The Role of Nucleolin Phosphorylation by CK2 in Regulating Cellular Fate Under Normal and Stress Conditions

Shu Xiao
The Graduate Center, City University of New York

How does access to this work benefit you? Let us know!
Follow this and additional works at: https://academicworks.cuny.edu/gc_etds
Part of the Biology Commons

Recommended Citation
Xiao, Shu, "The Role of Nucleolin Phosphorylation by CK2 in Regulating Cellular Fate Under Normal and Stress Conditions" (2017). CUNY Academic Works.
https://academicworks.cuny.edu/gc_etds/2412

This Dissertation is brought to you by CUNY Academic Works. It has been accepted for inclusion in All Dissertations, Theses, and Capstone Projects by an authorized administrator of CUNY Academic Works. For more information, please contact deposit@gc.cuny.edu.
THE ROLE OF NUCLEOLIN PHOSPHORYLATION BY CK2 IN REGULATING CELLULAR FATE UNDER NORMAL AND STRESS CONDITIONS

by

Shu Xiao

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

2017
The role of nucleolin phosphorylation by CK2 in regulating cellular fate
under normal and stress conditions

by

Shu Xiao

This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction
of the dissertation requirement for the degree of Doctor of Philosophy.

Anjana Saxena

__________________________
Date Chair of Examining Committee

Cathy Savage-Dunn

__________________________
Date Executive Officer

Supervising Committee:

Frida Kleiman

Xinyin Jiang

Jimmie Fata

James Borowiec

THE CITY UNIVERSITY OF NEW YORK
ABSTRACT

The role of nucleolin phosphorylation by CK2 in regulating cellular fate under normal and stress conditions

by

Shu Xiao

Advisor: Dr. Anjana Saxena

Nucleolin (NCL or C23) is an abundant genotoxic stress-responsive RNA binding phosphoprotein. NCL constitutes 10% of total nucleolar protein that has functions in multiple biological processes, including ribosome biogenesis, DNA/RNA metabolism, cellular response to DNA damage, cell growth, proliferation and death. In this dissertation, I elucidate the role of nucleolin phosphorylation by casein kinase 2 (CK2) in controlling cellular fate by regulating p53 checkpoint under normal and stressed conditions. First, I demonstrate that the six consensus CK2 sites on the N-terminus of NCL are important for cell survival and proliferation. Expression of CK2 phosphorylation-deficient NCL mutant leads to dominant negative effects on cell proliferation and triggers p53 checkpoint activation to induce expression of pro-apoptotic markers downstream. Next, I present evidence that lack of NCL phosphorylation by CK2 increases cells’ sensitivity to apoptosis upon genotoxic stress (UV irradiation and topoisomerase II inhibitor etoposide). The substantially upregulated PUMA expression in dephosphorylated-NCL expressing cells may play a pivotal role in the increased cells’ sensitivity to apoptosis. In addition, phosphorylation-deficient NCL mutant expression also induces significant upregulation of AIF. Last but not least, in collaborate with Dr.Kleiman’s lab, we further demonstrate that
NCL phosphorylation by CK2 regulates p53 checkpoint post-transcriptionally through affecting NCL interaction with PARN deadenylase and the target gene TP53 mRNA, as well as with ARE-binding protein HuR to mediate HuR availability to their common target TP53 mRNA. Dr. Kleiman’s lab determined that NCL can bind to the 3’UTR ARE of TP53 mRNA under non-stressed conditions. Moreover, in vitro assays show that NCL interacts directly with the N-terminus of PARN, however, while NCL-WT enhances PARN activity, phosphorylation-deficient NCL mutant abolishes PARN deadenylation. In this dissertation, I provide evidence that phosphorylation of NCL by CK2 not only plays a crucial role in NCL associating with TP53 mRNA both under non-stressed and stressed conditions, but also regulates the interaction of NCL with p53 protein, PARN deadenylase and HuR respectively in cell extracts. Post-transcriptional regulation of TP53 mRNA by the effect of functional interplay of NCL-PARN-HuR might ultimately link to the increased p53 protein levels and induction of apoptosis pathways. These data provide new insights into the role of NCL phosphorylation by CK2 in regulating gene expression post-transcriptionally to control cell fate during cellular stress response.
ACKNOWLEDGEMENTS

I would like to thank my mentor, Dr. Anjana Saxena, for her guidance throughout my graduate research. I am thankful for her open communication and discussion that she led in the lab and that created my outlook of productive collaborative work. I am also thankful to the awesome group of people I worked with in the lab. They include Elif Caglar, Dibash Das, Kenneth Ng, Zaineb Nadeem, Allana Rodriguez, and especially thank to Rachele Dolce Rameau, Esther Akinwunmi and Jingyuan Wang, who contributed tremendously to many aspects of my work.

My sincere gratitude to my committee member Dr. Frida Kleiman of Hunter College of City University of New York for providing PARN antibodies and allowing me to learn co-immunoprecipitation (CO-IP) and RNA-immunoprecipitation (RIP) technique in her lab. I am also thankful for the help from Dr. Kleiman’s group Xiaokan Zhang, Emral Devany and Jorge Baquero regarding the technique trouble shooting and analysis of CO-IP and RIP data. Much gratitude is also offered to my committee member Dr. Xinyin Jiang of Brooklyn College of City University of New York for making available her lab for my research and technical assistance on RT-PCR experiment.

I would like to thank Dr. Jimmie Fata of College of Staten Island of City University of New York and Dr. James Nishiura of Brooklyn College of City University of New York who were beyond ordinary committee members. I am grateful for their way of leading onto the question and insightful comments which helped me to pursue deeper understanding of my research and
science in general. I am honored to have Dr. James Boroweic of NYU School of Medicine to serve on my committee and grateful for his time and feedback on my work.

Finally, words alone cannot describe the gratitude I owe to my family, they have made me who I am by showing me the important values and beliefs of life. My deepest appreciation to my parents, Wenwang Xiao and Xiaoyan Wei, and to my dearest younger brother, Yifan Wei, for their continuous support throughout all my academic achievements.
TABLE OF CONTENTS

ABSTRACT ........................................................................................................................................ iv

ACKNOWLEDGEMENTS ................................................................................................................ vi

LIST OF FIGURES AND TABLES .................................................................................................. x

LIST OF ABBREVIATIONS ................................................................................................................ xi

SIGNIFICANCE .................................................................................................................................. 1

CHAPTER I Background .................................................................................................................. 5

1. Properties and functions of nucleolin domains ........................................................................... 6

   1.1 The N-terminal domain of nucleolin ....................................................................................... 9

   1.2 The RNA binding domain of nucleolin .................................................................................. 10

   1.3 The GAR domain of nucleolin .............................................................................................. 12

2. Regulation of p53 checkpoint by nucleolin at multiple levels ..................................................... 14

   2.1 Nucleolus as a stress sensor .................................................................................................. 14

   2.2 Regulation of p53 checkpoint by nucleolin ............................................................................ 15

   2.3 Regulation of cell fate by p53 dynamics ................................................................................ 18

3. Post-transcriptional regulation of mRNAs by nucleolin ............................................................... 19

CHAPTER II Effect of nucleolin phosphorylation by CK2 in regulating cell proliferation ...... 26

Introduction ........................................................................................................................................ 27

Results .............................................................................................................................................. 31

Discussion ......................................................................................................................................... 40

CHAPTER III Effect of nucleolin phosphorylation by CK2 in inducing apoptosis .................... 45

Introduction ........................................................................................................................................ 46

Results .............................................................................................................................................. 50

Discussion ......................................................................................................................................... 60
CHAPTER IV Effect of nucleolin phosphorylation by CK2 in regulating mRNA stability upon DNA damage ............................................................................................................................................... 65
  Introduction.................................................................................................................................................................................... 66
  Results.......................................................................................................................................................................................... 70
  Discussion .................................................................................................................................................................................. 75
CHAPTER V Future Directions ....................................................................................................................................................... 80
CHAPTER VI Experimental Procedures ........................................................................................................................................... 86
CHAPTER VII References ............................................................................................................................................................... 95
LIST OF FIGURES AND TABLES

Figure 1. The localization of nucleolin in the nucleolus.......................................................... 8
Figure 2. The modular structure of nucleolin. ................................................................. 13
Figure 3. Regulation of p53 checkpoint by nucleolin............................................................... 17
Figure 4. Targeting the consensus CK2 sites in NCL............................................................ 29
Figure 5. Inducible expression of NCL (WT or 6/S*A) result in an increased p53 protein levels. ........................................................................................................................................ 32
Figure 6. Half-life analyses of p53. ....................................................................................... 33
Figure 7. NCL-6/S*A expression causes p53-dependent inhibition of cell proliferation........ 35
Figure 8. NCL-WT and 6/S*A interact with endogenous NCL. ............................................ 37
Figure 9. NCL-6/S*A expression results in an increased expression of apoptotic markers downstream the p53 pathway..................................................................................................................... 39
Figure 10. Mechanistic model by which nucleolin phosphorylation by CK2 regulates cell proliferation..................................................................................................................................................... 44
Figure 11. Inducible expression of NCL-6/S*A results in an increased procaspase levels. .... 52
Figure 12. Inducible expression of NCL-6/S*A sensitizes cells to UV-induced apoptosis. ..... 54
Figure 13. Inducible expression of NCL-6/S*A increases the efficiency of etoposide-induced apoptosis. ........................................................................................................................................ 57
Figure 14. Inducible expression of NCL-6/S*A increases AIF levels in nucleus. ....... 59
Figure 15. Nucleolin dephosphorylation at CK2 sites sensitizing cells to apoptosis. .......... 64
Figure 16. NCL phospho-variants can associate with p53, PARN and HuR under different cellular conditions............................................................................................................................... 72
Figure 17. NCL phosphorylation by CK2 affects its interaction with TP53 and BCL2 mRNA. . 74
Figure 18. Post-transcriptional regulation of p53 by nucleolin phosphorylation. ................. 79

Table 1. Partial list of nucleolin target mRNAs encoding cancer-related proteins .............. 25
LIST OF ABBREVIATIONS

3’UTR 3’ untranslated region
5’ETS 5’external transcribed spacer
AIF apoptosis inducing factor
APS ammonium persulfate
ARE Adenine Uracil Rich Elements
ATM ataxia telangiectasia-mutated kinase
ATR ATM and Rad51 related kinase
AUBP ARE-binding proteins
AUF1 AU-rich Element-binding Factor 1
BAK bcl-2 antagonist/killer
Bcl-2 B-cell lymphoma 2
BID BH3 interacting-domain death agonist
CDK1 cyclin-dependent kinase 1
CHX cycloheximide
CICD caspase-independent cell death
CK2 casein kinase 2
CPT camptothecin
Dx doxycycline
DXR doxorubicin
ECM evolutionary conserved motif
ECL enhanced chemiluminescence
ETP etoposide
FACS fluorescence activated cell sorting
FL full-length
GAR/RGG domain Glycine-Arginine Rich /Arg-Gly-Gly residues domain
GFP green fluorescence protein
GM-CSF colony-stimulating factor
Hdm2 human homolog of Mdm2
HuR human antigen R
KSRP K homology splicing regulatory protein
Mdm2 mouse double minute 2 proto-oncogene
MOMP mitochondria outer membrane permeabilization
mRNP messenger ribonucleoprotein
NCL nucleolin
NE nuclear extract
NLS nuclear localization signal
NRE nucleolin recognition element
NT nucleolin N terminal domain
p53 human 53 kDa tumor suppressor protein
PARN poly(A)-specific ribonuclease (deadenylation nuclease)
PABP poly(A)–binding proteins
PKC-ζ protein kinase C Zeta
PMSF phenylmethylsulfonyl fluoride
PUMA p53 upregulated modulator of apoptosis
RBD1-4 nucleolin RBD1, RBD2, RBD3, and RBD4 domains
RBDs RNA binding domains
RBP RNA-binding protein
RPA replication protein A
RPL 26 ribosomal protein L26
TNF-α tumor necrosis factor alpha
TOPO II topoisomerase II enzyme
TTP Tristetraprolin
SIGNIFICANCE

Cancer is fundamentally a disease of deregulation of cell proliferation. Normally the balance between cell proliferation and programmed cell death, mostly in the form of apoptosis, is maintained to ensure homeostasis. The ability to evade programmed cell death and acquire immortalization is the most important hallmarks of cancers [1]. What makes nucleolin (NCL) a central role in cancer formation and a promising target for cancer therapy? It is the higher abundance of NCL, selective presence on plasma membrane, and multifaceted influence on initiation and progression of cancer, including carcinogenesis, proliferation, angiogenesis and metastasis [2]. Elevated levels of NCL expression are generally related to malignancy and is found in a variety of tumors [3, 4].

NCL localizes in multiple cellular compartments and can translocate within the cell, it has various features on cellular processes that favors cell proliferation [5]. NCL in the nucleolus, where it is predominantly localized, contributes to the increased rDNA transcription and ribosome assembly that is required to maintain high level of protein synthesis because of the high proliferation rate of cancer cells [4, 6]. The fraction of NCL pools in the nucleoplasm regulates gene transcription and favors the expression of proliferative and anti-apoptotic genes, e.g. IL-9R, VEGF, CD95 and MCL1 [7-9]. In the cytoplasm, NCL increases mRNA stability by interacting with the AU-rich elements (AREs) in 3’UTR of the mRNA targets, e.g. anti-apoptotic factors BCL2 and BCLXL [10-12]. More importantly, NCL in the cytoplasm not only facilitates the translation of many oncogenic factors e.g. SP1 and AKT1 [13, 14], but also represses the translation of TP53 – the famous tumor suppressor [15-17]. It is generally accepted that
regulation of p53 checkpoint plays a fundamental role in determining cellular fate. Once activated by stress signal, p53, as a transcription factor, can turn on the expression of over 3000 target genes that are involved in multiple cellular responses, including cell cycle arrest, DNA repair, apoptosis, senescence, metabolism, autophagy, mRNA translation and feedback mechanisms [18].

NCL is highly phosphorylated at its N-terminus, which has been reported to be positively correlated with increased rates of cell proliferation [4, 19-22]. Casein kinase 2 (CK2) phosphorylates NCL on serine during interphase, while cyclin-dependent kinase 1 (CDK1) phosphorylates it mostly on threonine during mitosis. Interphase is a critical phase accounting for 90% of the total time of cell proliferation, when cellular survival and growth decisions are made. Even though the regulatory role of NCL in cell proliferation seems essentially due to the role of NCL phosphorylation in regulating ribosome biogenesis, there are evidence showing that NCL may regulate cell proliferation independently of its role on ribosome biogenesis. For example, NCL regulates the p53 checkpoint at multiple levels. Under non-stressed conditions, overexpressed NCL stabilizes p53 by binding to Hdm2 via the N-terminal domain and the central RNA-binding domains (RBDs) of NCL, and inhibits Hdm2-mediated p53 degradation, leading to cell cycle arrest and apoptosis [23, 24]. Upon stress, the increased level of p53 promotes NCL-p53 complex formation, which shifts the majority of NCL pool from the nucleolus to the nucleoplasm where NCL sequesters Replication Protein A (RPA) to transiently inhibit DNA replication [25, 26]. Interestingly, NCL-TM (a NCL mutant with three dephosphorylated CK2 sites) readily relocates partial of NCL pool to the nucleoplasm and increases NCL-RPA association in unstressed cells, causing a “nucleolar stress” like conditions [27], which indicates
that phosphorylation of NCL by CK2 may have a crucial role in regulating DNA replication, p53 activation and cellular response to stress. However, whether NCL phosphorylation by CK2 activates p53 and/or regulates p53 dynamics, upregulates p53-responsive gene, and ultimately leads to cell proliferation inhibition is not elucidated. Besides, NCL represses p53 translation by forming NCL-NCL homodimers and directly binds to both of 3’ and 5’UTR of TP53 mRNA under non-stressed conditions, while NCL-RPL26 heterodimers switch on p53 translation by competitively binding to the same 3’ and 5’UTR region of TP53 mRNA in response to stress [15-17]. However, whether NCL regulates TP53 mRNA stability or NCL phosphorylation by CK2 has a role in its function of regulating TP53 mRNA stability is not known yet. Taken together, elucidating the role of NCL phosphorylation by CK2 in regulating p53 checkpoint and factors in the downstream pathway may provide new insight of NCL’s function in controlling cellular fate.

In this thesis, I use a unique cellular tool that express CK2-specific full-length NCL phosphorylation variants upon a Tet-off inducible promoter system which can stably express either NCL wild type (WT) or a CK2 phosphorylation-deficient mutant (6/S*A; containing six alanine substitutions at the consensus serine sites) in human osteosarcoma U2OS cells. By using this system, I demonstrate that the six consensus CK2 sites on the N-terminus of NCL are important for cell survival and proliferation. Expression of CK2 phosphorylation-deficient mutant (NCL-6/S*A) leads to cell proliferation inhibition by inducing a sustained p53 dynamics and upregulating expression of p53-responsive pro-apoptotic factors. In addition, dephosphorylation of NCL at CK2 sites increases cells’ sensitivity to apoptosis upon genotoxic stress (UV irradiation or etoposide poison), which is probably due to, at least in part, the
upregulated basal levels of pro-apoptotic factors in NCL-6/S*A cells. Moreover, in collaboration with Dr. Kleiman’s lab, we further investigated the effect of NCL phosphorylation by CK2 in post-transcriptional regulation of p53 checkpoint. We demonstrate that phosphorylation of NCL by CK2 plays a crucial role not only in NCL associating with TP53 mRNA, but also in regulating its functional interactions with an RNA binding protein HuR and an mRNA decay enzyme PARN to regulate p53 gene expression both under non-stressed and stressed conditions. Post-transcriptional regulation of TP53 mRNA by the effect of functional interplay of NCL-PARN-HuR might ultimately link to the increased p53 protein levels and induction of apoptosis pathways. The studies presented in this dissertation are significant at several levels. First, I provide evidence showing that lack of NCL phosphorylation by CK2 confers a dominant-negative effect on cell proliferation. Second, my results indicate that dephosphorylation of NCL at the CK2 sites on its N-terminus may be an attractive therapeutic target in improving chemotherapy efficacy in treating cancers. Last, my results provide new insights into the role of NCL phosphorylation by CK2 in post-transcriptional regulation of p53 checkpoint to control cell fate during cellular stress response.
CHAPTER I

BACKGROUND
1. Properties and functions of nucleolin domains

Nucleolin (NCL or C23) is a multifunctional phosphoprotein which was originally identified as a major nuclear phosphoprotein localizing primarily to the nucleolus, mainly in the dense fibrillar component (DFC) and the granular component (GC) (Figure 1) [4, 28]. Because of NCL’s localization, initially researchers focused on the role of NCL in ribosome biogenesis, and found that NCL is a key factor which participates in all the major steps of ribosome biogenesis (see review Cong, R., Das, S., & Bouvet, P. (2011)). NCL not only has important roles in regulating rDNA transcription, which occurs at the border between the Fibrillar component (FC) and the DFC [29-33], but also in rRNA maturation and ribosome assembly, both of which take place in the GC [6, 34, 35]. The control of ribosome biosynthesis by NCL is physiologically crucial in regulating protein synthesis which is needed for cell growth [36]. Nucleolin protein and phosphorylation levels are significantly higher in actively dividing cells when compared to resting cells [37].

Studies have shown that NCL is appreciated to undergo nucleolus/nucleoplasm-cytoplasmic shuttling and even to be present on the cell surface of various types of cancer cells [38]. It has become evident that NCL is extraordinarily multifunctional that involved in variety aspects of cellular processes, including DNA metabolism (DNA replication, repair and recombination), posttranscriptional and translational regulation of various mRNAs including p53, and cell surface NCL serving as a receptor of several ligands to either mediate tumorigenesis or to relay anti-carcinogenic effects. (see review Cong, R., Das, S., & Bouvet, P. (2011).) The complex roles of nucleolin in cellular processes is linked to its structure of multifunctional domains, post-
translational modifications (mostly phosphorylation in the N-terminal domain) and its ability of shuttling from and to different compartments of a cell.
Nucleolin (NCL/C23) is a multifunctional abundant nucleolar phosphoprotein that is localized in the dense fibrillar component (DFC) and the granular component (GC) of the nucleolus. NCL is a key factor that participates in all the major steps of ribosome biogenesis. NCL not only regulates rDNA transcription and rRNA processing, which occur at the border between the Fibrillar component (FC) and the DFC, but also has important roles in rRNA maturation and ribosome assembly, both of which take place in the GC.
1.1 The N-terminal domain of nucleolin

NCL has three major structural and multifunctional domains: the N-terminal domain with acidic stretches and many phosphorylation sites, the central four RNA binding domains (RBDs) and C-terminal domain, which is also known as the glycine/arginine-rich (GAR) domain [6]. The N-terminus is highly phosphorylated by numerous kinases, including casein kinase 2 (CK2) [39], cyclin-dependent kinase (CDK1) [40], protein kinase C (PKC)[41] and the stress activated protein kinase p38 [42]. Although the role of most of these phosphorylation events is still not well understood, many studies have reported a correlation between NCL phosphorylation and increased cell proliferation [4, 19-22]. CK2 phosphorylates NCL on serine during interphase while CDK1 phosphorylates it on threonine during mitosis. In vitro study shows that CK2 is inactive during mitosis on nucleolin phosphorylation and serine-dephosphorylated nucleolin is efficiently phosphorylated by CDK1, which indicates that serine phosphorylation is not required for achievement of threonine phosphorylation that is linked to mitotic reorganization of nucleolar chromatin [6, 30, 39, 40, 43]. The presence of CDK1 phosphorylation sites regulates the function of the bipartite nucleolin nuclear localization signal (NLS) of NCL that it promotes the nuclear import when they are dephosphorylated while enhances cytoplasmic localization when phosphorylated [44]. It can be envisioned that sequential CK2 and CDK1 phosphorylation could modulate NCL function in controlling cell proliferation (growth and division) between interphase and the mitotic phase of the cell cycle.

1.1.1 The role of phosphorylation on the N-terminus of nucleolin

Changes in NCL phosphorylation play roles in several critical cellular processes such as cell growth [36], DNA replication [27] as well as DNA damage response (DDR) [42, 45, 46]. For
example, Bouvet et al. generated conditional NCL-knockout cells using DT40 cell line and found that NCL is absolutely required for the cell proliferation and cell survival. In the absence of significant NCL expression, cell proliferation is showed down and cells die through apoptosis. Nevertheless, the expression of different NCL mutants showed that RBDs harbor redundant functions since DT40 cells survive and proliferate in the absence of two or three RBDs. However, cells deprived of NCL N-terminus stopped proliferation in day 2 or 3, which indicates that the function of the N-terminus of NCL plays a crucial role in regulating cell proliferation. In addition, they found that the amount of NCL similarly affects the proliferation rate and the rDNA transcription, which suggests that the function of nucleolin in ribosomal synthesis could be largely responsible for its effect on cell proliferation [36]. Nevertheless, it is still not clear whether phosphorylation of NCL on its N-terminus has a direct role in cell proliferation, or if it is only through the control of ribosome biogenesis.

Redistribution of NCL from nucleolus to nucleoplasm is a stress response that can be caused by cytotoxic agents, heat shock and gamma irradiation [25, 26, 47]. Interestingly, Kim et al. demonstrated that NCL with three deficient CK2 phosphorylation sites on the N-terminus (S34A/S184A/S206A) shifts partial NCL pools to nucleoplasm in unstressed cells which mimics the stress response [27], indicating phosphorylation of NCL by CK2 plays a crucial role in cellular response to stress, but the exact mechanism is largely unexplored.

1.2 The RNA binding domain of nucleolin

The central region of NCL contains four RNA binding domains (RBDs), which are also known as RNA recognition motifs (RRMs), confer NCL’s RNA binding properties [42].
Decades ago two major RNA sequences on pre-rRNA have been identified as binding sites for NCL. The first is a small stem-loop structure called nucleolin recognition element (NRE), the first two RBDs of nucleolin are necessary and sufficient for the specific interaction of NCL with the NRE motif [48, 49]. For example, NCL binds to the NRE in the 5’UTR of prostaglandin endoperoxide H synthase (PGHS-1) mRNA and represses its translation [50]. The second is a short single-stranded RNA sequence called the evolutionary conserved motif (ECM), which is located five nucleotides downstream of the first processing site in the pre-ribosomal RNA 5’ external transcribed spacer (5’ETS). All four RBDs of nucleolin are required to interact with these sequences [51, 52]. In addition, internal ribosome entry site (IRES) is the sequence which was found in the 5’-UTR of some viral mRNAs that NCL interacts with and stimulates IRES-mediated translation, e.g. poliovirus (PV) and hepatitis C virus (HCV) [53]. NCL also binds to the IRES of specificity protein-1 (Sp1) on its 5’UTR as to increase Sp1 translation, and the phosphorylation of nucleolin at Thr641/Thr707 by EGFR mediated Akt activation contributes to Sp1 IRES activity mediated during lung cancer formation [13]. Moreover, studies show that NCL is able to interact with AU-rich elements (AREs) with the 3’UTR of many mRNAs, such as Bcl-2 family apoptotic regulators BCL2 and BCLXL, and enhances mRNA stability [11, 54]. In addition, the RBDs of NCL are also reported to be involved in protein interactions to regulate p53 checkpoint. It interacts with Hdm2 leading to the inhibition of p53 ubiquitination and a p53 stabilization [24]. NCL binds to ribosomal protein L26 (RPL26) via its RBDs to compete with RPL26 in negatively controlling p53 translation [15-17]. The central RBDs of NCL is required for binding to p53 mRNA, forming NCL-NCL homodimer and binding to RPL26.
1.3 The GAR domain of nucleolin

The C-terminal domain of nucleolin is called GAR (Glycine- and Arginine-Rich) or RGG (Arg-Gly-Gly) domain, because it is rich in glycine, arginine and phenylalanine residues. The GAR domain is mainly involved in interactions with proteins and some specific nucleic acids as well [6, 55]. For instance, increased level of p53 upon DNA damage promotes NCL-p53 complex formation via the interaction of the GAR domain of NCL and p53 C terminal regulatory domain. This interaction shifts the majority of the NCL pool from the nucleolus to the nucleoplasm, where NCL sequesters RPA (human replication protein A), which is also through binding to NCL’s GAR domain, to transiently inhibit DNA replication [24-27]. Interestingly, GAR domain of NCL can interact with IRES element in the 5’-UTR of Sp1 mRNA and positively regulates the translation of Sp1 in tumorigenesis [13].
Nucleolin has three major structural and multifunctional domains: the N-terminal domain with acidic stretches and many phosphorylation sites, the central four RNA binding domains (RBDs) and the glycine/arginine-rich (GAR) C-terminal domain. The N-terminus is highly phosphorylated by either casein kinase 2 (CK2) during interphase or cyclin-dependent kinase (CDK1) during mitosis. The bipartite nuclear localization signal (NLS) is located at the border between the N-terminus and the central RBDs. RBDs render sequence-specific RNA binding (e.g. BCL2 and BCLXL) while GAR domain is mainly involved in protein-protein interaction (e.g. p53 and RPA).
2. Regulation of p53 checkpoint by nucleolin at multiple levels

2.1 Nucleolus as a stress sensor

p53 is the most important tumor suppressor which is known as the guardian of the genome, and about half of human cancers carry errors in this TP53 gene [56]. Nucleolus is a major stress sensor and transmits signals to the system for regulation of p53 activity [57, 58]. In recent years, modulation of the stress response (via activated p53 checkpoint) by nucleolar factors has been recognized to play an important role in tumorigenesis [59-63]. A major regulator of p53 is the E3 ubiquitin ligase MDM2 (also known as HDM2 in human) that directly interacts with the N-terminus of p53, and promotes p53 cytoplasmic degradation by the 26S proteasome to maintain the steady state of low p53 protein levels under normal physiological conditions [64, 65]. MDM2 gene, at the same time, is a transcriptional target of p53, this Mdm2-mediated p53 ubiquitination forms an auto-regulatory feedback loop which keeps p53 levels in check to protect cells from excessive p53 activation.

Rubbi and Milner demonstrate that a common denominator of all p53-inducing stresses is that they all cause nucleolar disruption and compromise nucleolar function. They propose a model of p53 stabilization – prevention of p53 degradation by nucleolar disruption. Disruption of nucleolar structure alone can lead to p53 stabilization without induction of DNA damage or p53 phosphorylation [59]. The “nucleolar stress” causes partial redistribution of nucleolar proteins such as NCL [25, 26], ARF [66, 67], nucleophosmin/B23 [68], and ribosomal proteins L5 [69] and L11 [70], from the nucleolus to the nucleoplasm, where they interact with MDM2 to inhibit its activity and stabilize p53 [57].
2.2 Regulation of p53 checkpoint by nucleolin

Figure 3 summarizes the current knowledge of the role of NCL in regulating p53 checkpoint at multiple levels. Saxena et al. reported that overexpression of NCL in unstressed cells increases p53 level by binding to Hdm2 to inhibit Hdm2-mediated p53 degradation. The stabilized p53 causes cell-cycle arrest by increasing p21 expression, and induce a reduced rate of cellular proliferation and an increase in apoptosis [23]. Bhatt et al. later identified that it was both the N-terminal domain and the central RBDs of NCL that interact with Hdm2 leading to the inhibition of p53 ubiquitination and p53 stabilization [24]. Under stress conditions, Bhatt et al. also reported that the increased level of p53 promotes NCL-p53 complex formation via the interaction of the GAR domain of NCL and p53 C-terminal regulatory domain [24], and thus shifts the majority of the NCL pool from the nucleolus to the nucleoplasm, where NCL sequesters RPA to transiently inhibit DNA replication. The translocation of NCL is concomitant with the increase of NCL-RPA complex formation. Interestingly, although p53 is not required for NCL-RPA complex formation, nucleolin mobilization is strongly dependent on NCL-p53 complex formation, but independent of the transcriptional transactivation function of p53 [25, 26]. The mechanism for NCL translocation mediated by p53 upon stress needs to be further studied.

The role of NCL regulating p53 checkpoint is not limited to post-translationally, but also post-transcriptionally due to the characteristics of NCL as a RNA-binding protein (RBP). NCL and several other RBPs, including HuR (Human antigen R) and RPL26, are shown to regulate TP53 mRNA at multiple levels [15, 17, 71]. NCL oligomerizes and forms NCL-NCL homodimer, RPL26 interacts with NCL to disrupt the NCL dimerization and forms NCL-RPL26
heterodimer. Upon DNA damage, NCL-RPL26 competes with NCL-NCL in binding to the same 5’ and 3’UTR regions of $TP53$ mRNA and oppositely regulate p53 translation, NCL represses p53 translation while RPL26 enhances it [15-17]. In addition, HuR not only increases mRNA stability by binding to the 3’UTR ARE of $TP53$, but also promotes p53 translation [71, 72]. Nevertheless, it is not clear whether NCL also can interact with the ARE on the 3’UTR of $TP53$ and regulate $TP53$ mRNA stability, and if NCL and HuR are competitors in binding to the ARE on the 3’UTR of $TP53$. 
Figure 3. Regulation of p53 checkpoint by nucleolin.

1) Under non-stressed conditions, p53 protein levels are kept low mainly by Hdm2-mediated degradation. NCL regulates p53 checkpoint at multiple levels. Over expression of NCL increases p53 protein level by binding to Hdm2 and inhibiting Hdm2-mediated p53 degradation. Activated p53 transactivates its downstream targets causing p53-dependent cell-cycle arrest and apoptosis. 2) Under stressed conditions, the increased level of p53 promotes NCL-p53 complex formation, which shifts the majority of the NCL pool from the nucleolus to the nucleoplasm, where NCL sequesters RPA to transiently inhibit DNA replication. 3) Besides, NCL represses the translation of p53 mRNA by forming NCL-NCL homodimer and directly binding to the 3' and 5' UTR of TP53 mRNA under non-stressed conditions, while RPL26 disrupts the NCL-NCL homodimer upon stress, and turns on p53 translation by forming NCL-RPL26 heterodimers and binding to the same region of the 3' and 5' UTR of TP53 mRNA.
2.3 Regulation of cell fate by p53 dynamics

Much attention has been focused on the different post-translational modifications of p53 leading to stabilization of p53 and activation of different downstream pathways that regulate cell fate [73, 74]. Nevertheless, a growing number of studies recently are revealing that cells can send and receive information by controlling the temporal behavior (dynamics) of their signaling molecules, and the dynamics of p53 plays a pivotal role in determining cell fate [75, 76]. p53 dynamics in response to DNA damage were originally observed from a population of cells and described as damped oscillations when measured by western blot [77]. The average dynamical behavior of a population cells often represents a distorted version of individual cells’ behavior which can mask true dynamical responses [78, 79]. Recently from single cell analyses, Lahav’s group revealed that p53 dynamics are “pulsed vs. sustained” which varies depending on the stimulus. For example, double-stranded breaks caused by $\gamma$-irradiation triggers a series of excitable pulses with fixed amplitude and duration which is independent of the damage dose, but the number of pulses increases with higher damage dose. Single-stranded breaks induced by UV elicits a sustained p53 dynamics which is a single pulse with a damage dose-dependent amplitude and duration [80]. Later their work showed that the pulsed vs. sustained p53 dynamics controls cell fate by regulating downstream target genes, pulsed p53 favoring transient response such as DNA repair or temporary cell cycle arrest, while sustained p53 triggering irreversible fates such as program cell death (apoptosis) or permanent cell cycle arrest (senescence) [81].

The stereotyped nature of the p53 pulses was one of the most striking features about them when they were first discovered by Lahav et al [78], even though the mechanism for achieving the precision in timing and amplitude is still largely unknown, the p53 dynamic behaviors
controlling cell fate is a new model that gains more attentions in the studies of potential functional roles for transmitting cellular information through the dynamics of signaling molecules and possible applications for the treatment of disease [75, 76, 80-82].

3. Post-transcriptional regulation of mRNAs by nucleolin

NCL is one of the most multifunctional RNA-binding proteins (RBPs) that participates extensively in RNA regulatory mechanisms, including transcription of rRNA, ribosome assembly, mRNA stability and translation, and microRNA processing [83]. Specifically, the N-terminus of NCL is required for an interaction with the component of the pre-RNA processing complex that catalyzes the cleavage of pre-RNA occurring in the 5’external transcribed spacer (5’ETS) [84-86]. The central RBDs of NCL bind to a stem-loop structure found in nascent pre-RNA and play a chaperon function by facilitating the proper folding of pre-RNA [49, 52, 87]. On the other hand, the RBDs of NCL are crucial in binding with mRNAs and regulating mRNA stability and translation in response to stresses [14, 42]. The RGG in the C-terminus of NCL can cooperate with the RBDs and interact with target mRNAs as well [14]. In addition, NCL was also reported to interact with microprocessor complex to affect the biogenesis of the pro-tumorigenic microRNAs 15a and 16 [88].

Gene expression can be intensively regulated at the level of RNA, which occurs through alterations in translational efficiency [89] and/or in messenger RNA (mRNA) stability [90, 91]. NCL is identified as a stress responsive RBP that regulates the stability and translation of numerous mRNAs during cellular stress response. NCL can not only globally enhance the translation of mRNAs with G-rich elements in coding and non-coding regions, but also interact
with the AU-rich elements (AREs) in the 3’UTR of numerous mRNAs and enhance their stability [14, 42, 83]. Much evidence shows that in most cases NCL plays positive roles and favors the expression of proliferative and anti-apoptotic genes. Table 1 shows some best characterized mRNA targets of NCL that encode cancer progress-related proteins, e.g. anti-apoptotic factor \textit{BCL2} and \textit{BCLXL} [11, 92], tumor suppressors \textit{CDKN2A (ARF)} [93] and \textit{TP53} [15-17].

The ARE is the most well-studied \textit{cis}-acting element that is responsible for rapid mRNA decay. Majority of mRNA decay is through the deadenylation-dependent pathway that initiates with removal of the poly(A) tail by deadenylases, which is the first and rate-limiting step in the degradation of ARE-containing mRNAs. Poly(A)-specific ribonuclease (PARN) is one of the major deadenylases in mammalian cells that can regulate the stability of mRNAs involved in DNA damage response, such as c-myc, c-fos and c-jun [94]. Specific ARE-binding proteins (AUBPs) including NCL are believed to play a fundamental role in the recognition of mRNAs and recruitment of other \textit{trans}-acting factors and/or factors that modulate the poly(A) tail such as PARN and poly(A)–binding proteins (PABPs). For example, AU-rich Element-binding Factor 1 (AUF1) binds to the ARE in the 3’UTR of \textit{BCL2} mRNA and recruits the exosome to facilitate rapid mRNA decay after deadenylation [54, 95]. Overexpressed NCL can competitively bind to the \textit{BCL2} mRNA ARE which decreases the binding of AUF1 with \textit{BCL2} mRNA ARE and blocks the recruitment of the exosome, leading to increased \textit{BCL2} mRNA stability and protein levels in response to UV irradiation [54, 95]. Similarly, NCL stabilizes \textit{BCLXL} mRNA by a mechanism where the stabilizing effect was dependent upon the presence of poly(A) tail. The interaction of NCL and PABPs blocks the access of PARN and therefore slows down the
deadenylation process [11]. Tristetraprolin (TTP) destabilizes ARE-containing mRNA targets e.g. tumor necrosis factor alpha (TNF-α) [96] and granulocytemacrophage colony-stimulating factor (GM-CSF) [97], by stimulating PARN deadebylation and promoting the removal of the poly(A) tails [98]. K homology Splicing Regulatory Protein (KSRP) accelerates mRNA degradation by interacting with AREs and recruiting PARN and exosomes [99-103]. On the contrary, Human antigen R (HuR), which is an ubiquitously expressed AUBP and a stabilizer of ARE-containing mRNAs, can stabilize mRNAs by blocking the binding of those destabilizing AUBPs, e.g. TTP and KSRP, to the AREs in the 3’UTR of mRNA targets [104-106]. HuR is predominantly localized within the nucleus under non-stressed conditions. Many stress stimulators including UV radiation can induce HuR shuttling from nucleus to cytoplasm, where it binds and stabilizes a large subset of ARE-containing mRNAs through its RNA recognition motifs (RRMs) and/or enhance the translation of target mRNAs [107, 108].

Interestingly, studies have shown that HuR and NCL are both predominantly nuclear RBPs that shuttle from the nucleus to the cytoplasm upon stress, and regulate ARE-containing mRNAs [104]. Moreover, they bind to many common mRNA targets and plays antagonistic or synergistic roles on their shared mRNA targets e.g. $ARF$, $TP53$, $BCL2$, $H1F1A$ and $MMP9$ etc., which are hallmark genes of cancer progress [92, 93, 109, 110], but the functional interactions between HuR and NCL have not been well studied.

Focusing on the p53-checkpoint signaling, NCL competes with HuR to regulate translation of $ARF$ mRNA, the upstream regulator of p53. In the absence of HuR, NCL associates with $ARF$ mRNA in the nucleolus and enhances its translation, while in the presence of HuR, the interaction of HuR with $ARF$ mRNA in the nucleoplasm represses the translation of $ARF$ mRNA
In the case of TP53, NCL-NCL homodimers binds to a region of double-strand RNA formed by base-pairing interactions of sequences in the 5' and 3' UTRs of TP53 mRNA, keeping a steady low rate of p53 translation under non-stressed conditions. Upon stress, RPL26 becomes available and forms NCL-RPL26 heterodimer that are competitively bound to the same region in 5' and 3' UTRs of TP53 mRNA, which disrupts the NCL-NCL homodimers and switches on p53 translation [15-17]. On the other hand, HuR not only increases TP53 mRNA stability by binding to the ARE on TP53 mRNA 3’UTR after stress [72], but also promotes p53 translation by binding to TP53 3’UTR after UV exposure, although it is a result of sequestration of TP53 mRNA on polysomes, rather than binding to the ARE sequence of TP53 3’UTR per se. [71]. It seems that NCL and HuR have opposite roles in regulating TP53 since NCL repress p53 translation while HuR enhances it, but further elucidation is still needed. Interestingly, studies from our collaborator Dr. Kleiman’s lab show that PARN is recruited to the TP53 mRNA and decreases the stability of TP53 mRNA under non-stressed conditions, while HuR competes with PARN for binding with TP53 mRNA, resulting in an increase in TP53 expression following UV treatment [111-113]. Nevertheless, whether NCL also plays a role in the PARN regulated deadenylation of TP53 mRNA remains to be determined. In addition, it is reported that HuR can work synergistically with NCL to stabilize BCL2 mRNA and enhance the translation of BCL2 by binding concurrently to the ARE in the 3’UTR of BCL2 mRNA. HuR and NCL are both present in common BCL2 messenger ribonucleoprotein (mRNP) complexes and this coprecipitation is RNA dependent [12, 54]. Similarly, NCL binds to the 3' UTR of MMP9 mRNA and enhances its translation [114], HuR also binds to the 3' UTR of MMP9 mRNA but increases its stability [115], although it is not yet known if HuR and NCL may regulate MMP9 expression.
competitively or cooperatively. NCL physically binds to the 3’UTR of \textit{HIF1A} and increases its stability [116], while HuR associates principally with the 5’UTR of \textit{HIF1A} to promote its translation [117]. Taken together, these indicate that NCL and HuR might have functional interplay to control the expression of the NCL/HuR common mRNA targets, but the biological significance of the HuR-NCL interaction is still not clear currently.

AUBPs can be subjected to post-translational modifications which regulates their functions in response to signals or cellular changes upon DNA damage. It is reported that phosphorylation of NCL affects its ability in binding with mRNA targets [42]. For instance, NCL phosphorylation by p38 increases its RNA-binding activity and enhance interaction of NCL with a subset of mRNAs encoding stress-response proteins following exposure to stresses like UV light and ionizing radiation [42]. UV increases the binding capacity of NCL to AREs in the 3’-UTR of \textit{BCLXL} mRNA in a p38 MAPK-dependent manner, because phosphorylation of NCL by p38 prevents the recruitment of other destabilizing factors, e.g, TTP and KSRP, to \textit{BCLXL} mRNA [11, 118]. The Syk protein kinase binds robustly to NCL and phosphorylates it on tyrosine, enhancing its ability of binding with \textit{BCLXL} mRNA as well [119]. Similarly, phosphorylation of HuR by p38 following ionizing radiation also increases its binding activity with target mRNAs e.g. \textit{CDKN1A} (encoding the cdk inhibitor p21) [120]. KSRP phosphorylated by p38 displays compromised binding to ARE-containing transcripts and fails to promote their rapid decay, although it retains its ability to interact with the mRNA degradation machinery [121, 122]. TTP phosphorylation by MK2 (substrate of p38 MAPK) and subsequent interaction with adaptor molecules reduces ARE-containing mRNA affinity and/or impairs the recruitment of the mRNA decay machinery (e.g., exosome) to its mRNA targets, while dephosphorylation of TTP can
increase its apparent binding affinity for its ARE-containing mRNA targets [98, 123]. However, whether NCL phosphorylation by CK2 plays a role in regulating its binding capacity with mRNA targets as well as mediating mRNA stability are currently unknown.
### Table 1. Partial list of nucleolin target mRNAs encoding cancer-related proteins

<table>
<thead>
<tr>
<th>Target after DNA damage</th>
<th>mRNA DNA</th>
<th>Binding region on mRNA</th>
<th>Effect on mRNA</th>
<th>Implication in Cancer</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>mRNA Stability</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BCL2</td>
<td>3’-UTR ARE</td>
<td>Stabilization</td>
<td>Anti-apoptosis</td>
<td>[10, 124]</td>
<td></td>
</tr>
<tr>
<td>BCLXL</td>
<td>3’-UTR ARE</td>
<td>Stabilization</td>
<td>Anti-apoptosis</td>
<td>[11]</td>
<td></td>
</tr>
<tr>
<td>HIF1A</td>
<td>3’-UTR ARE</td>
<td>Stabilization</td>
<td>Angiogenesis</td>
<td>[116]</td>
<td></td>
</tr>
<tr>
<td>IL2</td>
<td>5’-UTR JNK response element</td>
<td>Stabilization</td>
<td>Immunosurveillance</td>
<td>[125]</td>
<td></td>
</tr>
<tr>
<td>GADD45A</td>
<td>3’-UTR</td>
<td>Stabilization</td>
<td>Stress response</td>
<td>[126, 127]</td>
<td></td>
</tr>
<tr>
<td>OSM</td>
<td>3’-UTR GC-rich cis-elements (GCREs)</td>
<td>Stabilization</td>
<td>Metastasis</td>
<td>[128]</td>
<td></td>
</tr>
<tr>
<td>GAST</td>
<td>3’-UTR</td>
<td>Stabilization</td>
<td>Inflammation</td>
<td>[129, 130]</td>
<td></td>
</tr>
<tr>
<td>Translation Efficacy</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TP53</td>
<td>3’-UTR and 5’-UTR</td>
<td>Repression</td>
<td>Tumor suppressor</td>
<td>[15-17]</td>
<td></td>
</tr>
<tr>
<td>CDKN2A (ARF)</td>
<td>G-rich elements</td>
<td>enhancement</td>
<td>Tumor suppressor</td>
<td>[14, 93]</td>
<td></td>
</tr>
<tr>
<td>MMP9</td>
<td>3’-UTR</td>
<td>enhancement</td>
<td>Invasion</td>
<td>[114]</td>
<td></td>
</tr>
<tr>
<td>AKT1</td>
<td>G-rich elements</td>
<td>enhancement</td>
<td>Survival</td>
<td>[14]</td>
<td></td>
</tr>
<tr>
<td>CCNI</td>
<td>G-rich elements</td>
<td>enhancement</td>
<td>Anti-apoptosis</td>
<td>[14]</td>
<td></td>
</tr>
<tr>
<td>SPI1</td>
<td>5’-UTR internal ribosome entry site (IRES)</td>
<td>enhancement</td>
<td>Tumorigenesis</td>
<td>[13]</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER II

EFFECT OF NUCLEOLIN PHOSPHORYLATION BY CK2 IN REGULATING CELL PROLIFERATION
**Introduction**

Previous studies report that the increased level of p53 upon stress promotes NCL-p53 complex formation via the interaction of NCL GAR domain and p53 C-terminal regulatory domain [24], and shifts the majority of the NCL pool from the nucleolus to nucleoplasm, where NCL sequesters RPA (human replication protein A) to transiently inhibit DNA replication [25, 26]. Kim et al. later reported that NCL-TM, a NCL mutant with three CK2 sites (S34A/S184A/S206A) [30] on its N-terminal converted to non-phosphorylatable alanines, mimics endogenous NCL’s cellular response to stress conditions, where NCL-TM has significant nucleoplasmic localization in unstressed cells and interacts with RPA to inhibit DNA replication [27]. Follow up study from our colleague Maldonado show that the NCL-TM shows decreased binding to the NRE sequence of pre-rRNAs and a concomitant increase in mobility within the nucleus by translocating to nucleoplasm as compared with NCL-WT, suggesting that dephosphorylated NCL reduces its affinity with pre-rRNAs which leads to increased free NCL pool. But it does not completely abrogate the main nucleolar accumulation of NCL because NCL-TM still has appreciable affinity for pre-rRNA. In addition, preliminary data show that expression of NCL-TM causes cell proliferation inhibition and sensitization to apoptosis (Priscilla Maldonado. Regulation of the proliferative activites of Nucleolin by CK2. PhD thesis 2011). Taken together, these studies indicate that phosphorylation on the N-terminus of NCL by CK2 regulates NCL mobility within the nucleus which may correlate with the role of NCL in regulating DNA replication, p53 activation and cellular response to stress. However, the mechanism is needed to be further elucidated.
To further elucidate the role of NCL phosphorylation by CK2 in regulating cell fate, the Saxena laboratory later genetically modified human NARF6 cells through retroviral infection, allowing induced stable expression of either NCL wild type (WT) or a CK2 phosphorylation-deficient mutant (6/S*A; containing six alanine substitutions at the consensus serine sites: S28A/S34A/S145A/S153A/S184A/S206A; see Figure 4,) by a Tet-off promoter system. The NARF6 cells which were originally derived from U2OS cells, express wild-type p53 as well as support the IPTG (isopropyl b-D-thiogalactopyranoside) - regulated expression of the p14ARF (Alternate Reading Frame), an upstream regulator of p53 during oncogenic stimulation [131]. Hereafter I refer to these modified cells as inducible NCL (WT or 6/S*A) cells.
The six consensus CK2 sites (all serine) that were mutated to alanine in the 6/S*A construct are denoted by asterisks (*). An enlarged schematic of the N-terminal domain is also shown.

Figure 4. Targeting the consensus CK2 sites in NCL.
We demonstrated that NCL-6/S*A is only 16% phosphorylated as compared to WT, which indicates that the mutation of the six CK2 sites greatly reduces NCL phosphorylation. Moreover, NCL-6/S*A is readily localized in the nucleoplasm as compared to NCL-WT in unstressed cells [132]. These data indicate that the NCL-6/S*A mutation mimics the effect of stress by causing partial NCL translocation from the nucleolus to the nucleoplasm under non-stressed conditions. In addition, NCL phosphorylation by CDK1 remains unchanged between NCL-WT and 6/S*A (K. Ng and A. Saxena, unpublished data) which strongly suggests that CK2 is the major kinase that phosphorylates NCL during interphase, confirming the earlier study which had already shown that serine phosphorylation by CK2 is not required for achievement of threonine phosphorylation of NCL and serine-dephosphorylated NCL is efficiently phosphorylated by CDK1 during mitosis [43].

To extend the study, in this chapter I demonstrate the six consensus CK2 sites on the N-terminus of NCL are important for cell survival and proliferation. Expression of CK2 phosphorylation-deficient NCL mutant (NCL-6/S*A) leads to dominant negative effects on cell proliferation and triggers p53 stabilization to induce expression of pro-apoptotic markers downstream.
Results

Inducible expression of nucleolin phospho-variants activate p53 checkpoint

Earlier Dr. Saxena reported that exogenous NCL expression stabilizes p53 and regulates its transcriptional activity [23]. Therefore, first I examined the effects of NCL-WT and 6/S*A expression on p53 protein levels. Here I present data from inducible NCL cells when exogenous NCL expression was induced by removal of doxycycline (DX) for a range of 7–28 days. Both variants (NCL-WT and NCL-6/S*A) resulted in upregulation of p53 protein levels but greater increase was observed with NCL-6/S*A expression (Figure 5A). Interestingly, NCL-WT showed fluctuating levels of p53 (with periodic variation), while NCL-6/S*A resulted in more persistent (sustained) p53 protein levels. Corresponding to the p53 levels, increases in p21 protein, the downstream target of p53, were also observed. The scatter plot representing the p53 and p21 protein levels during the 7 to 28 days of induced expression of WT or 6/S*A expression as compared to the Ctrl cells strongly indicated that both p53 and p21 levels were higher in NCL-6/S*A expressing cells (Figure 5B).

Expression of nucleolin phosphorylation-deficient mutant increases p53 protein half-life

Next I determined how NCL dephosphorylation affected p53 protein half-life. The p53 protein half-life is clearly increased with NCL-6/S*A expression as compared to NCL-WT as indicated by a representative Western blot (Figure 6A). As indicated in Figure 6B, the p53 half-life is lower in cells expressing NCL-WT (~30–40’) as compared to NCL-mutant (~1h), while control cells have normal half-life of p53 (~15–20’).
Figure 5. Inducible expression of NCL (WT or 6/S*A) result in an increased p53 protein levels.

(A) Western blot analyses for cells grown without doxycycline (Dx) for the indicated period indicating inducible NCL-expression (WT or 6/S*A). Both WT and 6/S*A lead to net increase in p53 protein levels and corresponding p21 protein levels, the downstream target of p53. (B) Plots of p53 and p21 protein levels shown in 5A. The quantification was done by NIH Image J software. Values were first corrected for the β-actin levels and then compared to Ctrl (no exogenous NCL, no Dx day 7) cells. The graph is representative of at least three independent experiments.
Figure 6. Half-life analyses of p53.

(A) Inducible NCL-cells grown without Dx for 17 days to express either NCL-WT or NCL-6/S*A. Cells were then treated with cycloheximide (CHX, 40 µg/ml) for indicated times. Lysates were prepared and analyzed by Western blotting for p53, FLAG (for NCL expression) and the β-actin loading control. (B) Plot of p53-expression levels following CHX treatment corrected for the β-actin levels. The graph is representative of three independent experiments done in duplicates. Half-life of p53 is ~60 min for NCL-6/S*A, ~40 min for NCL-WT and ~20 min for ctrl cells.
Nucleolin mutant, defective in CK2 phosphorylation, causes dominant-negative effect on cell proliferation in a p53-dependent manner

To understand the physiological consequences in terms of cellular fate upon the stabilized p53 protein levels, I examined cell cycle progression and cell proliferation upon induced NCL-WT and 6/S*A expression. Cell cycle distribution revealed a significant decrease in the fraction of cells in S-phase upon NCL-6/S*A expression (Figure 7A, **p < 0.005). Employing a more sensitive method to determine the S-phase population, EdU positive cells were reduced from 47% (in WT) to 34% in 6/S*A expressing cells (Figure 7B). The decreased proportion of cells in S-phase strongly suggest that cells with continuous expression of NCL-6/S*A delay the cell cycle, failing to progress through the S-phase. Indeed, the MTS cell proliferation assays demonstrate that NCL-6/S*A is inhibitory to cell proliferation as compared with WT or Ctrl cells (Figure 7C).

To determine if the NCL-6/S*A mediated inhibition of cell proliferation is p53-dependent, I used HCT116 p53-wt and null cells [133]. As depicted in Figure 7D, there is no significant difference in proliferation rate with expression of NCL (WT or 6/S*A) in p53-null background although lesser viability is evident with 6/S*A. In the presence of p53-wt, however, NCL-6/S*A expression showed a significantly lower rate of cell proliferation as compared to NCL-WT (Figure 7D). These data decisively demonstrate that NCL phosphorylation-deficient mutant inhibits cell proliferation in a p53-dependent manner. Intriguingly, the inhibitory effect by NCL-6/S*A occurs in the presence of endogenous NCL-WT. Thus, expression of phosphorylation deficient NCL mutant may exert dominant-negative effect on cell proliferation.
Figure 7. NCL-6/S*A expression causes p53-dependent inhibition of cell proliferation.

(A) DNA content of cells expressing vector, NCL-WT or NCL-6/S*A using propidium iodide staining and flow cytometry analyses. NCL-6/S*A expressing cells have significantly (p value ≤0.005) low % of cells in the S-phase as compared to cells expressing WT or V. (B) Reduced % of cells in S-phase with 6/S*A expression for 10d as assayed by Click-iT EdU flow cytometry kit (Invitrogen). Analyses were performed using ModFit software. (C) Continuous expression of 6/S*A (17d) is inhibitory to cell-proliferation as analyzed by MTS assay. (D) HCT116-p53 wt or ko cells were transfected with NCL-WT or -6/S*A and assayed for cell proliferation using MTS solution. NCL-6/S*A expressing cells require p53 to inhibit cell proliferation.
Exogeneous-NCL (WT and 6/S*A) associate with endogenous-NCL

Earlier it was shown that NCL oligomerizes through its RNA binding domain [17]. To further explore the mechanism of dephosphorylated NCL exerting the dominant-negative effect on cell proliferation, I investigated whether NCL-6/S*A can associate with endogenous-NCL. My co-ip experiment showed that endogenous-NCL is precipitated along with both Flag-tagged NCL-WT and 6/S*A (Figure 8). This indicates that NCL-6/S*A might apply its dominant-negative effect by antagonizing endogenous-WT through protein-protein Interactions.

Lack of nucleolin phosphorylation by CK2 activates expression of apoptotic markers downstream in the p53 pathway

To further elucidate NCL-6/S*A mediated inhibition of cell proliferation, I analyzed markers in the apoptotic pathway to obtain information about the observed cellular fate. As depicted in Figure 9, I present cumulative data from day1 (24h) to day24 after induced NCL expression by the removal of Doxycycline. It is observed that p53 levels are increased with both NCL-WT and NCL-6/S*A expression as early as 24 h of NCL induction, and p53 levels are fluctuating with NCL-WT expression (day1~24), while NCL-6/S*A expression induces a sustained upregulation of p53 levels (day1~24) (Figure 9A). Moreover, expression of NCL-6/S*A resulted in an increased level of Bcl-2 homology 3 (BH3)-only BID (BH3 interacting-domain death agonist) and PUMA (p53 upregulated modulator of apoptosis), anti-apoptotic protein of Bcl-2 (B-cell lymphoma 2) family BAK, and mitochondria factor AIF. But these are not observed with Ctrl or NCL-WT expressing cells, although NCL-WT expression causes a slightly upregulation of p21 levels (Figure 9B and 9C), which is probably due to the NCL-WT induced fluctuating p53 levels.
Figure 8. NCL-WT and 6/S*A interact with endogenous NCL.

Nuclear extracts (NE) were prepared from cells grown without Dx for at least 10 d for NCL-WT or 6/S*A expression. Ctrl represents control cells without exogenous NCL expression. Equal amounts of NE protein from these cells were then subjected to co-immunoprecipitation using anti-FLAG M2 beads. Western analyses of NE and bound fractions were analyzed by anti-NCL (to detect: -exogenous 3xFlag-tagged NCL, upper band and –endogenous NCL, lower band), anti-Flag, anti-p53 and antip21. Anti-TOPOII β blot serves as the loading control for NE. The data is representative of three independent experiments performed with 10 d–20 d of WT or 6/S*A expression. This data supports the hypothesis that NCL (both WT and 6/S*A) can associate with endogenous NCL (i.e. NCL-NCL interactions).
Figure 9. NCL-6/S*A expression results in an increased expression of apoptotic markers downstream the p53 pathway.

(A) Graph of p53 protein levels from day 1 to day 24 of induction of NCL-expression in NCL-WT or NCL-6/S*A cells, compared with control cells. (B) Western blot analyses for cells grown without Dx for the indicated period indicating inducible NCL-expression (WT or 6/S*A). Both WT and 6/S*A expression resulted in an increase in p53 and p21 protein levels. Increased expression of BH3-only pro-apoptotic markers (BID, BIM and PUMA) were observed as early as 1 d or 4 d of induced NCL-6/S*A expression. (C) Plots of p53, p21 and BH3-only protein levels shown in panel A. The quantification was done by NIH Image J software. Values were first corrected for the β-actin levels and then compared to Ctrl (no exogenous NCL, no Dx day 1) cells. The graph is representative of at least two independent experiments.
Discussion

In the current chapter, by using a new cellular tool that express CK2-specific full-length NCL phosphorylation variants upon a Tet-off inducible promoter system, I make the novel observation that NCL phosphorylation by CK2 at the six consensus sites is required for cell survival and cell proliferation. Loss of phosphorylation at these CK2 sites results in increased p53 levels activating the signaling pathway downstream to p53. Expression of CK2 phosphorylation-deficient NCL mutant causes a reduced fraction of cells in S-phase that ultimately leads to an inhibition of cell proliferation presumably by initiating apoptosis pathway (see the model in Figure 10). In contrast, NCL-WT expression causes pulsatile p53 and p21 protein levels that allow cells to survive probably by resuming the cell cycle progression (Figure 9).

In recent years, modulation of the stress response (via activated p53 checkpoint) by nucleolar factors has been recognized to play an important role in tumorigenesis [59-63]. NCL, the major nucleolar protein regulates p53-signaling at multiple levels: in exponentially dividing cells, heightened NCL levels can trigger a p53-checkpoint by NCL binding to Hdm2 and reducing p53 ubiquitination [23, 24]. This may reflect a feedback mechanism that serves to prevent hyperproliferation induced by abnormally high NCL levels. During the DNA damage response, NCL can translocate from its usual nucleolar locations to nucleoplasm where it can modulate the translation of p53 through NCL-RPL26 or NCL-NCL oligomerization [15-17] and/or regulate p53 protein through direct interactions with p53 [24, 26, 27] or its antagonist Hdm2 [23, 24].

In this study, I extend the regulation of p53 by NCL phosphorylation demonstrating that dephosphorylation at CK2 sites leads to “nucleolar stress” like conditions that result in a dominant-negative effect on cell proliferation which is predominantly p53-mediated, and activate
the apoptotic factors in p53 signaling pathway. I envision that NCL-6/S*A negates the effects of endogenous NCL through oligomerization and indeed I observe interactions between endogenous-NCL and exogenous-NCL (WT or 6/S*A) in our co-IP assays to support this concept (Figure 8). Future stoichiometric analyses between NCL and its different phosphorylation forms will provide more insights about NCL oligomerization and its physiological implications.

Recently from single cell analyses, the phenomenon of pulsed versus sustained p53 levels and different cellular fate has provided useful insights about p53 control of gene expression in DNA repair, cell cycle arrest, apoptosis and senescence pathways [75, 80-82]. In fact, both NCL-WT and NCL-6/S*A expression increase the p53 protein levels from day1 and I observe an increased p53 protein half-life associated with NCL-mutant expression. Nevertheless, I frequently observe fluctuating p53 levels with NCL-WT expression while persistent elevation of p53 levels with NCL-6/S*A expression. Even though it is not single-cell analysis, but this is the first time that p53 expression pattern of a cells population are recorded over a long period of time (from day1 to day28 after induced NCL expression), which should be a reliable way of reflecting p53 dynamic behaviors upon inducible NCL expression. My results show that sustained p53 levels caused by phosphorylation-deficient NCL (NCL-6/S*A) expression results in significant upregulation of all p53-mediated proapoptotic factors that have been tested e.g. BID, BAK, PUMA and AIF, while fluctuating p53 with NCL-WT expression leads to upregulation of cell cycle regulators e.g. p21 but not the proapoptotic factors (Figure 8). This is consistent with Lahav et al’s studies that regulation of p53 dynamics – “pulsed vs. sustained” leads to expression of a different set of downstream genes determining the phenotypic outcomes of cell fate, pulsed
p53 favoring transient response such as DNA repair or temporary cell cycle arrest, while sustained p53 triggering irreversible fates such as apoptosis or senescence [75, 80-82]. Besides, although my results strongly support that NCL mutant requires p53-mediated signaling in restricting cell proliferation, a p53-independent increase of PUMA cannot be completely ruled out. It is also possible that targeting NCL phosphorylation by CK2 could synergistically initiate a broader range of cell death pathways that leads to more efficient retardation of proliferation. Future investigation should determine the status of p53 transcription function and provide evidence whether the upregulation of proapoptotic factors is directly p53 mediated.

Together, hypophosphorylated NCL with more nucleoplasmic localization presumably causes “nucleolar stress” like conditions that can trigger multiple events: NCL binds to p53-protein and RNA directly or modulates p53 levels through interactions with other p53-regulators (e.g. Hdm2 or RPL26); other nucleolar factors can similarly sense this stress to regulate p53 checkpoint [57]. Thus, increases in p53-levels in NCL-mutant expressing cells could be the net result of these direct and indirect events. Future studies with single cell analyses will prove highly useful in validating the functional impacts of NCL hypophosphorylation in regulating the kinetics of the p53 signaling pathway. It is worth mentioning that NCL is also an important RNA binding protein that regulates gene expression through direct NCL/RNA interactions or indirectly by recruiting other molecules in RNA metabolism. Importantly, the N-terminus and the RNA binding domains of NCL are positioned adjacently and hence can influence NCL protein conformation and/or functional properties [134]. In spite of this association, due to the highly-acidic nature of the N-terminus and technical issues of purifying full-length NCL, the role of NCL phosphorylation has been overlooked while studying many of its physiological
functions. These NCL constructs and clones are therefore valuable in facilitating the full-length NCL purification through its tag (His, Flag). Additionally, these cells have an ability to express p14ARF tumor suppressor protein that lies upstream to the p53 checkpoint activation, more commonly during oncogenic stimulation. Using the inducible expression of NCL in this study I reveal for the first time how CK2-mediated NCL phosphorylation provides necessary quality control surveillance on the major cellular decision of cell survival.
Figure 10. Mechanistic model by which nucleolin phosphorylation by CK2 regulates cell proliferation.

NCL-WT and phosphorylation-deficient mutant (6/S*A) activate p53 checkpoint and increase corresponding p21 levels. However, NCL-WT expression allows cells through S-phase progression and resumes cell cycle. On the other hand, NCL-6/S*A acts as a dominant-negative mutant that negates NCL-WT functions possibly through oligomerization causing inhibition of cell proliferation and initiating apoptotic pathway.
CHAPTER III

EFFECT OF NUCLEOLIN PHOSPHORYLATION BY CK2
IN INDUCING APOPTOSIS
Introduction

In previous chapter I demonstrate that lack of NCL phosphorylation by CK2 causes a ‘‘nucleolar stress’’ like conditions in unstressed cells and has a dominant negative effect on cell proliferation by stabilizing p53, resulting in higher expression of proapoptotic p53 target genes [27, 132], including BH3-only proteins PUMA [135] and BID [136], Bcl-2 family proapoptotic factor BAK [137] and mitochondrial factor AIF [138]. BH-3 only proteins are essential for initiating the mitochondrial-mediated apoptosis, by triggering oligomerization of pro-apoptotic members of the Bcl-2 family BAX and/or BAK and forming channels to induce mitochondrial membrane permeabilization (MOMP), releasing apoptogenic factors e.g. cytochrome c and AIF into the cytoplasm, which is viewed as a point of no return to trigger programmed cell death (PCD). [139-143]. Cytochrome c forms apoptosome (with active caspase-9 and Apaf-1) in the cytoplasm to induce classical caspase-dependent cell death, while AIF is known to play the central role in initiating caspase-independent cell death (CICD), by translocating from the mitochondrial to the nucleus, where it causes reactive oxygen species (ROS) production, chromatin condensation and large-scale DNA fragmentation [144-151].

Interestingly, my results show that cytochrome c is not detectable in these inducible NCL cells (NCL-6/S*A and NCL-WT) (data not shown), which implies that NCL dephosphorylation may have not triggered the irreversible cellular event MOMP to release the mitochondrial apoptogenic factors in unstressed cells. The observed elevation of AIF levels is possibly solely due to transcriptional upregulation by p53. In fact, the hallmark event of late stage of apoptosis – caspase cleavage is not present in NCL-6/S*A or NCL-WT cells either (data no shown), which confirms that in spite of the upregulated basal levels of proapoptotic factors induced by NCL-
6/S*A expression, full-scaled apoptosis is not yet triggered in unstressed cells. Nevertheless, I observe upregulation of total levels of the procaspases in NCL-6/S*A cells, including major caspase initiators procaspase-8 and -9 and caspase effector procaspase-3 (Figure 11). It is reported that total levels of the procaspases can tip the scales between cell survival and death, and elevated levels of procaspases plays a key role in sensitizing cells to apoptosis [152]. The basal expression patterns of apoptosis and cell cycle regulators play the essential role in determining the cell fate -- life or death in response to stress [153, 154]. My results show that dephosphorylation of NCL at CK2 sites upregulates the basal levels of many proapoptotic p53-responsive genes, however, whether dephosphorylation of NCL at CK2 sites affect cell fate in response to apoptotic stimuli is not known yet.

Previous studies show that the re-localization of NCL to the nucleoplasm in U2OS cells is stress-specific. It is only observed with heat shock, camptothecin (CPT) treatment or ionizing radiation, but not with UV exposure [26, 27]. In our recent publication Xiao et al have shown similar results that both NCL variants (NCL-WT and NCL-6/S*A) translocate completely to the nucleoplasm upon treatment with CPT (2 mM for 2 h), while exposure to UV (50 J/m²) had a reduced effect on each variant, with WT and 6/S*A both showing a combination of nucleolar and nucleoplasmic localization [132]. UV radiation does not induce DNA strand breaks directly, but it can cross-link consecutive pyrimidine bases leading to the exposure of single-stranded DNA (ssDNA) and also produce free radicals. Once unrepaired lesions are converted into DNA double-strand breaks (DSBs), it can lead nucleolar disruption and induce apoptosis [155, 156]. It was reported that UV-induced apoptotic responses in U2OS are p53-dependent [157]. Dephosphorylation of NCL at CK2 sites leads to fraction of the NCL pool translocating to
nucleoplasm and causing a ‘‘nucleolar stress’’ like conditions, including p53 stabilization and upregulation of proapoptotic factors downstream [27, 132]. Whether NCL dephosphorylation affect cells’ response to UV-induced apoptosis needs to be further explored.

Etoposide is a clinically active anticancer drug, which belongs to a class of topoisomerase II (Topo II) poisons that can cause both DNA single-strand breaks (SSBs) and topoisomerase II-linked DNA double-strand breaks (DSBs) [158]. Studies have shown that etoposide can induce apoptosis in various types of cancer cells, including osteosarcoma U2OS cells by activating p53 and the caspase cascade [159-164]. Although etoposide is not a potent chemotherapeutical agent in treating osteosarcoma due to the drug-resistant characteristics of osteosarcoma, it is commonly used in combination with other agents, e.g. the MAP (methotrexate, doxorubicin and cisplatin) regimen [165-168]. To improve the ability to disrupt pathways in cancer cells and lead to an improved clinical response, the important concept is to design new ways of using Topo II -targeting agents in combinations with agents that target other cellular processes [169, 170]. NCL is known to participate in multiple biological processes and is fundamental to the survival and proliferation of cells [4, 36]. Dephosphorylation of NCL at CK2 sites is inhibitory to cell proliferation and causes p53 stabilization and upregulation of proapoptotic p53 target genes [132], it would be interesting to investigate whether dephosphorylation of NCL at CK2 sites affects cells’ sensitivity to etoposide-induced apoptosis.

To extend the current study, in this chapter I investigate the effect of NCL phosphorylation by CK2 in inducing apoptosis in response to different types of genotoxic stresses, ultraviolet (UV) irradiation and the topoisomerase II (Topo II) poison etoposide. My results show that expression of CK2 phosphorylation-deficient NCL mutant not only sensitizes U2OS cells to UV-
induced apoptosis but also increases total percentage of apoptotic cells under etoposide treatment. In addition, even after apoptosis is triggered by etoposide in these inducible NCL cells, robust upregulation of PUMA and AIF is only present in cells with NCL-mutant expression but not NCL-WT, implying a mechanism of p53-independent increase of proapoptotic factors may be synergistically activated by NCL dephosphorylation.
Results

Expression of dephosphorylated-nucleolin mutant increases the levels of procaspases

Apoptosis comprises a complex interplay of cellular events. Caspases, a family of cysteine aspartic acid proteases, are degradative enzymes that play multiple roles during the initiation and execution of apoptosis [171]. My previous results show expression of dephosphorylated-nucleolin mutant (NCL-6/S*A) increases basal levels of major proapoptotic p53-responsive genes. Therefore, I next determined whether the hallmark event of apoptosis – caspase cascade activation is being triggered by NCL dephosphorylation. Exogenous NCL expression (NCL-WT and NCL-6/S*A) was induced by removal of doxycycline (Dx) for a range of 2–24 days. Interestingly, although caspase cleavages are not detectable in these inducible NCL cells, there is an obvious increase of the total levels of procaspases in NCL-6/S*A cells, including major caspase initiators procaspase-8 (extrinsic pathways) and procaspase-9 (intrinsic pathways), and caspase executioner procaspase-3 (Figure 11). The scatter plot represents procaspase-3, -8 and -9 levels during the 2 to 24 days of induced expression of WT or 6/S*A as compared to the Ctrl cells (Figure 11B).

Expression of dephosphorylated-nucleolin mutant sensitizes cells to UV-induced apoptosis

It was reported that total levels of the procaspases can tip the scales between cell survival and death, and elevated levels of procaspases plays a key role in sensitizing cells to apoptosis [152]. To determine whether these inducible NCL cells show different phenotypic outcomes to genotoxic stress, I assessed the apoptotic response in these cells to two major types of DNA damage conditions, UV irradiation and exposure to the topoisomerase II poison etoposide as
representative of single-strand and double-strand breaks in the DNA, respectively. These cells were exposed to UV irradiation (40 J/M²) and harvested 24 hours post-UV. Western blot results show that cleavage of caspase-3 and PARP are clearly present in NCL-6/S*A cells but absent in NCL-WT cells upon UV stress (Figure 12A and 12B). Interestingly, even though p53 is significantly increased in all cells after UV exposure, the upregulation of PUMA and AIF is still only observed with NCL-6/S*A expression but not in NCL-WT or control cells (Figure 12C). These indicate that dephosphorylated-NCL mutant sensitizes cells to UV-induced apoptosis, and p53-independent increase of proapoptotic factors might also be activated by the expression of NCL-mutant.
Figure 11. Inducible expression of NCL-6/S*A results in an increased procaspase levels.

(A) Western blot analyses for cells grown without doxycycline (Dx) for the indicated period indicating inducible NCL-expression (WT or 6/S*A). Total cell lysates were harvested at different days of -Dx and analyzed by Western blot. NCL-6/S*A cells express higher levels of procaspase-3, -8 and -9. (B) Plots of cumulative data of procaspases levels during day2 to day24 of induced expression of NCL-WT or 6/S*A. The quantification was done by NIH Image J software. Values were first corrected for the β-actin levels and then compared to Ctrl (no exogenous NCL, no Dx day 2) cells. The graph is representative of at least three independent experiments.
Apoptotic markers (Arbitrary Units)

Dx 40days

- UV + UV - UV + UV - UV + UV - UV + UV - UV + UV

p53 PUMA AIF Caspase-8 Caspase-9 Caspase-3

Ctrl NCL-WT NCL-6/S*A

Cleaved-Caspase-3 Levels
(17/19KDa)

Caspase-8-FL

PARP-FL

Cleaved-PARP

Caspase-9-FL

p53

PUMA

AIF

β-Actin

Cleaved-PARP Levels
(UV treatment)

Ctrl WT 6/S*A

Apoptotic markers (Arbitrary Units)
Figure 12. Inducible expression of NCL-6/S*A sensitizes cells to UV-induced apoptosis.

(A) Cells were grown without doxycycline for 40 days (-Dx 40 days) of inducible NCL-expression (WT or 6/S*A). Total cell lysates were harvested post-UV 24 hours and analyzed by Western blot. NCL-6/S*A cells show increased levels of procaspase-8 and -9, p53, PUMA, and AIF under no UV (non-stress), while only p53 level is upregulated in NCL-WT cells. Post-UV 24 hours, cleaved-caspase-3 and cleaved-PARP is clearly present in NCL-6/S*A cells but not in Ctrl or NCL-WT cells. (B) Graphs of cleaved-caspase-3 levels shown in A. Values were first normalized with β-actin levels and then compared to Ctrl cells. The graph is representative of at least three independent experiments. (C) Graphs of cleaved-PARP levels shown in A. Values were first normalized with β-actin levels and then compared to Ctrl cells. The graph is representative of at least three independent experiments. (D) Graphs levels of apoptotic markers in these inducible NCL cells under non-stress (no UV) and stress conditions (post-UV 24 hours). The levels of p53, PUMA, AIF, and procaspase-8 and -9 are significantly upregulated in NCL-6/S*A cells as compared with Ctrl and NCL-WT under no UV (non-stress), while only a modest upregulation of p53 is present in NCL-WT cells. Post-UV 24 hours, p53 levels are greatly increased in all these cells, but upregulation of AIF and PUMA is still only present in NCL-6/S*A cells. Caspases levels (caspase-3, -8 and -9) are comparable in NCL-WT and -6/S*A cells. The graph is plotted from multiple western blot results. Values were first normalized with β-actin levels and then compared to Ctrl cells.
Expression of dephosphorylated-nucleolin mutant increases the efficiency of etoposide-induced apoptosis

Next, I investigated how these inducible NCL cells respond to etoposide-induced apoptosis. Cells were treated with 100 µM etoposide and harvested at different time point 12h, 24h and 48h. Cleavage of caspase-8, -9 and -3 and PARP are present in all these cells, suggesting that apoptosis is triggered by etoposide treatment (Figure 13D), which is consistent with the reports that etoposide can induce caspases cascade activation of both intrinsic (caspase-9 activation) and extrinsic pathway (caspase-8 activation) in cancer cells [160] [161-164]. The expression of NCL-6/S*A and -WT (3×flag-NCL) both remain unchanged after 72 hours of doxorubicin (DXR) treatment. Interestingly, similar as UV exposure, significant upregulation of PUAM and AIF is only observed in NCL-6/S*A cells after etoposide treatment. Furthermore, to better quantify the efficiency of apoptosis, I use Annexin V/PI staining assay by Flow cytometry to examine the percentage of cells undergoing early apoptotic cellular event – phosphatidylserine (PS) translocation. Early apoptotic cells that translocate PS from the inner face of the plasma membrane to the cell surface have the affinity binding to Annexin V but exclude propidium iodide (PI), since the integrity of the plasma membrane of cells undergoing apoptosis is preserved and most functions of the membrane remain unchanged. [172, 173]. My result shows that apoptotic positive cells (Annexin V + / PI -) is more with NCL-6/S*A expression (34.55%) than with NCL-WT (26.38%) after etoposide treatment (Figure 13A, B and C). Together these results suggest that etoposide-induced apoptosis is more efficient with NCL-6/S*A expression,
and a broader range of apoptotic pathways might also be triggered synergistically with p53-mediated apoptosis by NCL dephosphorylation.
Figure 13. Inducible expression of NCL-6/S*A increases the efficiency of etoposide-induced apoptosis.

(A) Cells were grown without doxycycline for 6 days (-Dx 6 days) of inducible NCL-expression (WT or 6/S*A). Cells were harvested after 48 hours of 100µM etoposide treatment. Samples were prepared follow the protocol of Annexin V-FITC/PI apoptosis assay of Cellometer®. The results are showing in the quadrant gating format. LL: lower left (live cells); LR: lower right (apoptotic cells); UR: upper right (necrotic or dead cells); UL: upper left (debris cells) (B) A summary of the quadrant-gating results showing in A. % of Apoptotic cells is higher in NCL-6/S*A cells (34.58%) as compared with NCL-WT cells (26.38%) after etoposide treatment. (C) Graphs of apoptotic positive cells in these inducible NCL cells. (D) Cells were grown without doxycycline for 8 days (-Dx 8days) of inducible NCL-expression (WT or 6/S*A). Cells were harvested after 12 h, 24 h and 48 h of 100µM etoposide treatment, total cell lysate were analyzed by Western blot. Cleaved-caspase-3, -8 and -9 and cleaved-PARP is observed in all inducible NCL cells after etoposide treatment, but upregulation of AIF and PUMA is only present in NCL-6/S*A cells. The expression of NCL-6/S*A and -WT (3×flag-NCL) remains unchanged after 72 hours of doxorubicin (DXR) treatment.
Lack of nucleolin phosphorylation by CK2 increases AIF levels in the nucleus

Upregulation of AIF is observed only in cells with NCL-6/S*A expression. To determine whether AIF mediated caspase-independent cell death (CICD) is triggered by NCL dephosphorylation, I examined the expression levels of AIF in the nuclear fraction of these inducible NCL cells. My preliminary data shows that AIF is detectable in the nuclear extracts of NCL-6/S*A cells after 14 days of induced NCL expression but not in WT or ctrl cells (Figure 14A). Although AIF levels in the nuclear fractions are upregulated in all cells after 40 days, it is still higher in NCL-6/S*A cells (Figure 14A and 14B). This implies that NCL dephosphorylation might induce AIF nuclear translocation to induce CICD, but future studies are needed to provide direct evidence of AIF nuclear translocation and large-scale DNA fragmentation to support this preliminary notion.
Figure 14. Inducible expression of NCL-6/S*A increases AIF levels in nucleus.

(A) Western blot analyses for AIF expression in nuclear extracts. AIF is detectable in nuclear fraction after 14 days of NCL-6/S*A expression (-Dx 14d) but not with NCL-WT expression or in ctrl cells. AIF levels are increased in nuclear fractions of all cells but still higher in NCL-6/S*A cells after 40 days of NCL expression induction. (B) Graph of AIF protein levels of nuclear extracts of inducible NCL cells shown in A (-Dx 40d). The quantification was done by NIH Image J software. Values were first normalized with Histone H3 levels and then compared to Ctrl cells.
Discussion

In this chapter, I demonstrate that NCL dephosphorylation at CK2 sites increases cells’ sensitivity to apoptosis in response to different types of genotoxic stress. Lack of NCL phosphorylation by CK2 not only sensitizes cells to UV irradiation induced apoptosis but also increases the total percentage of apoptotic cells after topoisomerase II inhibitor etoposide treatment by activating caspases cascade. Interestingly, p53-transactivated proapoptotic factors PUMA and AIF are significantly upregulated only in cells with phosphorylation-deficient NCL expression even after p53 is activated by genotoxic stress, implying a mechanism of p53-independent increase of proapoptotic factors may be synergistically activated by NCL dephosphorylation at CK2 sites. PUMA as a potent apoptosis-sensitizer may play a pivotal role in the increased apoptosis sensitivity of cells with phosphorylation-deficient NCL expression. In addition, AIF-mediated caspase-independent cell death (CICD) might be also triggered by NCL dephosphorylation at CK2 sites, and contribute to p53-mediated apoptosis.

Our previous studies show that dephosphorylated NCL translocates from the nucleolus to nucleoplasm and causes a “nucleolar stress” like conditions in unstressed cells [27, 132]. This mimics stress response of cells that causes p53 stabilization and longer half-life, resulting in heightened expression of various p53-responsive proapoptotic genes, e.g. PUMA, BID, BAK and AIF (Figure 8). Interestingly, I observe upregulation of total levels of the procaspases in NCL-6/S*A cells, including major caspase initiators procaspase-8 and -9 and caspase effector procaspase-3 (Figure 10), which suggests that the stress-like conditions in NCL-mutant cells also causes upregulation of apoptosis regulators that are not transcriptionally regulated by p53. It has been reported that total levels of the procaspases can tip the scales between cell survival and
death, and elevated levels of procaspases also plays a role in sensitizing cells to apoptosis [152]. The basal expression patterns of apoptosis and cell cycle regulators play the essential role in determining the cell fate -- life or death in response to stress [153, 154]. In addition, my data show that the percentage of cells undergoing early apoptosis process (Annexin V + / PI -) is 1.30% with NCL-6/S*A expression and 1.07% with NCL-WT under non-stressed conditions (Figure 14). This suggests that although it is slightly higher with NCL-6/S*A expression, full-scaled apoptosis is not yet triggered in unstressed cells, which also explains why caspases cleavage is not present in these inducible NCL cells. However, upon UV exposure, cleavage of caspase-3 and PARP are clearly present in NCL-6/S*A cells but still absent in NCL-WT cells (Figure 11), strongly indicating dephosphorylation of NCL at CK2 sites sensitizes cells to UV induced apoptosis. Although apoptosis is triggered both in NCL-6/S*A and WT cells by etoposide treatment, since caspases cascade activation (caspase-8, -9 and -3) and PARP cleavage are present (Figure 13), the percentage of apoptotic cells is higher with NCL-mutant expression (34.58%) than with NCL-WT (26.38%) (Figure 14). This indicates that etoposide-induced apoptosis is more efficient in cells when NCL is dephosphorylated at CK2 sites. Taken together, these results strongly suggest that dephosphorylation of NCL at CK2 sensitizes cells to apoptosis upon genotoxic stress, probably through upregulation of basal levels of proapoptotic factors, which are predominantly p53-mediated.

PUMA is transactivated by p53 in response to genotoxic stress such as UV irradiation and topoisomerase inhibitors, e.g., etoposide [174, 175]. Interestingly, NCL-mutant cells not only present a substantial upregulation of basal levels of PUMA in unstressed cells but also a further significant increase of PUMA expression after stress (UV or etoposide), while NCL-WT cells
shows a slight increase of PUMA expression only after stress (Figure 12 and 13). This indicates that dephosphorylation of NCL by CK2 may not only activate p53-regulated activation of PUMA, it might trigger p53-independent upregulation of PUMA as well, and this should be further explored in future studies. PUMA is one of the most powerful “killers” among the BH3-only proteins, since it is able to bind and antagonize all known antiapoptotic factors of the Bcl-2 family, including Bcl-2, Bcl-XL, Mcl-1, Bcl-w and A1/Bfl1[176, 177]. Moreover, PUMA is an essential mediator of DNA damage-induced apoptosis that can work independently of p53 status [178]. Numerous studies have shown that exogenous PUMA is a potent sensitizer of inducing apoptosis in drug-resistant tumors to chemotherapeutic agents including etoposide [179-185]. Therefore, PUMA-mediated apoptosis may also play a crucial role in the increased sensitivity of dephosphorylated-NCL expressing cells to apoptosis.

Studies have shown that the AIF gene is a transcriptional target of p53, and its expression is positively regulated mainly by the basal levels of p53 while not much by the stress-activated p53. AIF can contribute to p53-mediated apoptosis through activating caspase-independent cell death (CICD) pathways [138, 150]. My results show that the basal level of AIF is significantly upregulated in unstressed cells with dephosphorylated NCL expression but not with NCL-WT expression (Figure 8). AIF levels are further increased slightly in NCL-mutant cells but not in NCL-WT or control cells after stress (UV or etoposide) (Figure 12 and 13). These data are consistent with the report that AIF gene is regulated by basal levels of p53, and that activation of p53 by genotoxic stress does not result in a significant further increase in AIF expression [138]. Besides, my preliminary data show that AIF expression is higher in nuclear extracts of NCL-mutant cells. Taken together, these results imply that deficient phosphorylation of NCL at CK2
sites might activate AIF-mediated CICD through p53-regulated AIF upregulation, synergistically with p53-mediated, caspase-dependent cell death, both of which contribute to the observed higher apoptosis rate of NCL-mutant cells. Further investigation should determine whether dephosphorylation of NCL by CK2 triggers the hallmark events of CICD, such as AIF nuclear translocation, reactive oxygen species (ROS) production, chromatin condensation and large-scale DNA fragmentation [144-151].

Taken together, I propose a model of NCL dephosphorylation at CK2 sites sensitizing cells to apoptosis (Figure 15). Lack of NCL phosphorylation by CK2 causes “nucleolar stress” like conditions in unstressed cells that dephosphorylated NCL readily relocates from nucleolus to nucleoplasm, the mobilization of NCL is probably dependent on p53 by forming NCL-p53 complex. The stress like condition caused by dephosphorylated NCL stabilizes p53, which in turn transcriptionally upregulates a series of downstream proapoptotic factors, including BH3-only proteins PUMA and BID, proapoptotic factor of Bcl-2 family BAK and mitochondrial factor AIF. Upon stress (UV radiation and cytotoxic agent etoposide), the upregulated basal levels of the proapoptotic factors greatly enhances the efficacy of apoptotic responses in cells by activating caspases-dependent cell death, and possibly caspase-independent cell death as well. I provide evidence in this the study that targeting NCL phosphorylation by CK2 may be an attractive target of developing new strategy to increase the effectiveness of chemotherapy in treating cancers. Genome-wide analyses of these mutations in NCL in variety of cancers should be investigated in future study.
Figure 15. Nucleolin dephosphorylation at CK2 sites sensitizing cells to apoptosis.

Lack of NCL phosphorylation by CK2 causes “nucleolar stress” like conditions in unstressed cells that dephosphorylated NCL readily relocates from nucleolus to nucleoplasm, the mobilization of NCL is probably dependent on p53 by forming NCL-p53 complex. The stress like condition caused by dephosphorylated NCL stabilizes p53, which in turn transcriptionally upregulates a series of downstream proapoptotic factors, including BH3-only proteins PUMA and BID, proapoptotic factor of Bcl-2 family BAK and mitochondrial factor AIF. Upon stress (UV radiation and cytotoxic agent etoposide), the upregulated basal levels of the proapoptotic factors greatly enhances the efficacy of apoptotic responses in cells by activating caspase-dependent cell death, and possibly caspase-independent cell death as well.
CHAPTER IV

EFFECT OF NUCLEOLIN PHOSPHORYLATION BY CK2 IN REGULATING MRNA STABILITY UPON DNA DAMAGE
Introduction

NCL is a stress responsive RNA-binding protein (RBP) that plays multiple roles in post-transcriptional regulation of gene expression by regulating both mRNA stability and translation during cellular stress response [14, 83]. Transcriptome-wide analyses reveal that NCL globally enhances translation of mRNAs with G-rich elements in coding and non-coding regions, many of which encode cell growth- and cancer-related proteins [14]. For example, NCL can associate with mRNA of tumor suppressor ARF, the upstream regulator of p53, in the nucleolus and enhance its translation [14, 93]. Moreover, NCL-NCL homodimer can interact with 3’ and 5’ UTRs of TP53 mRNA to repress p53 translation under non-stressed conditions. Disruption of the NCL-NCL homodimer by RPL26 switches on p53 translation upon stress [15-17]. On the other hand, NCL is reported to interact with the AREs in the 3’UTR of numerous mRNAs and enhance their stability, such as anti-apoptotic factors in Bcl-2-family e.g. BCL2 and BCLXL [11, 54, 92]. Nevertheless, post-translational modification of NCL by phosphorylation affects its ability in binding to its mRNA targets [42]. For example, UV increases the binding capacity of NCL to AREs in the 3’-UTR of BCLXL mRNA in a p38 MAPK-dependent manner, because phosphorylation of NCL by p38 prevents the recruitment of other destabilizing factors, e.g, TTP and KSRP, to BCLXL mRNA [11, 118]. Wang et al. later showed that the Syk protein kinase binds robustly to NCL and phosphorylates it on tyrosine, enhancing the binding of NCL to BCLXL mRNA as well [119]. My results from previous chapters show that NCL phosphorylation is vital for cell survival under non-stress conditions, phosphorylation-deficient NCL-6/S*A confers a dominant-negative effect on cell proliferation by stabilizing p53 and upregulating p53-responsive pro-apoptotic factors, which mimics cellular stress response [132]. However, whether
phosphorylation of NCL by CK2 affect its binding ability with TP53 mRNAs as well as mRNAs in apoptotic pathways is currently unknown. Therefore, it is important to investigate whether dephosphorylation of NCL at CK2 sites affects its function in regulating mRNAs of TP53 and apoptotic factors, e.g., BCL2 and BCLXL, which might ultimately link to the increased p53 protein levels and induction of apoptosis pathways.

HuR, a ubiquitously expressed RBP that belongs to the Hu (ELAV) family, also can recognize, bind, and stabilize a large subset of ARE-containing mRNAs through its RNA recognition motifs (RRMs) [104]. More importantly, NCL and HuR have many common target mRNAs and exert antagonistic or synergistic influences on their shared target mRNAs. However, the biological significance of the HuR-NCL interaction is not well studied [109, 110]. For instance, HuR competes with NCL to regulate translation of ARF mRNA. NCL enhances it, while HuR represses it [93]. In the case of TP53, HuR not only increases TP53 mRNA stability by binding to the ARE in the 3’UTR of TP53 mRNA [72], but also promotes p53 translation upon stress, although it is a result of sequestration of TP53 mRNA on polysomes, rather than binding to the ARE sequence of TP53 3’UTR per se [71]. It seems that NCL and HuR also have opposite roles in regulating TP53 mRNA since NCL repress p53 translation while HuR enhances it, but the roles of HuR and p53 in regulating TP53 mRNA remains to be determined. On the contrary, it is reported that HuR can work synergistically with NCL to stabilize BCL2 mRNA and enhance the translation of BCL2 by binding concurrently to the ARE in the 3’UTR of BCL2 mRNA. HuR and NCL are both present in common BCL2 messenger ribonucleoprotein (mRNP) complexes and this co-precipitation is RNA dependent [12, 54]. Taken together, it would be interesting to investigate whether NCL and HuR have functional interplay to control the
expression of NCL/HuR common mRNA targets in p53 signaling and apoptotic pathway during cellular stress response.

Deadenylation modulates the poly(A) tail length in mRNAs. This is a widespread strategy to control mRNA stability and has the potential to offer rapid changes in gene expression under different cellular conditions [105, 186-188]. PARN is one of the three major poly(A) specific 3’ exoribonucleases identified in mammalian cells [189, 190], which deadenylates the mRNA poly (A) tail and triggers ARE-mediated mRNA decay [191]. Studies from Dr. Kleiman’s lab have shown that PARN plays an important role in regulating the p53-checkpoint. PARN is recruited to the TP53 mRNA and decreases the stability of TP53 mRNA under non-stressed conditions, while HuR competes with PARN for binding with TP53 mRNA, resulting in an increase in TP53 expression upon UV treatment [111-113]. More importantly, in our collaborative project, Dr. Kleiman’s lab showed in vitro reconstituted assays that NCL and PARN both bind to ARE sequence in the 3’UTR of TP53 mRNA (unpublished data). NCL (both WT and 6/S*A) interacts with the N-terminal domain of PARN, but only when NCL in its phosphorylated state (NCL-WT) can activate PARN deadenylase activity. These results indicate that NCL might be one of the AUBPs that recruit PARN deadenylase to TP53 mRNA, and NCL phosphorylation by CK2 is a necessary condition that activates PARN deadenylation, representing a potential novel regulatory mechanism of TP53 gene expression.

To extend our current study, in this chapter I determine that phosphorylation of NCL by CK2 not only plays a crucial role in NCL associating with TP53 mRNA both under non-stressed and stressed conditions, but also modulates the interaction of NCL with p53 protein, PARN deadenylase and the ARE-binding protein HuR respectively in cell extracts. Post-transcriptional
regulation of TP53 mRNA resulting from the functional interplay of NCL-PARN-HuR might ultimately link to increased p53 protein levels and induction of apoptosis induced by NCL-6/S*A expression. These studies provide new insights into the function of phosphorylation of NCL at CK2 consensus sites in post-transcriptional regulation of gene expression to control cell fate through its altered interactions with other AUBPs, deadenylases as well as the mRNA targets.
Results

NCL phosphorylation by CK2 regulates its interactions with proteins that are involved in mRNA 3’ processing

To assess NCL-PARN interactions in the NCL inducible cell lines, we performed co-IP assays under non-stress conditions and upon UV exposure. As revealed from the input levels, p53 protein levels are increased post-UV exposure with NCL-WT, while p53 level is upregulated in NCL-6/S*A cells even under non-stressed conditions (Figure 16A and 16B), which is consistent with my previous result that NCL dephosphorylation causes a stress-like condition in unstressed cells by stabilizing p53 [132]. In addition, PARN levels are increased in NCL-WT cells after UV exposure, which is in accordance with the earlier report that UV-treatment upregulates PARN expression [112]. In fact, NCL-6/S*A cells increases PARN levels under non-stress condition (Figure 16A compare lanes 1-4 to 5-6 and Figure 16B), this again strengthens my previous finding that NCL-6/S*A mimics cellular stress conditions [132]. On the other hand, Flag-NCL co-IPed fraction shows that NCL-WT associations with p53, PARN and HuR proteins decrease upon UV-stress, while these associations are enhanced with NCL-6/S*A (Figure 16C). Interestingly, although PARN interaction with NCL-6/S*A decreases under non-stress condition, the NCL-mutant interactions with p53 and HuR are increased in unstressed cells (Figure 16C). These data indicate that NCL-6/S*A may not just simply mimicking the stress-response of NCL-WT, but that a distinct mechanism of regulating p53 expression might be also induced by NCL-6/S*A expression. Although it was reported that NCL and HuR interaction is RNA dependent [12, 54], my results show that NCL can associate with HuR in the absent of RNAs since I have added RNase in my experiment procedure to eliminate the effect of RNA on
the protein interactions. Future investigation should determine whether NCL-HuR is a direct interaction or in a complex that involves other factors as well.
Figure 16. NCL phospho-variants can associate with p53, PARN and HuR under different cellular conditions.

(A) Co-IP assays were performed using FLAG-M2 beads to IP FLAG-tagged NCL (WT and 6/S*A) along with other protein interactors. Equal amount of proteins were used in the assay. 5% input of total cell lysates and bound fractions were analyzed by Western blot for p53, FLAG, PARN, and HuR. Tubulin was used as a loading control. The image is a representative Western blot from at least three independent assays. (B) The bar graphs show the relative intensities for p53, PARN and HuR proteins in the input samples. Protein bands were quantified with Image J software and normalized to its corresponding loading control. To analyze the samples, the levels observed for each protein were further normalized to the levels observed in cells expressing NCL-WT in non-stress conditions (lane 3, panel A). The errors represent the SD derived from at least three independent experiments. (C) The bar graphs show the relative intensities of p53, PARN and HuR co-IPed with different phospho-variants of FLAG-NCL in the Western blot shown in panel A. The effect of UV treatment was also analyzed. The levels of the bound interactors were first normalized to the levels of either FLAG-NCL-WT or FLAG-NCL-6/S*A. Further normalization and quantification were done as in panel B.
NCL phosphorylation by CK2 alters NCL interaction with its target mRNAs

To understand how NCL phosphorylation by CK2 regulates its RNA-binding properties, next I determine NCL binding to its known target mRNAs, TP53 [15] [16, 17] and BCL2 [54], both of which are also HuR regulated mRNA targets [12, 110]. The NCL inducible cells were treated with or without UV irradiation, bounded RNAs were then extracted and purified from pulled-down Flag-tagged NCL (both NCL-WT and NCL-6/S*A) by RIP assays. RT-PCR were used to analyze the RNA samples using primers specific for TP53 and BCL2 genes [192, 193]. My results show that the binding of NCL-6/S*A with TP53 mRNA is dramatically decreased as compared with that of NCL-WT in both non-stress and stress conditions (Figure 17A), which suggests that NCL phosphorylation on CK2 is essential for its binding with TP53 mRNA. Nevertheless, it seems that dephosphorylation of NCL does not affect its binding ability with BCL2 mRNA, moreover, the binding to BCL2 mRNA is more with NCL-6/S*A than NCL-WT in unstressed cells (Figure 17C). In addition, the binding of NCL-WT with TP53 mRNA and BCL2 mRNA are both increased after UV exposure, which is consistent with previous report that stress conditions increase RNA-binding activity of NCL [42].
Figure 17. NCL phosphorylation by CK2 affects its interaction with TP53 and BCL2 mRNA.

(A & C) RIP assays were performed in these inducible NCL cells (WT or 6/S*A). Samples were harvested post-UV 2hours and IPed with anti-FLAG antibodies. Bound RNAs were then purified by Direct-zol RNA kit. RNAs IPed were quantified with qRT-PCR. The graph is representative from three independent RIP analyses. (B & D) The bar graph shows the ratio of the fold change for UV-treated/non-stress mRNA values for TP53 (B) and BCL2 (D) in NCL phospho-variants.
Discussion

In this chapter, I demonstrate that deficient phosphorylation of NCL at CK2 consensus sites affects its interaction with PARN deadenylase and the RNA-binding protein HuR both under normal and stressed conditions. Moreover, the phosphorylation of NCL by CK2 is crucial for NCL binding with TP53 mRNA, but does not affect as much on its binding with BCL2 mRNA (Figure 17 A&C). Here I propose a model (Figure 18) that provides a mechanism of NCL phosphorylation by CK2 regulates p53 checkpoint post-transcriptionally both under non-stressed and stressed conditions, potentially through regulating PARN deadenylase and the target gene TP53 mRNA, as well as the functional interplay with HuR. To the best of my knowledge this is the first report demonstrating NCL-HuR protein interaction in RNA-free cellular samples suggesting that mRNA target is not required for these associations.

Devany et al. reported that PARN deadenylase is recruited to TP53 mRNA and decreases its stability under non-stress conditions [112]. In the collaborative project with Dr. Kleiman, we show in vitro assay that NCL can bind to the 3’UTR ARE of TP53 mRNA under non-stressed conditions. More importantly, NCL interacts directly with the N-terminus of PARN, however, only NCL-WT, but not dephosphorylated NCL mutant (NCL-6/S*A), can activate PARN deadenylase activity (Zhang et al., unpublished data). On the other hand, studies show that NCL-NCL homodimer renders a “switch off” effect on p53 translation by binding to the double-stranded RNA structure containing complementary sequences of the 5’ and 3’ UTRs of TP53 mRNA under non-stress conditions [15-17]. Therefore, it seems that NCL-WT plays an important role in keeping a low p53 level under non-stress by destabilizing TP53 mRNA and repressing its translation. The observed modest upregulation of p53 in NCL-WT cells in my
results from previous chapter (Figure 5, 6 and 9), is possibly due to post-translational regulation of p53 by NCL-WT, since it is reported that overexpressed NCL can stabilize p53 by binding to MDM2 and inhibiting its activity of degrading p53 [23, 24].

On the other hand, however, dephosphorylated NCL releases target TP53 mRNA from PARN deadenylation since the binding of NCL-6/S*A with TP53 mRNA is dramatically decreased (Figure 17A), which may increase TP53 mRNA stability. Besides, my previous co-IP data show NCL-6/S*A can bind to endogenous NCL (Figure 8). The homodimer of dephosphorylated NCL-NCL may not as efficient as NCL-NCL in repressing p53 translation due to the dominant negative effect of dephosphorylated NCL, as a result, possibly switching on translation of partial p53 pools under non-stressed conditions. These results strongly suggest that lack of NCL phosphorylation by CK2 may increase TP53 mRNA stability as well as translation to upregulate p53 expression, which explains my observation from previous chapter that cells expressing dephosphorylated NCL (NCL-6/S*A) have significant heightened p53 protein levels even under non-stress conditions. It is important to investigate how dephosphorylation of NCL affect the TP53 mRNA decay rate and translational rate in future studies to provide explicit mechanism of the proposed working model.

Under stressed conditions, HuR comes into play as an important stabilizer of p53, since it can not only enhance p53 translation but also stabilize TP53 mRNA by binding to the ARE in the 3’UTR of TP53 upon stress [71, 72]. Zhang et al. reported that HuR can compete with other destabilizing AUBPs and prevent the recruitment of PARN to its target mRNAs e.g. TP53 mRNA, resulting in increased p53 expression [105, 111]. Decreased interaction of NCL-HuR (Figure 16C) may release partial HuR pools to compete with NCL in binding to ARE in 3’UTR
of TP53 mRNA and block the recruitment of PARN to TP53 mRNA, resulting in increased TP53 mRNA stability. Moreover, decreased interaction of NCL-PARN (Figure 16C) attenuates the deadenylation effect on TP53 mRNA which may lead to stabilization of TP53 mRNA as well. Besides, p53 translation can be greatly increased upon stress, since NCL-RPL26 heterodimer switches on p53 translation, and the free HuR enhances p53 translation as well. However, increased interaction of dephosphorylated NCL-HuR (Figure 16C) may decrease free HuR availability. Even though the increased association of dephosphorylated NCL-PARN (Figure 16C) may lead to increased TP53 mRNA stability, without the positive regulation of free HuR on TP53 mRNA stabilization and translation, p53 expression may not be upregulated as much upon stress. This partially explains my observation that after UV exposure p53 protein level is increased in cells expressing NCL-WT but not with dephosphorylated NCL (NCL-6/S*A) (Figure 16B).

In addition, NCL is known to stabilize anti-apoptotic BCL2 mRNA [92]. NCL-WT binding to BCL2 mRNA is significantly higher upon UV stress (Figure 17C), which is consistent with the earlier report showing RNA-binding properties of NCL are greatly activated during cellular response to genotoxic stress [42]. Interaction of NCL-6/S*A with BCL2 mRNA is increased under non-stress, indicating dephosphorylation of NCL mimics cellular stress response. Although NCL-6/S*A and BCL2 mRNA interaction increases further upon UV treatment, the dominant negative effect of NCL-6/S*A may decrease the stability of BCL2 mRNA, which may ultimately contribute to the induction of apoptosis. Future studies should further investigate the effect of dephosphorylation of NCL on mRNA stability of factors in apoptosis pathway.
Taken together, the results present in this chapter indicates that NCL phosphorylation by CK2 may control cell fate by regulating expression of p53, through mediating functional play of NCL-PARN-HuR to affect TP53 mRNA stability and translation. The phosphorylation on the N-terminus of NCL by CK2 is a necessary condition that may be needed to keep p53 levels low under non-stressed conditions so that cells can proliferate and survive. In addition, NCL phosphorylation by CK2 may have roles in regulating factors, e.g., BCL2 in apoptotic pathways to control cell fate as well. This study provides new insights into the role of NCL phosphorylation by CK2 in controlling cell fate by regulating tumor suppressor TP53 gene expression post-transcriptionally.
Figure 18. Post-transcriptional regulation of p53 by nucleolin phosphorylation.

(A) Under non-stress conditions, NCL-WT activates PARN activity to deadenylase TP53 mRNA while NCL-6/S*A abolish PARN deadienlylation (Zhang et al unpublished data). Moreover, the dramatically decreased association of TP53 mRNA with NCL-6/S*A may free TP53 mRNA from deadienlylation by PARN. Therefore, NCL-6/S*A may increase TP53 mRNA stability. On the other hand, the homodimer of NCL - NCL-6/S*A may not as efficient as NCL-NCL in repressing p53 translation due to the dominant negative effect of NCL-6/S*A, as a result, possibly switching on translation of partial p53 pools. Together, dephosphorylation of NCL may upregulate p53 expression by increasing TP53 mRNA stability and translation.

(B) Upon stress, HuR comes into play as an important stabilizer of p53. However, increased interaction of HuR with NCL-6/S*A may decrease free HuR availability. Even though the increased association of PARN with NCL-6/S*A may lead to increased TP53 mRNA stability, without the positive regulation of free HuR on TP53 mRNA stabilization and translation, p53 expression may not be upregulated in NCL-6/S*A cells as much as in NCL-WT cells.
CHAPTER V

FUTURE DIRECTIONS
In this dissertation, I demonstrate that the six consensus CK2 sites on the N-terminus of NCL are important for cell survival and proliferation. Expression of a hypophosphorylated-NCL mutant (6/S*A, six consensus serine altered to alanine) leads to dominant negative effects on cell proliferation and triggers p53 checkpoint activation to induce expression of downstream p53-responsive proapoptotic markers, including BH3-only proteins PUMA and BID, proapoptotic factor of Bcl-2 family BAK and mitochondrial factor AIF. The upregulated basal levels of these proapoptotic factors increases cells’ sensitivity to apoptosis upon genotoxic stress. Dephosphorylation of NCL at CK2 sites not only sensitizes NCL-mutant cells to UV irradiation-induced apoptosis but also increases total percentage of apoptotic cells in response to topoisomerase II inhibitor etoposide. The substantially upregulated PUMA expression in dephosphorylated-NCL expressing cells may play a pivotal role in the increased cells’ sensitivity to apoptosis. In addition, phosphorylation-deficient NCL mutant also induces significant upregulation of AIF, which may contribute to p53-mediated apoptosis through activating caspase-independent cell death (CICD) pathways. Moreover, in collaborate with Dr.Kleiman’s lab, we further demonstrate that NCL phosphorylation by CK2 regulates p53 checkpoint post-transcriptionally through affecting NCL interaction with PARN deadenylase and the target gene TP53 mRNA, as well as with ARE-binding protein HuR to mediate HuR availability to their common target TP53 mRNA. The effect of functional interplay of NCL-PARN-HuR on TP53 mRNA stability and translation might ultimately link to the increased p53 protein levels and induction of apoptosis pathways. These data provide new insights into the role of NCL phosphorylation by CK2 in regulating gene expression post-transcriptionally to control cell fate during cellular stress response. However, there are still some aspects that should be explored in
future studies so as to elucidate the complete mechanism of phosphorylation of NCL by CK2 in regulating cellular fate. The following proposed studies may help to better understand the working model shown in this dissertation.

**Further characterization of the role of nucleolin phosphorylation by CK2 in regulating p53 protein function**

Dr. Saxena reported previously that increased NCL leads to p53 stabilization by inhibiting of Hdm2-mediated p53 degradation. Both N-terminal domain and the central RBDs of NCL are associated with Hdm2 to inhibit its function [23, 24]. My results in this manuscript show that lack of NCL phosphorylation by CK2 induces significant p53 stabilization, since a longer half-life of p53 (~60 min) is observed with the expression of dephosphorylated-NCL mutant (NCL-6/S*A) (Figure 6). Subsequently I demonstrated that the expression of dephosphorylated-NCL is inhibitory to cell proliferation in a p53-dependent manner (Figure 7). Therefore, it would be important to investigate whether NCL phosphorylation by CK2 differentially regulates p53 protein function. Interestingly, unpublished data from Dr. Saxena’s lab suggested that NCL-6/S*A has greater association with Hdm2 as compared to NCL-WT (data not shown), which indicates that status of NCL phosphorylation by CK2 may play a role in regulating Hdm2-mediated p53 degradation to activate p53 checkpoint. It is important to further investigate the status of p53 ubiquitination in these inducible NCL cell lines so as to determine whether increased p53 protein levels and longer half-life in NCL-6/S*A cells is due to, at least in part, decreased p53 degradation.
Earlier Dr. Saxena reported that exogenous NCL expression stabilizes p53 and regulates its transcriptional activity [23]. My data in this dissertation show that upregulation of proapoptotic factors e.g. PUMA, BID, BAK and AIF, which are p53-responsive genes, are present only in NCL-6/S*A cells but not in NCL-WT cells (Figure 8), even though a modest p53 stabilization (half-life is about 40 min) is present in NCL-WT cells (Figure 6) which is consistent with Dr. Saxena’s earlier report. These results imply that dephosphorylation of NCL at CK2 sites might differentially regulates p53 transcriptional activity. Further investigation should focus on whether dephosphorylation of NCL stabilized p53 is transcriptional active, and how it affects the transcription activity of p53 on the downstream targets, e.g., PUMA, BID, BAK and AIF. This will provide information of whether upregulation of those proapoptotic factors in NCL-6/S*A cells is solely p53-mediated, or p53-independent mechanisms are also involved.

**Characterization of the role of nucleolin phosphorylation by CK2 in regulating mRNA levels of factors involved in apoptotic pathways during cellular stress response**

NCL has been identified as a stress responsive RNA binding protein, which regulates the stability and translation of numerous mRNAs in various pathways [83]. More importantly, NCL binding to TP53 mRNA negatively affects its translation, it achieves this negative regulation by competing out binding of ribosomal protein L26 to TP53 mRNA. My results show that expression of dephosphorylated-NCL mutant (NCL-6/S*A) not only inhibits cell proliferation under non-stressed conditions, but also increases cells’ sensitivity to apoptosis upon genotoxic stresses (UV or etoposide), which is due to, at least in part, upregulated basal levels of proapoptotic factors. Even though most of the proapoptotic factors are p53-responsive genes e.g.
PUMA, BID, BAK and AIF, some are not reported to be transcriptionally regulated by p53, such as caspases-3, -8 and -9. Therefore, NCL phosphorylation by CK2 might have an effect on the expression of p53 as well as other factor(s) involved in apoptosis. In collaboration with Dr. Kleiman’s lab, we determined that phosphorylation of NCL by CK2 plays a crucial role not only in NCL associating with TP53 mRNA, since the binding of NCL-6/S*A with TP53 mRNA is dramatically decreased as compared with NCL-WT (Figure 17A), but also in regulating its functional interactions with an RNA binding protein HuR and an mRNA decay enzyme PARN to regulate p53 gene expression both under non-stressed and stressed conditions. NCL-WT enhances PARN deadenylyase activity while NCL-6/S*A mutant failed to activate PARN deadenylyase activity in vitro. NCL-WT binding to PARN is reduced upon cellular DNA damage (UV). NCL-6/S*A interactions with PARN is reduced under non-stress conditions while increased upon DNA damage (Figure 16). However, whether NCL and its phosphorylation state play a role in promoting PARN recruitment to the target mRNA as well as increasing p53 mRNA degradation under non-stress conditions need to be further investigated. Post-transcriptional regulation of TP53 mRNA by the effect of functional interplay of NCL-PARN-HuR might ultimately link to the increased p53 protein levels and induction of apoptosis pathways. Nevertheless, we have not provided direct evidence of whether TP53 mRNA half-life/decay and translational rate are affected by dephosphorylation of NCL at CK2 sites. To better understand the working model in this dissertation, it would be important in future studies to investigate how expression of dephosphorylated-NCL mutant regulates TP53 mRNA half-life/decay and translational rate.
In addition, numerous evidence shows that NCL plays positive roles and favors the expression of proliferative and anti-apoptotic genes [14, 83]. For example, NCL interacts with AREs of mRNAs of anti-apoptotic factors in Bcl-2-familly e.g. BCL2 and BCLXL, and renders mRNA stability under genotoxic stress [11, 54]. Besides, HuR can work synergistically with NCL to stabilize BCL2 mRNA and enhance translation of BCL2 by binding concurrently to ARE in the 3’UTR of BCL2 mRNA [12, 54]. My results show that both NCL-WT and -6/S*A can bind to BCL2 mRNA. NCL-WT binding to BCL2 mRNA is significantly higher upon UV stress, and interestingly, it seems that dephosphorylation of NCL does not affect its binding ability with BCL2 mRNA, the association of NCL-6/S*A with BCL2 mRNA is increased as compared with NCL-WT in unstressed cells (Figure 17C), which again suggests NCL-6/S*A mimics cellular stress response. Since NCL-WT stabilizes anti-apoptotic BCL2 mRNAs, the dominant-negative NCL-6/S*A may negate NCL-WT’s function, resulting in decreased BCL2 mRNA stability which may contribute to the increased apoptosis rate. The follow-up studies should further investigate BCL2 mRNA half-life/decay rate in these inducible NCL cells, and provide evidence of how lack of NCL phosphorylation by CK2 affects BCL2 mRNA expression. Moreover, it would be also important to extend the studies to examine the effect of NCL phosphorylation by CK2 on other known nucleolin mRNA targets e.g. BCLXL in apoptotic pathway.
CHAPTER VI

EXPERIMENTAL PROCEDURES
Cell Culture and Growth Medium

The mammalian cell line U2OS (osteosarcoma, p53-wt, ARF-null) cells were obtained from ATCC (American Type Cell Culture). NARF cells [131] were kindly provided by Dr. Gordon Peters. All cell lines were routinely cultures in cell adherent culture dishes of desired size and maintained at 37°C in 5% CO2 atmosphere in a humidified incubator. The cell lines were grown in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 units penicillin-streptomycin.

Cell Lines Used in the Experiments [27]

The expression constructs for human nucleolin (NCL, NCBI: NP_005372.2 or UniProt: P19338); full-length (FL, aa 1-710) containing an N-terminal GFP-tag was described previously [24, 27]. Earlier we have generated NCL phospho-mutants with three putative CK2 sites at positions S34, S184, and S206 converted to non-phosphorylatable alanine [3/S*A, earlier designated as NCLTM, triple mutant [27]. Subsequently, we mutated three additional CK2 phosphorylation sites to further lower phosphorylation by CK2 [194-196] and generated a novel reagent 6/S*A (six serine mutated to alanine: S28A, S34A, S145A, S153A, S184A, S206A). Sequential site-directed mutagenesis for NCL phospho-mutants was done using the QuikChange site-directed mutagenesis kit (Stratagene). Top strand primer sequences used were:

S28A (5’ TCCAAAGGAGGTAGAAGAAGGCTAGAGATGAGGAAATGGC 3’)
S145A (5’ ATGCCGAGGAGGAAGACGCTGAGGAGGATGAGGAAGGATG 3’)
S153A (5’ AAGAGGAGGATGACGCTGAGGAGGATGAGGAGGAGG 3’)

87
Constructs with different tags (e.g. His, FLAG and GFP) were created for NCL (-WT or 6/S*A). PCR reactions (for WT and 6/ S*A constructs) were performed using 3xFlag-tagged NCL as a template and generated full-length NCL coding sequence with the primers that contain Not I and BamH I sites in the forward and reverse primers respectively. PCR products were subcloned into the Not I/BamH I sites on the pRetro-Off retroviral vector (Clontech). Production of retroviruses containing the NCL (WT or 6/S*A) expression cassettes were performed in Phoenix cells (Retroviral Helper dependent protocol, https://www.stanford.edu/group/nolan/protocols/pro_helper_dep.html). Subsequently, NARF6 cells were infected with retroviral constructs (NCL-WT or 6/S*A). Stable clones that allow inducible expression of either 3xFlag tagged NCL-WT or -6/S*A driven by a Tet-Off inducible promoter were isolated and expanded as described elsewhere [197]. Multiple clones that can turn on NCL expression by removal of doxycycline (Dx, semi-synthetic tetracycline) from the culture medium were selected. Addition of doxycycline in contrast, shuts off exogenous NCL expression, 90% in these selected clones. These NCL-clones were grown without doxycycline in the medium for the indicated time (hours or days) for NCL-expression and used in subsequent biological and biochemical assays as described. In each selective assay, analyses of multiple days of NCL-induction were included to obtain a representative outcome and possible insights into dose-dependent response.

Antibodies and Reagents

The primary antibodies used for co-immunoprecipitation and western blotting were as follows: rabbit polyclonal anti-FLAG monoclonal anti-p53 peroxidase clone DO-1, EZview™
Red FLAG® M2 Affinity Gel (Sigma-Aldrich, St. Louis, MO, USA); mouse monoclonal anti-p53(DO-1), rabbit polyclonal anti-nucleolin H250, mouse monoclonal β-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); Pro-Survival Bcl-2 Family Antibody Sampler Kit #9941, Pro-Apoptosis Bcl-2 Family Antibody Sampler Kit #9942, Stress and Apoptosis Antibody Sampler Kit #8357, DNA Damage Antibody Sampler Kit #9947, Initiator Caspases Antibody Sampler Kit #12675, mouse monoclonal anti-p21, rabbit monoclonal anti-AIF, rabbit monoclonal anti-Cytochrome c, rabbit monoclonal anti-TOPO II β, mouse monoclonal anti-α-Tubulin (HRP conjugate) (Cell Signaling Technology, Inc. Danvers, MA, USA); Clean-Blot IP Detection Reagent (AP) #21233 (Thermo Fisher Scientific Biosciences); anti-rabbit PARN (donated by Dr. Kleiman’s lab); The secondary antibodies used were anti-mouse and anti-rabbit HRP-conjugated antibodies (GE Healthcare Bio-Sciences Corp. Piscataway, NJ, USA). RNA extracting reagents: Trizol reagent (Sigma-Aldrich, St. Louis, MO, USA); DNA-free RNA kit #R1013 or R1015 (ZYMO RESREACH, Orange County, CA, USA).

**DNA-damaging agents**

The 90% confluent cultures were exposed to UV and harvested at the indicated time points. UV doses (40 J/m²) were delivered in two pulses using a stratinker (Stratgene) as described [111-113]. Then the cells were allowed to grow in 5% CO2 until time for harvesting or fixing at indicated time points post-UV treatment. Etoposide (ETP) were added to cells at 100μM and treated with the required time 12h, 24, and 48h.
**Cell proliferation assays**

MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay was used to determine cell proliferation rate. Cells were split at 5000/well into 96-well plates, and harvested at 24 h, 48 h, 72 h, and 96 h. Following harvesting, CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS) solution (Promega) was added at 20 ml/well and incubated 4 h at 37°C. OD values were measured at 490 nm by BioTek plate reader.

**Flow Cytometry Analyses**

Cells were trypsinized by 0.25% Trypsin-EDTA solution and then washed with PBS twice. Cells were fixed by drop-wise addition into ice-cold 70% ethanol and incubated on ice for 30 min. Cells were then stained with 0.5 ml of a solution containing PBS, 100 mg/ml propidium iodide (Sigma), 0.1% (v/v) Triton X-100, and 200 mg/ml RNase A, overnight at 4°C. Cells were analyzed next day by a BD Accuri C6 flow cytometer (BD Biosciences). For detection of cells specifically in the S-phase, Click-iT EdU Alexa Fluor 488 flow cytometry kit (Invitrogen) was used as per manufacturer’s instructions. We used Becton-Dickinson FACSort to measure the fraction of cells in S-phase using CellQuest Pro software (BD Biosciences). Flow data were then analyzed using FlowJo 9 and positive EdU signal is indicated as the S-phase cell population.

**Annexin V-FITC/PI Apoptosis Assay**

Follow the Cellometer manual protocol for apoptosis. Reagent product numbers: CS1-0114, CS0-0115-100ML, CS1-0116. Briefly, harvest the cells using trysin and wash in cold PBS. Spin
down the washed cells at 1000-2000 rpm for 5 min. Discard the supernatant and re-suspend cells in 1mL of 1XPBS. Verify the concentration of positive/negative controls and cell samples using the Vision CBA Analysis System. Use the Cellometer Sample Adjustment Calculator to generate the sample volume to obtain the target of 2,000,000 cells. Spin down cell sample at 1000-2000 rpm for 5 min, discard the supernatant. Re-suspend cells in 40μL of Annexin V Binding Buffer for a final concentration of \(~2\times10^6\) cells/ml. Prepare a sufficient volume to have 100μl per assay. Mix by pipetting up and down at least 10 times. Add 5ul of Annexin V-FITC and 1ul of the 100ug/ml PI working solution to each 100ul of cell suspension. Gently pipette the cells up and down ten times, then incubate for 15 min at RT in the dark. After incubation period, add 250ul of 1× PBS to the sample and spin down the cell sample at 1000-2000 rpm for 5 min. Carefully aspirate off the medium and re-suspend cell pellet in 50ul of Annexin V Binding Buffer. Mix gently by pipetting up and down 10 times. Keep the samples until ready to analyze by Cellometer Nexcelom Bioscience LLC.

**Western blot assay**

Cells were directly lysed in 2×SDS dye and separated by SDS-PAGE. Proteins were then immobilized onto nitrocellulose membranes (0.2 mm pore size). After blocking in non-fat dry milk (0.5%, w/v) for 1 h at RT, the membrane was incubated with primary antibodies at 4°C overnight, HRP-conjugated anti-mouse IgG or anti-rabbit IgG was used as secondary antibody. The membranes were visualized by ECL-plus reagent (Perkin-Elmer, Wellesley, MA, USA), scanned and analyzed using ImageQuant LAS 4000 biomolecular imager (GE Healthcare Bio-Sciences AB, Sweden). NIH Image J software was used to quantitate band intensities of Western
blots. Values were first corrected for the corresponding loading control and the normalized value of a particular marker as against the experimental control is then presented below each blot, as indicated. Lighter intensity blots were used in analyses by Image J. Darker exposure blots are also provided, when required. Multiple blots were used to quantitate various markers and different samples. Spliced out and combined lanes are clearly indicated with the vertical lines in the figures.

**Protein half-life assay**

Cells were continuously grown in DMEM medium without Dx to induce expression of 3xFlag-tagged NCL-WT or -6/S*A for indicated period. Protein synthesis was inhibited by incubating cells with cycloheximide (CHX, Sigma-Aldrich) at 40 mg/ml for indicated time periods. Cells were lysed with 2xSDS-PAGE dye at various times post-CHX incubation as indicated. Lysates were then analyzed by Western blotting for p53, FLAG (for NCL expression) and the β-actin loading control.

**Co-Immunoprecipitation**

For all co-immunoprecipitation experiments cell lines were grown in 150mm cell culture dishes at 100% confluency washed and stored at -80°C until ready to harvest. Adherent cells were collected in 15ml centrifuge tube using cold 1x phosphate buffered saline (PBS) containing 1% phenylmethanesulfonylfluoride (PMSF) solution. Cell suspension was centrifuged at speed 2000g for 10 minutes at 4°C. Total cell lysates (TCL) were prepared from harvested cells using modified standard protocols [23, 132, 198]. Briefly, cells were lysed by lysis buffer [50 mM Tris
pH 8.0, 150mM NaCl, 1 mM EDTA, 1% Triton X-100, 1× protease inhibitor cocktail, 0.5 mM PMSF, 1mM NaF, 1mM sodium vanadate Na$_3$VO$_4$, 20ug/ml RNase], do hand homogenizer 20 x on ice to help the extraction. Then incubate samples on a rotator for 1 hour at 4°C. Centrifugation at 13,000rpm for 15 minutes at 4°C, collect supernatant as TCL. Protein estimation is performed by Coomassie Plus (Bradford) Protein Assay (Thermo Fisher Scientific Biosciences). 5% of TCL was saved as input, 95% (1.5-2mg protein) were immunoprecipitated using 50 µl of EZview™ Red FLAG® M2 Affinity Gel (Sigma) to pull-down Flag-tagged NCL following manufacturers’ recommendations. Aliquots of IPed samples were analyzed by SDS-PAGE and immunoblotting. The results from three independent samples were analyzed and quantified using Image J program.

**RNA immunoprecipitation (RIP) assays**

The IP of nuclear RNA-protein complexes was performed as described [199]. Briefly, post-experimental treatment cells were cross-linked with 1% formaldehyde and the NEs were prepared. Extracts were treated with DNase (TURBO DNA-free Kit, Ambion), and the resulting material was IPed with polyclonal antibodies against FLAG (Sigma) to specifically pull-down NCL phospho-variants or control rabbit IgG (Sigma). Protein-RNA complexes were treated with proteinase K and reversal of cross-linking. The RNA was extracted from the IPs with Trizol reagent (sigma cat#: T9424) and DNA-free RNA kit (ZYMO cat: R1013 or R1015), and analyzed by RT-qPCR assays.

**RT-qPCR Assays**
As described previously [113, 200], equivalent amounts (0.5-1 μg) of input total RNA and IPed RNA were reverse transcribed using ProtoScript® II Reverse Transcriptase system (NEB) with oligo d(T)23VN and d(N)9. Relative levels were calculated using the ΔCt method.
CHAPTER VII

REFERENCES


resistant ovarian cancer cells to cisplatin by lowering the threshold set by Bcl-x(L) and Mcl-1. Mol Med 2011, 17(11-12):1262-1274.


