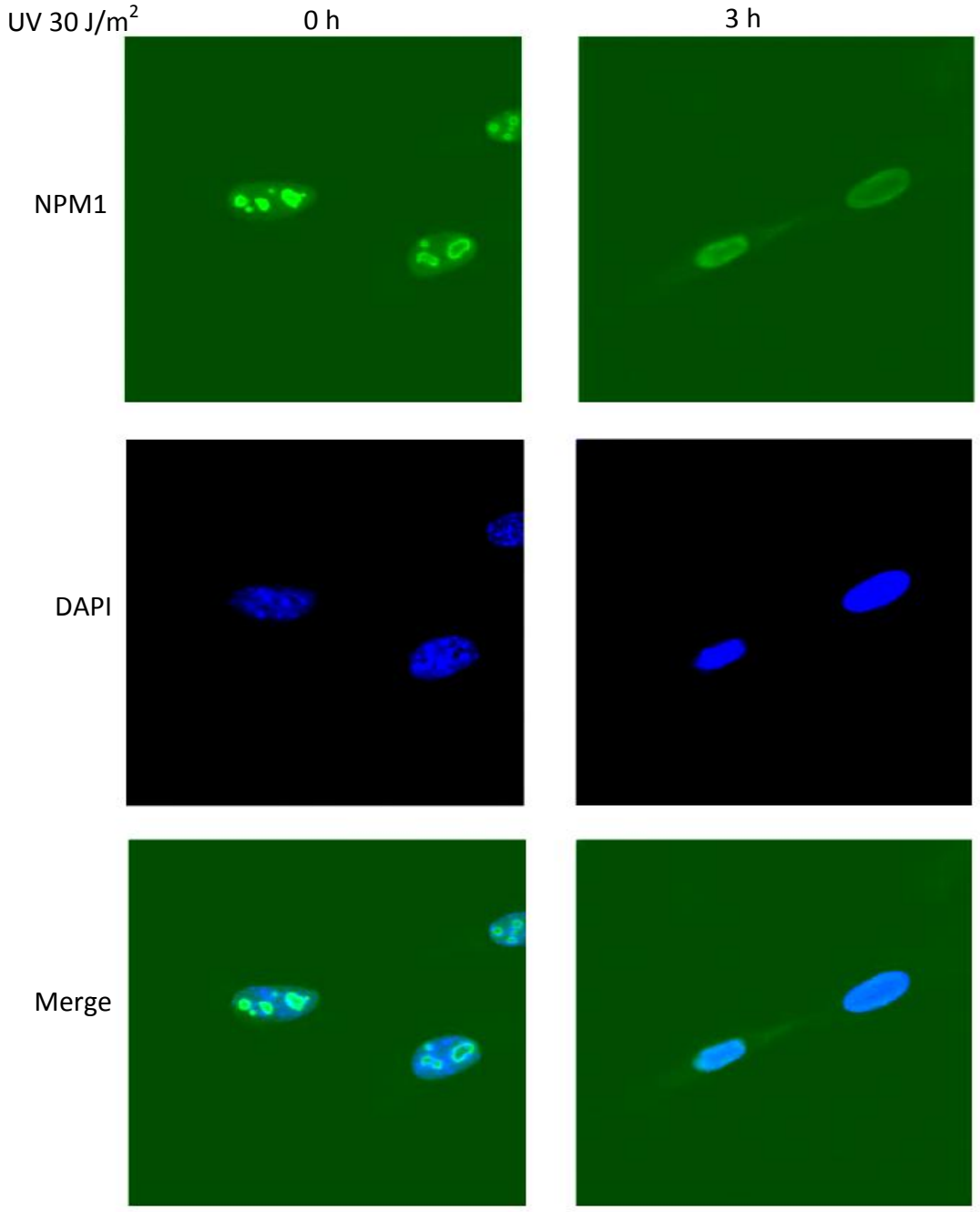


Figure A4.2 NPM1 temporary diffuses into nucleoplasm after UV irradiation.

NIH3T3 cells were grown on slides overnight and treated with UV irradiation (30 J/m^2). After UV treatment, cells were fixed with 2 % formaldehyde in PBS at the indicated times. Cells were then labeled with anti-NPM1 monoclonal antibody and FITC-conjugated secondary antibody to detect localization of NPM1 by using confocal microscope. DAPI staining was also performed to mark the nuclear region.

A4.4.4 PCNA is upregulated after NPM1 overexpression

To further determine whether NPM1 was required for PCNA upregulation, we first analyzed the mRNA expression level of PCNA in cells with NPM1 being transiently overexpressed. Here, we used HeLa cells with high transfection efficiency than NIH3T3 cells for the transient transfection experiments. Cells were transfected by different concentrations of pEGFP-N3-NPM1 plasmid as indicated. Interestingly, NPM1 overexpression increased the mRNA expression level of PCNA with a trend in dose-dependent manner (Figure A4.3A). It was consistent with previous report that PCNA expression level were increased upon NPM1 overexpression (Weng and Yung, 2005). To elucidate whether the increased PCNA expression was mediated by the transcriptional regulation of NPM1, the transcription level of PCNA in NPM1 overexpressed condition was measured by the reporter gene assay. As shown in Figure A4.3B, our results indicate that the transcription level of PCNA was dramatically increased in NPM1 overexpression. Collectively, NPM1 overexpression increased *PCNA* activation via a transcriptional regulation manner.

Figure A4.3: PCNA gene expression and promoter activity ensures increase in NPM1 overexpressed condition.

(A) HeLa cells were transiently transfected with the indicated doses of NPM1 expression vector (pEGFP-N3-NPM) and control vector pEGFP-N3. The total amount of plasmid DNA was kept constant by the addition of control vector. After 24 h transfection, cells were harvested and total RNA was extracted by TRIzol. The relative mRNA level of pEGFP-NPM, total NPM, PCNA and β-actin were measured by quantitative RT-PCR with specific primers as shown in Table 3.1. Detailed procedures of RT-PCR are described in chapter three.

(B) Effect of NPM1 overexpression on PCNA promoter activity. HeLa cells were cultured in 6-well plates with 80–90% confluency. Cells were cotransfected with 0.5 μg pGL3-wt-PCNA reporter gene, 0.1 μg of pCMV-SPORT-β gal (as a control for transfection efficiency) and the indicated amount of pEGFP-N3-NPM or pEGFP-N3 (control vector). The total amount of plasmid DNA was kept constant by the addition of pEGFP-N3. Twenty-four hours after transfection, cells were harvested and subjected to reporter gene assay by using equal amounts of proteins. The values of PCNA promoter activity from the control vector group were presented as 1. The results showed that significantly ($*P < 0.05$) higher PCNA promoter activities were found at pEGFP-NPM groups, but no significant differences were seen in pEGFP control group. Bars are means of triplicates ± SDs.

A4.4.5 C-terminal region of NPM1 is crucial for PCNA activation

To further assess the role of NPM1 in transcriptional activation of PCNA, we then determined the effects of NPM1 deletions on *PCNA* promoter activity. Figure A4.4A presents the schematic diagram of full-length NPM1 and its deletion mutants. The N-terminal deletion mutants, NPM (58–294) and NPM (131–294), could activate *PCNA* promoter as the full-length NPM1 did (Figure 4.4B). Notably, the N-terminus deletion mutant of NPM1 (remaining residues 131–294) had higher PCNA induction effect when compared with full-length NPM1 plasmid. However, C-terminal deletion mutants [NPM (1–222), NPM (1–187) and NPM (1–137)] abrogated the induction effect on *PCNA* promoter (Figure A4.4B). These data demonstrated that C-terminal region of NPM1 is crucial for PCNA activation. Furthermore, analysis of NPM1 sequence revealed that C-terminus of NPM1 (residues 189–243) is enriched with alkaline amino acids in sequence such as lysine and arginine residues. This characteristic of NPM1 C-terminus prefers to attract acidic nucleotides including DNA and RNA molecules (Hingorani et al., 2000). Whereas the electrostatic property of NPM1, it might interact with RNA or DNA molecules such as the *PCNA* gene promoter.

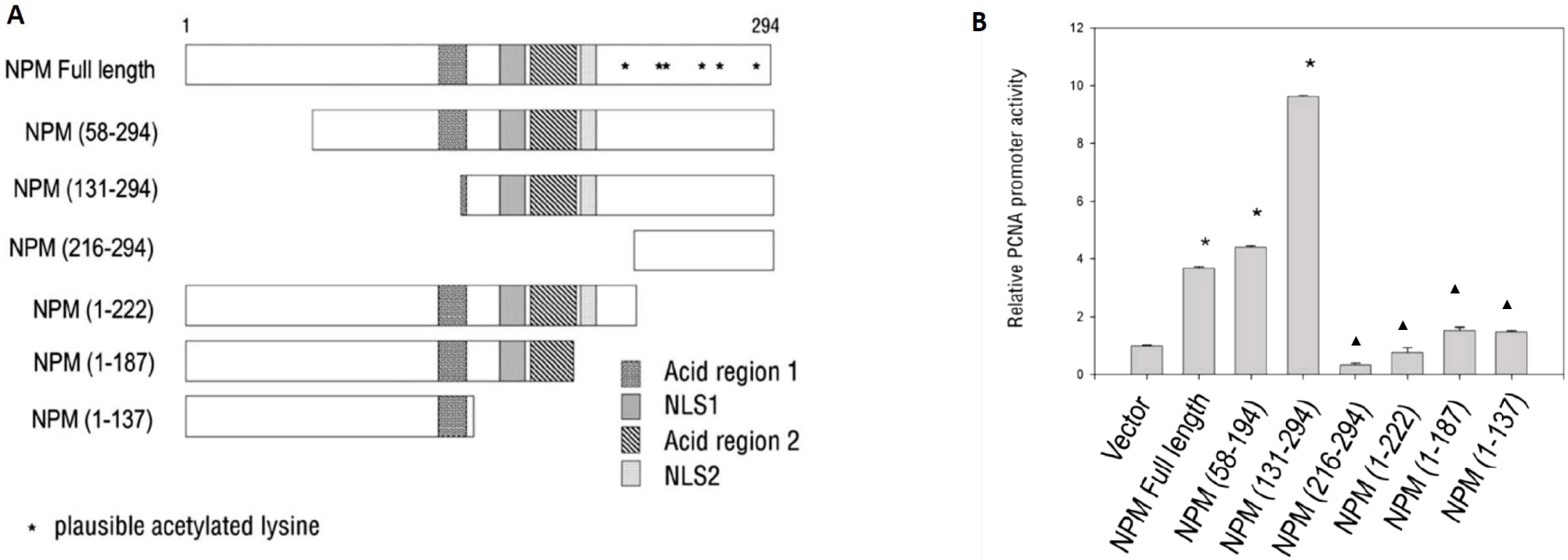


Figure A 4.4: C-terminal deletion mutants of NPM1 abolish the transactivation of PCNA.

(A) Schematic representation of the deletion variants of NPM1 plasmids.

(B) A Reporter gene assay was carried out with HeLa cells, which were co-transfected with wild-type *PCNA* reporter gene, empty control (vector) and different NPM1 deletion variants as indicated in the figure. Bars are triplicates ± SD. *PCNA* promoter activities were significantly ($*P < 0.05$) increased when compared with groups which were co-transfected with the *PCNA* reporter gene and the control vector. In contrast, *PCNA* promoter activities were significantly ($▲P < 0.05$) decreased when compared with NPM1 overexpressed group which was co-transfected with the *PCNA* reporter gene and pEGFP-N3-NPM1 (full-length NPM).

A4.4.6 Characterization of the localization of NPM1 deletion mutants

As described above, different NPM1 deletion mutants have various effects for *PCNA* transactivation. The localization of NPM1 deletion mutants might interrupt the interaction with PCNA and subsequently abolish *PCNA* gene promoter activity. To verify the possibility that the NPM1 deletion mutants were transfected into NIH3T3 cells, the localizations of NPM1 mutants in cells were identified by anti-NPM1 antibody. As consistent with previous reports, full-length of NPM1 expressed in nucleolus; whereas NPM1 mutants with various deletions in N terminus both expressed in nucleolus and nucleoplasm (Figure A 4.5A). However, NPM1 C-terminal deletion mutants represented nuclear and cytoplasmic diffusion in cells (Figure A 4.5B).

From figure of representation of NPM1 functional domains (see Figure 1.2 in Chapter One), the C-terminal region of NPM1 is enriched of alkaline amino acids in sequence and contains one nuclear localization sequence. When this C-terminal region is deleted, all NPM1 mutants with C-terminal deletion lost their nuclear localization sequence and localized in cytoplasm. Moreover, these C-terminal deletion mutants might lost their DNA/RNA binding ability resulting in disassociation with *PCNA* gene and attenuation of *PCNA* gene promoter activity (Figure A4.4B). N-terminal region of NPM1 contains two nuclear export signals that are required for nucleo-cytoplasmic shuttling of NPM1. Our results from Figure A4.5A showed that NPM1 N-terminal deletion mutants (NPM1[58-294] and NPM1[131-294]) expressed in whole nucleus, not restricted in nucleolus. However, a NPM1 N-terminal deletion mutant (NPM1[216-294]), which was deleted whole N-terminal region, acidic domain and partial DNA/RNA binding domain, represented the localization mainly in cytoplasm and diminished *PCNA* promoter activity. These data suggested that the localizations of NPM1 deletion mutants are corresponding with the transactivation of PCNA. The cellular location of NPM1 deletion mutants were listed in Table 4.1.

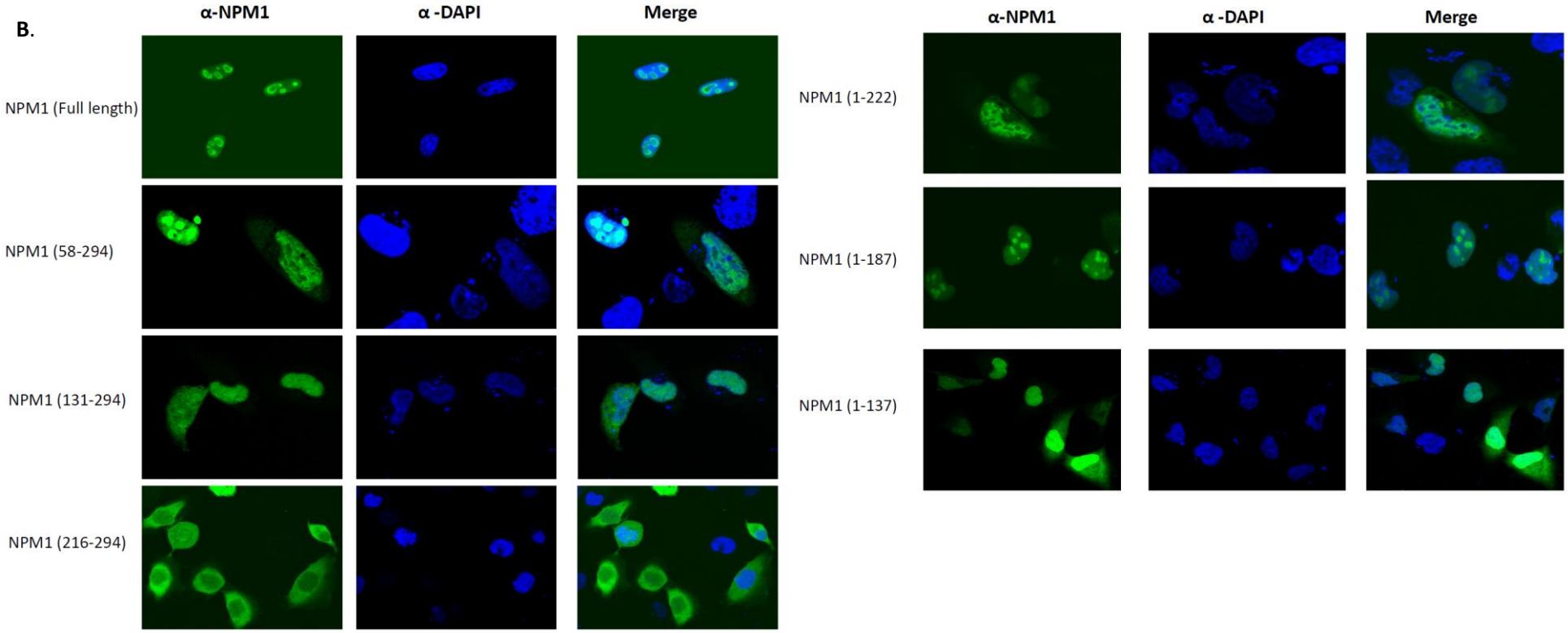


Figure A 4.5 Characterizations of the localization of NPM1 deletion mutants.

(A) Localization of NPM1 mutants with various deletions in N-terminal region. NIH3T3 cells were cultured in 100mm dishes with cover slides for overnight and transfected with NPM1 deletion mutants as indicated. Cells were fixed and co-stained with anti-NPM1 antibody and DAPI for nucleic acid detection.

(B) Localization of NPM1 C-terminal deletion mutants in cells. Similar culture conditions were performed as described above. Cells were transfected with different NPM1 C-terminal deletion mutants. Cells were fixed and co-stained with anti-NPM1 antibody and DAPI for nucleic acid detection.

NPM1 deletion mutants		Cellular signal intensity of NPM1 location		
		nucleolus	nucleoplasm	cytoplasm
	NPM1 (full length)	strong	weak	none
N-terminal deletion	NPM1 (58-294)	strong	strong	weak
	NPM1 (131-294)	strong	strong	weak
	NPM1 (216-294)	weak	weak	strong
C-terminal deletion	NPM1 (1-222)	strong	strong	weak
	NPM1 (1-187)	strong	strong	weak
	NPM1 (1-137)	strong	strong	weak

Table 4.1: The summary of cellular locations of NPM1 deletion mutants.

All NPM1 deletion mutants mainly expressed in nucleus excepted NPM1(216-294), which mainly located in cytoplasm.

A4.4.7 NPM1 Bound to YY1-responsive site on PCNA promoter region

To understand the underlying mechanism of *PCNA* transactivation, we suggested that NPM1 might interact with other binding partners or directly bind to *PCNA* promoter region for *PCNA* activation. In the last decade, the *PCNA* promoter region has been reported with YY1 binding site which reside between nucleotides –1 to +3 and required for transcription initiation (Labrie et al., 1995) and *PCNA* activation (Weng and Yung, 2005). First, we attempted to examine the role of the transcription factor YY1 and YY1-responsive site on *PCNA* promoter for *PCNA* activation by using chromatin immunoprecipitation assay (ChIP assay). HeLa cells-extracted chromatin was immunoprecipitated with antibodies to against YY1 and NPM1, respectively. Final PCR amplification was used for detecting YY1 and NPM1 in samples that had been immunoprecipitated with anti-YY1 and anti-NPM1, respectively (Figure A4.6A). The results demonstrated that both YY1 and NPM1 were recruited to *PCNA* promoter region. *Vimentin* is not regulated by YY1 and NPM1, and hence could not be detected in the immunoprecipitates (Figure A4.6A). Furthermore, HeLa cells were transiently transfected with pGL3-wt-PCNA (pcna-wt) or pGL3-mtYY1-PCNA (pcna-myy1) reporter to elucidate the role of YY1 responsive site in *PCNA* activation. Mutation at the YY1-responsive site resulted in a decrease of YY1 and NPM1 binding on *PCNA* promoter when compared to that with the remaining YY1 site (Figure A4.6B), suggesting that YY1-responsive site is essential for *PCNA* activation.

To further investigate the functional role of YY1-responsive site in *PCNA* activation, we tested whether NPM1 was directly bound to YY1-responsive site as well as YY1 by using the biotin-streptavidin pull-down assay. The biotin-

labeled double-stranded oligonucleotides corresponding to –107 to –57 of the *PCNA* promoter were incubated with nuclear extracts from HeLa cells. Then, the incubated mixtures were pulled down by streptavidin (the detailed method description see "Section A4.3.5"). The protein complexes bound to the biotin-labeled oligonucleotides were subsequently analyzed by immunoblotting assay with antibodies against YY1 and NPM1. Our results showed that the presence of YY1 and NPM1 on the probe was dependent on an intact YY1-binding motif (Figure A4.6C). As expected, the probe with YY1 site mutation abrogated the interacting with YY1 and NPM1. Consistent with *in vivo* ChIP assay data, the YY1-responsive site is essential for the NPM1 binding and recruitment. Here, we demonstrated that NPM1, a sequence-nonspecific protein, bound to YY1-binding site on the *PCNA* promoter region, resulting in *PCNA* activation.

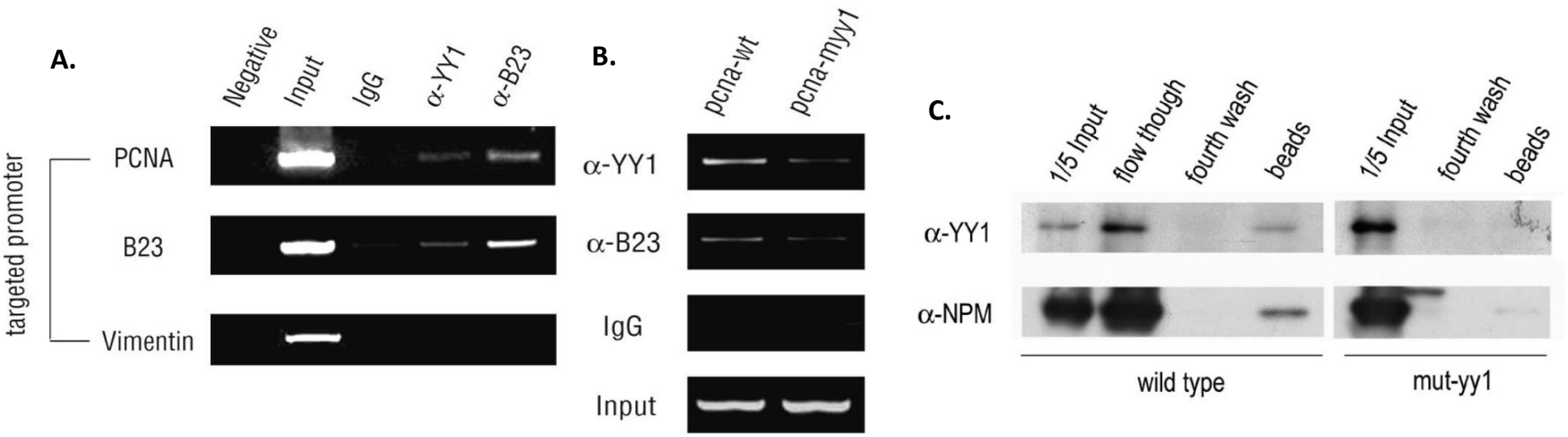


Figure A4.6: NPM1 binds to YY1 recognition site on PCNA promoter in vivo

(A) ChIP assay was performed as described in the "materials and methods" section. The chromatin sample was prepared from HeLa cells, fixed by formaldehyde, and then sonicated. Immunoprecipitation was done with a control antibody (immunoglobulin G [IgG]) or an antibody against YY1 (α-YY1) or NPM1 (α-NPM). Products from PCR amplification using primers specific to endogenous *PCNA*, *NPM1* and *Vimentin* promoter region were resolved by 1.5% agarose gel. DNA input (1/10 of immunoprecipitate) is shown on the left side.

(B) HeLa cells were transfected with wild type or YY1 site-mutated *PCNA* reporter indicated as pcna-wt and pcna-myy1, respectively. After 24 h transfection, cell lysates were prepared from ChIP assay and immunoprecipitation with control (IgG), YY1 and NPM1 antibodies were carried out. Products from PCR amplification using primers specific to exogenous *PCNA* reporters were resolved by 1.5% agarose gel.

(C) Total proteins were extracted from HeLa cells for pull-down assay. Wild type and YY1 site-mutated *PCNA* (mut-yy1) probes with biotin labeling were pre-incubated with streptavidin beads, and subsequently mixed with 1mg protein extracts for overnight incubation. Final eluted samples (flow through), washed fluid (fourth wash), streptavidin beads (beads) and lysate input (1/5 of input) were probed with specific antibodies as indicated in figure A4.4.C.

A4.4.8 NPM1 overexpression maintains the basal activity of PCNA with YY1 site mutation

As described above, NPM1 overexpression induced *PCNA* activation via its occupancy of YY1-responsive site on the *PCNA* promoter region. We then further investigated the promoter activities of the *PCNA* reporter with YY1 site mutation in the context of NPM1 overexpression. The reporter gene assay showed that the activity of the *PCNA* promoter with YY1 site mutation was significantly attenuated when compared with the wild-type *PCNA* reporter. Noteworthily, NPM1 overexpression maintained the basal activity of YY1 site-mutated *PCNA* reporter gene (Figure A4.7A). In parallel, we performed ChIP assay to verify the recruiting manifestation of YY1 and NPM1 on YY1 site-mutated *PCNA* promoter under NPM1 overexpressed condition. In this assay, the Flag-tagged NPM1 and endogenous YY1 were both recruited to wild-type *PCNA* promoter region. In contrast, endogenous YY1 lost its binding and ectopic expressed NPM1 had little binding on YY1 site-mutated *PCNA* promoter (Figure A4.7B). Taken together, we suggested that NPM1-induced PCNA activation through its recruitment on YY1-responsive site. However, NPM1 still could bind to the *PCNA* promoter with YY1 site-mutation and maintained the basal activity of *PCNA*.

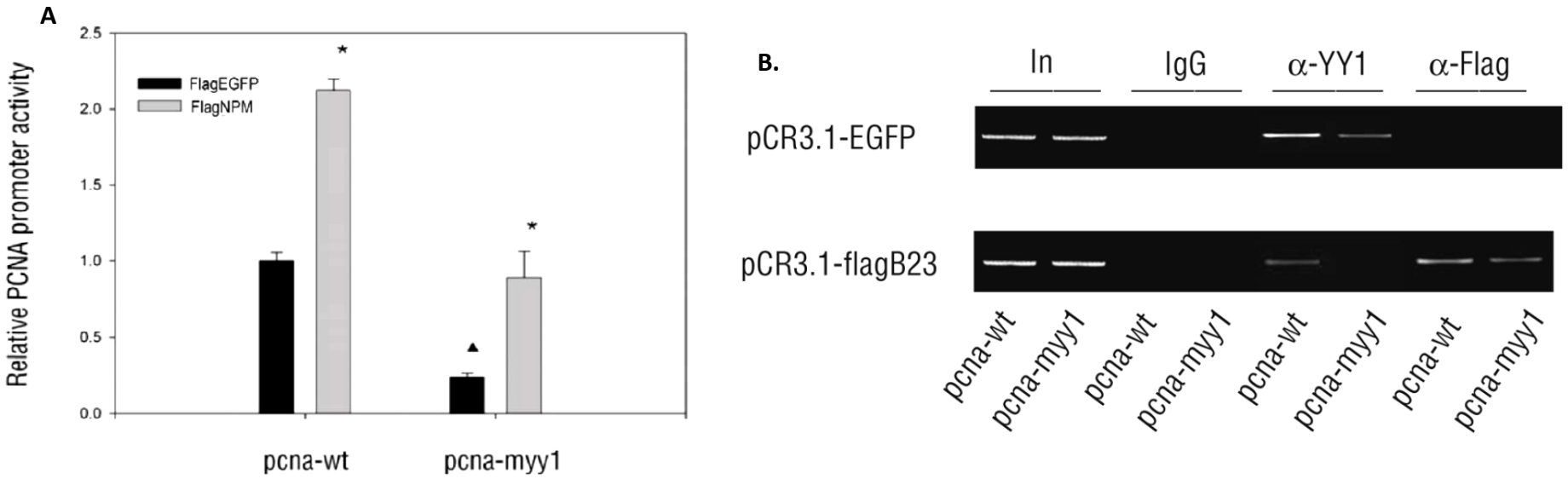


Figure A4.7: YY1 site-mutated PCNA promoter retards NPM1 bindings and maintains basal activity in NPM1 overexpressed condition.

(A) *PCNA* promoter reporter assay was performed on HeLa cells which were co-transfected with pGL3-PCNA reporters (pcna-wt and pcna-myy1) and ectopic Flag-tagged NPM1 constructs (Flag-EGFP and Flag-NPM). Bars are means of triplicates ± SD. Significantly (∗P<0.05) evoked wild-type *PCNA* (pcna-wt) promoter activity in NPM1 overexpression when compared with the control group (FlagEGFP). Mutated *PCNA* promoter activity was dramatically (▲P < 0.05) decreased when compared with group co-transfected with wild-type *PCNA* reporter gene and empty vector (Flag-EGFP). NPM1 overexpression maintained basal activity of *PCNA* promoter with YY1 site mutation (pcna-myy1).

(B) Similar transfection conditions were carried out in HeLa cells as described above. Cells were harvested and chromatin extracts were prepared for ChIP assay. Immunoprecipitation was done with control antibody (IgG), anti-YY1 antibody and anti-Flag antibody to recognize the Flag-tagged ectopic NPM1. Products (~400 bp) from final PCR analysis by using primers specific to exogenous *PCNA* promoter region were fractionated by 1.5% agarose gel.

A4.4.9 ***Assembly of NPM1 and YY1 on the PCNA promoter region***

We have demonstrated that NPM1 upregulates PCNA via YY1-responsive site, meanwhile YY1 was also recruited to *PCNA* promoter region and bound to YY1-responsive site. Yet the mechanism of this regulation is not well understood. Both NPM1 and YY1 are DNA-binding proteins. NPM1, a sequence-nonspecific DNA interacting protein, played an important role for *PCNA* activation. In particular, the C-terminal region of NPM1 had a decisive effect on the *PCNA* promoter activity (Figure A4.4). As previously reported, YY1 is a well-known sequence-specific DNA binding protein containing four C_2H_2-type zinc finger motifs (Gordon et al., 2006). We hypothesized that NPM1 could locate to regions near *PCNA* promoter in a sequence non-specific manner and influence the binding of YY1 to *PCNA* promoter so as to modulate the promoter activity. To verify this hypothesis we first set out to confirm the interaction of NPM1 C-terminus and YY1 with *PCNA* promoter by *in vitro* electrophoresis gel mobility shift assay (EMSA). We prepared two recombinant proteins: NPM1 C-terminus (residues 206–294, indentified as construct 5) and YY1 zinc finger domain region (containing four zinc finger motifs with 336 amino acids in sequence) for the electrophoretic mobility shift assay (EMSA). The double-stranded *PCNA* oligonucleotide (ds*PCNA*, 400 bp in length) containing YY1-responsive site was prepared from the wild-type *PCNA* reporter (pGL3-wt-PCNA) by cutting with two restriction enzymes, SmaI and NcoI.

First, we tested our recombinant protein extracts of YY1 zinc finger domain and NPM1 C-terminal region by EMSA with the 400-bp ds*PCNA* oligonucleotide (0.25 pmol) in an agarose gel system. YY1 proteins in increasing amounts

(Figure A4.8A, lane 2 to lane 9) gave distinct band shifts with ds*PCNA* when compared with control (Figure A4.8A, land 1: ds*PCNA* alone). This result provided a positive control for our EMSA and extractions of recombinant proteins because YY1 has been reported to bind on its recognition site of ds*PCNA* oligonucleotide. Second, the recombinant NPM1 C-terminal region protein in increasing amounts also showed obvious band shifts with ds*PCNA* (Figure A4.8B, land 2 to land 16) when compared with control (Figure A4.8B, land 1: ds*PCNA* alone). Yet instead of the distinct shift pattern observed for YY1, the shift pattern for NPM1 is smeared, probably due to the sequence non-specific nature of NPM1-ds*PCNA* oligonucleotide interaction. Next, we performed EMSA on the ds*PCNA* probe (400 bp) with the recombinant YY1 zinc finger domain, followed by the recombinant NPM1 in increasing amounts. This experiment was intended to investigate whether NPM1 and YY1 bind cooperatively, competitively or independently on *PCNA* promoter. Recombinant YY1 with small amount (0.4 pmol) showed rather slightly a band shift in our EMSA analysis (Figure A4.9, land 2). Remarkably, addition of NPM1 in increasing amounts to the YY1-ds*PCNA* mixture resulted in super-shifted bands, (Figure A4.9, land 3 to land 12), demonstrating that NPM1, and YY1 can assemble together with ds*PCNA*.

The classical EMSA is performed in non-denaturing polyacrylamide gel electrophoresis (PAGE). However, the size of 400-bp ds*PCNA* oligonucleotide is too long and easily trapped and obstructed in wells of PAGE gel. To further verify the EMSA data obtained using the non-conventional agarose-based approach, we cut down the size of ds*PCNA* probe to 30 bp, which also harbors an YY1 recognition site near transcription starting site to replace the 400 bp ds*PCNA*

probe (sequence of short ds*PCNA* oligonucleotide probe is listed in "*section3.9 of chapter three*"). First, we tested our short ds*PCNA* oligonucleotide and recombinant YY1 zinc finger domain by EMSA in PAGE system. Recombinant YY1 protein gave distinct band shifts with the short ds*PCNA* oligonucleotide when compared with ds*PCNA* alone (Figure A4. 10A). Consistent with previous results demonstrated in agarose gel (Figure A4.8A), YY1 recognizes its own YY1 binding on ds*PCNA* probes with distinct bandshifts. For determination of multi-components assembly on ds-DNA, we presented that NPM1 C-terminus bound to the complex of recombinant YY1 and ds*PCNA* and the multi-component complex produced super-shifts in the PAGE system and the agarose gel system. Recombinant YY1 protein associated with ds*PCNA* probe with clear bandshifts; however, the addition of NPM1 protein made the complex with smeared pattern (Figure A4.10B). The data observed from figure A4.10B, NPM1 might associate with YY1-ds*PCNA* complex in sequence-nonspecific pattern. Moreover, the size of 30bp ds*PCNA* probe might be not appropriate for NPM1; the 400 bp ds*PCNA* probe might provide enough three-dimensional space for DNA folding and proteins binding to represent a clear multiple complex in agarose gel (Figure A4.9). We suggested that endogenous NPM1 protein could synergistically associate with YY1-ds*PCNA* complex to mediate PCNA activation in cellular response to UV damage.

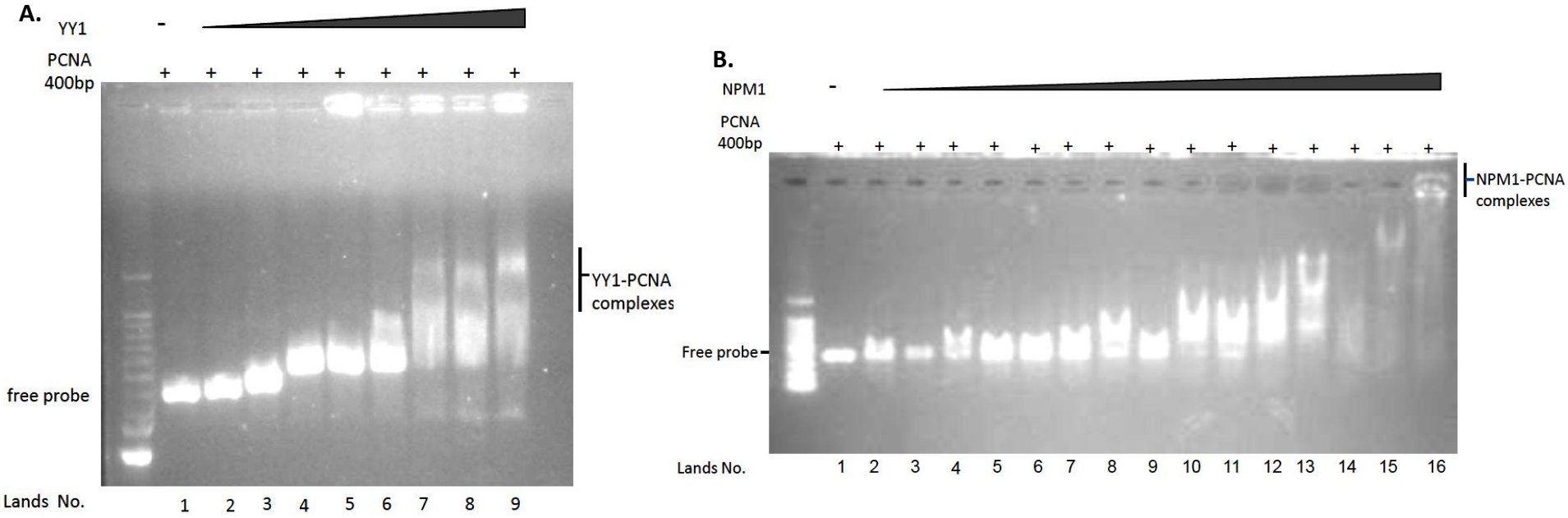


Figure A4.8: Recombinant YY1 and NPM1 protein are associated with 400bp double strand PCNA probe in electrophoretic mobility shift assay (EMSA) system.

(A) The EMSA was performed and detail described in "materials and methods". The 400-bp double-stranded *PCNA* oligonucleotide (ds*PCNA*) was prepared from pGL3-wt-PCNA reporter by cutting with two restriction enzymes, SmaI and NcoI. The *PCNA* probe (land 1, 0.25 pmol) was used to test the recombinant protein extracts of YY1 zinc finger domain (YY1 land 2, 0.25 pmol). Protein was added in two-fold increasing amounts. Samples were resolved by 1.5% agarose gel.

(B) EMSA of 400 bp ds*PCNA* (land1, 0.25 pmol) with NPM1 C-terminal protein region (NPM1 land2, 0.25 pmol). Protein was added in two-fold increasing amounts. Samples were also resolved by 1.5% agarose gel.

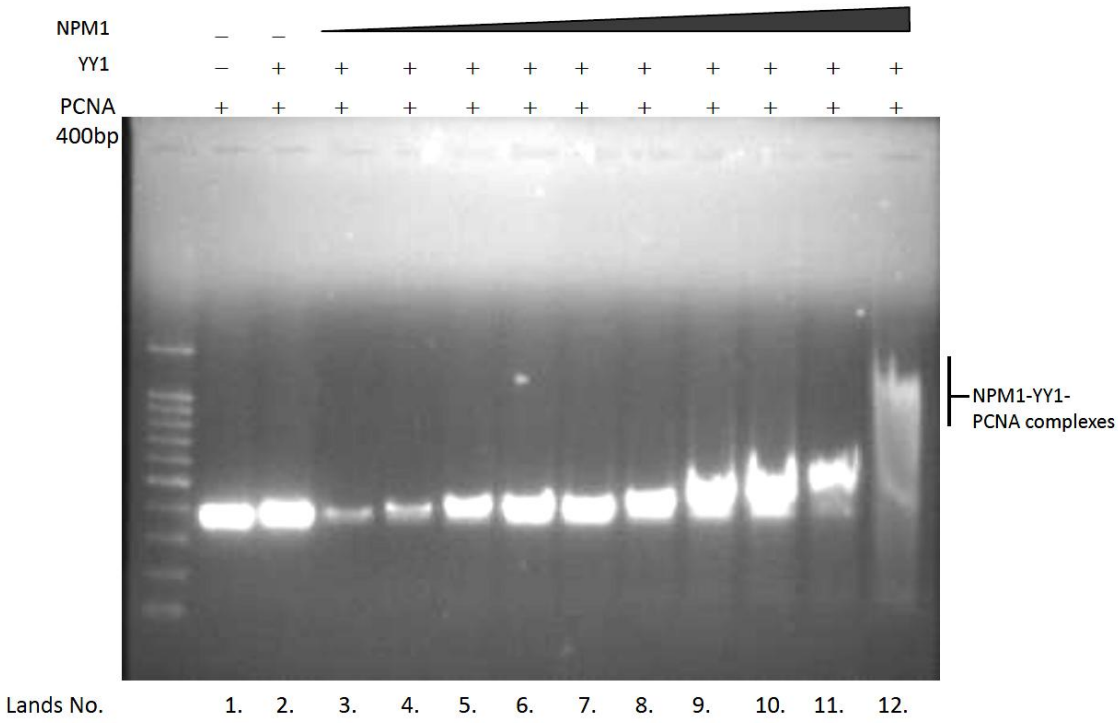


Figure A4.9: Synergic association of NPM1 on PCNA-YY1 complex is determined by multi-component mobility shift assay in agarose gel.

Multi-component mobility shift assay is an alternative EMSA system used to study multi-component assembly of DNA-binding complexes to determine the assembly of recombinant YY1 and NPM1 protein on double-stranded *PCNA* probe. The amounts of the YY1 protein (0.25pmol) and the ds*PCNA* probe (0.25pmol) were kept constant and the recombinant NPM1 C-terminus (starting concentration is 0.25pmol) was subsequently added in two-fold increasing amounts to the YY1-*PCNA* binding reaction. This resulted in super-shifts of *PCNA*-YY1-NPM1 complexes in 1.5% agarose gel.

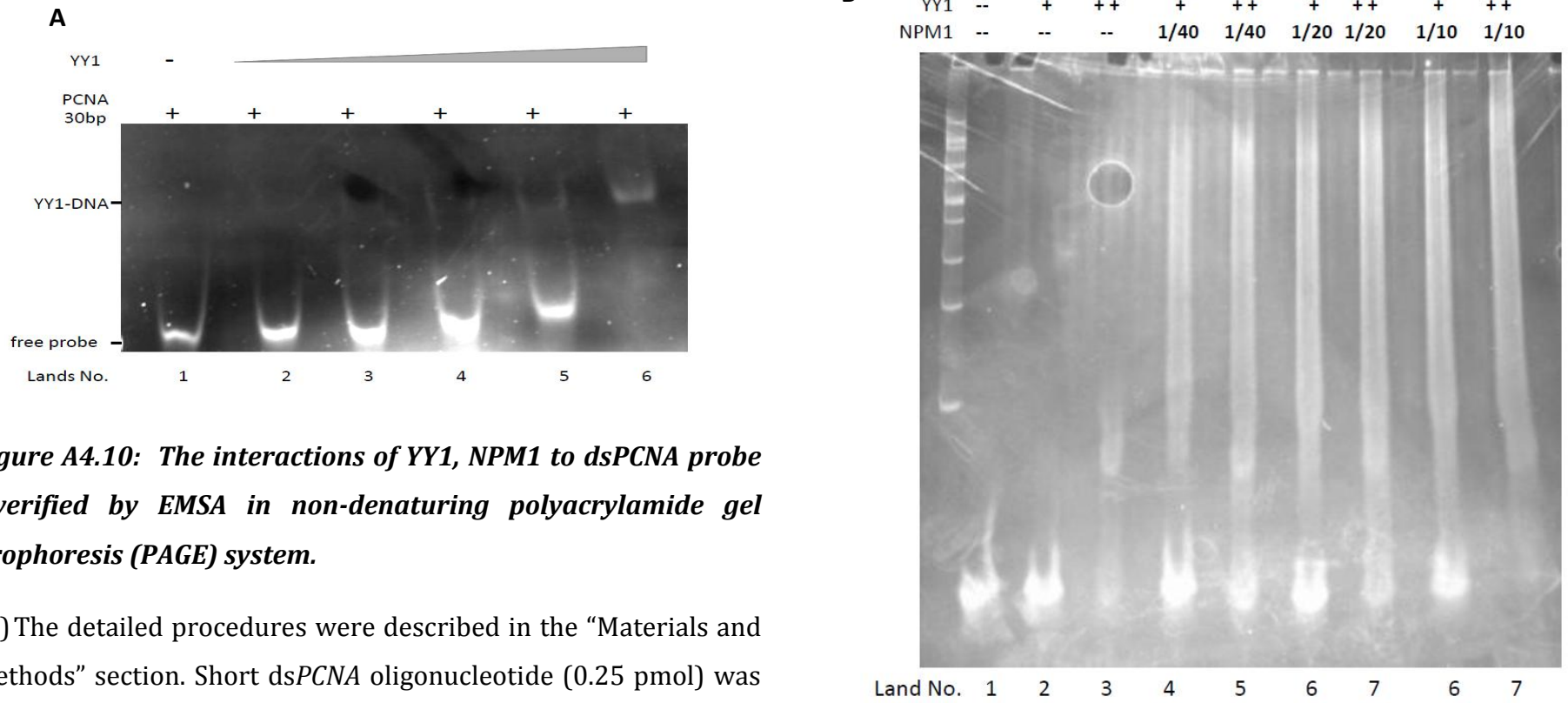


Figure A4.10: The interactions of YY1, NPM1 to dsPCNA probe are verified by EMSA in non-denaturing polyacrylamide gel electrophoresis (PAGE) system.

(A) The detailed procedures were described in the "Materials and methods" section. Short ds*PCNA* oligonucleotide (0.25 pmol) was incubated with recombinant YY1 protein in two-fold increasing amounts (starting concentration is 0.25pmol). The complexes were subsequently fractioned by 7.5% PAGE.

(B) In an alternative multi-component mobility shift assay, final sample complexes were analyzed in 7.5% PAGE. The EMSA of recombinant YY1 protein (0.4 pmol) and short ds*PCNA* oligonucleotide (0.25 pmol) were incubated in constant amounts, and NPM1 C-terminus protein was added in two-fold increasing amounts (starting concentration is 0.25pmol). This resulted in super-shifts in 7.5% PAGE.

A4.5 Discussion

As a multifunctional protein, NPM1 may exert its functions in apoptosis inhibition through modulating other regulatory proteins in addition to PCNA. For example, NPM1 can be activated by cellular hypoxia condition. During hypoxia, NPM1 inhibits hypoxia-induced p53 phosphorylation and interacts with p53 in hypoxic cells. NPM1 inhibits the activation of p53 and dampens p53-mediated apoptotic activation (Li et al., 2004). In addition, NPM1 has been reported to be induced upon UV irradiation. NPM1 overexpression in NIH3T3 cells result in increased cellular resistance to UV-induced DNA damage. This consequence depends on the activation of the DNA repair-associated gene, PCNA, which is upregulated subsequent to NPM1 overexpression after UV irradiation (Wu et al., 2002a; Wu et al., 2002b). Recently, NPM1 has been reported to interact with YY1 in modulating *PCNA* activity (Weng and Yung, 2005).

Here, we uncover the underlying mechanism how NPM1 interacts with YY1, binds to YY1-responsive site and consequently up-regulates PCNA expression. YY1 is a sequence-specific DNA binding protein that binds to the *PCNA* promoter via its responsive site, and NPM1 recruitment might have a synergic effect in enhancing the stability of *PCNA*-YY1 complexes, resulting in up-regulation of PCNA. Recently, reports have indicated that NPM1 can act as a histone chaperone participating in conformational change of nucleosomes and regulating transcription of rRNA genes (Lindstrom, 2011; Murano et al., 2008; Swaminathan et al., 2005). This chaperone activity may enforce more NPM1 binding on *PCNA*-YY1 complexes and subsequently modulate PCNA activity.

Moreover, our attempts to characterize the functional role of NPM1 in PCNA activation reveal that NPM1 and YY1 were recruited to and bound on the YY1 recognition site of the *PCNA* promoter (Figure A4.6). Notably, the YY1 site-mutated *PCNA* promoter retarded the NPM1 binding to promoter region and maintained its basal activity upon NPM1 overexpressed condition (Figure A4.7) This observation suggests two inferences about NPM1-induced PCNA activation. First, NPM1 may bind to other transcription factor binding sites for PCNA activation except YY1 recognition site. Second, NPM1 might also interact with other transcription factors or bind to *PCNA* directly, resulting in PCNA upregulation.

To further verify previous inferences, we performed a series of experiments. The results of NPM1 deletion mutants' experiments showed that C-terminal region of NPM1 was essential for PCNA activation (Figure A4.4B). Remarkably, we further identified the interacting patterns of protein (NPM1 or YY1) -DNA (ds*PCNA* probe) complexes by electrophoresis gel mobility shift assay (EMSA). At the beginning, we used a ds*PCNA* nucleotide probe with 400-bp in length harboring one YY1 recognition site near the transcription start site of the *PCNA* promoter. The data showed that NPM1-ds*PCNA* interacting reaction was similar with that of YY1-ds*PCNA* because two recombinant proteins both bound to long ds*PCNA* probes, resulting in band-shifts in agarose gel (Figure A4.8A&B). This observation confirms that C-terminal domain of NPM1 could interact with DNA molecule. Moreover, the result of multi-component mobility assays observed from figure A4.8B, NPM1 was associated to the YY1-*PCNA* complexes with smeared pattern (Figure A4.8B). This 400bp ds*PCNA* probe might provide

sufficient DNA length and spaces for NPM1 enforcement and DNA folding, resulting in *PCNA* activation.

To verify the interacting reactions of proteins-DNA fragment, we reduced the size of ds*PCNA* probe to 30bp while the short probe still contained a YY1 recognition site. This small ds*PCNA* probe could be used in PAGE-based EMSA experiments. Consistently, recombinant YY1 zinc finger domain still bound to small ds*PCNA* probe via its recognition site (Figure A4.10A); however, the NPM1 C-terminus protein could not make a clear trimolecular complex with YY1 and ds*PCNA* (Figure A4.8.B). Taken together, we suggested that NPM1 attracts and interacts with acidic molecules such as DNA and RNA fragments via its C-terminal domain which is enriched alkaline amino acid residues in sequence. Furthermore, the NPM1-mediated PCNA activation might have a restriction in DNA length because the 30 bp ds*PCNA* probe retarded the NPM1 association to YY1-*PCNA* complex.

In summary, we have obtained a few important findings in this study: (1) NPM1 evoked its acetylation status upon low-dosage of UV irradiation; (2) NPM1 directly bound to ds*PCNA* probe and formed with YY1 and ds*PCNA* a trimolecular complex and this resulted in PCNA upregulation; (3) YY1 site mutated PCNA promoter remained small amount of NPM1 binding for maintaining basal activity of PCNA. Collectively, we conclude that endogenous NPM1 binds to YY1-responsive site and associates with transcription factor YY1 and *PCNA* gene promoter region, and this directly leads to PCNA upregulation in cellular response to UV damage.

Future experiments would be carried out to identify the potential factors that induce NPM1 acetylation, and the function of acetylated NPM1 in PCNA activation. The results and findings are described in the following section.

Part B: The role of post-translational modification of NPM1 for PCNA activation

B4.1 Introduction

Nucleophosmin (NPM1, B23 or NPM) is an abundant nuclear phosphoprotein with multiple functions. NPM1 is involved in various cellular processes including cell cycle regulation, cell differentiation, proliferation and ribosomal synthesis (Lim and Wang, 2006; Okuwaki, 2008). Its expression level and activity are intimately related to carcinogenesis because NPM1 is often found overexpressed or mutated in cancer cells. Recently, studies have indicated that NPM1 is highly expressed in malignant hepatocyte tissues (Grisendi et al., 2006; Yun et al., 2007). Moreover, some environmental stimulations such UV irradiation can affect the protein expression level of NPM1 or result in post-modification of NPM1 (Lin et al., 2010; Okuwaki, 2008; Tawfic et al., 1995). The detailed functions of NPM1 post-translational modification are described in "*section 1.3.8"* of chapter one. In last decade, it has been documented that NPM1 overexpression renders the cells resistant to UV irradiation-induced apoptosis through mediating the tumor suppressor interferon regulatory factor-1 (IRF-1) in NIH3T3 cells (Kondo et al., 1997; Wu and Yung, 2002). Intriguingly, the effect of PCNA upregulation is possibly one of the reasons that NPM1 makes the cells resistant to UV-induced cell death (Wu et al., 2002b). When PCNA is being upregulated, cells increase DNA repair capacity and consequently are resistant to UV-induced cell death.

Proliferating cell nuclear antigen (PCNA) is an important regulatory protein involved in nucleotide excision pathway (NER), which is responsible

for removing bulky DNA, which is induced by low-dosage of UV irradiation. Recent reports have revealed the role of PCNA in carcinogenesis: (1) Elevated expression level of PCNA is highly associated with advanced tumor stages in prostate cancer and prostatic intraepithelial neoplasia (PIN) (Wang et al., 2010b); and (2) PCNA expression level also reveals a statistically significant correlation with NPM1 elevated expression in hepatocellular carcinoma (HCC) tissues when compared with non-malignant hepatocytes and normal liver tissues (Yun et al., 2007). For carcinogenesis, NPM1 has been reported to interact with and impede tumor suppressors p53 and IRF-1, and hence lead to high cell proliferation (Colombo et al., 2002; Kondo et al., 1997; Kurki et al., 2004a). NPM1 also increases DNA repair capacity through activating *PCNA* promoter activity, which is concurrently modulated by YY1 and NPM1 (Weng and Yung, 2005). Remarkably, NPM1 is an important target for anti-cancer therapy.

In part A, we report a novel finding about post-translational modification of NPM1 – acetylation status is evoked upon low-dose UV irradiation. A research group has demonstrated that NPM1 acetylation occurs at particular C-terminal lysine residues of NPM1 and is triggered by histone acetyltransferase (HAT) p300 (Eckner et al., 1994; Giordano and Avantaggiati, 1999; Swaminathan et al., 2005). HAT is a transcription coactivator interacting with various transcription factors involved in various cellular processes, such as differentiation and growth control. *Hasan* et al. (2001a) reported that p300 was colocalized to nucleus speckled structure and might participate in DNA repair synthesis via interacting with PCNA. This

endogenous p300-PCNA complex can promote DNA synthesis *in vitro*. Moreover, p300 can be associated with newly synthesized DNA after low-dosage of UV irradiation. All results suggest that p300 might be an important factor in facilitating PCNA function at DNA lesion sites, although the role of p300 in DNA repair synthesis has not yet been clearly demonstrated (Hasan et al., 2001a). However, p300 also functions as an acetyltransferase to trigger acetylation of all core histones in nucleosomes (Ito et al., 2000a; Ito et al., 2000b). Several lines of evidences indicate that p300 is directly involved in DNA metabolism via forming a complex with and acetylating flag structure-specific endonuclease 1 (Fen1), which is responsible for removing 5′ overhanging flaps in DNA repair and processing the 5′ ends of Okazaki fragments in the lagging strand during DNA synthesis. Acetylated Fen1 reduces its DNA binding affinity and endonuclease activity in impairing flag structure cleavage (Hasan et al., 2001b). Furthermore, p300 could also activate Dna2 (DNA replication helicase 2) through acetylation modification. Dna2 acetylation activates its DNA-dependent ATPase activities and increases its binding affinity for DNA nucleotides (Balakrishnan et al., 2009). We suggest that p300 induces NPM1 acetylation so as to increase its binding affinity with *PCNA* and up-regulate PCNA. Collectively, we would study the functional role of NPM1 in PCNA upregulation upon low-dose UV irradiation. Attempts would be made to verify and elucidate whether p300 triggers NPM1 acetylation upon UV irradiation and whether p300-induced NPM1 acetylation affects *PCNA* promoter activity and leads to changes of DNA repair capacity.

In the previous project (Part A), we conclude that the synergetic role of NPM1 is to stabilize the complex of YY1-ds*PCNA* and hence lead to PCNA upregulation. In Part B, we would show that p300 was directly recruited to the *PCNA* promoter region together with YY1 and NPM1 upon UV irradiation. Site-directed mutation of specific lysine residues of NPM1 mimics NPM1 deacetylation status, leading to the inhibitory effect on *PCNA* promoter activity and subsequently influencing DNA repair capacity.

B4.2 Specific questions to be addressed in Part B are as follows:

Previous study project, we demonstrated that NPM1 revealed post-translational modification upon UV irradiation (Figure A4.1C). The factor triggered NPM1 acetylation is an important issue. Further, whether this factor directly is involved in UV-induced PCNA activation, we have following questions:

1. What regulatory proteins are recruited to *PCNA* promoter region involved in UV-induced PCNA upregulation?
2. Is there evidence that the function of histone acetyltransferase of p300 will lead to post-translational modification of NPM1 upon low-dosage of UV irradiation?

Histone acetyltransferase p300 triggers NPM1 acetylation at particular lysine residues in C-terminal region (Swaminathan et al., 2005). To testify the NPM1 acetylation has transactivation effect on PCNA, the NPM1 acetylation-defective mutants had been generated for *PCNA* gene promoter measurement.

3. Is there evidence that NPM1 mutants mimicking the deacetylated state affect the *PCNA* promoter activity?

4. Is there evidence that NPM1 mutants mimicking the deacetylated state inhibit the transcription of PCNA via reducing its binding affinity with *PCNA* promoter?

Final, to elucidate the functional role of acetylated NPM1 in *PCNA* regulation, the measurement of remaining amount of UV-induced thymine dimer is a critical indicator for DNA repair capacity.

5. Is there evidence that NPM1 mutants mimicking the deacetylated state have inhibitory effect on DNA repair capacity?

B4.3 Materials and methods

The common "materials and methods" are listed and described in chapter three. Here we present the specific materials or methods that are performed in this section.

B4.3.1 Reagents and Antibodies

Anti-p300 polyclonal antibody (pAb, sc-584), anti-YY1 pAb (sc281) and anti-B23 pAb (sc-5564) were purchased from Santa Cruz (Santa Cruz, CA). Anti-PCNA mAb was from BD Bioscience (San Diego, CA), and horseradish peroxidase-conjugated (HRP) anti-mouse IgG antibody and anti-rabbit IgG antibody were from Promega (Madison, MI). Acetyl-lysine pAb (AB3879), acetyl-histone H4 pAb (06-598) and dimethyl-histone H3 (Lys9) pAb (07-441) were purchased from Millipore (VA, USA). To achieve obvious immunoblotting of YY1, the molecular weight of YY1 is similar to the heavy chain of immunoglobulin. A TrueBlot anti-rabbit HRP-IgG antibody was used in the corresponding immunoblotting experiments to avoid masking the signal, due to this antibody only recognizing the native form of the heavy chain of immunoglobulin.

B4.3.2 Plasmid and site-directed mutagenesis

The construction of sip300 plasmid was based on an earlier publication (Sankar et al., 2008). The DNA sequence is 5′-ACCAGATGATGCCTCGAATAA-3′ corresponding to AUG codon of p300 from +2501 to +2521, and contains shRNA sequence directly against p300, subsequently cloned into pSuper_neo vector with EGFP tag.

To further identify which lysine residue of NPM1 C-terminus is important for PCNA activation, an appropriate set of oligonucleotide primers were used for site-directed amino acid substitutions (lysine [K] to arginine [R]). This served to generate the plausible deacetylated lysine residues of NPM1 (Swaminathan et al., 2005) to mimic the constitutive deacetylated NPM1 mutants (K212R, K250R and K257R variants).

First, we used pCR3.1-FLAG-B23 as a PCR template to construct EGFP-tagged plasmid with lysine residues mutation. The cloning procedures and backbone vector is pEGFP-N3-NPM1 which is cloned and used in Part A study (the detailed method description see "section A4.3.2"). The full-length reverse primer with HindIII recognition cutting site as shown in underlined letters (5′-CCCCCC AAGCTTGAAGAGACTTCCTCCACTGCCA-3′) and the forward primers listed below were used to perform PCR for creating a set of oligonucleotide primers which were referred to as the mega-primers with desired mutations (underlined boldface italic letters).

For pEGFP-N3-NPM1-K212R: 5′-ATCAGAATGGAA***G***AGACTCAA AACCATCAT-3′
(pCR3.1-FLAG-NPM1-K212R)

For pEGFP-N3-NPM1-K229/230R:5′-CAAGAATCCTTCA***G***GA***G***ACAGGAAAAAAC T-3′

For pEGFP-N3-NPM1-K250R: 5′-GAAGACATTAAAGCA A***G***AATGCAAGCAAGT-3′
(pCR3.1-FLAG-NPM1-K250R)

For pEGFP-N3-NPM1-K257R:5′-ATAGAAA***G***AGGTGGTTCTCTTCCCAAAGTGGA-3′
(pCR3.1-FLAG-NPM1-K257R)

The amplified PCR products were isolated and purified for next round of PCR. The gel-purified mega-primers and full-length forward primer 5′-CCGCCGCTCG

AGATGGAAGATTCGATGGACATG-3′ with XhoI recognition cutting site were used to amplify the full-length NPM1 cDNA containing introduced mutated sequences. Notably, to construct pEGFP-N3-NPM1-K292R, full-length forward primer and reverse primer 5′-CCCCCCAAGCTTGAAGAGAC***C***TCCTCCACTGCCA-3′ with desired mutation (underlined boldface italic letters) were used to perform PCR (HindIII cutting site as underlined letters). Similarly, this amplified NPM1 PCR fragment with K292R mutation was purified by gel purification. Next, all purified PCR products were cloned into HindIII and XhoI sites of pEGFP-N3-NPM1 as mentioned in "section A4.3.2". The orientation of pEGFP-N3-NPM1 lysine mutants was analyzed by restriction enzyme mapping and full-length nucleotide sequencing.

The advantage of EGFP-tagged NPM1 deacetylated mutants is easier recognized by fluorescent microscopy after transient transfection. However, the EGFP site is large that might mask near DNA sequences and affect transcriptional activity of interested gene. Based on this, we constructed FLAG-tagged of NPM1 plasmids with desired lysine residues mutations by using similar procedures and primers as mentioned above. Briefly, we kept using full-length NPM1 cDNA in plasmid pCR3.1-FLAG-NPM1 as a PCR template. The full-length reverse primer and forward primers for cloning K212R, K250R and K257R variants listed above were used to perform PCR for creating the mega-primers with desired mutations. The amplified PCR products were isolated and purified for next round of PCR. The gel-purified mega-primers and full-length forward primer were used to amplify the full-length NPM1 cDNA containing introduced mutated sequences. Last, all purified PCR products were cloned into BsrGI and

BglII sites of pCR3.1-FLAG-B23. The orientation of pCR3.1-FLAG-B23 lysine mutants was analyzed by restriction enzyme mapping and full-length nucleotide sequencing.

B4.3.3 Recombinant proteins: cloning, expressions and purifications

The recombinant NPM1 proteins with acetylated-site mutation were generated by using an appropriate set of oligonucleotide primers for site-directed amino acid substitutions (lysine [K] to arginine [R] or glutamine [Q]). The primer sequences for cloning recombinant NPM1protiens (K212R, K250R, K257R, K212Q, K250Q and K257Q) are listed as follows:

For K212R: 5′-GAATGGAAGAGACTCAAAACC-3′(sense),

For K250R: 5′-CATTAAAGCAAGAA TGCAAGC-3′ (sense),

For K257R: 5′-CAAGTATAGAAAGAGGTGGTTC-3′ (sense),

For K212Q: 5′-GAATGGA CAAGACTCAAAACC-3′ (sense),

For K250Q: 5′- CATTAAAGCACAAATGCA AGC-3′ (sense),

For K257Q: 5′-CAAGTATAGAAAGAGGTGGTTC-3′ (sense).

All PCRs used the T7 terminator sequence as the corresponding reverse primer and the sequences of T7 terminator is as follows: 5′-TGCTAGTTATTGCTCA GCGG-3′. Subsequently, we used the full-length pCR3.1-FLAG-B23 as the PCR template for generating different recombinant NPM1 protein variants. The cloning steps of the recombinant NPM1 protein variants, proteins expression and purification were similar and described in detail in "*section 3.8 of chapter three*".

B4.3.4 Electrophoresis gel mobility shift assay (EMSA)

We performed EMSA in PAGE system by using ds*PCNA* oligonucleotides with 30-bp. The *PCNA* oligonucleotide sequence is 5′-GCGGACGCGGCGGCATTAAACGG TTGCAGG-3′, and anneals with complementary *PCNA* oligonucleotide by heating at 95°C for 10 min and then slow cooling to room temperature. The binding reactions contained a constant amount of the short ds*PCNA* probe at 0.25 pmol, and the protein concentration varied from 0 pmol to 2 pmol for NPM1 C-terminus, different NPM1 acetylated [Q] and deacetylated [R] variants and YY1 zinc finger domain. All reactions were prepared on ice in the EMSA buffer (20 mM HEPES pH 7.5, 40 mM KCl, 10 mM $MgCl_2$, 10 mM $CaCl_2$, 10 mM β-mercaptoethanol, 8% glycerol, 0.25 mg/ml bovine serum albumin), and the reaction mixture was gently mixed and incubated on ice for 15 min. After incubation, the reaction mixtures were then loaded into a 7.5% (36.5:1 acrylamide:bisacrylamide) polyacrylamide gel (PAGE gel pre-run at 85 V, 1 h) in 1x TBE (Tris-borate EDTA) buffer and run at 4°C for 4 h at 200 V. After electrophoresis, the resulting bands were visualized with the Kodak digital camera 4000 mm system.

Ethidium bromide (EtBr) was added into the nondenaturing PAGE gel for image capture instead of using radioactive labeling. In addition, we did not add bulk carrier DNA such as poly (dI: dC) for the binding reaction because all protein samples were purified by, for example, HPLC.

B4.3.5 Measurement of thymine dimer

To estimate the DNA repair capacity of the acetylation-defective NPM1 constructs *in vivo*, the amounts of thymine dimers (TMD) were measured in UV-irradiated cells by enzyme-linked immunosorbent assay (ELISA) as described previously (Lin et al., 2010; Zhai et al., 2005). The antibody against thymine dimers (NB600-1141) was purchased from Novus. Briefly, mouse NIH 3T3 cells in 10-cm culture dishes were transiently and separately transfected with 5 μg of each acetylation-defective NPM1 construct (K212R, K250R, and K257R) by LipofectAMINE reagent (Invitrogen). After 24 h transfection, cells were exposed to UV irradiation (30 J/m^2) and harvested immediately (time 0 h) and at specific post-UV treatment time points for DNA extraction to measure the amounts of thymine dimers. DNA was extracted from the cells harvested at the indicated time points after UV irradiation and bound to 96-wells microplate (200 ng/well in triplicate) which was precoated with protamine sulfate (Sigma). Subsequently, the plate was preincubated with 5% (v/v) FBS in PBS to avoid nonspecific binding of antibodies, and then incubated with 100 μl of desired antibody (TMD antibody, 1:1000 dilution), which was followed by incubation with 100 μl of goat anti-mouse IgM conjugated with peroxidase (Amersham Biosciences). Finally, thymine dimers were detected using color reaction with absorbance at 490 nm by peroxidase. Absorbance was measured using an ImmunoReader (Molecular Devices, Sunnyvale, CA). The mean values of three wells were calculated, and the background value without DNA was subtracted. The results were expressed as means ± SD.

B4.3.6 Isothermal titration calorimetry (ITC)

Biological macromolecular recognitions (such as protein-DNA, antibody-antigen, and hormone-receptor) require energy transfer to drive the interaction and stabilize the conformational change. The energy transfer provides the base for the thermodynamic characterization biophysically. Isothermal titration calorimetry (ITC) is used to quantitatively measure the thermodynamic properties of macromolecular interactions between ligands such as proteins and peptides, or proteins and other nucleotides (Pierce et al., 1999). Heat capacity is a significant feature for structural information. For instance, a decrease in the exposure of hydrophobic surface results in a reduction in heat capacity. Endothermic and exothermic reactions are manifested by the heat absorption or release during the titration (Moyzis et al., 1988). ITC can determine all binding parameters (binding constants [K_B], reaction stoichiometry [n], enthalpy [ΔH] and entropy [ΔS]), thereby providing a complete thermodynamic profile of the macromolecular interaction for calculating the binding affinities and elucidating the mechanism of ligand interaction in a single experiment. We used ITC to compare the binding affinities between recombinant acetylation-defective NPM1 variants [R] and ds*PCNA* oligonucleotide or between acetylation-constitutive NPM1 variants [Q] and ds*PCNA* oligonucleotide. For YY1 zinc finger domain (YY1)/DNA interaction, titration was performed in titration buffer [1.5 mM $ZnCl_2$, 100 mM Bis-Tris, 50 mM KCl, 100 mM NaCl, 20 mM $MgCl_2$, pH 7.0]. YY1 (200 μM) was titrated into titration buffer containing 30-bp ds*PCNA* oligonucleotide (10 μM). For NPM1 (wt-B23)/DNA interaction, titration was performed in the titration buffer without $ZnCl_2$ supplement. The assay conditions of wild-type NPM1 (wt-B23, 200 μM) was described above. For comparing the binding

affinities of acetylation-defective NPM1 constructs [K212R, K250R and K257R] and acetylation-constitutive NPM1 constructs [K212Q, K250Q and K257Q], these NPM1 constructs (20 μM each of [Q] and [R] constructs) were incubated with ds*PCNA* (10 μM) at room temperature for 30min, and subsequently the recombinant YY1 zinc finger domain (100 μM) was titrated into these pre-incubated titration buffer.

Some detailed descriptions about common or general "materials and methods" have seen in chapter three

B4.4 Results

B4.4.1 PCNA promoter activity is inhibited in p300 knockdown condition

According to *Hasan* et al. (2000a), p300 is involved in chromatin remodeling at DNA damage lesions to augment PCNA function in the DNA repair pathway, particularly in nucleotide excision repair (NER). To characterize the functional role of p300 in PCNA activation, we generated a si-p300 plasmid (referred as *pSuper.neo*-GFP-siP300) to knockdown the endogenous p300 expression. First, we tested our p300 knockdown effect in transient transfection and then detected the p300 expression level by using western blot analysis (the detailed method description see "section 3.4 in Chapter three"). The data showed that the endogenous p300 expression level could be attenuated by si-p300 plasmid in dose-dependent manner (Figure B4.1A). Next, we sought to determine the *PCNA* promoter activity in p300 knockdown condition. NIH3T3 cells were transiently co-transfected with pGL3-wt-PCNA reporter and *pSuper.neo*-GFP-siP300 in various concentrations. After forty-eight hours transfection, cells were harvested for reporter gene assay. The data revealed that *PCNA* promoter activity was decreased in the context of p300 knockdown condition (Figure B4.1B). We suggested that PCNA promoter activity might be regulated by p300 and NPM1 mutually.

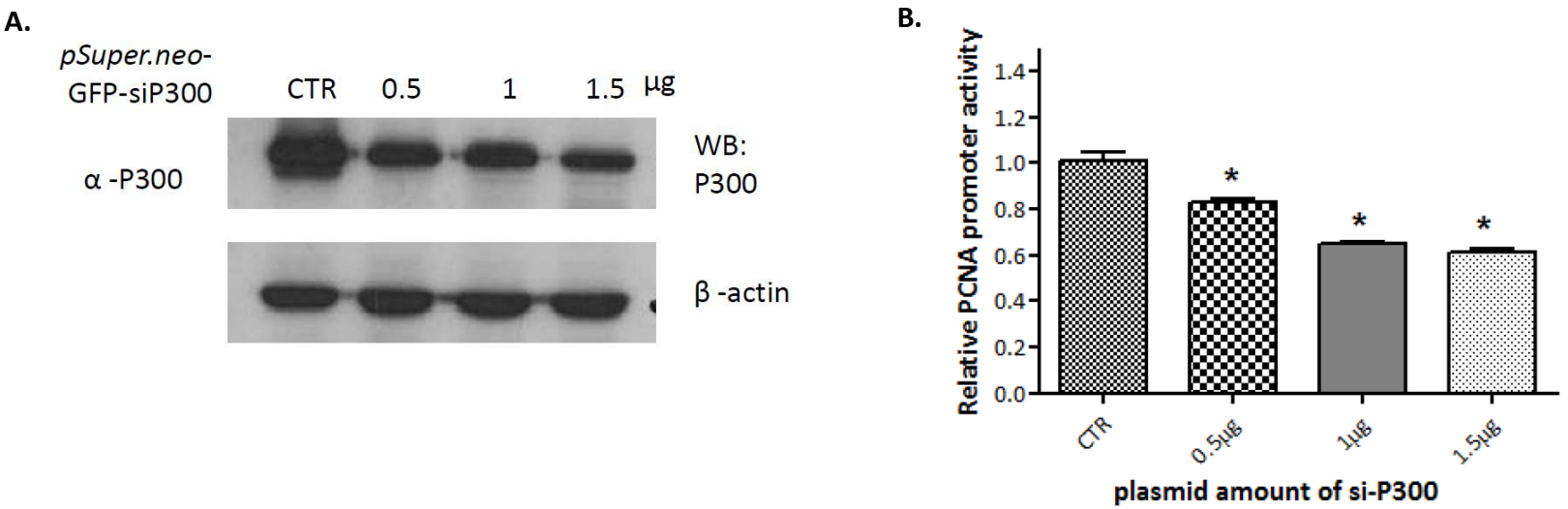


Figure B4.1: Decreased PCNA promoter activities in p300 knockdown condition.

(A) NIH3T3 cells were transiently transfected with *pSuper.neo*-GFP-siP300 (0 or control, 0.5, 1 and 1.g μg) and harvested at 48 h after transfection. Equal amounts of lysate proteins were separated by 7.5% SDS-PAGE and subjected to western blotting with the antibodies indicated.

(B) NIH3T3 cells were co-transfected with pGL3-wt-PCNA reporter gene and *pSuper.neo*-GFP-siP300 (amount as indicated) and harvested at 48 h after transfection for reporter assay (see *Materials and methods* in chapter three). The promoter activities of *PCNA* were measured and presented as means of triplicates ± SDs. *$P < 0.05$.

B4.4.2 ***Increased occupancy of NPM1, p300 and YY1 on PCNA gene promoter upon UV irradiation***

In the previous study (Part A), we concluded that NPM1 bound to the YY1 recognition site of the *PCNA* promoter region and this resulted in PCNA upregulation. Recent reports have also demonstrated the reciprocal modulations of PCNA and p300 activities by each other *in vivo* (Cazzalini et al., 2008; Hong and Chakravarti, 2003; Naryzhny and Lee, 2004; Swaminathan et al., 2005). In addition, p300 is recruited to DNA damage lesions so as to facilitate PCNA function (Hasan et al., 2001a). Next, we sought to further delineate the underlying mechanism of UV-induced NPM1 overexpression that resulted in PCNA activation.

To verify the recruitment of NPM1, YY1 and p300, we performed chromatin immune-precipitation (ChIP) with NPM1, YY1 and p300 antibodies to determine the relative promoter binding of these proteins after UV irradiation. The experimental results showed that there was a significant time-dependent increase in the recruitment of NPM1 and p300 to the *PCNA* gene promoter after UV irradiation (Fig B4.2A). Similarly, the binding of YY1 to the *PCNA* gene promoter was observed at 6 h post-UV treatment (Fig B4.2A). These data observed from figure B4.2A, further quantitative their relative binding on PCNA promoter region, the Real-time PCR were performed and showed in figure B4.2B. The results from Real-time PCR quantification demonstrated that NPM1 and p300 significantly increased their binding to the *PCNA* gene promoter after UV treatment. That was consistent with our previous arguments regarding the intimate relationship between PCNA and p300. The recruitment of YY1 was

shown at 6 h post-UV treatment and sustained to 9 h. These data indicated that NPM1 and p300 were UV-induced immediate response genes to turn on the expression of downstream genes for rapidly repairing UV-induced DNA damage. The functional role of YY1 might facilitate NPM1 binding to the *PCNA* gene promoter and extend *PCNA* gene activation.

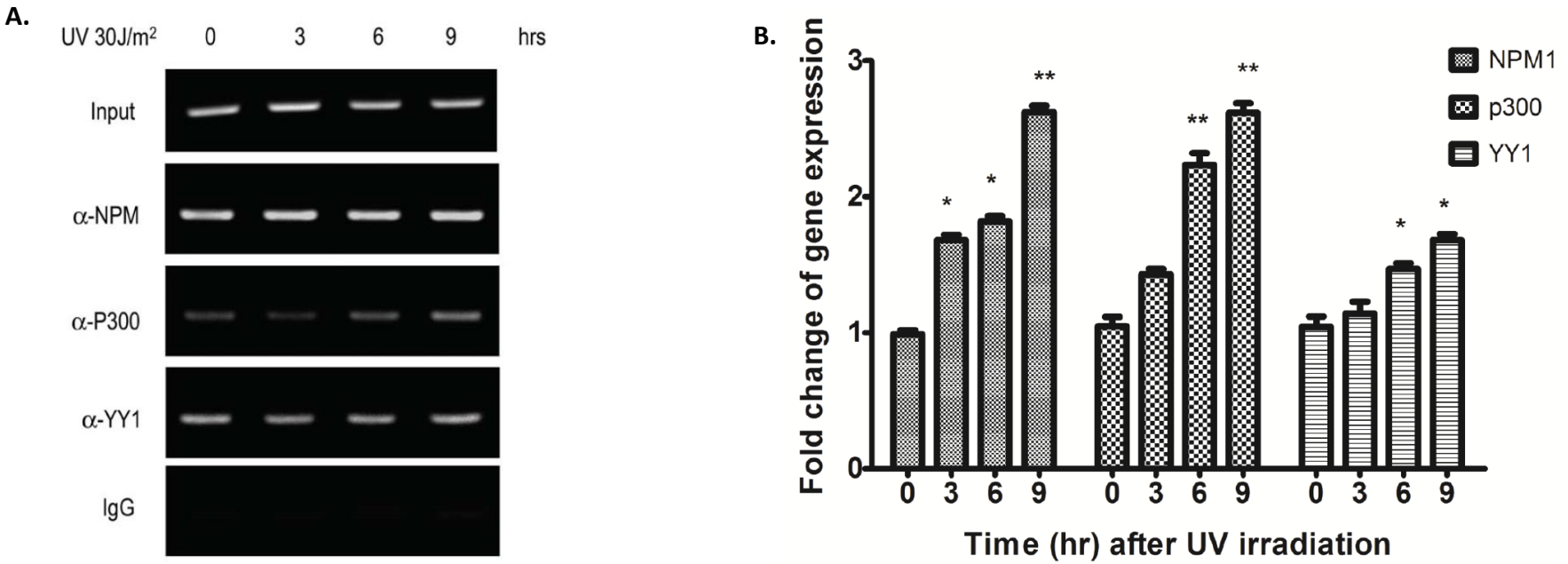


Figure B4.2: Increased occupancy of NPM1, p300 and YY1 on PCNA promoter in cellular response to UV irradiation

(A) NIH3T3 cells were grown to 80% confluence in 10-cm cultural dish and exposed to UV irradiation (30 J/m^2). Non-irradiated and irradiated cells were harvested for ChIP assay (see *section 3.7* of chapter three) at the indicated post-treatment time points. Bound DNA was extracted and then subjected to polymerase chain reaction (PCR) amplification using primers corresponding to the *PCNA* gene promoter sequence (listed in *Table3.1* of chapter three). After 30 cycles of amplification, PCR products were run on 2% agarose gel.

(B) Bound DNA was subjected to quantitative RT-PCR analysis as described in *section 3.6 of chapter three* to quantify the binding of NPM1, YY1 and p300 to the *PCNA* promoter (*$P < 0.05$, **$P < 0.0001$). Relative promoter binding, expressed as normalized ratio of IP to input, are shown (the values of the 0 hr post-UV treatment group are represented as 1).

B4.4.3 PCNA promoter activity is inhibited by NPM1 acetylation-defective mutants

Histone acetyltransferase p300 is able to acetylate four histones and other non-histone proteins for chromatin remodeling or activation of downstream genes (Balakrishnan et al., 2009; Ito et al., 2000b; Naryzhny and Lee, 2004; Ogryzko et al., 1996). NPM1 is one of the non-histone proteins acetylated by p300, which recognizes particular lysine residues on the C-terminus of NPM1 to enhance the histone chaperone ability of NPM1 (Swaminathan et al., 2005). In part A, we had a novel finding that NPM1 was acetylated upon low-dose UV irradiation (Fig A4.1C). We suggested that p300 induced NPM1 acetylation, which in turn led to chromatin remodeling to enable *PCNA* gene activation. Next, we sought to verify the effects of NPM1 acetylation on *PCNA* gene activation.

First, we constructed several NPM1 deacetylated mutants, in which specific C-terminal lysine [K] residues were substituted by arginine [R] to mimic NPM1 deacetylation. HeLa cells were co-transfected with a *PCNA* reporter gene and wild-type NPM1 (B23EGFP or Flag-NPM1), or different NPM1 deacetylated mutants (EGFPK212R, EGFPK229/230R, EGFPK250R, EGFPK292R, flagK212R, flagK250R and flagK257R). The activity of the *PCNA* gene promoter was measured at 24 h post-transfection. *PCNA* gene promoter activity was highly increased in NPM1 overexpression condition (Fig B4.3A&B). However, Figure B4.3A shows that, in the presence of NPM1 deacetylated mutants, *PCNA* promoter activity dropped to around 40% of that of the NPM1 overexpressed groups (B23EGFP). Remarkably, since lysine residue K292 in NPM1 cannot be acetylated *in vivo* condition, we then used other lysine residues to measure *PCNA*

promoter activity in similar experimental condition (Fig B4.3B). *PCNA* promoter activity was abolished in presence of NPM1 deacetylated mutants (flagK212R and flagK250R). Interestingly, flagK257R didn't reveal similar inhibitory effect on *PCNA* promoter activity when compared with other deacetylated mutants. These data indicate that deacetylated NPM1 mutants have an inhibitory effect on *PCNA* promoter activity. Similarly, NPM1 acetylation might have an activating effect on *PCNA* promoter. This has been proved by previous experiments that UV-induced NPM1 overexpression and increased acetylation status subsequently lead to *PCNA* gene activation (Fig A4.1, A4.2 & B4.2).

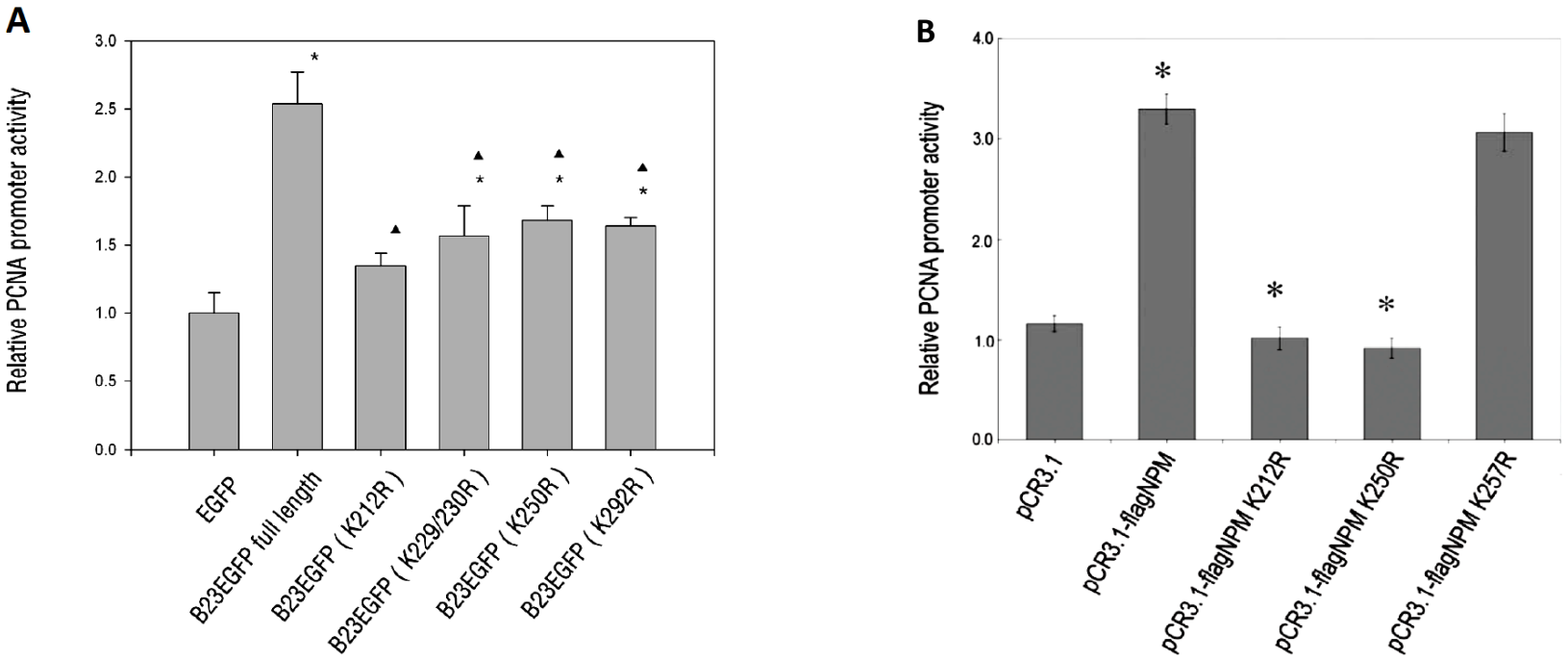


Figure B4.3: PCNA promoter activities are attenuated by exogenous NPM1 acetylation-defective mutants.

(A) HeLa cells were transfected with control vector (pCR3.1) or the indicated EGFP-tagged NPM1 (B23EGFP) acetylation-defective constructs (K212R, K229/230R, K250R and K292R represent site-directed mutants in which the respective lysine residues were altered to arginine), along with *PCNA* reporter. After 24hr transfection, cells were harvested and performed in reporter gene promoter assay (see "*section 3.6 of Chapter Three"*). The values are the averages of three independent sets with means of triplicates ± SD. **P*<0.05.

(B) Transiently transfection was performed in HeLa cells with the indicated Flag-tagged NPM1 deacetylated mutants. Assay conditions are as described for panel A.

B4.4.4 In vitro association complex with NPM1, YY1 and dsPCNA

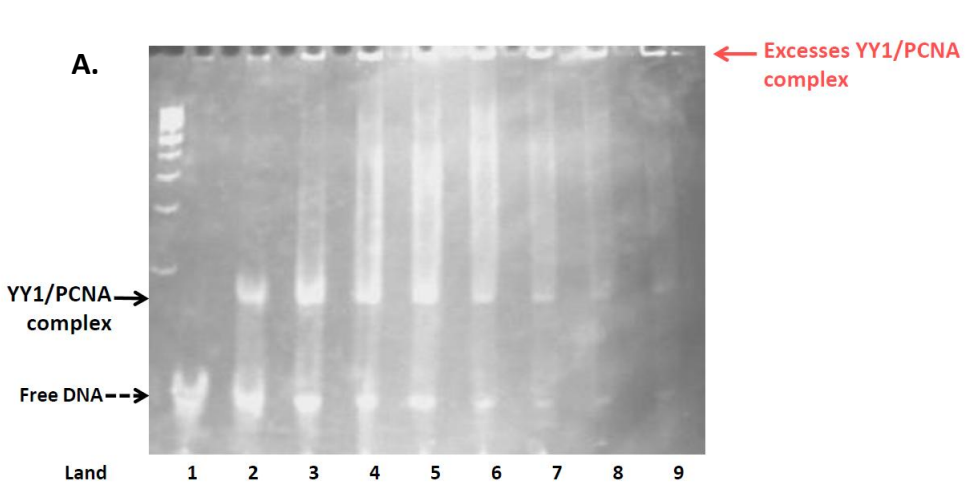
To gain further insight into the protein-DNA interactions leading to PCNA upregulation, we tested whether various NPM1 post-translational modification forms (site-directed amino acid substitutions for changing lysine [K] to arginine [R] mimicking the acetylation-defective state of NPM1; lysine [K] to glutamine [Q] mimicking the acetylation-constitutive state of NPM1) have different interacting affinity to ds*PCNA* oligonucleotide and YY1 zinc finger domain when compared with that of recombinant NPM1 C-terminus (wt-B23) by using electrophoresis gel mobility shift assay (EMSA) and isothermal titration calorimetry (ITC). The detailed procedures of EMSA and ITC are described in "*section B4.3"*; the sequence of ds*PCNA* oligonucleotide probe is listed in "*section 3.9 of chapter three*".

First, we tested our recombinant extract of YY1 zinc finger domain by EMSA and ITC with the 30bp ds*PCNA* oligonucleotides probe to verify the sequence-specific interaction between YY1 and the ds*PCNA* probe that contained a YY1 recognition site. We observed that recombinant YY1 zinc finger domain gave distinct band shits with *dsPCNA* probe in 7.5% non-denaturing polyacrylamide gel (Fig B4.4A). Addition of YY1 protein in increasing amounts to the binding reaction with ds*PCNA* probe resulted in a shift, demonstrating that YY1 zinc finger domain was able to bind to this 30bp ds*PCNA* probe. Notably, excess recombinant YY1 zinc finger domain protein formed aggregates and remained in the wells of gel. That might be self-interaction of YY1 zinc finger domain results in YY1 aggregation. For protein-DNA/protein interactions, ITC can provide a quantitative measure of the affinity (K_d), entropy (ΔS) and enthalpy (ΔH) by measuring the heat absorption or release while a interested protein is titrated

into a solution with a macromolecule (Parker et al., 2009). Here, we performed ITC to determine the stoichiometry and thermodynamic parameters for YY1 zinc finger domain binding to the short ds*PCNA* probe (Fig B4.4B). Form a fit to the binding isotherm generated from titration of YY1 zinc finger domain into a solution containing ds*PCNA* probe, the following parameters were calculated for the reaction at room temperature (23-25°C): an endothermic ΔH of -1.711×10^{11} ± 1.52.5 Kcal/mol^{-1}, an apparent equilibrium association constant (Ka) of 2.11×10^{3} ± 7.83×10^{3} M^{-1} and a stoichiometry of 1.52×10^{-4} ± 13.5 YY1: ds*PCNA*. The titration profile suggests that YY1 can interact with ds*PCNA* specifically, although the binding affinity of this interaction cannot be readily calculated due to the weak nature of the interaction. Based on these values, there is a slight heat capacity change for YY1 binding to ds*PCNA* probe. These data pointed out the interaction of YY1-ds*PCNA* probe is sequence-specific association.

Next, we also examined the interaction between recombinant NPM1 C-terminal protein region (as referred to wt-B23) and 30-bp ds*PCNA* oligonucleotide probe in EMSA and ITC system. We did not observe obvious band shifts in our EMSA results. However, addition of wt-B23 in two-fold increasing amounts to the binding solution with ds*PCNA* probe resulted in quite negligible and smeared band shifts, particularly at high concentration of wt-B2 (Fig B4.5A, lane 5 to lane 9). There might be a sequence-nonspecific association between wt-B23 and ds*PCNA* probe. The stoichiometry and thermodynamic parameters for wt-B23 binding to ds*PCNA* probe were determined by ITC (Fig B4.5B). However, there was no significant change in the heat capacity for wt-B23 binding to dsPCNA probe. Collectively, these data confirm prior reports that YY1 is a sequence-specific DNA binding protein recognizing its binding site on

ds*PCNA* probe, and NPM1 is a RNA/DNA interacting protein via its electrical attraction ability.



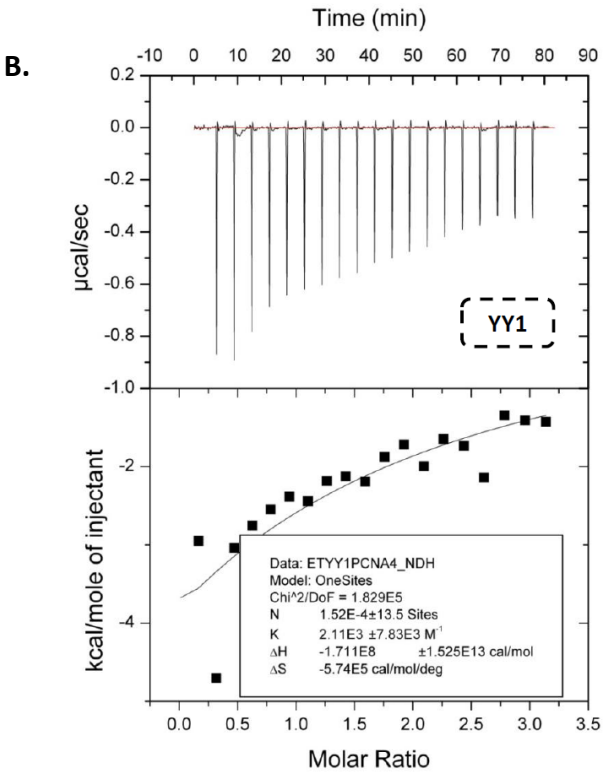
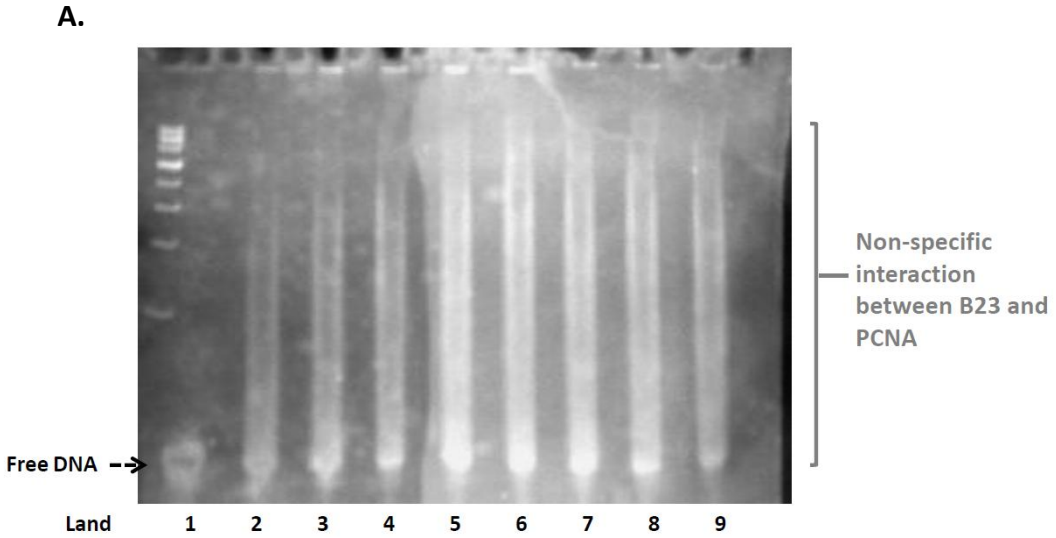


Figure B4.4: Interaction between recombinant YY1 zinc finger domain protein and dsPCNA oligonucleotide probe

(A) EMSA showing the effect of binding between YY1 and ds*PCNA* probe. Recombinant YY1 protein in 2-fold increasing amounts (starting concentration from 0pmol to 2pmol) was incubated with ds*PCNA* probe at 4°C for 15 min. The DNA-YY1 protein complexes were resolved on a 7.5% non-denaturing polyacrylamide gel as described in "*section B4.3.4 of materials and methods*".

(B) ITC titration of YY1 zinc finger domain to ds*PCNA* probe. Data were fit using software to yield thermodynamic parameter (ΔH) $-1.711\times10^{11} \pm 1.52.5$ Kcal/mol^{-1}, equilibrium association constant (Ka) $2.11\times10^{3} \pm 7.83\times10^{3}$ M^{-1} and stoichiometry (n) $1.52\times10^{-4} \pm 13.5$ YY1: ds*PCNA*. The titration condition was described in "*section B4.3.5 of materials and methods*".



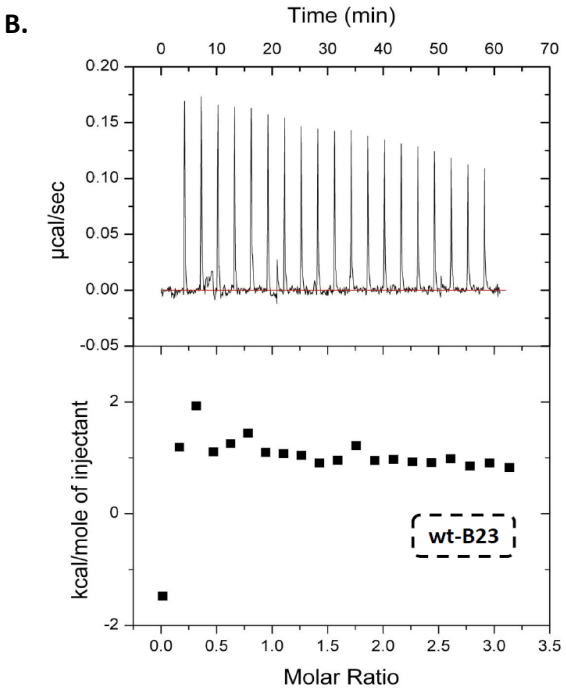


Figure B4.5: Interaction between recombinant NPM1 C-terminus to dsPCNA oligonucleotide probe

(A) EMSA results show the association pattern of NPM1 C-terminal protein (B23) to ds*PCNA* probe. The NPM1 protein in 2-fold increasing amounts (starting concentration from 0pmol to 2pmol) was incubated with dsPCNA at 4°C for 15min. Assay conditions are similar to those described in panel A of Fig B4.4.4.

(B) Wild type-NPM1 (wt-B23) C-terminal titration (0–200µM) was titrated into a solution containing the double-stranded 30-bp *PCNA* oligonucleotide (10 µM). For a detailed description of ITC see "*section B4.3.5 of materials and methods*".

B4.4.5 ***Binding affinity of acetylated and deacetylated NPM1 to dsPCNA are verified by ITC and EMSA***

We have performed EMSA and ITC experiments to measure the binding affinity of deacetylation-mimic [R] and acetylation-mimic [Q] NPM1 constructs to ds*PCNA* probe for supposition verification. Our EMSA results cannot clearly distinguish the differences of binding affinities between NPM1 acetylation-defective mutants and acetylation-constitutive mutants. All of recombinant NPM1 mutants ([Q] and [R]) showed similar association pattern to ds*PCNA* probe (Fig B4.6). Notably, K257Q seems preferred to aggregate by itself that might reduce its binding association to ds*PCNA* probe and thus lose its inhibitory effect on *PCNA* gene promoter activity (Fig B4.6C& B4.3B). We suggested that lysine 257 residue of NPM1 might be important for the structural stability of NPM1.

We sought to design a competition experiment to determine whether the ternary YY1-NPM1-ds*PCNA* complex formation led to upregulation of the *PCNA* gene promoter activity. Since the binding affinity of various NPM1 mutants for ds*PCNA* probe was crucial for PCNA upregulation, we performed ITC to measure the thermodynamic parameters for YY1: NPM1/ds*PCNA* binding. In the first experiment, recombinant NPM1 C-terminus (wt-B23) was pre-incubated with the 30-bp ds*PCNA* oligonucleotide probe at 4°C for 15 min, and YY1 zinc finger domain was subsequently titrated into the reacting solution for performing ITC (Fig B4.7G). The prior ITC results for NPM1-ds*PCNA* binary complex showed that titrating NPM1 into ds*PCNA* probe solution presented a slight endothermic reaction (Fig B4.5B). However, when YY1 was titrated into the solution

containing ds*PCNA* probe and NPM1 protein, there was only negligible change in the heat capacity (Fig B4.7G). These data indicate that the YY1 recruitment may loosen the chromatin structure and the stable association between NPM1 and ds*PCNA* probe to recruit more NPM1 protein to bind to the promoter region. Moreover, in NPM1-induced PCNA activation, YY1 may act as an indirect activator by attracting other transcription activating factors to fasten *PCNA* promoter and NPM1 together and initiate PCNA activation.

Next, we performed similar experiments to compare the binding affinity of various NPM1 mutants for ds*PCNA* probe (Fig B4.7A to B4.7F). Our ITC data showed that NPM1 deacetylation-mimic mutants ([R] mutants) and acetylation-mimic mutants ([Q] mutants) have similar binding affinity to 30-bp ds*PCNA* probe. At the beginning, we suggested that NPM1 deacetylation-mimic mutants [R] might be strongly associated with ds*PCNA* oligonucleotide probe. Consequently, the recruitment of YY1 was impeded and the *PCNA* gene promoter activity was thus decreased (Figure B4.3). Conversely, the interaction between ds*PCNA* oligonucleotide probe and NPM1 acetylation-mimic mutants [Q] might be in a quite weaker association, and therefore YY1 binding to the *PCNA* promoter region was enhanced and led to increasing *PCNA* promoter activities. However, these unexpected results could not support our hypotheses. That might be the length of 30-bp dsPCNA probe which is too short for protein occupancy and enforcement. That was why we could not observe the obvious heat capacity changes while recombinant YY1 zinc finger domain was titrated into the solution containing ds*PCNA* probe and NPM1 mutated proteins. The other possibility might rely on recombinant proteins. Because all deacetylated

acetylated NPM1 protein are not full length constructions which are only expressed in C-terminal domain. The incompletely recombinant proteins may lost their interacting and functional activity to associate with other molecules.

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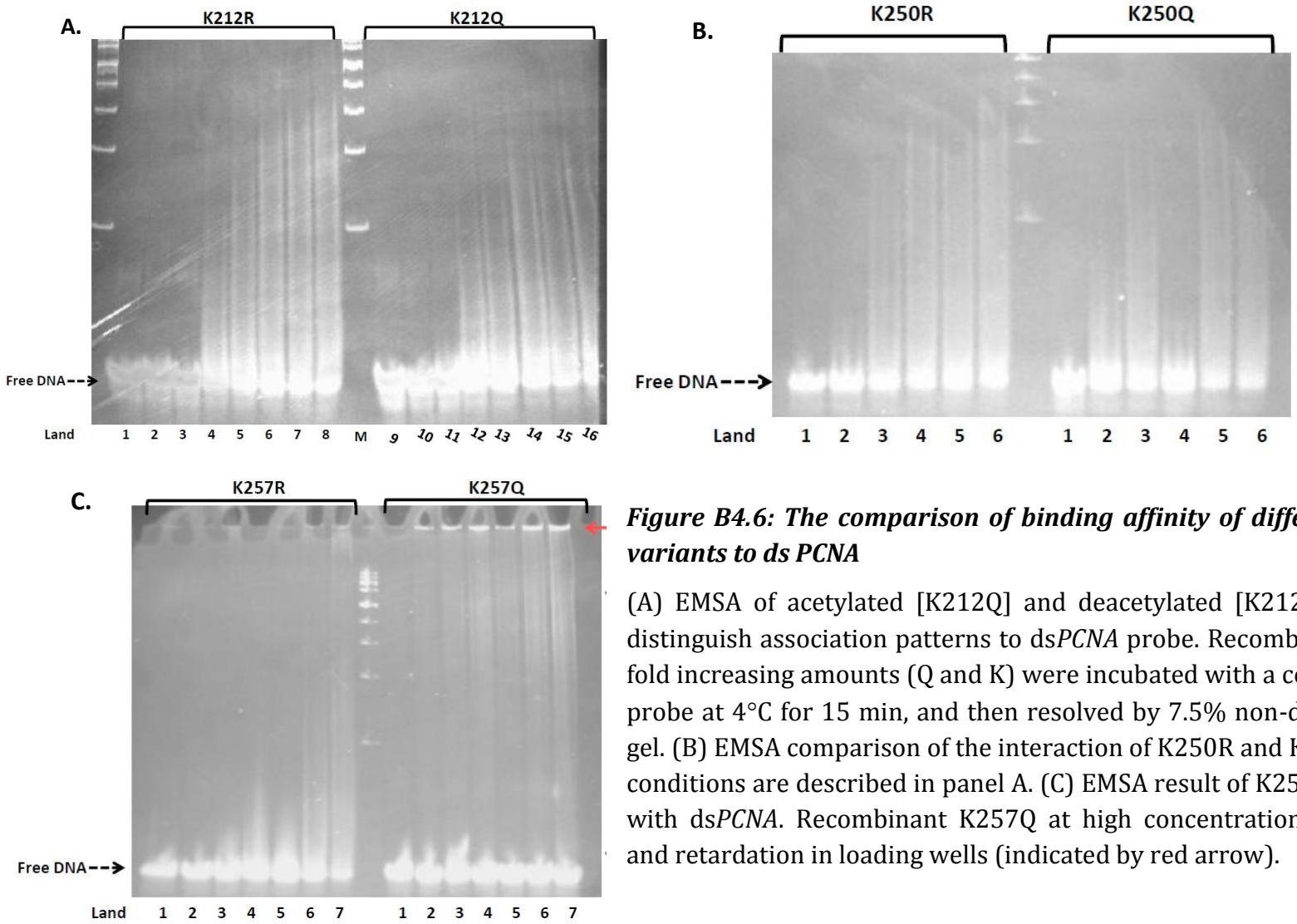
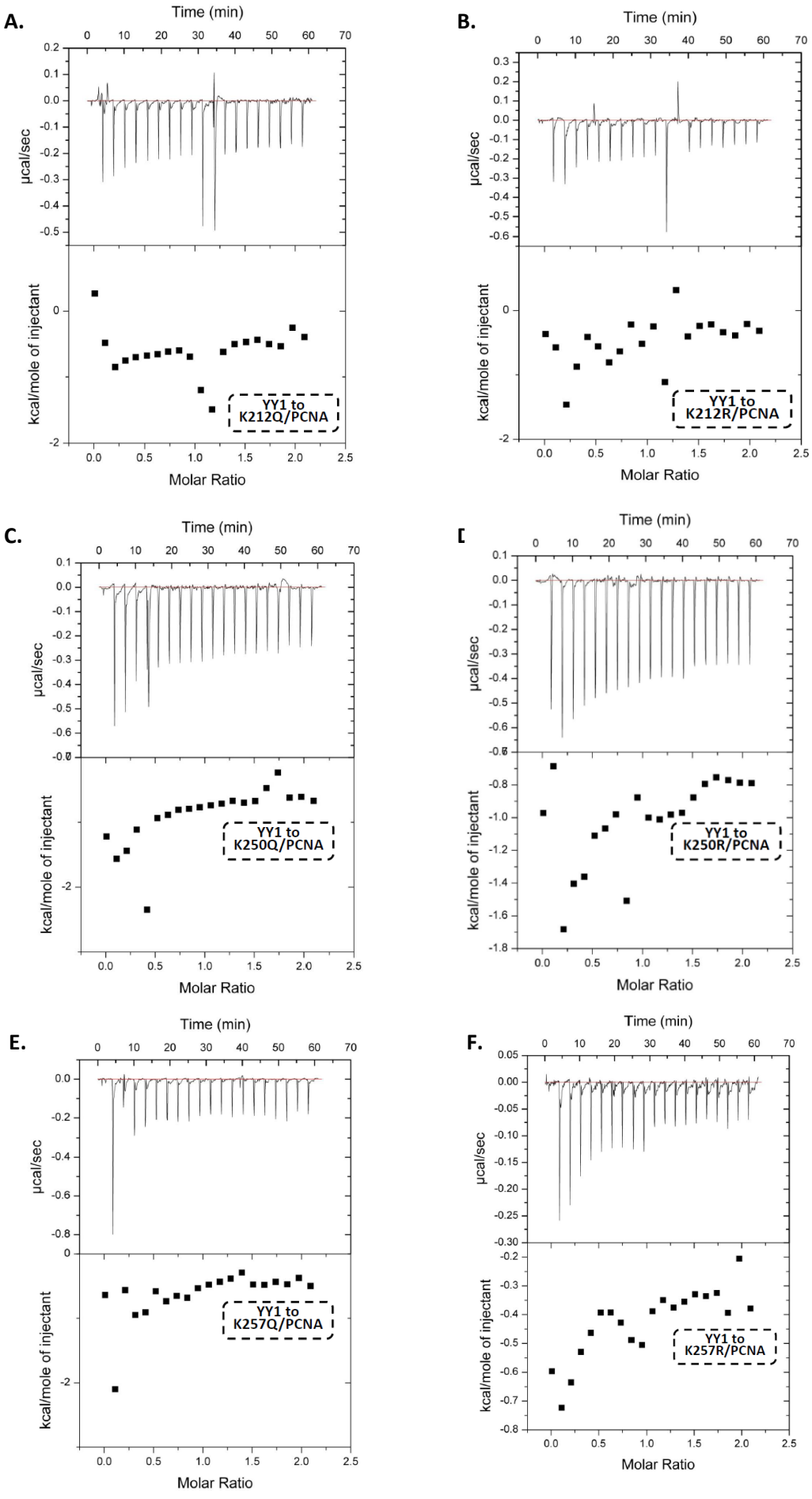


Figure B4.6: The comparison of binding affinity of different NPM1 modification variants to ds PCNA

(A) EMSA of acetylated [K212Q] and deacetylated [K212R] NPM1 mutants try to distinguish association patterns to ds*PCNA* probe. Recombinant NPM1 variants in 2-fold increasing amounts (Q and K) were incubated with a constant amount of ds*PCNA* probe at 4°C for 15 min, and then resolved by 7.5% non-denaturing polyacrylamide gel. (B) EMSA comparison of the interaction of K250R and K250Q with ds*PCNA*. Assay conditions are described in panel A. (C) EMSA result of K257R and K257Q interacting with ds*PCNA*. Recombinant K257Q at high concentration showed self-aggregation and retardation in loading wells (indicated by red arrow).


A.
Time (min)
µcal/sec
kcal/mole of injectant
YY1 to K212Q/PCNA
Molar Ratio
B.
Time (min)
µcal/sec
kcal/mole of injectant
YY1 to K212R/PCNA
Molar Ratio
C.
Time (min)
µcal/sec
kcal/mole of injectant
YY1 to K250Q/PCNA
Molar Ratio
Time (min)
µcal/sec
kcal/mole of injectant
YY1 to K250R/PCNA
Molar Ratio
E.
Time (min)
µcal/sec
kcal/mole of injectant
YY1 to K257Q/PCNA
Molar Ratio
F.
Time (min)
µcal/sec
kcal/mole of injectant
YY1 to K257R/PCNA
Molar Ratio

G.

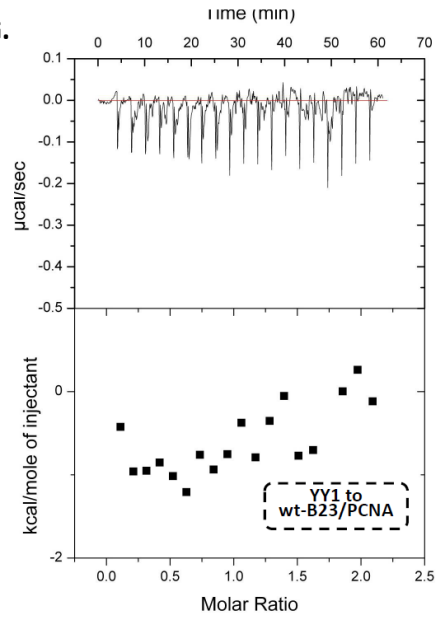


Figure B4.7: Measuring the binding affinity of YY1 for dsPCNA-wtNPM1 and NPM1 post-translational modification variants by isothermal titration calorimetry (ITC)

Recombinant YY1 zinc finger domain (100 μM) was titrated into a solution containing 10 μM ds*PCNA* and 20 μM of a recombinant NPM1 mutant.
(A) YY1 to K212Q/PCNA complex; (B) YY1 to K212R/PCNA complex; (C) YY1 to K250Q/PCNA complex; (D) YY1 to K250R/PCNA complex; (E) YY1 to K257Q/PCNA complex; (F) YY1 to K257R/PCNA complex; (G) YY1 to wt-B23/PCNA complex. The measurements do not show significant difference in the binding affinity of YY1 for ds*PCNA*-acetylated [Q] or ds*PCNA*-deacetylated [R] NPM1 complexes.

B4.4.6 DNA damage repair capacity is inhibited by deacetylated NPM1

To further investigate the transcriptional repression of NPM1 deacetylation mutants, we performed CHIP assay with antibodies against gene activation and silencing (H3K9me2 [referred to as Dimethyl H3 in figure]) markers. We found that cells expressed deacetylated NPM1 mutants, K212R and K250R, were detected high levels of gene silencing marker H3K9me2 in promoter region, whereas gene activation makers (H3K14ac [AcH3] and acH4 [AcH4]) were attenuated in promoter region (Figure B 4.8A). This histone profile results were correlated with the deacetylation effect of NPM1 in *PCNA* down-regulation (Figure B4.3).

Finally, to clarify the functional consequences of PCNA down-regulation repressed by NPM1 deacetylated mutants, we measured the remaining thymine dimers amount by using ELISA assay. Thymine dimers are produced when adjacent thymine residues are covalently linked by exposure to UV irradiation. The covalent linkage of thymine dimer results in the dimer being replicated as a single base, which leads to a frameshift mutation. Moreover, cells will repair these lesions by removing thymine dimers through nucleotide excision repair (NER).

PCNA is a critical protein involved in NER, thus that we measured remaining thymine dimer amount to represent the DNA repair capacity of PCNA. As shown in Figure B4.8B, when compared with control (referred as vector), cells expressing NPM1 deacetylated mutants (K212R and K250R) exhibited slight

effect to remove thymine dimers after UV treatment. However, exogenous expression of FlagNPM1 (FlagB23) and K257R mutants were enhanced the cellular ability to remove thymine dimers after UV treatment. Taken together, the above results demonstrated that NPM1 deacetylated mutants decrease the *PCNA* promoter activity by changing histone signature profiles and subsequently decreasing DNA repair capacity. We concluded that DNA repair capacity is repressed by NPM1 deacetylated mutants.

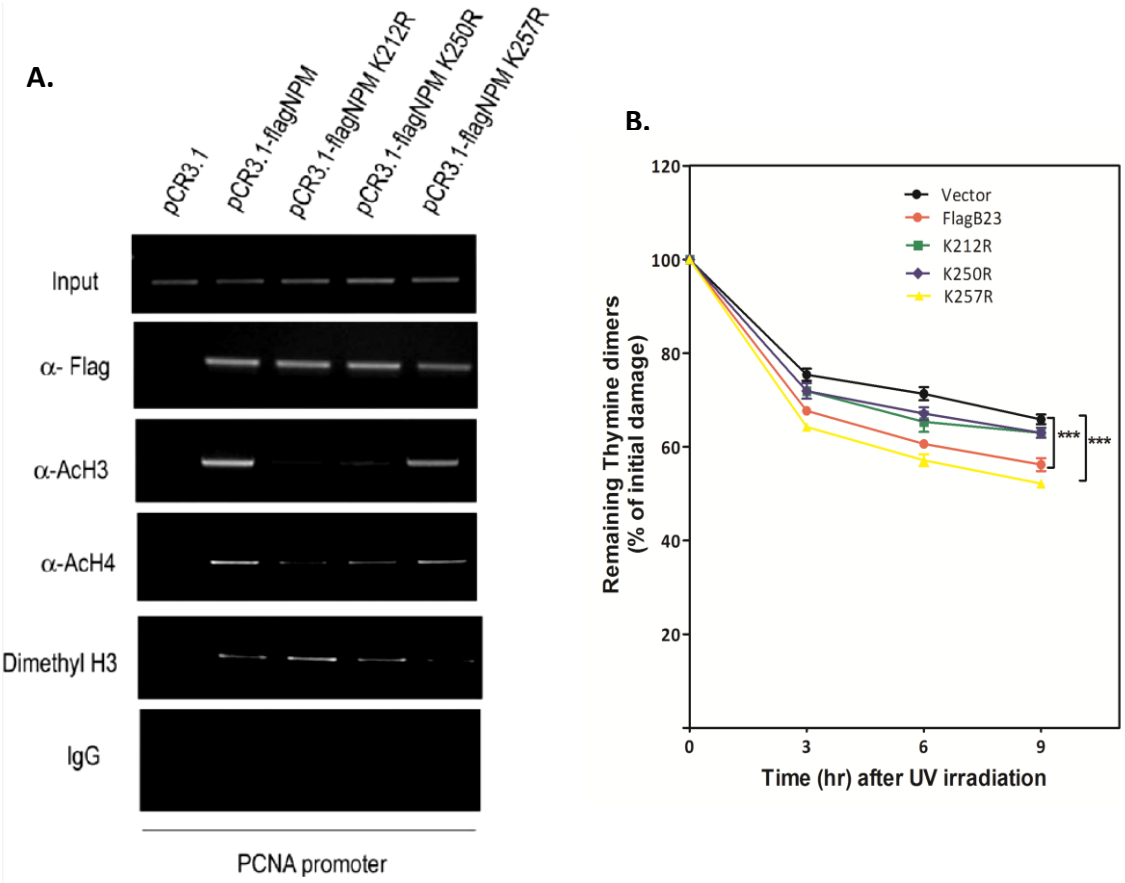


Figure B4.8: NPM1 deacetylated mutants attenuate the acetylation of histone tails and decrease the PCNA-mediated DNA repair capacity

(A) ChIP assay for epigenetic markers and the recruitment of Flag-tagged NPM1 constructs was performed as described in "*section 3.7 of chapter three*". HeLa cells were transfected with the indicated constructs as described in A. Bound DNA was subjected to PCR to monitor the binding of NPM1 (as recognized by the anti-Flag antibody α-Flag) and histone signatures (α-AcH3 and α-AcH4 as gene activating markers; α-Dimethyl H3 as gene silencing marker) on the endogenous *PCNA* promoter.

(B) DNA repair capacity was determined by measuring the amount of thymine dimers with ELISA as described in "*section B4.3.5"*. First, acetylation-defective NPM1 mutants decrease the capacity of cells to remove thymine dimers. NIH3T3 cells were transfected with control vector (vector: black line) or the indicated Flag-tagged NPM1 (FlagB23: red line) constructs (K212R: green line, K250R: blue line and K257R: yellow line represent site-directed mutants in which the Lys212, Lys250 and Lys257 residues were respectively replaced by arginine [R]), and subsequently irradiated with UV (30J/m^2) and lysed immediately (at time 0) or at the time points indicated. The remaining amount of thymine dimers were presented as percentage to those at time 0. Bars presented are means ± SD of three independent experiments. ***$P < 0.001$, the groups of FlagB23 and K257R versus the vector group which were used by two-way ANOVA: Bonferroni post-tests.

B 4.5 Discussion

Here, we address a model for the biological role of NPM1 in transcriptional activation of PCNA through p300 recruitment upon UV irradiation. In earlier time, *Hasan* et al. (2000a), has reported that endogenous p300 is involved in chromatin remodeling at DNA damage lesions to augment PCNA function in the DNA repair pathway, particularly in nucleotide excision repair. Recent studies have also demonstrated that p300 and PCNA influence their biological functions, activities, even leading to post-translational modifications by each other. PCNA, p300 and p21 (cell-cycle inhibitor, CDKN1A) have been shown to be recruited to the UV-induced DNA damage lesions, and p21 regulates the interaction between PCNA and p300 *in vivo*. Indeed, knockdown of p21 or disrupting its interaction with PCNA leads to an increased association of p300 with PCNA (Cazzalini et al., 2008). Moreover, the results of immunoprecipitation also show that p300 and histone deacetylase (HDAC1) interact with PCNA, suggesting that they are likely responsible for acetylation and deacetylation of PCNA (Naryzhny and Lee, 2004). However, the interaction between PCNA and p300 results in an inhibitory effect on the HAT activity of p300, thereby repressing transcription via the hypo-acetylation of chromatin (Hong and Chakravarti, 2003). Collectively, the activities of PCNA and p300 have a reciprocal modulation. We performed *PCNA* gene promoter assay in the context of p300 knockdown. When compared with control group, cells lacking p300 exhibited diminished PCNA promoter activity in a dose-dependent manner (Fig. B4.1). Furthermore, low-dose UV treatment enforced the binding of NPM1 and p300 to the *PCNA* gene promoter region (Fig B4.2). These data indicate that p300 might play a role with NPM1 in UV-induced PCNA activation. Remarkably, NPM1 acetylation triggered by p300 was initially

reported by *Swaminathan* et al. (2005). They demonstrated that NPM1 acetylation was induced by p300 at specific lysine residues by using in vitro acetylation assay. Their results are consistent with our finding that p300 and NPM have reciprocal effect on PCNA activation.

Next, we further aimed to pinpoint whether the NPM1 acetylation-defective mutants are responsible for mediating UV-induced PCNA activation. According to *Swaminathan* et al. (2005), for deacetylated NPM1 mutants (K212R, K250R and K257R), specific ablation of individual deacetylated mutants' expressions was achieved by site-directed mutagenesis. We found that ectopic expression of NPM1 acetylation-defective mutants (EGFP-tagged NPM1K212R, K229/K230R, K250R, K292R and Flag-tagged NPM1K212R, K250R) exhibited reduced *PCNA* promoter activity when compared with wild-type NPM1 and the Flag-NPM1K257R mutant (Fig B4.3). The diminished *PCNA* promoter activities further reduced cellular ability to remove thymine dimers, resulting in decreased DNA repair capacity (Fig B4.8A). Moreover, the ChIP assay revealed specific histone signature profiles in each NPM1 deacetylated mutants groups that were consistent with results of the promoter activity (Figure B4.3). We observed that all NPM1 acetylation-defective mutants and Flag-NPM1 could be recruited to the *PCNA* gene promoter region to mediate PCNA activation. Notably, *PCNA* promoter activities were increased by NPM1 and K257R mutant respectively that were recognized by an absence of the gene silencing marker H3K9me2 in the promoter region. The gene activating markers H3K14ac and acH4 were also detected in the promoter region of these cells.

Our findings showed direct evidence that *PCNA* promoter activity is repressed by deacetylated NPM1. We represented a model to illustrate that NPM1 acetylation might activate *PCNA* gene expression by opening chromatin structure and recruiting transcriptional activators (Figure B 4.9).

Furthermore, we assessed and compared the binding affinity of acetylation-defective [R] and acetylation-constitutive [Q] NPM1 constructs for ds*PCNA* probe by using EMSA and ITC. The resulting bands of EMSA revealed negligible differences between acetylation mutants (K212Q, K250Q and K257Q) and their corresponding deacetylation mutants (K212R, K250R and K257R) (Fig B4.6). Theoretically, we suggested that NPM1 acetylation-defective mutants might stably associate with ds*PCNA* probe and impede YY1 binding, and NPM1 acetylation-constitutive mutants have converse effect. However, our ITC results could not support our hypothesis (Fig B4.7). In NPM1 acetylation-defective mutants, changing lysine [K] residue to arginine [R] did not affect the electrical property of NPM1. Lysine and arginine are both basic and polar while glutamine is neutral and polar. Based on this theory, the electrical charge of NPM1 acetylation-constitutive mutants would tend to be neutral polar when compared with acetylation-defective mutants. Subsequently, NPM1 acetylation-defective mutants should be more attracted by acidic molecules such as DNA or RNA molecule. The tight complex structure of deacetylated NPM1 and dsPCNA might retard the chaperone function of NPM1 and transcription factors recruitment and inhibit PCNA promoter activity. Taken together, we suggested that NPM1 deacetylation might have an inhibitory effect on *PCNA* gene promoter activity. Meanwhile, NPM1 acetylation might activate *PCNA* gene promoter activity and

thus increase the DNA repair capacity. A recent study also demonstrated the importance of NPM1 acetylation level, which is significantly associated with malignancy of cancer stages (Shandilya et al., 2009). Consequently, we concluded that NPM1 acetylation triggered by p300 might transactivate PCNA and thus increase DNA repair capacity, resulting in cancer resistance to radiotherapy or anti-cancer drugs.

Notably, recent reports have also demonstrated that HAT inhibitors can be used as drugs in different disease models including in cancer, asthma, chronic obstructive pulmonary disease (COPD) and viral infection (Dekker and Haisma, 2009). For instance, *Choi* et al. (2011) have showed that an HAT inhibitor, procyanidin B3, directly inhibited the p300-mediated acetylation of androgen receptor, resulting in growth arrest in prostate cancer cells. Furthermore, the newly developed Spermidinyl-CoA-based HAT inhibitors (Spd-CoA) reveal potent effects in the inhibition of histone acetylation. The data showed that the Spd-CoA-mediated histone acetylation inhibition led to transient arrest of DNA synthesis, cell cycle delay in S-phase, and inhibition of nucleotide excision repair and the repair of DNA double strand breaks (Bandyopadhyay et al., 2009). These effects potentially increased the cellular sensitivity to DNA-targeted chemotherapeutic drugs (e.g., cisplatin [Platinol™] and 5-fluorouracil), DNA damaging drugs (camptothecin) and UVC irradiation. Collectively, the combination treatment with HAT inhibitors and anti-sense of NPM1 could therefore provide a new therapeutic strategy and supplement conventional cancer treatments.

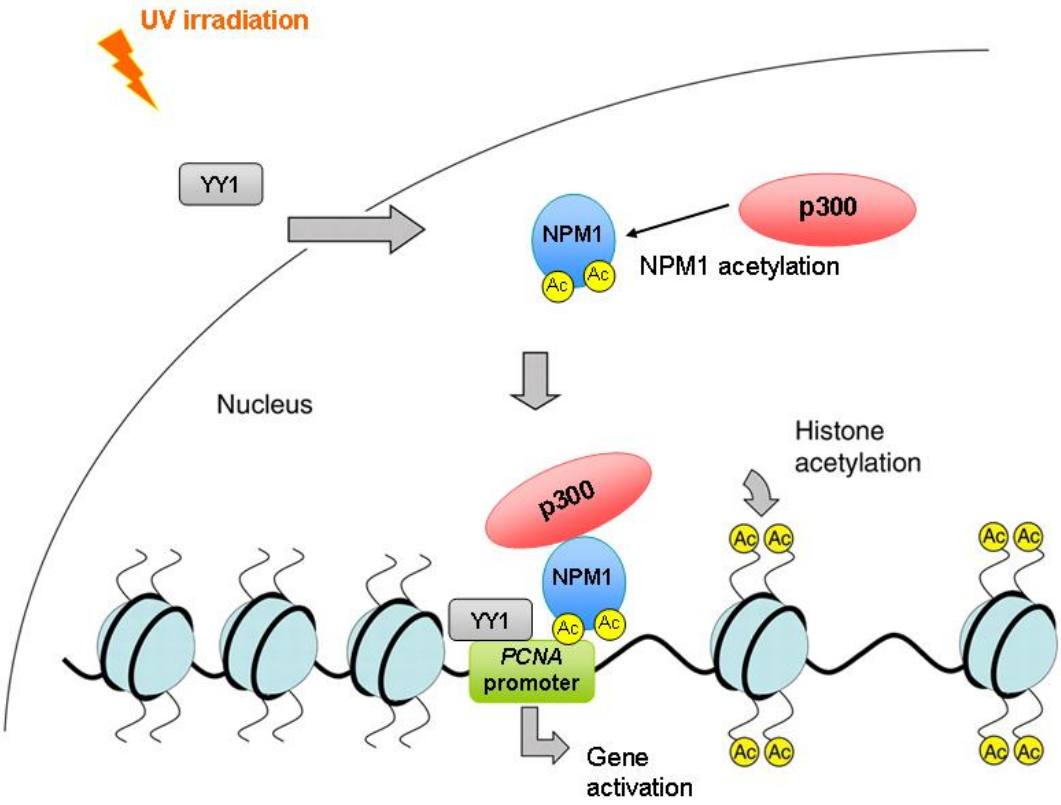


Figure B4.9: A model of NPM1 acetylation activates PCNA gene expression.

In cellular response to UV damage, NPM1 acetylation is induced by histone acetyltransferase p300. Acetylated NPM1, YY1 and p300 increase occupancy on PCNA gene promoter region. Subsequently, p300 induces acetylation of histone tails and leads to a temporary open modification of chromatin structure. The opening chromatin structure recruits transcription factor YY1 and acetylated NPM1 to access the DNA and initiate *PCNA* transcriptional process [This figure was adapted from (Pons et al., 2009)].

Chapter Five:

Summary of the Main Findings

To recap, the present study had three major points of focus, each with specific questions to be addressed (as initially presented in chapter two). The foci, questions and main findings of this study are summarized below.

Focus 1. The interaction between NPM1 and YY1 are responsible for up-regulation of PCNA

1. What is the expression pattern of PCNA and YY1 in the context of NPM1 overexpression?
    a. The mRNA and protein expression level of PCNA in ectopic NPM1 overexpression
    b. Transcriptional level of PCNA in ectopic NPM1 overexpression

Ectopic NPM1 overexpression induced gene expression and activated promoter activity of PCNA.

2. Is there evidence that NPM1 through YY1 and YY1 recognition site up-regulates the *PCNA* promoter activity?

Main finding: NPM1 was co-localized with endogenous YY1 and bound to YY1 recognition site of *PCNA* promoter, resulting in upregulation of PCNA. Human endogenous *PCNA* promoter region has a YY1 recognition site near the transcription start site. The recruitment of YY1 and NPM1 to the endogenous *PCNA* promoter region was verified by ChIP assay in the context of YY1 site-mutated *PCNA* promoter overexpression (pcna-myy1). These data showed that

NPM1 bound to both wild-type *PCNA* promoter region and that with YY1 site-mutation. However, YY1 could only recognize its binding site on the wild-type *PCNA* gene promoter region. Notably, the biotin-labeling pull-down assay revealed that NPM1 was associated with YY1 and bound to YY1-binding site of the *PCNA* gene promoter, resulting in PCNA activation.

Focus 2. The role and function of YY1 in NPM1 upregulation of PCNA

3. Is there evidence that ectopic NPM1 overexpression still has an effect on YY1 site-mutated *PCNA* promoter?

NPM1 overexpression maintained the basal activity of YY1 site-mutated *PCNA* promoter while NPM1 still bound to the *PCNA* promoter region with YY1 site mutation.

4. Is there evidence to prove which domain of NPM1 is responsible for upregulation of PCNA?

Main finding: The C-terminus of NPM1 was crucial for PCNA upregulation. Ectopic expression of NPM1 deletion mutants with different lengths revealed that the C-terminal region of NPM1 was essential for PCNA upregulation.

5. Is there evidence that the C-terminus (residues 206–294) of NPM1 domain is responsible for binding to *PCNA* promoter?

Main finding: The recombinant NPM1 C-terminus protein could interact with ds*PCNA* oligonucleotide probe in a non-specific interaction manner. Our EMSA results indicated that NPM1 C-terminus was a sequence-nonspecific DNA

binding region which interacted with ds*PCNA* probe via electronic attraction between basic amino acid residues of NPM1 C-terminus and acidic DNA nucleotides of ds*PCNA* probe.

Summary: According to the literature, NPM1 has been identified as a sequence-nonspecific DNA binding protein (Chang and Olson, 1989). We suggested that NPM1 overexpression induced PCNA activation potentially through interacting with the transcription factor YY1. However, experimental results showed that recombinant NPM1 C-terminus protein could interact with ds*PCNA* probe as determined by EMSA and ITC. We suggested that the recruitment of NPM1 might have synergistic and reinforcing effect on the YY1 binding to the *PCNA* gene promoter region.

6. What is the expression pattern of NPM1 and PCNA after low dose UV treatment at up to 3, 6, and 9 h?

The mRNA and protein expression levels of NPM1 and PCNA were both increased in a time-dependent manner after UV irradiation. PCNA upregulation followed after UV-induced NPM1 overexpression.

7. What regulatory proteins are recruited to the *PCNA* promoter region, resulting in UV-induced PCNA up-regulation?

Main finding: As a transcriptional coactivator, p300 was another important factor involved in UV-induced PCNA upregulation. The ChIP assay results

showed that UV-induced PCNA activation was mediated by recruitment and reinforcement of NPM1, p300 and YY1 on *PCNA* promoter after UV irradiation.

8. Is there evidence that the histone acetyltransferase function of p300 will lead to post-translational modification of NPM1?

Main finding: This is a novel finding that p300 recruitment increased the acetylation status of NPM1 upon UV irradiation. In accordance with the literature, our research also verified that NPM1 was a downstream substrate for p300 which acetylated NPM1 at specific lysine residues.

Focus 3. The physiological function of NPM1 acetylation-defective or acetylation-constitutive constructs in modulating PCNA activation.

9. Is there evidence that NPM1 acetylation-defective constructs affect the *PCNA* gene promoter activity?

We found that NPM1 acetylation-defective constructs with EGFP-tagged and Flag-tagged dramatically reduced the *PCNA* gene promoter activity when compared with the group in wild-type NPM1 overexpression. Consistent with *PCNA* gene promoter assay, the ChIP assays data showed that Flag-tagged NPM1 deacetylation mutants (Flag-NPMK212R and Flag-NPm1K250R) exhibited an inhibitory effect on *PCNA* activation because gene silencing marker H3K9me2 was detected in *PCNA* promoter region.

10. Is there evidence that NPM1 acetylation-defective constructs inhibit *PCNA* activation, resulting in decreased DNA repair capacity?

Main finding: NPM1 acetylation-defective constructs (Flag-NPM1K212R and K250R) exhibited diminished cellular ability to remove thymine dimers when compared with that of groups with NPM1 and K257R overexpression. Cells were transfected with different NPM1 deacetylated mutants for 24 h and then exposed to UV irradiation. After UV treatment, cells were immediately harvested (time 0 hr) and at different post-UV treatment time points. To determine the DNA repair capacity, the amounts of thymine dimers were measured by ELISA. Consistent with *PCNA* promoter assay, the NPM1 deacetylated mutants revealed diminished ability removal to remove thymine dimers.

Summary: It is a novel finding that NPM1 acetylation could be induced by UV irradiation. Interestingly, NPM1 deacetylation might be able to inhibit PCNA activation, resulting in diminished DNA repair capacity. We suggested that NPM1 acetylation might be able to enhance and enforce PCNA activation, resulting in increased DNA repair capacity. Notably, a recent report has indicated that elevated NPM1 acetylation is significantly associated with malignancy stages of oral cancer (Shandilya et al., 2009). Consequently, elevated NPM1 acetylation in malignancy stages potentially results in resistance to radiotherapy or anti-cancer drugs. According to this manifestation, a treatment with NPM1 antisense and HAT inhibitor combination might provide a new therapeutic strategy in cancer treatment.

Chapter Six:

Discussion and Limitations of the Overall Study and Suggestions for Further Research

5.1 Discussion

As a multifunctional protein, NPM1 has been well-documented regarding its functions. One of NPM1's functions is involved in transcriptional regulation by interacting with various proteins and thus participates in a broad spectrum of cellular activities, such as cell cycle regulation and cellular differentiation. For example, NPM1 acts as a coactivator of NF-κB in regulating the expression of the antioxidant enzyme, MnSOD (Dhar et al., 2004). NPM1 protein has been found to interact with a transcription factor AP2α, resulting in reduced gene promoter activities of *c-myc* and *NPM1* during the retinoic acid-induced cell differentiation in human leukemia HL-60 cells (Liu et al., 2007a; Yung, 2004). In this study, we found that NPM1 overexpression resulted in PCNA upregulation by binding to YY1 recognition site on the *PCNA* gene promoter region. Moreover, we also found that PCNA upregulation followed UV-induced NPM1 overexpression (Fig A4.1). UV irradiation also increased NPM1 acetylation which is one of NPM1 post-translational modifications. Indeed, NPM1 has been reported to carry various types of modifications including phosphorylation, acetylation and SUMOylation etc. For detailed descriptions and functions of NPM1 posttranslational modification, see "*section 1.3.8* in chapter one".

NPM1 acetylation was initially reported in 2005 by *Swaminathan* et al. who proved that NPM1 could be acetylated by histone acetyltransferase (HAT) p300

at specific lysine residues at the C-terminus by using in vitro acetylation assay. Previously, *Hasan* et al. (2001) reported that endogenous p300 could interact with PCNA and participate in chromatin remodeling, resulting in enhanced PCNA function in DNA repair synthesis. Additionally, p300 also acted as a co-repressor via interacting with YY1 and HDAC3 (histone deacetylase 3), then this ternary complex was recruited to the YY1 site of *c-Myc* to repress gene transcription of *c-Myc* (Sankar et al., 2008). However, the repressive function of p300 was not through its intrinsic HAT activity (Baluchamy et al., 2007). Instead, it relied on the C-terminal domain of p300, which served a repressive function by facilitating the recruitment of HDAC3 to the YY1 site (Sankar et al., 2008).

We found that p300 might act as a co-activator and directly bound to the *PCNA* gene promoter region with NPM1. Recruitment of p300 and NPM1 occurred in a time-dependent manner upon UV irradiation. Consequently, p300 was directly involved in UV-induced PCNA activation (B4.2). As previously described, YY1 is one of the p300-interacting proteins and is involved in PCNA upregulation (Weng and Yung, 2005). We then tried to elucidate the reciprocal relationship between NPM1, p300 and YY1 in PCNA activation. Biotin-labeling pull-down assay showed that NPM1 recognized and bound to the YY1-binding site of the *PCNA* promoter, resulting in PCNA activation (Fig A4.4C). On the other hand, mutation of the YY1 site resulted in the loss of PCNA activation, and YY1 recruitment and only negligible binding of NPM1 were detected in *PCNA* promoter region (Fig A4.5). We also presented that NPM1 acetylation-defective mutants had inhibitory effect on the *PCNA* promoter activity (Fig B4.3) and subsequently reduced DNA repair capacity (Fig B4.8A). However, the underlying

repressive mechanism of NPM1 acetylation-defective mutants-mediated *PCNA* repression was still unclear. We suggested that p300 recruitment triggered NPM1 acetylation and disentangled chromatin structure to facilitate the recruitment of YY1 and NPM1 to the YY1 recognition site for *PCNA* activation. We used ds*PCNA* oligonucleotide probes of different lengths (400bp and 30bp in length) to verify our hypothesis. In our EMSA performed with two ds*PCNA* probes, we observed that recombinant human YY1 zinc finger domain and NPM1 C-terminal region bound to both ds*PCNA* sequences (Fig A4.7 & A4.8B). Therefore, it is likely that the C-terminal of NPM1 served a synergic role to facilitate YY1 and NPM1 recruitment for upregulation of PCNA. These results were consistent with PCNA activation in the context of NPM1 overexpression (Fig A4.2). However, we further distinguished the reciprocal interaction of NPM1 acetylated mutants, deacetylated mutants and YY1 with ds*PCNA* probe by EMSA and ITC. The data revealed only negligible differences in their binding affinity when compared with NPM1 acetylated mutants and deacetylated mutants (Fig B4.6 & B4.7). It might be limited by the length of the ds*PCNA* probe because NPM1 is a sequence-nonspecific DNA binding protein. The 30-bp ds*PCNA* probe might be too short for NPM1 recruitment and increasing NPM1 protein amounts to excess level. Notably, NPM1 acetylation is an important marker for cancer progression. Recently, several reports have demonstrated that NPM1 acetylation level is significantly associated with malignancy stages of cancer (Shandilya et al., 2009). Increased PCNA expression level in HCC (hepatocellular carcinoma) patient tissues is associated with high NPM1 expression level and correlated with clinical cancer stages (Yun et al., 2007). Moreover, the elevated PCNA expression level has also been observed in prostate cancer, and PCNA expression level is highly correlated with cancer stages (Wang et al., 2010b). Collectively,

UV-induced DNA damage would trigger the intrinsic HAT activity of p300, leading to evoked NPM1 expression and acetylation status. Meanwhile, the consequence of PCNA activation occurred subsequent to NPM1 overexpression, being related to increased DNA repair capacity. It is likely to explain that NPM1 overexpression in certain cancers is required for inducing a resistance pathway against anti-cancer drugs or cancer-radiotherapy. For effective cancer therapy, NPM1 could be an important anti-cancer target. For prediction of prognosis and recurrence, NPM1 acetylation level would be a referable index.

5.2 Limitations of overall study and suggestion for further research

In this research project, we encountered one difficulty regarding the size of the ds*PCNA* probe because it would affect the experimental methods and results. Thus, we performed EMSA and ITC experiments with two different ds*PCNA* oligonucleotide probes. Initially, the 400-bp ds*PCNA* was prepared from pGL3-wt-PCNA reporter gene by restriction enzyme cutting and PCR amplification for EMSA experiments (the detailed preparation sees "*section 3.9* in chapter three"). However, the size of ds*PCNA* probe was not appropriate for non-denaturing PAGE gel, and therefore we used agarose gel for this alternative ESMA system (Fig A4.6 & A4.7). The disadvantage of agarose gel is the gel resolution, which is insufficient to clearly distinguish the differences in the binding affinity of deacetylated and acetylated NPM1 mutants for a long ds*PCNA* probe. For better resolution and separation of various NPM1 mutants, we synthesized a new 30-bp ds*PCNA* probe for analysis in non-denaturing PAGE gel EMSA system. This short ds*PCNA* probe was used in ITC experiments to detect the dynamic thermal changes between protein-DNA interactions by ITC experiments (Fig B4.6 & B4.7).

However, this 30-bp ds*PCNA* probe used in EMSA and ITC could not provide satisfactory results for determining the differences in binding affinity. It is likely that NPM1 binds to DNA sequences with a minimal sequence. The biological effects of NPM1 point mutations are thus not clearly detected by *in vitro* systems.

For further study, double or triple point-mutations of lysine residues of NPM1 could be constructed for detection of protein-DNA interaction and *PCNA* promoter activity. The anti-proliferation and anti-resistant effect in cancer cells by measuring the cancer cell viability or invasion in combination with NPM1 knockdown and HAT inhibitors condition could also be verified. Recently, HATs are the new targets for drug development research. For example, curcumin, a specific inhibitor of the p300/CREB-binding protein HAT activity, has been reported to inhibit p300-mediated acetylation of p53 in cellular condition and HAT-dependent chromatin transcription (Balasubramanyam et al., 2004). Notably, a recent article has been reported that curcumin treatment enhances the therapeutic effect of doxorubicin via induction of p53-p300 cross-talk, and subsequently enhancing the transcription activity of p53 and intrinsic cell death cascade in breast cancer cells (Sen et al., 2011). Curcumin also can be applied in heart failure treatment. A study demonstrated that curcumin treatment exerted beneficial effect on rat with myocardial infarction (Sunagawa et al., 2011). Because some of hypertrophy-responsive transcription factors, GATA4, MEF2 and SRF are regulated by histone deacetylases and HATs, particularly by p300. In cellular response to cardiomyocyte hypertrophy, the expression level of p300 and its HAT activity are enhanced. Therefore, GATA4 would be acetylated by p300 and resulted in the increase of DNA-binding capacity and up-regulation of

hypertrophy-relative genes. These results demonstrated that HAT inhibitor treatment by curcumin could inhibit the HAT activity of p300 and hypertrophy of cardiomyocytes (Sunagawa et al., 2011). Another HAT inhibitor, procyanidin B3 effectively inhibited p300-mediated androgen receptor (AR) acetylation in both *in vivo* and *in vitro* condition and subsequently inhibited acetylation-dependent prostate cell proliferation (Choi et al., 2011). Thus, treatments of HAT inhibitors or p300-specific inhibitors could potentially decrease or attenuate NPM1 acetylation for alternative therapeutic strategy and efficiently anti-cancer effects.

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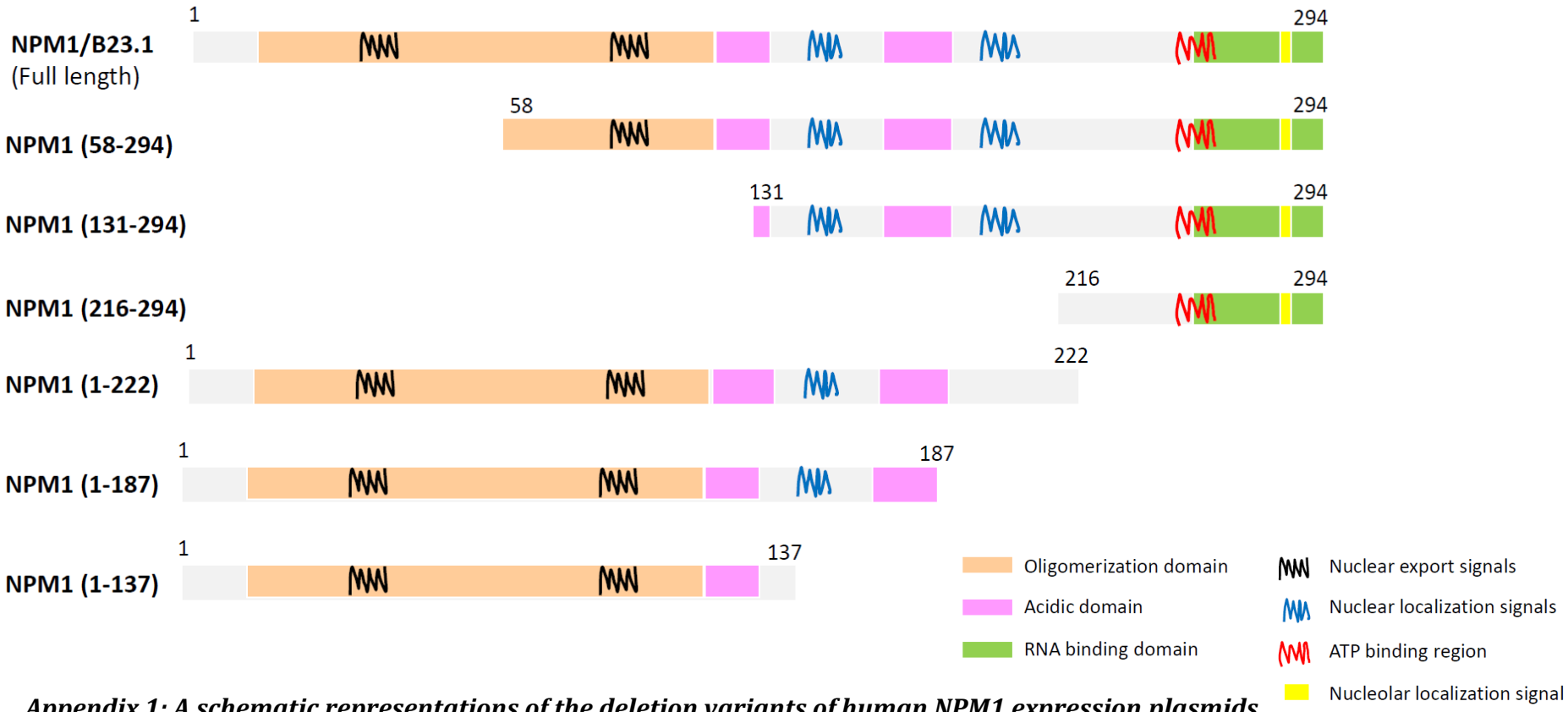
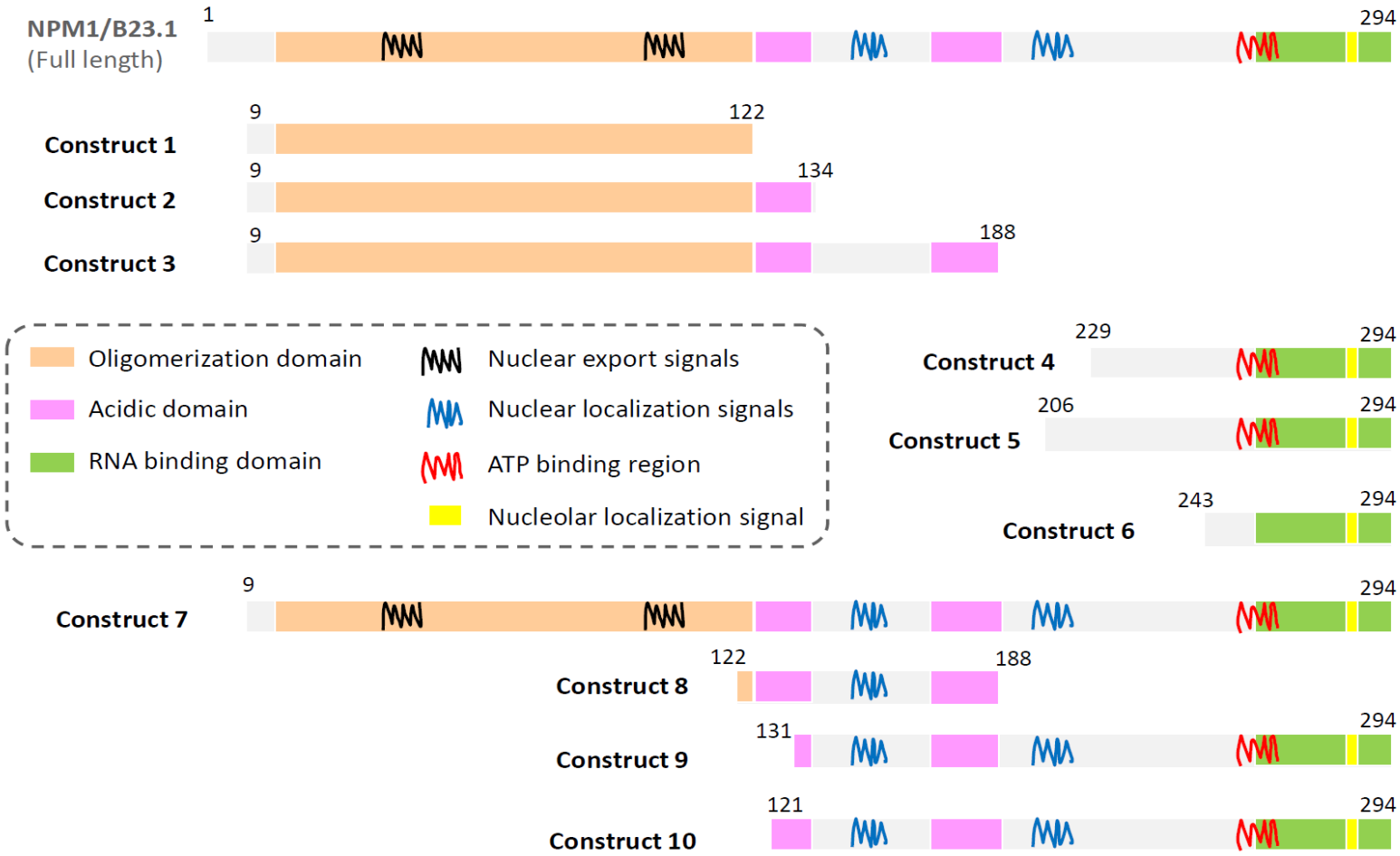


Appendix 1: A schematic representations of the deletion variants of human NPM1 expression plasmids.

EGPF-tagged NPM1 deletion mutants were used to verify which interaction domain of NPM1 is essential for PCNA activation measuring by reporter gene assay.



Appendix 2: Schematic representations of different lengths of NPM1 recombinant constructs.

According to previously experimental results, NPM1 C-terminus is essential for PCNA activation and involves in NPM1 acetylation. Therefore, construct 5 of NPM1 (as referred as wild-type NPM1) is used in electrophoresis mobility sift assays and isothermal titration calorimetry.