Mechanisms Of Deadenylation Regulation Under Different Cellular Conditions

Xiaokan Zhang
Graduate Center, City University of New York

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MECHANISMS OF DEADENYLATION REGULATION
UNDER DIFFERENT CELLULAR CONDITIONS

by

XIAOKAN ZHANG

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

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

Dr. Frida Kleiman, Hunter College of CUNY

Date Chair of Examining Committee

Dr. Edward Kennelly, The Graduate Center of CUNY

Date Executive Officer

Dr. Diego Loayza, Hunter College of CUNY

Dr. Kevin Ryan, City College of CUNY

Dr. Serafin Piñol-Roma, City College of CUNY

Dr. Hualin Zhong, Hunter College of CUNY

Supervisory Committee

THE CITY UNIVERSITY OF NEW YORK
ABSTRACT

Mechanisms of Deadenylation Regulation Under Different Cellular Conditions

By

Xiaokan Zhang

Adviser: Professor Frida Esther Kleiman

Control of gene expression by regulating mRNA stability after DNA damage has the potential to contribute to the cells rapid response to stress. The main focus of this dissertation is to elucidate the role(s) of nuclear PARN deadenylase in controlling mRNA stability, hence gene expression, of factors in the p53 signaling pathway during the DNA damage response (DDR). Understanding the mechanisms of these regulatory pathways will provide new insights on how the control of gene expression upon DNA damage decides cellular fate, offering new opportunities for therapeutics. In Chapter II, I presented evidence that PARN along with the cleavage factor CstF-associated tumor suppressor BARD1 participates in the regulation of endogenous transcripts in different cellular conditions. In Chapter III, I identified the mRNA targets of PARN in non-stress conditions, and contributed to describing a feedback loop between p53 and PARN, in
which PARN deadenylase keeps p53 levels low by destabilizing p53 mRNA through its 3′ untranslated region (3’UTR) in non-stress conditions, and the UV-induced increase in p53 activates PARN, regulating gene expression during DDR in a transactivation-independent manner. In Chapter IV, I presented evidence that PARN deadenylase has a specific effect on the steady-state levels of not only AU-rich element-containing but also microRNA (miRNA)-regulated nuclear mRNAs. I showed that the functional interaction of PARN with miRNA-induced silencing complex contributes to p53 mRNA stability regulation. These studies provide the first description of PARN deadenylase function in miRNA-dependent control of mRNA decay and of miRNA-function in the nucleus. Finally, in Chapter V, I determine that nucleolin is one of the RNA binding proteins that recruits PARN to the p53 mRNA and this can be regulated by phosphorylation, representing a novel regulatory mechanism for p53 gene expression. The data presented in this dissertation has contributed to describe and comprehend some novel mechanisms behind the regulation of gene expression during DDR.
SIGNIFICANCE

Cells are constantly exposed to stress caused by environmental factors such as oxidation, hydrolysis, alkylation, radiation and toxic chemicals. As a consequence, each cell in our body experiences more than 74,000 damages per day. If the damage is not repaired, DNA mutations might occur and proteins harmful to the cell might be expressed. The regulation of gene expression during the DNA damage response (DDR) is a fundamental cellular process that is controlled at multiple levels, such as transcription, mRNA processing and translation. While the study of gene expression regulation during DDR has been traditionally focused at the transcriptional level, it has recently become apparent that the role of post-transcriptional control may be equally important.

The dynamic nature of the mRNA 3'-end processing machinery allows the regulation of the steady-state levels of different mRNAs and has the potential to contribute to the cells rapid response to stress. The DDR involves changes in mRNA steady-state levels and, consequently, in gene expression of different genes. For example, while the steady-state levels of genes involved in cell cycle and DNA repair change at different points of the response to allow DNA repair, the steady-state levels of most housekeeping genes are transiently decreased to avoid the formation of erroneously processed truncated mRNAs that might generate harmful proteins.

mRNA polyadenylation and deadenylation are important processes that allow rapid regulation of gene expression in response to different cellular conditions. As the mRNA poly(A) tail is important in the regulation of mRNA steady-state levels, determination of how the poly(A) tail length of a particular mRNA and, consequently, its
stability are determined and regulated constitutes a major challenge in understanding control of gene expression in different cellular conditions. It has been established that modulation of the length of poly(A) tail of an mRNA by the polyadenylation/deadenylation machinery is a widespread strategy used to control mRNA stability and protein production in different cellular conditions such as development, mRNA surveillance, inflammatory response, cell differentiation, cancer, and especially during the DDR. One of the mechanisms underlying the transient decrease of cellular poly(A)$^+$ mRNA levels after DNA damage involves the formation of a complex between the cleavage stimulation factor CstF, the deadenylation factor poly(A)-specific ribonuclease (PARN), and the tumor suppressors BARD1 (BRCA1-associated RING domain 1), BRCA1 and p53. The formation of BARD1/CstF1/PARN complex has a role in activating the deadenylase activity of PARN, while at the same time it inhibits the 3’ cleavage step of the polyadenylation reaction. PARN along with the CstF/BARD1 complex participates in the regulation of the steady-state levels of endogenous transcripts under different cellular conditions. Any mutations or functional defects in those polyadenylation/deadenylation factors are prone to result in deregulation of proper gene expression, which may cause cancer.

mRNA steady-state levels are regulated by microRNAs (miRNAs), AU-rich elements (ARE)-binding proteins (BP), polyadenylation/deadenylation factors, and RNA-BP that recognize cis-acting sequences in the target mRNA. The dynamic change of these RNA-protein complexes regulates deadenylation signaling different checkpoints during DDR. The relevance of the functional connection between these cis-acting elements and the 3’ processing machinery is highlighted by changes in the length of the 3’UTR of
different mRNAs in cancer cells and during cell differentiation by the use of alternative 3′ cleavage and polyadenylation signals (APA). Changes in the length of 3′UTRs of mRNAs can eliminate or include several cis-acting elements, such as miRNA target sites and AREs, and these changes can affect the stability, hence the abundance, of different transcript isoforms in different subtypes of tumors. The cis-acting elements are frequently present in genes that encode proteins involved in cell growth regulation, cell differentiation and DDR. Increasing evidence has been presented to show the important roles that ARE- and miRNA-mediated deadenylation plays in different biological processes. Interestingly, a functional overlapping in the regulation of mRNA stability by ARE- and miRNA-mediated regulatory pathways has also been described. The role of PARN deadenylase in these regulatory processes has not been elucidated.

PARN has been shown to be involved in ARE-mediated deadenylation, and PARN expression has an effect on the steady-state levels of ARE-containing mRNAs, such as c-fos and c-myc, in non-stressed and UV-treated cells. In this thesis, I am investigating the role of cis-acting signals and trans-acting factors in regulating the stability of PARN target mRNAs. The working model in this dissertation is that PARN deadenylase plays a role in nuclear mRNA degradation of one of its targets, the p53 mRNA, through both ARE-mediated and miRNA-mediated regulatory pathways, and that miRNA-loaded miRISC contributes to the specific recruitment of nuclear PARN to its target mRNA in mammalian cells. In that scenario, I also investigated the role of nucleolin as one of the ARE-BPs involved in the recruitment of PARN deadenylase to its target mRNAs. The studies presented in this dissertation are highly significant at several levels. First, my studies were aimed at understanding control of gene expression by
regulation of mRNA stability during DDR, a field that remains largely unexplored. 

Second, my preliminary data provided first insights about the underlying molecular ties for such regulation. I identified that nucleolin, an RB protein that is highly expressed in cancers, interacts with PARN, an mRNA decay enzyme. Third, PARN has been extensively studied at the biochemical level but very little is known about its biological targets under different cellular conditions. Finally, I explored new regulatory mechanisms in the p53 pathway. While most of the studies on the expression of genes involved in stress response pathways have traditionally focused on transcription as a major regulator, it has recently become apparent that the posttranscriptional control of mRNA steady-state levels may play an equally important role. The mechanism(s) behind the regulation of p53 mRNA levels under non-stressed as well as DNA-damaging conditions has(ve) not been well characterized. My results indicate that PARN deadenylase keeps p53 levels low in non-stress conditions by interacting with both AREs and miRNA targeting sites in the 3’UTR and destabilizing the p53 mRNA. Importantly, these studies support the functional overlap between ARE- and miRNA-mediated mRNA turnover pathways, increasing the complexity of the signaling present in the 3’UTR of different genes. This is consistent with the idea that cell-specific 3’ processing profiles, and hence gene expression patterns, depends on the complexity of the signaling in the 3’UTR of the genes and the functional/dynamic interaction of the 3’ processing machinery and the DNA damage response/tumor suppression factors, providing functional connection between mRNA processing and cancer subtypes. The studies presented in this dissertation will serve as a valuable framework both for understanding these critical
biological processes and for developing appropriate therapeutic approaches to a variety of disorders, including cancer.

Finally, evidence is presented in this dissertation of a novel regulatory miRNA-mediated pathway in the nucleus. While most studies traditionally have focused on cytoplasmic miRNA-mediated pathways, miRNA’s nuclear functions have begun to emerge in recent years. Although miRNA-mediated gene silencing in the nucleus has been described, the mechanism(s) and deadenylase(s) involved in this process have not been elucidated. The data presented here is the first to address the mechanism behind miRNA-dependent control of deadenylation in the nucleus, showing the functional interplay among PARN deadenylase, the AREs in the 3’UTR, miRNA abundance and Ago-2 cellular localization. Together the studies presented here provide new insights of a regulatory pathway that involves a novel nuclear function of miRISC in mammalian cells as well as of the p53 pathway.
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CHAPTER I

BACKGROUND
NUCLEAR POLYADENYLATION/DEADENYLATION MACHINERIES

Modulation of the length of poly(A) tail of an mRNA by the polyadenylation/deadenylation machinery is a widespread strategy used to control mRNA stability and gene expression in different cellular conditions, such as development, mRNA surveillance, inflammatory response, cell differentiation, cancer, and the DNA damage response (DDR). The dynamic nature of the mRNA 3’-end processing machinery allows the regulation of the steady-state levels of different mRNAs and has the potential to contribute to the cells rapid response to stress. Almost all eukaryotic mRNA precursors undergo a co-transcriptional cleavage followed by polyadenylation at the 3’ end. After the signals are selected, polyadenylation occurs to full extent, suggesting that this first round of polyadenylation is a default modification for most mRNAs. However, the length of these poly(A) tails changes by the activation of deadenylation, which might regulate gene expression by affecting mRNA stability, mRNA transport or translation initiation. The mechanisms behind deadenylation activation are highly regulated and associated with different cellular conditions. After deadenylation, depending on the cellular response, some mRNAs might undergo an extension of the poly(A) tail or degradation. The polyadenylation/deadenylation machinery itself, microRNAs (miRNAs) or RNA binding factors are involved in the regulation of polyadenylation/deadenylation. Studies on the interplay between polyadenylation and deadenylation are providing critical information required for a mechanistic understanding of several diseases, including cancer development.
Figure 1. Schematic representation of the mammalian mRNA 3’ end formation. The cleavage step of the 3’ end processing is initialed by the assembly of cleavage complex through a cooperative binding of CstF at the G/U- and U-rich region and CPSF at the AAUAAA signal. CPSF-160 directly interacts with CstF3 (77kD) and PAP. CF I, CF II and RNAP II also play a role in the cleavage reaction. After the cleavage step, CPSF and PAP remain bound to the cleaved RNA and elongate a 200-adenosine residue poly(A) tail to the 3’end of the cleaved product in the presence of PABP. Taken from Zhao et al. (1999)
Almost all eukaryotic mRNA precursors, with the exception of histones, undergo a co-transcriptional modification at the 3’ end. The 3’ end formation includes a two-step reaction, an initial cleavage step followed by the synthesis of a 200-adenosine residue tail to the 3’ end of the upstream cleavage product (Figure 1; reviewed in (Zhao et al., 1999) (Shatkin and Manley, 2000; Shi et al., 2009). In mammalian cells, the poly(A) tail length is approximately 200-250 nucleotides long (Brawerman, 1981; Wahle and Winkler, 2013). Polyadenylation plays a fundamental role in regulating mRNA stability, translation and nuclear export, and thus is essential for the proper control of mRNA levels and of gene expression in eukaryotes (Colgan and Manley, 1997; Zhao et al., 1999). It has been shown that regulation of 3’ end formation plays crucial role in cell growth control (Chuvpilo et al., 1999; D’Ambrogio et al., 2013; Takagaki and Manley, 1998; Takagaki et al., 1996) and perhaps in diseases, especially in tumor cells (Scorilas, 2002). One of the first steps of the reaction is the recognition of the highly conserved hexamer AAUAAA located at 10 to 30 nucleotides upstream of the cleavage site by the cleavage and polyadenylation specific factor (CPSF) and of the G/U- and U-rich region located further downstream by cleavage stimulation factor (CstF(Takagaki and Manley, 1997). While a relatively simple signal sequence in the precursor mRNA is required for the reaction, many diverse and specific interactions between a large number of protein factors are involved in the formation of polyadenylation complex and regulation of 3’ end processing in different tissues and in different cellular conditions. While CPSF, CstF, cleavage factors 1 and 2 (CF I and CF II, respectively), RNA polymerase II (RNAP II) and poly(A) polymerase (PAP) play a role in the endonucleolytic cleavage step and help to specify the site of processing; CPSF, PAP, symplekin and poly(A) binding protein
(PABP) are involved in the polyadenylation step. CstF is one of the essential polyadenylation factors. CstF is active most likely as a dimer with each subunit consisting of three protein factors called CstF1 (55 kDa), CstF2 (64 kDa) and CstF3 (77 kDa). CstF2 is largely responsible for RNA binding (Takagaki and Manley, 1997). Both CstF1 and CstF3 subunits interact with the C-terminal domain (CTD) of the largest subunit RNAP II LS, likely facilitating the RNAP II-mediated activation of 3’-end processing and linking transcription and RNA processing (Hirose and Manley, 1998; McCracken et al., 1997; Mirkin et al., 2008). CstF1 plays important roles in regulation of mRNA processing by interacting with other factors (Figure 2). It contains seven WD-40 (tryptophan-aspartic acid) repeats, which are characteristic of regulatory proteins involved in protein-protein interactions (Neer et al., 1994; Takagaki and Manley, 1992), and an N-terminal hydrophobic region.

All organisms are constantly exposed to a variety of environment agents that damage the DNA, such as UV-light, ionizing radiation and chemicals, which may lead to

**Figure 2. The 3’ processing factor CstF1.** The CstF1 subunit of the CstF complex contains seven WD-40 (tryptophan-aspartic acid) repeats and an N-terminal hydrophobic region.
replication and transcription blockages, mutagenesis, and cellular cytotoxicity (Friedberg, 1995; Wood, 1996). The maintenance of genome integrity and fidelity is essential for the proper function and survival of all cells, and requires the coordinated control of gene expression and DNA repair mechanisms to recognize and correct DNA lesions (Lindahl, 1993; Sancar, 1994). One example is provided by the transient decrease of cellular poly(A)$^+$ mRNA levels following UV-irradiation and its normal recovery as part of the DDR (Hanawalt, 1994; Ljungman, 1999). Although the mechanism involved in this response is still not clear, it has been suggested that the decrease of mRNA level is due to UV-induced transcription inhibition (Donahue et al., 1996). This indeed is likely a significant part of the mechanism. However, those studies have not considered the important role of RNA processing on mRNA levels. Consistent with this, following UV-irradiation, the 3’ cleavage step of the polyadenylation reaction is repressed as a result of the direct interaction between the polyadenylation factor CstF1 with the tumor suppressor factor BARD1 (Kleiman and Manley, 1999). The UV-induced inhibition of mRNA 3’ processing occurs in a similar time frame that the UV-induced decrease of poly(A)$^+$ mRNA levels. BARD1 is a 97 kDa nuclear protein that associates with the breast cancer susceptibility gene product BRCA1 (Wu et al., 1996). Both proteins share structural features, they possess N-terminal RING finger motifs, which are responsible for the BRCA1/BARD1 interaction, three ankyrin repeats that are involved in protein-protein interactions, and two BRCA1 C-terminal (BRCT) domains that are involved in DNA repair and cell cycle regulation. BRCA1/BARD1 stabilizes each other and their association enhances their functions (Baer and Ludwig, 2002). It has been shown that the DNA-damage induced inhibition of polyadenylation correlates with increasing amount of
a BRCA1/BARD1/CstF complex formation (Kleiman and Manley, 2001), providing a link between transcription-coupled RNA processing and DNA repair. Moreover, mRNA 3’ end processing can also be repressed following DNA damage as a result of the proteasome-mediated degradation of RNAP II, representing another possible redundant, mechanism to explain the UV-induced inhibition of 3’ processing (Kleiman et al., 2005).

Interestingly, the mechanism underlying the regulation of 3’ end cleavage in response to DNA damage also involves the functional interaction of the deadenylation/polyadenylation machineries. As discussed in Chapter II, my studies have shown that following UV-induced DNA damage, the polyadenylation factor CstF1 directly interacts with the deadenylation factor poly(A)-specific ribonuclease (PARN) inhibiting the 3’ cleavage step of polyadenylation and activating deadenylation in the nucleus, suggesting the existence of alternative mechanisms to regulate gene expression under different cellular conditions (Cevher et al., 2010). Although most of the polyadenylation factors have been described and the reaction is now relatively well understood, the mechanisms underlying poly(A) removal are much less defined. In mammalian cells, the removal of the mRNA poly(A) tail is the earliest and rate-limiting step in mRNA decay (Chen and Shyu, 2003; Wilusz et al., 2001). Several mRNA decay pathways have been studied in eukaryotic cell, most of which are deadenylation dependent (Parker and Song, 2004). Two major PARN-mediated pathways of mRNA degradation have been defined: the 3’-5’ decay pathway and the deadenylation-dependent decapping pathway (Figure 3; (Beelman and Parker, 1995; von der Haar et al., 2004). In the 3’-5’ decay pathway the degradation occurs in the cytoplasmic exosome in the 3’-5’ direction after the removal of the poly(A) tail, while in the deadenylation-dependent decapping pathway degradation is
initiated by deadenylation followed by DCP1:DCP2-mediated decapping and 5’-3’ exonucleolytic degradation of mRNA by the Xrn1p 5’ exonuclease (Caponigro and Parker, 1996; Mukherjee et al., 2002). In addition to these general mRNA decay pathways, other degradation pathways exist. For example, another well-known mRNA decay pathway is nonsense mediated mRNA decay (NMD). NMD is a quality control mechanism which


**Figure 3. PARN-mediated degradation of mRNA in the cytoplasm.** Two major PARN-mediated pathways of mRNA degradation have been defined: 1) After the removal of the poly(A) tail, mRNA is first decapped by DCP followed by exonuclease XRN1-dependent degradation in the 5’→3’ direction. 2) After deadenylation, mRNA degradation occurs in the 3’→5’ direction by the exosome followed by decapping of the 5’ end. Modified from Dr. Wilusz lab webpage http://www.cvmbs.colostate.edu/mip/wiluszlab/whatwedo.html
selectively eliminates mRNAs harboring premature termination codons (Karam et al., 2013; Li and Wilkinson, 1998; Maquat, 1995). NMD is believed to occur not only in the cytoplasm but also in the nucleus since some ribosomal and translational activities have been detected in that compartment (Ishigaki et al., 2001; Trcek et al., 2013). Interestingly, PARN copurifies with essential NMD factors and PARN down-regulation abrogated nonsense-mediated decay (Lejeune et al., 2003).

The steady-state levels of cellular mRNAs are determined by the balance between their biosynthesis and turnover. Different mRNAs within the same cell have distinct lifetimes. In mammalian cells, mRNA lifetimes range from several minutes to days, depending on various genes and the cellular conditions (Khodursky and Bernstein, 2003). The mechanisms controlling deadenylation are dynamic and highly regulated. Deadenylation processes are a widespread strategy that allows rapid control of mRNA stability, and represent additional checkpoint targets in the regulation of gene expression. Deadenylases are the key catalytic exoribonucleases that are required for proper regulation of poly(A) tail length by degrading poly(A) tail in a 3’ to 5’ direction. In mammalian cells, a number of deadenylases have been identified and studied in detail. There are two superfamilies of deadenylases: DEDD (Asp-Glu-Asp-Asp)-type enzyme, and EEP (endonuclease-exonuclease-phosphatase)-type deadenylase (Goldstrohm and Wickens, 2008). All known deadenylases belong to either superfamilies, for example, POP2, PARN, poly(A) nuclease (PAN2) and CAF1 deadenylase belong to DEDD superfamily, while CCR4 and Nocturnin deadenyase belong to EEP superfamily (Goldstrohm and Wickens, 2008; Thore et al., 2003; Zuo and Deutscher, 2001). Interestingly, some multi-subunit deadenylases are composed from subunits from both
families (Doidge et al., 2012). It has been shown that deadenylases localize both in the nucleus and the cytoplasm and are involved in different cellular processes. The best characterized deadenylases so far are the CCR4-POP2-NOT multi-subunit complex, which is the predominant deadenylase in eukaryotes, PARN, a divalent metal-ion dependent, processive and cap-interacting exoribonuclease, and (PAN), which is involved in early steps of poly(A) tail metabolism.

PARN is one of the three major poly(A) specific 3’ exoribonucleases identified in mammalian cells (Mitchell and Tollervey, 2000; Wu et al., 2005). PARN is expressed ubiquitously in all tissues of most eukaryotic organisms (Copeland and Wormington, 2001). In calf thymus cell free extracts and in Xenopus oocytes two isoforms of PARN have been described with molecular sizes of approximately 74 kDa and 62 kDa, both of which have shown enzymatic activity and different nuclear-cytoplasmic distribution. While the 74 kDa isoform of PARN is exclusively nuclear, the 62 kDa isoform is cytoplasmic (Korner et al., 1998; Martinez et al., 2000). So far, it has remained obscure how the subcellular localization of PARN is regulated, although it appears likely that proteolytic cleavage plays a role. While the cytoplasmic PARN’s activity has been extensively studied, the nuclear functions of PARN are not completely understood. PARN is found mostly in the nucleus and it does not seem to play a key role in cytoplasmic mRNA decay (Ota et al., 2011). PARN has been identified as an oligomeric, highly processive, metal-ion dependent and cap-interacting poly(A) specific 3’ exonuclease (Grishin, 1998). PARN consists of a nuclease domain that performs the deadenylation reaction, a R3H domain, which is constituted by an invariant arginine that is separated by three residues from a highly conserved histidine and binds single stranded
nucleotides, and a RNA recognition motif (RRM) (Figure 4; (Wu et al., 2005). While the R3H domain is responsible for PARN’s specificity for single-stranded poly(A) (Martinez et al., 2001), the RRM harbors the cap binding properties (Nilsson et al., 2007). It also has been shown that its deadenylation activity and processivity is enhanced by the mRNA 5’-end-located cap structure (Astrom et al., 1992; Dehlin et al., 2000; Korner et al., 1998; Martinez et al., 2001). On the other hand, PARN activity is inhibited by cap binding protein (CBP80) or nuclear poly(A) binding protein-1 (PABPN-1) (Balatsos et al., 2006; Gao et al., 2001). It has been suggested that PARN simultaneous interaction with the cap structure and the 3’ end located poly(A) tail shields the 5’ from decapping enzymes and initiates the deadenylation process. Interestingly, the communication between both the 3’ and 5’ ends of mRNA is very important because it integrates the initiation of translation, translation and mRNA turnover (Martinez et al., 2001).

**Figure 4.** PARN consists of a nuclease domain, a R3H domain, and a RRM motif. R3H domain, a single-stranded nucleic-acid-binding domain, is responsible for PARN’s specificity for single-stranded poly(A), while the RRM harbors the cap binding properties. Modified from He et al. (2013).
Besides, PARN can promote deadenylation of AU-rich elements (AREs)-containing mRNAs in the presence of tristetraprolin (TTP; (Lai et al., 2003). Interestingly, it has been described that UV induces stabilization of ARE-containing mRNAs; such as c-fos, kin17, c-jun, IκB and c-myc (Blattner et al., 2000). It has also been shown that the UV-induced transcript stabilization and enhanced protein levels of short basal half–life, such as oncogenes, apoptosis and cell-cycle related genes, growth factors and cytokines, is due to the inhibition of cytoplasmic mRNA deadenylation and degradation (Gowrishankar et al., 2005).

AU RICH ELEMENT MEDIATED DEADENYALATION

About 54% of human genes have more than one conserved polyadenylation sites that show different efficiencies of polyadenylation (Tian et al., 2005). Several cis-acting elements within the 3’ untranslated region (3’UTRs) are responsible for the selection among these alternative polyadenylation (APA) sites. Although a direct connection between APA and the polyadenylation/deadenylation machinery has not been described, the selection between the distal or proximal alternative signals might cause the inclusion or exclusion of other cis-acting RNA elements which are involved in polyadenylation/deadenylation processes (Figure 5). The relevance of these regulatory processes is highlighted by the finding that changes in the length of the 3’ UTRs of different mRNAs due to alternative 3’ cleavage and polyadenylation changes the number of miRNA target sites and AREs in cancer cells (Mayr and Bartel, 2009; Shi, 2012; Singh et al., 2009), and during cell differentiation (Ji et al., 2009; Sandberg et al., 2008; Zlotorynski and Agami, 2008). Interestingly, deadenylation is often under the control of

cis-acting regulatory elements, which includes AREs and miRNA target sites, within the 3’UTRs of eukaryotic mRNAs.

About 12% of mammalian mRNAs bear an important regulatory signal known as AREs in their 3’UTRs, which has been shown to play significant roles in mRNA stability regulation (Guhaniyogi and Brewer, 2001). The ARE typically contains one or several AUUUA pentamer repeats within a U-rich region of the 3’UTR (Chen and Shyu, 1995; Lagnado et al., 1994; Zubiaga et al., 1995). ARE sequences are frequently present in genes that encode tightly regulated proteins involved in cell growth regulation, cell differentiation and responses to external stimuli. The destabilizing functions of AREs are

Figure 5. Schematic diagrams of cis-acting RNA sequences at the 3'UTR involved in polyadenylation/deadenylation processes. The selection between the proximal or distal alternative polyadenylation signals leads to the exclusion or inclusion of cis-acting RNA sequences, such as miRNA target sites and ARE, which might mediate polyadenylation/deadenylation processes. Taken from Zhang et al., (2010)(Zhang et al., 2010)(Zhang et al., 2010)(Zhang et al., 2010)(Zhang et al.,
important because in their absence proto-oncogenes, such as c-fos, c-myc and c-jun, could become oncogenes (Schiavi et al., 1992).

A number of trans-acting factors, known as ARE-binding proteins (ARE-BPs), regulate ARE-mediated decay and, subsequently, modulate the stability of ARE-containing mRNAs. ARE-BPs either recruit deadenylases to the target mRNAs promoting deadenylation or block the recruitment of deadenylases and exosome inhibiting deadenylation. The ARE-BPs that promote ARE-containing mRNAs decay include tristetraprolin (TTP), butyrate response factor 1 (BRF1), AU-rich binding factor 1 (AUF1) and KH-type splicing regulatory protein (KHSRP or KSRP); only Hu protein R (HuR) has been shown to play a role in stabilizing ARE-containing mRNAs (Barreau et al., 2005; Fan and Steitz, 1998; Ma et al., 1996; Westmark et al., 2005). For example, TTP directs its target ARE-containing mRNA tumor necrosis factor (TNF-α) for degradation by expediting removal of the poly(A) tail. Interestingly, the phosphorylation of TTP inhibits the recruitment of CAF1 deadenylase, and as a result, it reduces the ability of TTP to promote deadenylation (Dean et al., 2004; Lai et al., 1999; Marchese et al., 2010; Winzen et al., 2004). Supporting the idea that CAF1-CCR4-NOT deadenylase complex plays a critical role in ARE-mediated deadenylation, knockdown of CAF1 has been shown to abrogate deadenylation and decay of the ARE-containing α-globin mRNA (Schwede et al., 2008; Zheng et al., 2008). Interestingly, PARN deadenylase has also been shown to be involved in ARE-mediated deadenylation: KSRP recruits PARN to ARE-containing mRNAs to initiate the poly(A) tail shortening that precedes degradation by the exosome (Gherzi et al., 2004). PARN could also promote TTP-directed
Figure 6. Model of ARE-mediated regulation of deadenylation. The ARE-binding proteins mediate destabilization and/or stabilization of the ARE-containing mRNAs. ARE-BPs, such as AUF1, TTP, BRF1 and KHSRP, recruit deadenylases, such as PARN and CAF1-CCR4-NOT, to target ARE-containing mRNAs and initiate the deadenylation process that precedes degradation. Another ARE-BP, HuR, plays a role in stabilizing ARE-containing mRNAs by blocking the binding of ARE-BPs involved in the destabilization of ARE-mRNAs, such as AUF1, TTP and KHSRP. This competition stabilizes the association of PABP to the poly(A) tail or prevents the recruitment of deadenylases to the ARE-mRNA. Taken from Zhang et al., (2010)
deadenylation in vitro (Korner et al., 1998; Lai et al., 2003; Lin et al., 2007; Moraes et al., 2006). As discussed in Chapters II and III, my recent studies also indicated that PARN deadenylase has a role regulating the stabilities of ARE-containing mRNAs, such as c-fos and c-myc, in non-stressed cells (Cevher and Kleiman, 2010; Devany et al., 2013).

Nucleolin is a multifunctional ARE-BP with multiple subcellular localizations in the cell. Nucleolin is localized ubiquitously in the nucleolus, and is also found in other nuclear regions, as well as in the cytoplasm and the plasma membrane (Borer et al., 1989; Ginisty et al., 1999). Nucleolin protein contains multiple phosphorylation sites in its acidic N-terminal region, four RNA-binding domains mediating the interaction with mRNAs and pre-rRNA in the central region, and arginine/glycine-rich domain (RGG/GAR) in the C-terminal region, which can also interact with target mRNAs as well as with other proteins (Figure 7; Ghisolfi et al., 1992; Serin et al., 1996; Bouvet et al., 1998; Abdelmohsen et al., 2011; Bouvet et al., 1998; Ghisolfi et al., 1992; Serin et al., 1996). Phosphoprotein nucleolin could be phosphorylated by numerous kinases, such as Cdc2, casein kinase II (CKII), protein kinase C (PKC) and the stress activated protein kinase p38 (Dranovsky et al., 2001; Tediose et al., 2010; Yang et al., 2002; Zhou et al., 1997). The role of most of these phosphorylation events is not well understood. Nucleolin has been implicated in many different cellular processes, such as chromatin remodeling (Angelov et al., 2006), ribosomal RNA (rRNA) processing (Ginisty et al., 1998), ribosome assembly (Turner et al., 2009) and nucleo-cytoplasmic transport (Hovanessian et al., 2010). Besides, nucleolin participates extensively in the post-transcriptional fate control of mRNA targets, which typically contain AREs in their 3’UTR.
Nucleolin modulates the stability and translation of target mRNAs through multiple ways. First, nucleolin affects mRNA turnover, both increasing and decreasing mRNA half-life, by interacting with the 5’UTR and/or 3’UTR of target mRNAs. For example, in leukemia cells, nucleolin enhances the expression of pro-oncogenic protein B-cell lymphoma 2 (Bcl-2), which blocks apoptosis in cancer cells (Yang et al., 1997), by stabilizing BCL2 mRNA through the association with its ARE at 3’UTR (Sengupta et al., 2004). Another mRNA target of nucleolin is the amyloid precursor protein (APP), which is linked to Alzheimer’s disease. Nucleolin associates with the 3’UTR of APP mRNA, and accelerates its degradation in response to stress (Westmark and Malter, 2001). Nucleolin also has influence on the translation of a subset of associated mRNAs.

Figure 7. **Schematics of nucleolin domains:** The acidic N-terminal region of nucleolin protein contains multiple phosphorylation sites; the central region contains four RNA-binding domains, which are responsible for the interaction with mRNAs and pre-rRNA; and arginine/glycine-rich domain (RGG/GAR) in the C-terminal region can also interact with target mRNAs and protein factors. Taken from Bhatt et al., (2012)
Nucleolin has been found to bind to p53 mRNA and suppressed its translation (Takagi et al., 2005). Interestingly, nucleolin is highly expressed in proliferating cells, such as cancer and stem cells. Nucleolin’s ability to associate with mRNA of target genes, which are functionally involved in stress response, cell survival, as well as cancer, and regulate their expression through changes of either mRNA stability or translation efficiency, might explain its implication in human disease. Recently, nucleolin has also been reported to play a role in the regulation of the biogenesis of tumorigenic microRNAs, such as miR-15a and miR-16 (Pickering et al., 2011), suggesting new roles for nucleolin in controlling gene expression.

**miRNA MEDIATED DEADENYLATION**

miRNAs comprise a large family of small single-stranded non-coding RNAs (~21nt in length), which are encoded within the genome of species ranging from protozoans to plants to mammals. miRNAs play key roles in a broad range of biological processes. In mammals, it is predicted that the regulation of more than 60% of all protein-coding genes are mediated by miRNAs. They act at the post-transcriptional level to modulate gene expression, by imperfectly base-pairing to the target mRNAs. In most studied animals’ miRNAs, the hybrids are formed between the miRNA 5’- proximal “seed” region and the complementary sequences in the 3’UTRs of the target mRNA (Bushati and Cohen, 2007; Filipowicz et al., 2008; Friedman et al., 2009; Ghildiyal and Zamore, 2009; Mayr and Bartel, 2009). It has been shown that each mRNA could be regulated by more than one miRNA, and each miRNA could base-pair with more than one target mRNAs.
While most studies traditionally have focused on cytoplasmic miRNA-mediated pathways, miRNA’s nuclear functions have begun to emerge in recent years. Although miRNA-mediated gene silencing in the nucleus has been described (Nishi et al., 2013; Robb et al., 2005), the mechanism(s) and deadenylase(s) involved in this process have not been elucidated. Generally, miRNAs inhibit protein synthesis either through translation repression and/or through deadenylation activation, which leads to mRNA degradation (Chekulaeva and Filipowicz, 2009; Eulalio et al., 2008; Filipowicz et al., 2008). These two mechanisms have been identified as two distinct independent pathways: the ability of miRNAs to expedite deadenylation does not depend on decreased translation; nor does translational repression by miRNAs require a poly(A) tail (Wu et al., 2006). Recently, it is also reported that some miRNAs could also function as translation activator in specific situations (Henke et al., 2008; Orom et al., 2008; Vasudevan et al., 2008). miRNAs function in the form of ribonucleoprotein complexes, known as miRNA-induced silencing complex (miRISC), which deliver miRNAs to their mRNA targets. Argonaute (Ago) and GW182 family proteins are the best-characterized protein components involved in the miRNA-mediated gene expression control (Chekulaeva and Filipowicz, 2009; Eulalio et al., 2008).

The key components of miRISCs are Ago family proteins. Ago proteins contain three evolutionarily conserved domains, PAZ, MID and PIWI. Through these domains, Ago proteins are able to associate with 3’ and 5’ end of the miRNA (Jinek and Doudna, 2009; Peters and Meister, 2007). In mammals, four Ago proteins, Ago1 through Ago4, function in miRNA induced translation repression. Interestingly, Ago proteins are able to repress protein synthesis when artificially tethered to the mRNA 3’UTR (Pillai et al.,
2004; Pillai et al., 2005; Wu et al., 2008), indicating that their involvement in control of mRNA expression is miRNA-independent. It has been shown that some Ago proteins are more potent repressors than others and cell- and tissue-specificity has also been reported in some studies (Wu et al., 2008). It has also been described that knockdown of Ago2 in human HEK293 cells lead to a much more profound effect on miRNA-mediated repression than the knockdown of the other three Ago proteins, suggesting that in mammals Ago2 may have some specific functions that other Ago proteins cannot complement (Schmitter et al., 2006). GW182 proteins are another group of factors crucial for miRNA-mediated functions (Eulalio et al., 2009). There are three mammalian GW182 proteins, known as TNRC6A, -B and –C. They interact directly with Agos through their GW repeats located in the N-terminal portion of the protein, and act downstream of Agos (Eulalio et al., 2009; Till et al., 2007). It has been shown that the disruption of GW182-Ago interaction by point mutations or peptide competition results in abrogation of miRNA-mediated repression, indicating the crucial role of GW182-Ago interaction (Eulalio et al., 2009; Till et al., 2007)). Additional protein components of miRISCs have also been identified to function as regulators in miRNA-mediated repression (Peters and Meister, 2007).

Much evidence supports the idea that miRNAs destabilize target mRNAs through deadenylation and subsequent decay (Figure 8). Many studies have showed that the levels of specific miRNAs, or the activity of the miRNA machinery, have profound effect on the level of miRNA-targets and that the miRNA-mediated downregulation of the levels of target mRNA has important biological consequences (Johnson et al., 2005; Sampson et al., 2007; Wakiyama et al., 2007). The deadenylation and the subsequent decay of
mRNAs targeted by miRNAs require the Ago and GW182 components of the miRISC (Akao et al., 2006; Lazzaretti et al., 2009). The knockdown of human Ago2 abrogates miRNA-mediated deadenylation; while the disruption of GW182-Ago interaction also fails to activate mRNA deadenylation (Eulalio et al., 2008; Till et al., 2007). The cytoplasmic poly(A) binding protein 1 (PABPC-1) is an additional protein component that is critical for the miRNA-mediated deadenylation. It has been shown that a conserved motif in GW182 interacts with the C-terminal domain of PABPC-1 and that this interaction and the activity of PABPC-1 contribute to miRNA-mediated poly(A) removal (Fabian et al., 2009; Jinek et al., 2010; Zekri et al., 2009). It has been proposed that the miRNA-mediated degradation of mRNAs involves the association of Ago

![Figure 8. Model of miRNA-mediated deadenylation.](image)

miRISCs, which contain Ago, GW182, PABPC1 and either CAF1-CCR4-NOT (as indicated) or Pan2-Pan3 (not shown) deadenylases, deliver miRNAs to the target mRNAs and mediate deadenylation, which leads subsequently to mRNA degradation. Taken from Zhang et al., (2010)
proteins to the miRNAs and the recruitment of GW182 to the target mRNAs via its N-terminal domain; then the GW182 C-terminal silencing domain recruits the deadenylase complex through the interaction with PABPC-1 (Goss and Kleiman, 2013). One of the most studied deadenylases involved in miRISCs is the CAF1-CCR4-NOT1 complex, which contains two protein factors with deadenylase activity, CCR4 and CAF1. It has been shown that the CAF1-CCR4-NOT complex associates with PABPC-1, and the deadenylase activity of the CAF1-CCR4-NOT complex is necessary for the miRNA-mediated degradation (Zekri et al., 2009). Supporting these results, both the knockdown of CAF1 or NOT1 expression and the over-expression of CCR4 or CAF1 mutants significantly reduce miRNA-mediated deamination and mRNA decay, but not translational repression (Behm-Ansmant et al., 2006; Fabian et al., 2009; Piao et al., 2010). Although the miRISC has been shown to functionally interact with several deadenylases, its functional interaction with PARN has not been elucidated.

Interestingly, some of the seed signals recognized by miRNA overlap with AREs in the 3’UTR (Bhattacharyya et al., 2006; Jing et al., 2005). Although the exact contribution of miRNAs, miRISC, AREs and ARE-BPs to mRNA decay has not been elucidated yet, recent studies have described a functional overlapping on mRNA stability between ARE- and miRNA-mediated regulatory pathways (Figure 9. It has been shown that miRNAs can functionally interact with ARE-BPs, and factors involved in miRNA metabolism, such as Dicer and Ago, are required for ARE-mediated decay (Jing et al., 2005). For example, it has been shown that HuR can bind AREs present in c-Myc 3’UTR at a site proximal to that recognized by let-7 miRNA. HuR appeared to facilitate the targeting of let-7-loaded miRISC to an adjacent region of HuR binding site, and to mediate the
reduction of c-Myc mRNA levels (Kim et al., 2009). Another example is the cooperation of ARE-BP TTP and miR-16 in targeting tumor necrosis factor-α mRNA for ARE-mediated mRNA degradation (Jing et al., 2005). TTP does not bind directly miR-16 but it forms a complex with miRISC, and that complex recruits the deadenylase and the exosome for mRNA degradation. HuR can also relieve CAT-1 mRNA from miR-122 repression upon stress in human liver cells (Bhattacharyya et al., 2006).

Figure 9. Model of overlapping in the ARE - and miRNA-regulated deadenylation. Cooperation of ARE s-BPs, miRNAs, deadenylases and exosome is essential for the regulation of mRNA stability. The recruitment of the ARE-BPs HuR or TTP to the ARE sequence assists the targeting of let-7- or miR-16-loaded miRISC complexes, respectively, to the most proximal site to the ARE sequence. Taken from Zhang et al., (2010)
CHAPTER II

EFFECT OF PARN EXPRESSION ON ENDOGENOUS GENE EXPRESSION UNDER DIFFERENT CELLULAR CONDITIONS
INTRODUCTION

The steady-state levels of cellular mRNAs are determined by the balance between their biosynthesis and turnover. The turnover rates of individual mRNAs can vary in response to changes in the cellular environment and the mRNA poly(A) tail is one of the key structures required for correct regulation of mRNA degradation. The poly(A) tails are also critical for regulation of mRNA processing, translation and subcellular localization, such as nuclear export (Colgan and Manley, 1997; Mandel et al., 2008; Zhao et al., 1999). Thus, the poly(A) tail is a fundamental cis-acting element that is essential for proper control of gene expression at several different levels in eukaryotes. The poly(A) tail is synthesized in the nucleus through a two-step polyadenylation reaction; an initial cleavage step, which specifies the 3’ end of the mRNA, followed by the synthesis of a 200 adenosine residues tail to the 3’ end of the upstream cleavage product (reviewed in Shatkin and Manley, 2000; Zhao et al., 1999). The polyadenylation reaction is by itself a highly regulated event and is used for example to regulate tissue or developmental specific gene expression and for cell growth control (Chuvpilo et al., 1999; Takagaki and Manley, 1998; Takagaki et al., 1996). Several examples of cases are known which links deficiencies in the polyadenylation machinery to disease development, including tumor formation (reviewed by Scorilas, 2002).

The polyadenylation reaction requires the assembly of a rather large number of interacting protein factors that recognize a relatively simple set of cis-acting signal sequence elements in the mRNA precursor. Cleavage stimulation factor (CstF) is one of the essential polyadenylation factors. CstF is active most likely as a dimer with each subunit consisting of three protein factors called CstF1, CstF2, and CstF3. CstF3 interacts
directly with the down-stream located GU-rich cis-acting element. Both the CstF1 and CstF2 subunits interact specifically with the carboxy-terminal domain (CTD) of RNA polymerase II largest subunit (RNAP II LS), likely facilitating the RNAP II-mediated activation of 3’ end processing (Hirose and Manley, 1998; McCracken et al., 1997). Moreover, 3’ end processing can be repressed following DNA damage as a result of the interaction between CstF1 and BRCA1-associated RING domain protein (BARD1, (Kleiman and Manley, 1999) and of the proteasome-mediated degradation of RNAP II (Kleiman et al., 2005). Studies from Dr. Kleiman’s lab have recently shown that cells with reduced levels of CstF display decreased viability following UV treatment, reduced ability to ubiquitinate RNAP II, and defects in repair of DNA damage (Mirkin et al., 2008), supporting the idea that CstF plays a direct role in the DNA damage response.

Although most of the polyadenylation factors have been described and the reaction is now relatively well understood, the mechanisms behind poly(A) removal are much less defined. In mammalian cells, the earliest and rate limiting step in mRNA decay is the removal of the mRNA poly(A) tail (Chen and Shyu, 2003; Wilusz et al., 2001). PARN is one of the three major poly(A) specific 3’ exoribonuclease identified in mammalian cells and characterized thus far (Mitchell and Tollervey, 2000; Parker and Song, 2004; Wu et al., 2005). It is expressed ubiquitously in all tissues of most eukaryotic organisms (Copeland and Wormington, 2001) and localizes both to the nucleus and the cytoplasm. PARN shows high specificity for single stranded poly(A) (Korner and Wahle, 1997; Martinez et al., 2001), and its deadenylating activity is stimulated by the mRNA 5’ end located cap structure (Dehlin et al., 2000; Gao et al., 2001; Martinez et al., 2001; Nilsson et al., 2007; Wu et al., 2009). Although the exact function of PARN in the
nucleus is unknown, it has been established that the CBP80 (Balatsos et al., 2006) and the poly(A) binding protein 1 (PABPN1, Gao et al., 2001) both inhibit PARN activity. Interestingly, the cap binding complex (CBC) has also been shown to play a role in polyadenylation by stabilizing the RNA/CstF complex formed in the nucleus and the depletion of CBC reduces the mRNA cleavage reaction (Flaherty et al., 1997).

Dr. Cevher and colleagues (2010) found that the polyadenylation factor CstF1 interacts strongly with the same region of PARN, the C-terminal domain, as its inhibitor CBP80. Like the previously described CstF/BARD1/BRCA1 complex, the CstF/PARN complex formation is stimulated upon UV light–treatment and participates in the inhibition of the 3’ cleavage reaction of polyadenylation upon DNA-damaging conditions. More importantly, they also showed that the CstF1/PARN interaction activates deadenylation in vitro and that UV treatment can activate nuclear PARN deadenylase activity. They provided evidence that the tumor suppressor BARD1 strongly activates deadenylation by PARN in the presence of CstF1 and that the siRNA-mediated knockdown of BARD1 decreases the UV-induced activation of deadenylation. In addition, their data showed that CBP80 and CstF1 could compete for binding to PARN, providing a mechanism to regulate PARN deadenylase activity in different cellular conditions. As part of those studies, I determined that these functional interactions correlate with changes in both stability and polyadenylation of different mRNA precursors, such as housekeeping genes and some clinically significant genes, upon UV treatment and that reduced expression of PARN is sufficient to revert the observed changes. Taken together, these results indicate that the CstF/PARN complex plays a role in the inhibition of 3’ cleavage of polyadenylation and the activation of deadenylation in
the nucleus upon DNA-damaging conditions, suggesting the existence of alternative mechanisms to regulate gene expression in different cellular conditions. This manuscript was the first report describing a mechanism of gene expression regulation in response to DNA damage that involves the deadenylation/polyadenylation machinery.

RESULTS

The data presented by Cevher and colleagues (2010) provided evidence that DNA damage induces the activation of PARN-mediated mRNA deadenylation in the nucleus. To further investigate this, I determined the expression levels of different endogenous mRNAs in cells treated by UV irradiation and with siRNAs targeting PARN. Briefly, 24 h after transfection with the indicated siRNAs, cells were exposed to UV light and total nuclear RNA was purified at different time points after UV treatment. Gene expression was analyzed by real-time quantitative reverse transcription PCR (qRT–PCR). Random and oligo-(dT) primers were used for the RT reaction and qPCR reactions were performed using commercially available primers. First, the expression levels of two housekeeping genes, GAPDH and ß-actin, were analyzed under different cellular conditions. The qRT–PCR (Figure 10) analysis showed that the mRNA levels of these genes decreased under DNA-damaging conditions in cells treated with control siRNA. These data are consistent with earlier studies from Dr. Kleiman’s lab (Mirkin et al., 2008) and others previous observations (Dheda et al., 2004; Maccoux et al., 2007), which showed that GAPDH RNA expression can change significantly in different biological systems and under different conditions, and that these variations can lead to experimental error between analyzed samples when GAPDH is used as control. Interestingly, the UV-
induced decrease in the mRNA levels of endogenous housekeeping genes was lost when
the cells were treated with siRNAs targeting PARN (Figure 10).

To determine the role of the BARD1/CstF/PARN complex formation in the
regulation of mRNA levels, I used RNA samples from a stable cell line that expresses a
BARD1 mutant in the threonine 734 (Kim et al., 2006). This mutant has a defect in DNA
damage-induced BARD1 phosphorylation at the T734, which is important not only for
the BARD1/CstF1 complex formation but also for the UV-induced inhibition of both
mRNA 3’ end processing (Kim et al., 2006). A similar pattern of changes in the mRNA
levels of housekeeping genes was observed using the cell line expressing the BARD1
mutant T734A (Figure 10), which did not show BARD1/CstF/PARN complex formation
and activation of deadenylation after UV treatment (Cevher et al., 2010). These results
indicate that BARD1/CstF/PARN complex formation has an important role to decrease
the mRNA levels of housekeeping genes under DNA-damaging conditions, and thereby
might contribute to the UV-induced decrease in the cellular levels of total mRNA. As it
has also been shown that PARN can promote the deadenylation of AU-rich element
(ARE)-containing mRNAs (Lai et al., 2003; Moraes et al., 2006), I also analyzed two
ARE-containing mRNAs, that is, c-fos and c-myc, by qRT–PCR. Both mRNAs increased
transiently under DNA-damaging conditions in cells treated with control siRNA and in a
sarcoma cell line expressing WT BARD1 (Figure 10), in keeping with earlier studies that
indicate that ARE elements within the 3’UTR can control mRNA levels under different
cellular conditions. For example, ARE elements can decrease mRNA stability under non-
stress conditions and can increase mRNA stability after UV treatment in mammalian cells
(Blattner et al., 2000; Bollig et al., 2002; Gowrishankar et al., 2005; Wang et al., 2000).
Figure 10. Analysis of the effect of PARN expression on endogenous gene expression after UV treatment. Real-time RT-PCR analysis of GAPDH, b-actin, c-fos and c-myc expression after UV-treatment using RNA samples from cells treated with control/PARN siRNA and from cells expressing WT/T734A BARD1 mutant. As the RT products of GAPDH from cells treated with control siRNA and not treated with UV were used as endogenous control, the log value corresponding to this sample was zero. The values shown in the figure have been adjusted to avoid the presentation of negative values. The data shown are the mean±s.e.m. from three independent experiments. Equal amounts of cDNAs were used in qRT–PCR reactions using primers specific for GAPDH, b-actin, c-fos and c-myc mRNAs. Relative quantification was performed using standard curves of known amounts of total cDNA. The results shown are the
average of four PCRs from two different RNA extractions. Taken from Cevher et al., (2010)
Supporting my results, Blattner et al. (Blattner et al., 2000) showed that c-fos mRNA expression increased 45 min to 1 h after UV treatment and then dramatically decreased 2 h after UV treatment. Interestingly, PARN knockdown cells and cells expressing the T734A BARD1 mutant showed increase in the mRNA levels of both c-fos and c-myc in samples from cells not treated with UV (Figure 10). These results suggest that the BARD1/CstF/PARN complex has a role in decreasing the levels of short-lived mRNAs involved in the control of cell growth and differentiation, keeping their expression levels low under non-stress conditions. Both reduced expression of PARN and lack of BARD1/CstF/PARN complex formation have a slight effect on the UV-induced increase in the expression levels of these genes, suggesting that other mechanism(s) might be involved in determining the mRNA levels of these genes under DNA-damage conditions. Taken together, these results provide evidence that the BARD1/CstF/PARN complex has important roles in the regulation of the levels of different endogenous mRNAs under different cellular conditions. As proposed by Cevher and colleagues (Cevher et al., 2010), it is possible that the competition between CBP80 and CstF1 for binding to PARN under different cellular conditions could have a role in regulating the PARN activity and, therefore, the mRNA levels of different genes.

DISCUSSION

During the DNA repair process, control of gene expression either by transcription or by RNA processing is important to allow the access of the repair enzymes and to prevent the formation of deleterious proteins. Following UV irradiation, the cellular levels of mRNA are transiently decreased (Hanawalt, 1994; Ljungman, 1999). The cellular mechanisms involved in this response are unknown but imply a functional
interaction of the DNA repair, transcription, and RNA processing machineries. Supporting this idea, it has been described that polyadenylation is transiently inhibited upon UV treatment (Kleiman and Manley, 2001; Kleiman et al., 2005; Mirkin et al., 2008). As mRNA poly(A) tails are important for regulation of mRNA stability (Mandel et al., 2008; Shatkin and Manley, 2000; Zhao et al., 1999); changes in the polyadenylation levels either by activation/inhibition of the reaction or by controlling the balance between polyadenylation and deadenylation could account for some of the changes in mRNA levels after UV treatment.

Previous work from Dr. Kleiman’s lab indicated that the polyadenylation factor CstF1 plays a coordinating role in the nuclear response to UV-induced DNA damage through its interaction with different factors in different cellular environments (Kleiman and Manley, 1999, 2001; Kleiman et al., 2005; Mirkin et al., 2008). Cevher and colleagues (Cevher et al., 2010) discovered that CstF1 interacts with the deadenylation factor PARN and that this interaction plays a role in inhibition of 3’ cleavage of the polyadenylation reaction and activation of deadenylation upon DNA damage treatment. They also found that BARD1 is not only involved in the UV-induced inhibition of 3’ cleavage (Kleiman and Manley, 1999, 2001) but also in the UV-induced activation of deadenylation in the presence of CstF1. Furthermore, Cevher and colleagues (Cevher et al., 2010) found that the previously identified nuclear CBP80/PARN deadenylation inhibitory complex decreased significantly in abundance upon DNA-damaging conditions, whereas the complex containing CstF1/PARN increased and that CstF1 competed with CBP80 on binding to the same region of PARN. In fact, addition of CstF1 and BARD1 to in vitro deadenylation reactions reverted the CBP80 inhibition effect on
PARN activity. As part of those studies, I determined that PARN knockdown had an effect on the stability and polyadenylation of different genes in different cellular conditions (Figure 10). Taken together, these results suggest that an interplay between these factors might control gene expression under DNA-damaging conditions by regulating polyadenylation/deadenylation.

Based on the results presented in the work of Cevher and colleagues (Cevher et al., 2010), the following regulatory scenario was proposed (Figure 11). In the absence of DNA damage treatment, CBP80 binds to the C-terminal domain of nuclear PARN and inhibits its hydrolytic activity to ensure that PARN does not degrade the mRNA (Balatsos et al. 2006). In this situation, CBC is also known to enhance polyadenylation of pre-mRNAs by increasing the stability of the RNA/CstF complex (Flaherty et al., 1997). As a result of these functional interactions, polyadenylation takes place and normal levels of total mRNA are observed. After DNA damage the BRCA1/BARD1-containing complex is recruited to sites of DNA repair to inhibit mRNA processing by RNAP II ubiquitination followed by degradation of the large subunit of RNAP II, or by covalent modification of other element/s of the complex. This facilitates DNA repair and/or prevent polyadenylation of aborted nascent mRNAs. If the UV-induced inhibition of mRNA 3' cleavage is bypassed, the CstF/PARN interaction may provide a fall-back mechanism to ensure that erroneously polyadenylated mRNAs are eliminated by the activation of deadenylation. In this situation it has been proposed that CBP80 dissociates from PARN, allowing PARN to interact with the CstF1/BARD1 complex. This reorganization will result in an activation of deadenylation and contribute to the
Figure 11. A model of poly(A) tail dynamics after DNA damage. In the absence of DNA damage treatment, CBP80 binds to nuclear PARN, inhibiting its deadenylase activity. After exposure to UV treatment, the CBP80 protein dissociates from PARN, allowing binding of PARN to the CstF1/BARD1 complex. As a result of these functional interactions, polyadenylation is inhibited and a 5’ cap-dependent deadenylation decay pathway is activated, generating a decrease in the levels of total mRNA. Taken from Cevher et al. (2010)

inhibition of polyadenylation. The final outcome will therefore be that polyadenylation is inhibited and deadenylation activated, contributing to the observed decrease in the levels of total mRNA under DNA-damaging conditions. A similar mechanism for control of gene expression has been described by others. For instance, Kim and Richter (Kim and Richter, 2006) have shown that the length of the cytoplasmic poly(A) tail is regulated by polyadenylation/deadenylation under different cellular conditions by the direct interaction of PARN with the polyadenylation factor CPEB. Moreover, Mauxion et al. (Mauxion et al., 2008) have shown that the tumor suppressor BTG2 is a general activator of the
cytoplasmic deadenylyases Pop2/Caf1 and Ccr4, and that overexpression of BTG2 accelerates the deadenylation of several reporters and endogenous transcripts, such as GAPDH and β-actin.

It has been shown that PARN co-purifies with essential nonsense-mediated decay factors (NMD), and that siRNA mediated down-regulation of PARN abrogates NMD (Lejeune et al., 2003). Although those reports focused on cytoplasmic PARN, it is possible that the activation of deadenylation by the CstF/PARN/BARD1 complex formation in the nucleus might signal the degradation of those erroneously polyadenylated prematurely terminated mRNAs, providing a mechanism of nuclear mRNA decay. Consistent with this previous work from Dr. Kleiman’s lab showed that prematurely terminated polyadenylated mRNA transcripts can be detected in vivo following DNA damage, especially under conditions when the CstF/BARD1/BRCA1 checkpoint is not activated (Mirkin et al., 2008).

Control of polyadenylation/deadenylation in the nucleus could represent a mechanism to regulate gene expression, which could be important to allow a rapid response during development or after stress treatment. For example, UV-treatment induces a decrease in the cellular levels of total mRNA to avoid the expression of deleterious proteins that may be harmful to the cell (Ljungman, 1999). UV-treatment also induces stabilization of ARE-containing mRNAs; such as c-fos, kin17, c-jun, IκB and c-myc (Blattner et al., 2000), to induce the expression of some proteins involved in DNA repair and cell cycle. My results indicate that PARN can decrease the stability of housekeeping genes upon DNA-damaging conditions and of ARE-containing genes upon non-stress conditions (Figure 10). Consistent with my results, it has been shown that
ARE-dependent deadenylation plays an important role in the mRNA decay of several oncogenes involved in regulation of cell growth and differentiation (Blattner et al., 2000; Lai et al., 2003; Moraes et al., 2006). My results indicate that gene expression of different genes, such as housekeeping and ARE-containing genes, might be regulated in the nucleus by the functional interaction between CstF/BARD1, CBP80 and PARN under different cellular conditions.

As the tumor suppressor BARD1 is involved in this response, it is possible that malignant cells display altered levels of polyadenylation of certain mRNAs. Supporting this idea, enhanced polyadenylation has been detected in certain tumor cells (Scorilas, 2002), polyadenylation is inactivated in M phase (Colgan et al., 1996; Colgan et al., 1998), expression levels of poly(A) polymerase can interfere with cell growth (Zhao and Manley, 1998). Moreover, antiproliferative transcription factors, such as BTG2 and TOB, have been shown to enhance deadenylation, and the subsequent mRNA decay (Ezzeddine et al., 2007; Mauxion et al., 2008). Furthermore, the expression levels of certain ARE-containing genes, such as c-jun and c-fos, increase significantly in cancer cells (Andersen et al., 2002; Milde-Langosch, 2005; Zajchowski et al., 2001). Interestingly, microRNAs, which have been either directly involved in human cancers or described as oncogenes or tumor suppressors, can direct rapid deadenylation of mRNAs and subsequent decay (Wu et al., 2006; Zhang et al., 2007a).

Taken together, it can be concluded that regulation of the levels of 3’ end polyadenylation is an important event in controlling cell growth and in the response to certain stresses, such as UV treatment, and that polyadenylation/deadenylation process might represent a new mode of global regulation of gene expression.
CHAPTER III

IDENTIFICATION OF GENES THAT ARE REGULATED BY PARN DEADENYLATION IN DIFFERENT CELLULAR CONDITIONS
INTRODUCTION

Almost all eukaryotic mRNA precursors, with the exception of histones, undergo a cotranscriptional cleavage followed by polyadenylation at the 3’ end. This first round of polyadenylation is considered a default modification for most mRNAs and confers stability. In contrast, activation of deadenylation alters the length of poly(A) tails, affecting mRNA stability, transport, or translation initiation, and hence gene expression (Zhang et al., 2010). Thus, mechanisms controlling deadenylation are highly regulated and play key roles in cellular responses, such as mRNA surveillance, DNA damage response (DDR), and tumor progression, as well as cell development and differentiation (Cevher and Kleiman, 2010; Cevher et al., 2010; Ji et al., 2009; Mayr and Bartel, 2009; Singh et al., 2009). Poly(A)-specific ribonuclease (PARN) deadenylase is one of the major poly(A) specific 3’ exoribonucleases identified in mammalian cells (Copeland and Wormington, 2001) that plays a role in DDR (Cevher et al., 2010). PARN can interact with the cleavage stimulation factor 1 (CstF1), and the CstF1/PARN complex formation has a role in the regulation of gene expression by inhibition of mRNA 3’ cleavage and activation of deadenylation upon DNA damage (Cevher et al., 2010). PARN, an mRNA decay enzyme, has been studied extensively in vitro at the biochemical levels but very little is known of its biological targets and its role in different cellular conditions. Recently, it has been shown that PARN regulates the expression of genes involved in mRNA metabolism, transcription, and cell motility in mouse myoblasts (Lee et al., 2012). My previous studies indicate that the CstF/PARN complex can decrease the mRNA levels of housekeeping genes under DNA-damaging conditions and of genes involved in
cell growth and differentiation under non-stress conditions (Figure 10; (Cevher et al., 2010).

Deadenylation of mRNA is regulated by microRNAs (miRNAs), adenylate-uridylate–rich element (ARE) binding proteins, polyadenylation factors, and RNA binding (RB) factors that recognize cis-acting sequences in the target. About 12% of mammalian mRNAs bear important regulatory signal AREs in their 3’ untranslated regions (UTRs), which have been shown to play significant roles in mRNA stability regulation (Guhaniyogi and Brewer, 2001). PARN has been shown to be involved in ARE-mediated deadenylation and to promote tristetraprolin (TTP)-directed deadenylation in vitro (Lin et al., 2007). KH-type splicing regulatory protein recruits PARN to ARE-containing mRNAs to initiate the poly(A) tail shortening that is followed by exosome-mediated degradation (Gherzi et al., 2004). Interestingly, tumor suppressors, such as breast cancer type 1 susceptibility protein (BRCA1) and BRCA1-associated RING domain protein (BARD1), associated to the polyadenylation factor CstF1 have been shown to regulate deadenylation by functional interactions with PARN deadenylase (Cevher et al., 2010).

Recently, work form Dr. Kleiman’s lab showed that the polyadenylation factor CstF1 can also form a complex with another tumor suppressor and DNA repair factor with compromised expression on most cancers, p53, resulting in the inhibition of mRNA 3’ cleavage (Nazeer et al., 2011). Downstream signaling in the p53 pathway includes several cellular responses. The expression of a large number of genes involved in DNA repair, cell cycle arrest, and/or apoptosis is regulated by transactivating properties of p53. This occurs via specific DNA binding of the p53 protein to a p53 response element that is
found either in promoters or introns of target genes (Tokino and Nakamura, 2000). Transactivation-independent functions of p53 have also been described (Takwi and Li, 2009). For example, certain miRNAs are regulated by p53, and these miRNAs cause dramatic changes in gene expression, offering an indirect p53-mediated control of gene expression at the posttranscriptional level (Chang et al., 2007).

Extending those studies, work from Dr. Kleiman’s lab showed that p53 regulates not only mRNA 3′ cleavage (Nazeer et al., 2011) but also PARN-dependent deadenylation in different cellular conditions (Devany et al., 2013). As part of these studies, I also identified the mRNA targets of PARN in non-stress conditions. Together these results provide evidence of a unique feedback loop between p53 and PARN, in which PARN deadenylase keeps p53 levels low in non-stress conditions by destabilizing p53 mRNA through its 3′UTR, and the UV-induced increase in p53 activates PARN, representing a mechanism of gene expression regulation in a transactivation independent manner.

RESULTS

As PARN is involved in ARE-mediated deadenylation (Lin et al., 2007), promotes TTP-directed deadenylation (Lai et al., 2003), and decreases mRNA levels of ARE-containing genes under non-stress conditions (Figure 10; Cevher et al., 2010), I decided to extend these studies and determine which mRNAs might be regulated by PARN using microarray assays. These assays were performed using RNA samples from HeLa cells treated with control or PARN siRNAs under non-stress conditions. The RNA samples were analyzed for gene expression using Affymetrix GeneChip Human Gene 1.0
ST array, which contains up to 290,000 different transcripts, by the Yale Center for Genome Analysis. In collaboration with Dr. Bin Tian, UMDNJ-New Jersey Medical School, we performed pathway analysis of regulated genes using the Ingenuity Systems applications. Pathway analysis results indicated that the p53 signaling pathway is the most significantly affected by PARN knockdown in non-stress conditions (Figure 12). In

**Figure 12. PARN deadenylase significantly affects the cellular expression of genes of the p53 pathway under non-stress conditions.** Pathway analysis of significantly regulated genes by PARN. Nuclear RNA samples isolated from HeLa cells, treated with siRNAs targeting PARN or control, were analyzed using the Human Gene 1.0 ST GeneChip (Affymetrix) array. Significant genes were selected by t test (P value < 0.05). Analysis of canonical pathways was conducted by using data from Ingenuity Systems (www.ingenuity.com). The bar graph shows significance of pathway for regulated genes. P values were calculated using the Fisher’s exact test, and the −log (P value) values are displayed. Only the top five pathways are shown. Data represent three independent experiments. Taken from Devany et al., (2013)
addition, the p53-related gene network was found to be the most significantly regulated by network analysis and transcription factor analysis (Devany et al.; 2013). Approximately 75% of genes from the p53 signaling pathway, including p53, were affected by PARN knockdown, indicating that PARN expression has a specific effect on the expression of genes associated with p53-mediated signaling in non-stress conditions. Interestingly, under those conditions 20% of PARN mRNA targets in the p53 signaling pathway showed both miRNA target site(s) and an ARE(s) in their 3’UTRs (Table 1), suggesting that PARN might play a role not only in the ARE-mediated but also in miRNA-mediated deadenylation.

I further confirmed the effect of PARN knockdown on the abundance of several transcripts in the p53 signaling pathway by quantitative (q)RT-PCR (Figure 13). Although there was a good correlation between the change observed in the microarray (Table 1) and that determined by qRT-PCR (Figure 13), the qRT-PCR showed changes of a greater magnitude than the array. These results support my previous study that showed that PARN can promote deadenylation and mRNA instability of FBJ osteosarcoma oncogene and myelocytomatosis oncogene (c-myc; Figure 10; (Cevher et al., 2010), keeping their expression levels low under non-stress conditions. Because PARN is a deadenylase, I expected an increase in the steady-state levels of its targets by PARN knockdown. However, my results show both up- and down-regulation of transcripts, suggesting complex effects of PARN knockdown on the expression of these mRNAs.
### Table 1. Analysis for the presence of AREs and miRNA targeting sites in the 3’UTRs of genes from the p53 pathway affected by PARN expression in non-stress conditions.

The expression of 141 genes from the p53 pathway was affected upon siRNA-mediated knockdown of PARN (Devany et al., 2013). 20% of these genes, including p53, showed both target sites. ARED 3.0 database and miRWalk were used for the search of ARE-containing mRNAs and miRNAs validated targets (Dweep et al., 2011), respectively.
Figure 13. The effect of PARN knockdown on the abundance of several transcripts in the p53 signaling pathway by quantitative (q) RT-PCR. qRT-PCR analyzing c-fos, c-myc, BCL-XL, HDAC1 and RB1CC1 mRNA levels using total RNAs samples isolated from cells treated with control/PARN siRNA. qRT-PCR determinations give results similar to microarrays with regard to change in mRNA abundance after PARN knockdown. Taken from Devany et al., (2013)

The microarray analysis showed a 1.25 fold increase in p53 mRNA level of PARN- to control- siRNA treated HeLa cells (Table 1). Importantly, consistent with the pathway analysis results, PARN knockdown resulted in a significant increase not only of p53 mRNA steady-state levels (Figure 14) but also of p53 protein levels (compare lanes 1 and 2 in Figure 14), reaching expression levels similar to that observed after UV treatment. After UV treatment, the changes in the levels of p53 mRNA and p53 protein were PARN-independent, indicating there is (are) other mechanism(s) involved in the regulation of p53 expression during DDR.
Figure 14. PARN deadenylase significantly affects the cellular expression of genes of the p53 pathway under non-stress conditions. (A) p53 mRNA and (B) protein levels are affected by PARN expression. qRT-PCR and Western blot analysis of p53 expression after UV treatment using RNA or protein samples, respectively, from cells treated with control/PARN siRNA. A representative Western blot from three independent assays is shown. Topo II was used as loading control. The basal level of the proteins was arbitrarily set at 1.0 in the first lane, and relative fold change of each protein level is shown below each lane. Taken from Devany et al., (2013)
To determine the effect of PARN expression on the stability of p53 mRNA, I compared mRNA decay rates of p53 transcript in cells treated with control- or PARN-siRNA. The half-life of the p53 transcript was analyzed by qRT-PCR of nuclear RNA samples taken at different time points from PARN/control siRNA- and actinomycin D (Act-D)-treated cells (Figure 15). Previous observations have shown a similar half-life for the p53 transcript (Mazan-Mamczarz et al., 2003). My results indicate that PARN knockdown stabilized the p53 transcript (Figure 15). Because PARN is a deadenylase, the stabilization of p53 mRNA by its knockdown might be due to changes in the poly(A) tail length. Importantly, as shown in Figure 16, siRNA-mediated knockdown of PARN elongated the poly(A) tail length of p53 mRNA (quantification is shown in Figure 16B). Together, these results indicate that the PARN deadenylase affects p53 expression by regulating poly(A) length and hence mRNA stability in non-stress conditions.
Figure 15. PARN regulates p53 mRNA half-life. mRNA decay rates for p53 and ACTIN, a non-PARN target gene, were determined by qRT-PCR at different time points following PARN/control siRNA- and Act-D treatment. The relative half-life of the p53 transcript was calculated from three independent samples. Errors represent the SD derived from three independent experiments. Western blot analysis of PARN expression after PARN/control siRNA and Act-D treatment is also shown. Taken from Devany et al., (2013)
Figure 16. PARN regulates p53 mRNA poly(A) tail length. (A) Nuclear RNA from PARN/control siRNA-treated cells was reverse-transcribed using an oligo(dT)-anchor primer and amplified using an oligonucleotide that hybridizes within the 3' UTR of p53 mRNA. The products were separated on a nondenaturing PAGE and detected by ethidium bromide staining. An RT-PCR product from a non-PARN target gene (ACTIN exon 3–4) was used as a loading control. A representative PAGE from three independent assays is shown. Molecular weight standard (MWS, 100-bp ladder from Promega) is also included. (B) Quantification of poly(A) tail length was done by obtaining the density profile of control and PARN KD lanes using ImageJ software. Taken from Devany et al., (2013)
DISCUSSION

The studies presented in this dissertation contributed to a study that provided evidence of a unique feedback loop between p53 and PARN deadenylase, in which PARN keeps p53 levels low in non-stress conditions by destabilizing p53 mRNA, and the UV-induced increase in p53 activates PARN deadenylase regulating gene expression during DDR in a transactivation-independent manner. Several lines of evidence support this model. First, the C-terminal domain of p53 can activate PARN-dependent deadenylation in vitro and p53 expression levels correlate with levels of mRNA deadenylation (Devany et al., 2013). Second, those studies show the direct interaction of the C-terminal domain of p53 with the C-terminal domain of PARN and the existence of protein complexes of these factors in cellular nuclear extracts (Devany et al., 2013). Third, PARN significantly affects the cellular expression of genes in the p53 pathway under non-stress conditions and the stability and poly(A) length of the p53 mRNA (Figures 12-13 and 15-16). Fourth, PARN knockdown and UV treatment induce a similar increase in p53 expression (Figure 14). Finally, PARN regulates p53 expression through the ARE sequence present in the 3′ UTR of p53 mRNA (Devany et al., 2013). Taken together, these results provide insights into p53 function and the mechanisms behind the regulation of mRNA 3′ end processing in different cellular conditions.

Together these studies show an alternative mechanism to regulate the expression levels of p53 based on the control of the steady-state levels of p53 mRNA by PARN deadenylase under non-stress conditions (Figure 17A). Supporting this, my previous studies indicate that PARN has a role in decreasing the levels of short lived mRNAs involved in the control of cell growth, DDR, and differentiation, keeping their expression
levels low under non-stress conditions (Figure 10, (Cevher et al., 2010). Under stress conditions, the induction of p53 expression is associated with a decrease in the levels of total poly(A) mRNA (Ljungman et al., 1999). Because mRNA poly(A) tails are important for the regulation of mRNA stability, it is possible that these changes of poly(A) mRNA levels might represent another mechanism of p53-mediated control of gene expression. In fact, studies from Dr. Kleiman lab indicated that an increase in the expression of p53 inhibits the mRNA 3′ cleavage step of polyadenylation (Nazeer et al., 2011) and induces PARN deadenylase activity (Devany et al., 2013), suggesting that the p53 associated to the PARN/CstF/BARD1 complex might regulate gene expression by controlling the steady-state levels of mRNAs (Figure 17B). Considering that the p53 pathway is tightly controlled in cells (reviewed in (Vousden, 2006), the p53-associated control of mRNA 3′ processing machinery could represent an indirect mechanism to repress target gene expression at the posttranscriptional level. The antiproliferative factor BTG2 represents another example of a general activator of mRNA deadenylation by its direct interaction with the Pop2–Caf1 and Ccr4 deadenylases (Mauxion et al., 2008). This model is consistent with the idea proposed by Singh et al. (Singh et al., 2009) that the interaction of the 3′ processing machinery and factors involved in the DDR/tumor suppression might result in cell-specific 3′ processing profiles.

Control of deadenylation could represent a mechanism to regulate gene expression in different cellular conditions, such as development, stress treatment, or different metabolic conditions. Supporting this idea, recently it has been shown that PARN regulates the expression of genes involved in mRNA metabolism, transcription, and cell motility in mouse myoblasts, resulting in PARN-dependent regulation of cell
motility and wound healing in those cells (Lee et al., 2012). Indeed, the Gene Ontology analysis presented in this dissertation also revealed significant down-regulation of genes involved in similar pathways (Table 1, (Devany et al., 2013), such as structure morphogenesis, cell adhesion, cell migration, and so on. Consistently, the microarray data presented here showed a decrease in the abundance of mRNA for several genes involved in cell motility, such as adenosine A2b receptor, ankyrin repeat containing domain 54, and

Figure 17. Model for the regulation of expression of genes in the p53 pathway by PARN deadenylase associated p53 in different cellular conditions. (A) PARN deadenylase decreases the stability of the p53 mRNA in non-stress conditions. The AREs in the 3’ UTR of the p53 mRNA have an important role in this regulatory process. (B) Under DNA damage conditions, p53 protein accumulates, allowing its association to and activation of PARN deadenylase resulting in the decrease levels of target mRNAs in the p53-dependent DDR pathway. Taken from Devany et al., (2013)
collagen alpha-2 chain in PARN knock-down cells. However, the p53 signaling pathway was not reported by Lee et al. (Lee et al., 2012), suggesting cell-specific functions of PARN. Like Lee et al. (Lee et al., 2012), I also observed a decrease in the steady-state levels of some transcripts by PARN knockdown. However, it is not clear whether this reflects the function of PARN per se or is the indirect consequence of PARN’s effect on genes involved in other mRNA metabolic pathways, such as transcription and RNA processing factors.

The p53 pathway is tightly controlled in cells (reviewed by (Vousden, 2006). The microarray data presented in this dissertation showed that the expression of 141 genes from the p53 pathway was affected upon siRNA-mediated knockdown of PARN. Interestingly, 20% of these PARN target genes, including p53, showed both miRNA targeting sites and ARE regulatory sequences in their 3’UTR (Table 1). This data suggests that PARN might mediate the deadenylation of its target mRNAs through both cis-acting elements present at 3’UTR. This is consistent with the model proposed in Jing et al. 2005 (Jing et al., 2005) and by myself (Zhang et al., 2010) that both miRNAs and AREs functionally overlap and contribute to the control of mRNA stability. The characterization of the regulatory elements in the 3’ UTR of p53 and the factors involved in this PARN-dependent regulatory pathway may allow us to better understand the mechanisms that control p53 expression and to find alternative strategies for treating tumorigenesis and metastasis in various cancers.
CHAPTER IV

MECHANISMS BEHIND PARN-MEDIATED REGULATION OF THE STEADY-STATE LEVELS OF GENES INVOLVED IN STRESS RESPONSE PATHWAYS UNDER NON-STRESS CONDITIONS
INTRODUCTION

Modulation of the length of poly(A) tail of an mRNA by the polyadenylation/deadenylation machinery is a widespread strategy used to control mRNA stability and gene expression in different cellular conditions. The dynamic nature of the mRNA 3’-end processing machinery allows the regulation of the steady-state levels of different mRNAs and has the potential to contribute to the cell’s rapid response to stress. Nuclear poly(A) specific ribonuclease (PARN), a poly(A) specific 3’ exoribonuclease, has been shown to play a role in DDR. The association of PARN with the cleavage stimulation factor 1 (CstF1) inhibits mRNA 3’ cleavage and activates deadenylation upon UV-induced DNA damage (Cevher et al., 2010). Besides, PARN is also activated by tumor suppressors and DNA repair factors with compromised expression on most cancers, such as BARD1/BRCA1 (Cevher et al., 2010) and p53 (Devany et al., 2013). Interestingly, PARN regulates the stability of mRNAs of genes involved in DDR, such as c-myc, c-fos, c-jun, and transcripts in the p53 and BARD1/BRCA1 pathways, keeping their levels low under non-stress conditions (Cevher et al., 2010; Devany et al., 2013; Moraes et al., 2006).

Deadenylation, and consequently mRNA stability, is under the control of cis-acting regulatory elements present in the 3’ untranslated region (3’UTRs) of eukaryotic mRNAs, such as alternative polyadenylation (APA) signals, AU-rich elements (AREs) and microRNA (miRNA) targeting sites. The use of different APA signals generates a diversity of mRNA isoforms that carry different arrangements of AREs and miRNA target sites and exhibit different stabilities (Figure 5). Both sequence and location of these elements are between these cis-acting elements and the 3’ processing machinery is
highlighted in cancer cells (Mayr and Bartel, 2009; Singh et al., 2009) and during cell differentiation (Ji et al., 2009; Sandberg et al., 2008; Zlotorynski and Agami, 2008), where changes in the length of the 3’UTR of different mRNAs affect cell expression patterns. These sequence elements recruit trans-acting factors that regulate those processes and affect gene expression, such as miRNA-induced silencing complex (miRISC), ARE-binding proteins (ARE-BPs), polyadenylation factors, and RNA binding (RB) factors. Many studies support the idea that miRNAs destabilize mRNA through deadenylation pathways, while AREs function in mRNA stability by either preventing mRNA from degradation by exosome or by recruiting the exosome to decrease the mRNA stability (Zhang et al., 2010).

ARE sequences are frequently present in genes that encode tightly regulated proteins involved in cell growth regulation, cell differentiation and responses to external stimuli. The destabilizing functions of AREs are important because in their absence proto-oncogenes, such as c-fos, c-myc, c-jun, might become oncogenes (Schiavi et al., 1992). Other mRNAs, such as IL-3, need AREs in order to inhibit the growth of autocrine tumor mast cells by an immunosuppressant cyclosporin A (Nair et al., 1994). A number of trans-acting factors, known as ARE-BPs, regulate ARE-mediated decay by either inhibiting or activating deadenylation, and subsequently change the stability of ARE-containing mRNAs (Barreau et al., 2005). ARE-BPs regulate ARE-mediated decay of mRNAs by recruiting or blocking the recruitment of the deadenylases to the target mRNAs in different cellular conditions (Figure 6). PARN has been shown to be involved in ARE-mediated deadenylation and to promote tristetraprolin (TTP)-directed deadenylation in vitro (Lin et al., 2007). KH-type splicing regulatory protein (KSRP)
recruits PARN to ARE-containing mRNAs to initiate the poly(A) tail shortening that is followed by exosome-mediated degradation (Gherzi et al., 2004).

miRNAs comprise a large family of small, non-coding single-stranded RNAs, which are predicted to mediate more than 60% of all protein-coding genes in mammalian cells (Friedman et al., 2009). From protozoans to plants to mammals, miRNAs play key roles in a broad range of biological processes, in the form of ribonucleoprotein complexes, known as miRISCs, which deliver miRNAs to their mRNA targets. It is believed that miRNA function in gene expression regulation mostly through post-transcriptional events, either through translation regulation and/or through deadenylation activation, which leads to mRNA degradation, and consequently the modulation of gene expression (Figure 8). It is conventionally accepted that miRNAs function as a negative regulator of mRNA expression; however, under certain conditions, such as quiescence or in oocytes, miRNA-mediated upregulation of target mRNAs has also been demonstrated (Fehr et al., 2012; Truesdell et al., 2012). Interestingly, miRNA-expression profiles change during DDR (Pothof et al., 2009), suggesting a role of miRNA-mediated pathway in controlling gene expression during this response.

Briefly, miRISCs are assembled in the cytoplasm where one strand of the mature miRNA duplex is incorporated along with Argonaute (Ago) proteins, which represent the catalytic activity, and then the miRISC is directed to its mRNA targets 3’UTR through interactions with sites of imperfect complementarity (Bartel, 2009; Bushati and Cohen, 2007; Filipowicz et al., 2008; Friedman et al., 2009; Ghildiyal and Zamore, 2009; Hutvagner and Zamore, 2002; Liu et al., 2004; Martinez et al., 2002; Meister et al., 2004). Besides 3’UTRs, miRNAs can also modulate cellular gene expression by targeting
the 5’UTRs, coding regions promoters, and gene termini (Huang et al., 2012; Kim et al., 2008; Lytle et al., 2007; Place et al., 2008; Tay et al., 2008; Younger and Corey, 2011a, b). Additional protein components are critical for miRNA-mediated deadenylation. For example, Ago recruits the GW182 factor (TNRC6 in humans, (Lazzaretti et al., 2009; Takimoto et al., 2009; Zipprich et al., 2009), and cytoplasmic poly(A) binding protein 1 (PABPC1), both of which contribute to miRNA-mediated poly(A) removal (Fabian et al., 2009; Jinek et al., 2010; Zekri et al., 2009). One of the best studied deadenylases involved in miRISCs is the CAF1/CCR4/NOT1 complex (Zekri et al., 2009). Either the knockdown of CCR4 or NOT1 (Behm-Ansmant et al., 2006; Fabian et al., 2009) or the overexpression of CCR4 or CAF1 mutants significantly reduce miRNA-mediated deadenylation and mRNA decay (Piao et al., 2010). Pan2-Pan3 deadenylase has also been described to promote miRNA-mediated deadenylation and then trigger mRNA decay (Chen et al., 2009). However, the functional interaction of miRISC and PARN deadenylase has not been elucidated.

The role of miRNA-mediated deadenylation in different biological processes has been studied in different systems. For example, it has been shown that let-7-associated miRISCs directly activate deadenylation of target mRNAs in a cell-free system (Wakiyama et al., 2007), and that the overexpression of let-7 miRNAs decreases the mRNA levels of oncogenes, such as c-myc and RAS (Johnson et al., 2005; Sampson et al., 2007). These results indicate that a decrease in let-7 miRNA levels might lead to tumorigenicity in lung and colon cancers (Akao et al., 2006; Johnson et al., 2005)). The processing of let-7 precursors is blocked by lin-28B, a putative RNA-binding protein highly expressed in hepatocellular carcinoma (Wang et al., 2010). The overexpression of
lin-28B enhances tumorigenecity by increasing the expression of the oncogenic let-7 targets (Maeda et al., 2010). miR-125b has also been described to participate in miRNA-mediated deadenylation and in the reduction of cellular abundance of targeting mRNAs (Wu and Belasco, 2005). Interestingly, the overexpression of miR-125b correlates with the downregulation of lin-28 (Eda et al., 2009). These studies indicate that miRNAs might play crucial roles in multiple oncogenic pathways. In fact, it has been suggested that miRNAs might function as oncogenes and promote cancer development by negatively regulating tumor suppressor genes and/or genes that control cell differentiation of apoptosis (Zhang et al., 2007a; Zhang et al., 2007b).

While most studies traditionally have focused on cytoplasmic miRNA-mediated pathways, miRNA’s nuclear functions have begun to emerge in recent years (Cernilogar et al., 2011; Robb et al., 2005; Tan et al., 2009). Subcellular localization studies in mammalian cells have shown that elements from the miRISC, such as Ago-1 and Ago-2, are localized in the nucleus (Ahlenstiel et al., 2012; Ohrt et al., 2008; Tan et al., 2009). Nuclear-cytoplasmic shuttling proteins, such as TNRC6A and CRM1, navigate loaded miRISC into the nucleus, leading miRNA-mediated gene silencing (Nishi et al., 2013). It has also been shown that elements from the miRISC play a role in transcriptional regulation (Cernilogar et al., 2011), in alternative splicing (Liu et al., 2012), and in epigenetic regulation and chromatin organization (Pushpavalli et al., 2012). These findings reinforce the functional RISC components activity in the nucleus, suggesting miRNA pathways can be adapted to function in the mammalian nucleus. Deep sequencing analysis has shown that a subset of miRNAs is predominantly localized in the nucleus (Liao et al., 2010). In fact, nuclear miRISCs are also able to specifically cleave
their target mRNAs with high efficiency, resulting in miRNA-mediated gene silencing in the nucleus (Nishi et al., 2013). Interestingly, nuclear miRISCs are loaded in the cytoplasm and imported into the nucleus, and their nuclear accumulation depends on the presence of RNA targets (Ohrt et al., 2008). However, despite the significant progress made in documenting nuclear miRISC, the mechanism(s) underlying miRNA-mediated regulation of gene expression in the nucleus (is) are not clear and the participating nuclear deadenylase and other factors remain(s) to be elucidated.

Interestingly, some of the seed signals recognized by miRNA overlap with AREs in the 3′UTR (Figure 9). Although the exact contribution of miRNAs, miRISC, AREs and ARE-BPs to mRNA decay has not been elucidated yet, recent studies have described a functional overlap between ARE- and microRNA-mediated mRNA turnover pathways. It has been described that miRNAs can functionally interact with ARE-BPs, and that Dicer and Ago are required for ARE-mediated decay (Jing et al., 2005). Moreover, it has been shown that the ARE-BP HuR can bind AREs present in c-myc 3′UTR at a site proximal to that recognized by let-7 miRNA. HuR appeared to facilitate the targeting of let-7-loaded miRISC to an adjacent region of HuR binding site, and to mediate the reduction of c-Myc mRNA levels (Kim et al., 2009). Another example is the cooperation of ARE-BP TTP and miR-16 in targeting tumor necrosis factor-α mRNA for ARE-mediated mRNA degradation (Jing et al., 2005). TTP does not bind directly to miR-16 but it forms a complex with miRISC, and that complex recruits the deadenylase and the exosome for mRNA degradation. HuR can also relieve CAT-1 mRNA from miR-122 repression upon stress in human liver cells (Bhattacharyya et al., 2006).
As mentioned above, my results indicate that PARN regulates the stability of the p53 transcript keeping its levels low under non-stress conditions (Cevher et al., 2010; Devany et al., 2013; Moraes et al., 2006). The tumor suppressor p53 is a nuclear phosphoprotein that acts as a transcription factor in response to several stress stimuli. Downstream signaling in the p53 pathway includes numerous cellular responses. The expression of a large number of genes involved in DNA repair, control of cell cycle and apoptosis, differentiation, senescence and cellular homeostasis are regulated by transactivating properties of p53 (Lane and Levine, 2010; Reinhardt and Schumacher, 2012; Vousden and Prives, 2009; Wiesmuller, 2001). This occurs via specific DNA binding of the p53 protein to a p53 response element that is found either in promoters or introns of target genes (Tokino and Nakamura, 2000). The p53 pathway can be also regulated by signaling downstream from p53 (e.g., MDM2, MDM4, INK4/ARF), p53 isoforms (e.g., p63, p73), and microRNAs (e.g., miR-34). Transactivation-independent functions of p53 have also been described (Takwi and Li, 2009). For example, certain miRNAs are regulated by p53, and these miRNAs cause dramatic changes in gene expression, offering an indirect p53-mediated control of gene expression at the posttranscriptional level (Chang et al., 2007). Although many reports have been published about control of p53 protein expression and its effect on downstream pathways, very little is known of the mechanisms behind the control of p53 mRNA steady-state levels in different conditions (Vilborg et al., 2010).

In this study, I found that PARN deadenylase regulates the mRNA stability of one of its targets, the p53 mRNA (Devany et al., 2013), via a nuclear miRNA-mediated pathway. I found that Ago-2 activates PARN deadenylase activity by directly interacting
with the N-terminal domain of PARN and forming a complex in the nucleus. Interestingly, the binding of PARN to the p53 mRNA 3’UTR depends on cis-acting signals present in this region, such as AREs and miRNAs target sites, and Ago-2 expression. I showed that the miR-125b-loaded miRISC recruits PARN to the target p53 mRNA leading to a change in p53 mRNA steady-state level. These results reveal a novel regulatory pathway wherein PARN deadenylase plays a role in not only ARE- but also miRNA-mediated regulation of mRNA stability and, consequently, expression level. This regulatory pathway indicates a novel nuclear function of miRISC in mammalian cells as well as provides new insights in the p53 pathway.

RESULTS

PARN regulates p53 expression through its association with p53 3’UTR.

Previously, I have shown that PARN plays a role regulating p53 mRNA steady-state level through destabilizing p53 transcript (Figures 14-16 and Table 1, (Devany et al., 2013). In this part of study, I investigated the mechanisms underlying regulation of p53 mRNA stability by PARN deadenylase. First I tested if PARN could associate with p53 mRNA under non-stress conditions. RNA immunoprecipitation (RIP) assays using antibodies against PARN showed that p53 mRNA can form a complex with PARN in samples from cross-linked RKO cells (Figure 18), indicating that PARN can regulate p53 mRNA stability by, most probably, an indirect association to the 3’ UTR. RIP assays also showed that PARN can form a complex with c-myc RNA (Figure 18), which is another target of PARN deadenylase (Cevher et al., 2010).
As most of the regulatory elements involved in PARN-mediated regulation of mRNA stability are located in the 3’ UTR of the genes, I decided to determine whether the PARN-induced decrease of p53 mRNA levels under non-stress conditions is through this region of p53. Firefly luciferase plasmids were constructed in a way that luciferase gene is under the control of either the wild type p53 3′ UTR or the vector 3’UTR (Figure 19A). Recently, a G-quadruplex structure that protects the p53 mRNA from degradation

Figure 18. PARN can interact with p53 and c-myc under non-stress conditions. (A) RIP of endogenous genes from RKO cells. The extracts were immunoprecipitated with either anti-PARN or IgG antibodies. The endogenous nuclear RNA immunoprecipitated with the antibodies was quantified by qRT-PCR using primers specific for each gene. (B) The bar graph indicates the quantification of RNA immunoprecipitated with the indicated antibodies. The qRT-PCR values were calculated from three independent samples. Taken from Devany et al. (2013)
upon stress by binding to heterogeneous nuclear ribonucleoprotein H/F has been described (Decorsiere et al., 2011). This structure, which is located downstream of the 3’ cleavage site, was not included in this luciferase construct. To investigate the role of p53 expression in these luciferase assays, I used a group of isogenic cell lines that express different levels of p53: the colon cancer HCT116 and p53-null HCT116 cell lines and the colon carcinoma RKO and RKO-E6 (low p53 levels) cell lines. A significant increase in firefly/Renilla ratio for the construct with the p53 3′UTR relative to the control construct was detected in RKO/RKO-E6 and HCT116/HCT116 p53−/− cells treated with PARN siRNA (Figure 19B). RKO/RKO-E6 and HCT116/HCT116 p53−/− cells showed similar ratios for the expression of firefly/Renilla luciferase indicating that the PARN-mediated regulation of p53 expression is p53-independent.

Since PARN could associate with p53 transcript, and its role in the control of p53 expression is through p53 mRNA 3’UTR region, next I tested if PARN binds to p53 mRNA at its 3’UTR. Constructs carrying the p53 3′UTR (luc-p53) or not (luc-vector) were transfected into RKO cells. Then RIP assays using antibodies against PARN or IgG were performed followed by qRT-PCR with luciferase primers for quantification. The RIP assays indicate that PARN can form a complex with the luciferase mRNA carrying the 3’ UTR of p53 (Figure 19). Interestingly, the 3′ UTR of p53 mRNA also contains ARE that associates with ARE-binding proteins, such as wild-type p53-induced gene 1 (Vilborg et al., 2009) and HuR (Zou et al., 2006) and regulates p53 mRNA steady-state levels. Importantly, it has been shown in Dr. Kleiman’s lab that the replacement of the ARE sequence from the p53 3′ UTR (noARE construct) significantly increased the firefly/Renilla ratio compared with the WT p53 3′ UTR construct (Devany et al., 2013),
Figure 19. PARN regulates p53 expression through ARE sequence present in the 3′ UTR of p53 mRNA. (A) Diagram of firefly luciferase reporter constructs with the vector or 3′ UTR sequence from the p53 gene. Polyadenylation signals (PAS) are indicated. (B) Constructs carrying the p53 3′ UTR (p53) or not (vector) were transfected in cells treated with PARN or control siRNAs. The ratios of the firefly/Renilla values for the p53 construct relative to the vector construct are shown. The firefly/Renilla values were calculated from three independent samples. Errors represent the SD derived from three independent experiments. Taken from Devany et al., (2013)

showing that the AREs can decrease mRNA stability and hence expression of the luciferase-p53 3′ UTR construct. Interestingly, those studies showed that the siRNA mediated knockdown of PARN only increases the expression ratio of firefly/Renilla
luciferase from the constructs carrying the AREs but not from the constructs without the AREs, indicating that the AREs in the p53 3’ UTR are necessary for PARN-mediated regulation of p53 expression. Supporting this idea, RIP assays presented in this dissertation indicate that PARN can form a complex with the luciferase mRNA carrying the 3’ UTR of p53 and this is abolished when AREs are replaced by other sequences (Figure 20). Together, these results indicate that the ARE in the p53 3’UTR is important for the PARN-mediated regulation of p53 mRNA steady-state levels and that this regulation is p53-independent.

**Figure 20. PARN can interact with p53 3’UTR under non-stress conditions.**

RIP of cells transfected with luciferase constructs carrying either the p53 3’UTR (luc-p53) or not (luc-vector). A construct with the ARE-replaced in the p53 3’ UTR (noARE) was also analyzed. The extracts were immunoprecipitated with either anti-PARN or IgG antibodies. Nuclear RNA immunoprecipitated with the antibodies was quantified by qRT-PCR using primers specific for luciferase gene and actin gene.
The miRISC component Ago-2 binds to PARN deadenylase in the nucleus.

PARN regulates the stability of ARE-containing mRNAs keeping their levels low under non-stress conditions (Cevher et al., 2010; Devany et al., 2013; Moraes et al., 2006). Interestingly, 20% of PARN mRNA targets in the p53 signaling pathway showed not only AREs but also miRNA target sites in their 3’UTRs (Chapter III, Table 1). RNA-binding proteins, such as ARE-binding proteins and elements of the miRISC, are necessary to recruit an active deadenylase to its mRNA substrates leading to mRNA deadenylation and degradation. Although the miRISC has been shown to functionally interact with several deadenylases, its functional interaction with PARN has not been elucidated. To test the possibility that elements of the miRISC might also be involved in PARN-dependent deadenylation, I analyzed the potential interaction of PARN and Ago-2. First, the localization of Ago-2 and PARN in cellular fractions from HCT116 cells was analyzed (Figure 21). Both Ago-2 and PARN were present in the nuclear fraction. Topoisomerase II (Topo II) and actin were used as subcellular fractionation controls. As nuclear deadenylation is activated upon UV-induced DNA damage (Cevher et al., 2010), I have included stress conditions in this study. Interestingly, Ago-2 shuttled between the nuclear and cytoplasmic compartment in response to UV treatment, indicating a dynamic intracellular distribution of Ago-2 in response to DNA damage and suggesting a possible role of miRNA pathway in control of gene expression during DDR. The previously described UV-induced increase in nuclear PARN (Cevher et al., 2010) was also observed.

To test whether PARN physically associates with Ago-2 I performed co-immunoprecipitation (co-IP, Figure 22) and pull-down (Figure 23) assays. The co-IPs indicate that PARN can form (a) protein complex(es) with Ago-2 in nuclear extracts (NE)
Figure 21. Both PARN and the miRISC component Ago-2 are present in the nuclear fraction. Both PARN and Ago-2 are present in the nuclear fraction. Ago-2 shuttles between the nuclear and cytoplasmic compartment in response to UV irradiation. Equivalent amounts of cytoplasmic and nuclear fractions of HCT116 cells were subjected to SDS-PAGE and proteins were detected by immunoblotting using antibodies against Ago-2 and PARN. Both nuclear (nPARN) and cytoplasmic (cPARN) isoforms of PARN are shown. Topo II and actin are used as subcellular fractionation control. The basal level of the proteins was arbitrarily set at 1.0 in the first lane, and relative fold change of each protein level is shown below each lane. Quantifications were done with Image J software (http://rsb.info.nih.gov/ij/).

from HCT116 cells. Interestingly, the complex was detected in both non-stress conditions and after UV treatment. As samples were treated with RNase A, the observed interactions were probably not due to an RNA tethering effect. The results showed that PARN can interact directly with Ago-2 and that the N-terminal domain, which contains the nuclease activity and is responsible for cap-binding properties of PARN (Martinez et al., 2001), is
important for this interaction (Figure 23). Together these results indicate that PARN can interact with the miRISC component Ago-2 to form (a) complex(es) independently of miRNA and stress conditions.

**Figure 22.** The miRISC component Ago-2 interacts with PARN deadenylase to form a protein complex in the nucleus. PARN and Ago-2 could form (a) complex(es) in NEs of HCT116 cells independently of UV treatment. The NEs were immunoprecipitated with anti-PARN (Left) and anti-Ago-2 (Right). Equivalent amounts of the pellets (IP) and supernatants (SN) were resolved by SDS-PAGE and proteins were detected by immunoblotting using antibodies against PARN and Ago-2. Antibody against Topo II was used as a control.
Figure 23. The miRISC component Ago-2 interacts directly with PARN deacylase in vitro. Ago-2 interacts directly with the N-terminal domain of PARN. Immobilized His-PARN on nickel beads was incubated with GST-Ago-2 (left panel). Bound proteins were eluted, detected by Western blotting with antibodies against PARN or Ago-2. 5% of His-PARN and GST-Ago-2 used in the reaction are shown as input. Immobilized GST-Ago-2 or GST on glutathione beads were incubated with full-length (FL), N-terminal domain (NTD) or C-terminal domain (CTD) of His-PARN (right panel). Bound proteins were detected by immunoblotting as before. 5% of His-PARN derivatives used in the reaction are shown as input.
Ago-2 has a role in the UV-induced activation of nuclear PARN deadenylase activity.

To test whether Ago-2 has a direct influence on PARN deadenylase activity I performed in vitro reconstituted deadenylation assays, where I monitored the deadenylation of a radiolabeled L3(A30) RNA substrate in a reaction using limiting amounts of His-PARN and increasing amounts of GST-Ago-2. Addition of GST-Ago-2 enhanced the deadenylation activity of PARN up to 2.3 folds (Figure 24), indicating that Ago-2 is an activator of PARN activity in a cell-free system and in the absence of miRNA. Importantly, deadenylation activity was not detected when using GST-Ago-2 alone. Extending these results, I investigated the role of Ago-2 in nuclear deadenylation by performing siRNA mediated knockdown of Ago-2 in HCT116 cell line and then analyzing the UV-induced activation of deadenylation. As described in previous studies (Cevher and Kleiman, 2010; Devany et al., 2013), Figure 25 shows that deadenylation activity in NEs of HCT116 cells treated with control siRNA increased significantly after UV treatment. Interestingly, deadenylation, especially after UV-treatment, was completely abolished in samples from cells treated with siRNA targeting Ago-2. These results indicate that in the presence of Ago-2, which is involved in cytoplasmic deadenylation processes, PARN-mediated deadenylation is activated both in vitro and in samples from HCT116 cells. Together these results suggest that Ago-2/PARN complex formation activates PARN deadenylase and, therefore, might regulate gene expression.
Figure 24. Ago-2 enhances PARN-mediated deadenylation in vitro. Ago-2 can activate PARN-dependent deadenylation in vitro. Deadenylation assays were performed in the presence of radiolabeled capped L3(A30) RNA substrate as described (Cevher et al., 2010), using different concentrations of His-PARN and increasing amount of GST-Ago-2. Positions of the polyadenylated RNA L3(A30) and the L3 deadenylated product are indicated. Numbers beneath gel lane indicate relative deadenylation (RD), which was calculated as [L3 fragment/(L3(A30) + L3 fragment)] x 100. Quantifications were done with Image J software.
Figure 25. siRNA mediated knockdown of Ago-2 abolishes UV-induced activation of deadenylation. siRNA-mediated knockdown of Ago-2 abolishes UV-induced activation of deadenylation in HCT116 cells. The protein levels of Ago-2 and Topo II were analyzed by Western blotting after Ago-2/control siRNA treatment (Left). A representative deadenylation reaction from three independent assays is shown (Right). NEs from HCT116 cells treated with Ago-2/control siRNA and UV irradiation, and allowed to recover for 2 h were analyzed for radiolabeled L3(A30) deadenylation as described (Cevher et al., 2010). RNAs were extracted and deadenylation reactions were analyzed as in Figure 24.
Association of PARN to its target mRNAs requires Ago-2.

As Ago-2 is the core component of the miRISC complex, which delivers miRNAs and deadenylases to their mRNA targets, and my results indicate that it binds directly to PARN enhancing its deadenylase activity (Figures 22-25), I wonder whether Ago-2 plays a role in PARN-mediated expression regulation of p53, one of its targets, under non-stress conditions. To test these NEs from HCT116 cells treated with control, PARN, Ago-2 or both PARN/Ago-2 siRNAs were analyzed by Western blot for p53 expression. Interestingly, p53 protein levels increased in samples from cells treated with either Ago-2 or PARN siRNA (Figure 26), suggesting that both factors might be involved in the same

![Figure 26. siRNA-mediated knockdown of Ago-2 and PARN have similar effects on p53 expression levels.](image)

Figure 26. siRNA-mediated knockdown of Ago-2 and PARN have similar effects on p53 expression levels. NEs from HCT116 cells treated with control, PARN, Ago-2, or both PARN/Ago-2 siRNAs were analyzed by Western blot for p53 expression. Topo II was used as loading control (not shown). The p53 protein expression levels were calculated from three independent samples. The basal level of the proteins was arbitrarily set at 1.0 in the control siRNA-treated sample.
p53 regulatory pathway. In fact, the double knockdown of PARN and Ago-2 resulted in similar p53 protein levels to Ago-2 knockdown alone indicating that Ago-2 is necessary for PARN-mediated reduction of p53 protein levels.

Next, I tested whether Ago-2 could facilitate the recruitment of PARN to its mRNA targets. My previous studies indicated that PARN can regulate the steady-state levels of ARE-containing mRNAs, such as p53 and c-myc, by indirect association to their transcripts under non-stress conditions (Devany et al., 2013). To examine the possible role of Ago-2 in recruiting PARN deadenylase to its mRNA targets, RNA immunoprecipitation assays were performed using samples from HCT116 cells treated with either control or Ago-2 siRNA (Figure 27). Consistent with my previous results (Devany et al., 2013), using antibodies against PARN, the RIP assays showed that both p53 and c-myc mRNAs formed a complex with PARN in samples from control siRNA-treated cells (Figure 27). Interestingly, siRNA-mediated knockdown of Ago-2 reduced the interaction of PARN with both p53 mRNA and myc mRNAs. To confirm these results, one student at Dr. Kleiman’s lab, Emral Devany, performed RNA pull-down assays using an in vitro transcribed biotinylated RNA carrying the p53 3’UTR sequence and NEs from HCT116 cells treated with Ago-2 or control siRNAs (personal communication, not shown). Supporting my results, her studies indicate that RNA encompassing p53 3’UTR pulled-down PARN from samples of control siRNA-treated cells, and this RNA-PARN interaction significantly decreased when samples from Ago-2 siRNA-treated cells were used in the assay.
Figure 27. Ago-2 is required for the association of PARN to its target mRNAs under non-stress conditions. NEs from HCT116 cells treated with Ago-2/control siRNA were prepared after formaldehyde crosslinking. The extracts were immunoprecipitated with either antibodies against PARN or control IgG. The endogenous nuclear RNA immunoprecipitated was quantified by qRT-PCR using primers specific for p53 and myc gene. The qRT-PCR values were calculated from three independent samples.

To rule out the possibility that the effect of depletion of Ago-2 on reducing PARN association with p53 mRNA 3’UTR is due to the cell-wide response to low levels of Ago-2 expression, Emral Devany also performed RNA pull-down assays using an in vitro transcribed biotinylated RNA encompassing WT p53 3’UTR, NEs from HCT116 cells treated with Ago-2 siRNA and increasing amounts of recombinant Ago-2. Interestingly, increasing amounts of recombinant Ago-2 in the incubation system increased the amounts of PARN detected in the p53 3’UTR pull-down fraction (personal communication, not shown). Together, these results suggest
that Ago-2 might play a critical role in PARN-mediated regulation of gene expression by recruiting PARN to its target mRNAs.

PARN regulates p53 expression through not only ARE but also miRNA targeting site present in the 3’UTR of p53 mRNA.

It is known that PARN is involved in ARE-mediated deadenylation (Korner and Wahle, 1997; Lai et al., 2003; Lin et al., 2007). My studies have shown that Ago-2 activates PARN deadenylase through direct protein interaction (Figures 22-25) and participates in recruiting PARN to some of its mRNA targets (Figure 27), suggesting that PARN might play a role not only in ARE- but also miRNA-mediated deadenylation. Supporting this, others have shown the functional overlapping of ARE- and miRNA-mediated regulatory pathways (Bhattacharyya et al., 2006; Jing et al., 2005; Kim et al., 2009). Interestingly, p53 mRNA contains both miRNA targeting sites (miR-504, miR-125a/miR-125b, and miR25/miR30d) and AREs at its 3’UTR, and some of these signals overlapped as showed in Figure 28. To test whether both AREs and miRNA targeting sites are involved in the PARN-associated Ago-2-mediated regulation of p53 mRNA steady-state levels under non-stress conditions constructs were generated that have the luciferase gene under the control of WT p53 3’UTR or replacement mutants in AREs and/or miRNA targeting sequences that are in close proximity in the p53 3’UTR (Figure 29). More specifically, I focused my studies on miR-504 (Hu et al., 2010; Le et al., 2009)
Figure 28. Sequence of p53 mRNA 3’UTR. miR-binding sites and AU-rich elements (AREs) are shown. miR-125b binding site (red) and miR-504 binding site are adjacent to an ARE (blue).
and miR-125b (Le et al., 2009) that are located next to the ARE (Vilborg et al., 2009; Zou et al., 2006) in the p53 3’UTR that is important for PARN binding and PARN-mediated regulation of p53 mRNA steady-state levels (Figure 20; (Devany et al., 2013). Interestingly, the siRNA-mediated knockdown of PARN significantly increased the ratio of firefly/renilla luciferase activity from the constructs carrying the WT p53 3’UTR but the effect of PARN knockdown was completely abolished when the AREs (noARE), miRNA target site (nomiR) or both (noBOTH) signals are replaced by other sequences (Figure 30). Together, these results indicate that both regulatory signals at the p53 3’UTR are necessary for PARN-mediated regulation of p53 expression.

**Figure 29. Diagram of firefly luciferase reporter constructs with different 3’UTR sequences from the p53 gene.** Polyadenylation signals (PAS) are indicated.
Figure 30. Both miRNA targeting sites and AREs are critical for PARN-mediated regulation of p53 expression. Constructs carrying the p53 3’UTR (p53) or ARE (noARE), miR-125b targeting site (nomiR) or both signals (noBOTH) replaced p53 3’UTR were transfected into HCT116 cells. Luciferase assays were done in cells treated with control or PARN siRNA. The ratio of the firefly/Renilla values obtained for each construct in PARN knockdown cells relative to control siRNA-treated cells are shown. The firefly/renilla values were calculated from three independent samples. Errors represent the SD derived from three independent experiments.

Next, I examined whether PARN physically associates with p53 mRNA through ARE and/or miRNA targeting sites in the 3’UTR. First, several cell lines were used to test if ARE sequence present at the p53 mRNA 3’UTR is critical for the interaction of PARN with p53 mRNA. Consistent with my previous studies (Figure 20), the RIP assays indicated that PARN can form a complex with the luciferase mRNA carrying the 3’UTR of p53 and this is abolished when AREs are replaced by other sequences (Figure 31).
RKO/RKO-E6 and HCT116/HCT116 p53-/- cells showed similar ratios for the RIP assays with different constructs, indicating that the PARN-mediated regulation of p53 expression and PARN binding to AREs are p53 independent.

**Figure 31. ARE sequences present in the 3’ UTR are involved in the interaction of PARN with p53 mRNA under non-stress conditions.** RIP analysis of samples cells transfected with luciferase constructs carrying either the p53 3’ UTR (p53) or ARE-replaced p53 3’ UTR (noARE). Nuclear extracts were immunoprecipitated with either anti-PARN or IgG antibodies. The endogenous nuclear RNA immunoprecipitated with the antibodies was quantified by qRT-PCR using primers specific for the luciferase gene. The ratio of the fold change for p53/no-ARE RNA values obtained for each construct is shown. The qRT-PCR values were calculated from three independent samples.
Extending these studies, the RIP assays indicate that PARN cannot form a complex with the luciferase mRNAs carrying the 3’UTR of p53 with replaced sequences in the AREs or/and miRNA targeting sites (Figure 32). Importantly, the interaction of PARN with the luciferase mRNA carrying the 3’UTR of p53 is lost in HCT116 cells treated with Ago-2 siRNA, indicating that Ago-2 knockdown and the replacement of the 3’UTR signals had a similar effect on the interaction of with p53 3’UTR. Together these results support the idea that miRNA-associated Ago-2 and ARE might be part of the same PARN-mediated regulatory pathway. Moreover, RNA pull-down assays using RNAs encompassing either WT p53 3’UTR or the mutant variants of p53 3’UTRs, and NEs from HCT116 cells showed that the RNA-PARN interaction depended on the presence of both the ARE sequence and miRNA targeting site (Figure 33). As expected, the interaction of Ago-2 with the RNA was not decreased by the replacement of the ARE.
Figure 32. Ago-2 expression and cis-acting signals present in the 3’UTR of p53 mRNA are involved in the association of PARN with p53 mRNA 3’UTR. RIP assays were performed using samples from HCT116 cells treated with Ago-2/control siRNAs and transfected with the luciferase constructs described in Figure 29.

Figure 33. Both ARE and miR-125b targeting signal at the 3’UTR are necessary for PARN to target at p53 mRNA. RNA pull-down assays were performed using biotinylated RNA carrying WT or signal replaced (noARE, nomiR, and noBOTH) 3’UTR of p53 and NEs from HCT116 cells. A representative pull-down reaction from three independent assays is shown.
Then I tested whether miRNAs play a role in the PARN-mediated regulation of p53 mRNA stability in the nucleus of human cells. I studied miRNAs, such as miR-504, miR-125b, and miR25/30d, that have been previously shown to have a target site in p53 3’UTR and to downregulate p53 expression (Hu et al., 2010; Kumar et al., 2011; Le et al., 2009). First, the effect of UV-treatment on the abundance of these miRNAs was determined in samples from HCT116 cells by qRT-PCR analysis of total and nuclear RNA samples. Consistent with previous reports (Le et al., 2009; Pothof et al., 2009; Tan et al., 2012), the total amount of each miRNA showed a change of different magnitude after DNA damage (Figure 34). Changes in the abundance of these miRNAs were also observed in the nuclear RNA samples in response to DNA damage. As described before (Kim et al., 2008), these results also showed the enrichment of miR-320 in nuclear fractions.

To examine whether PARN associates with these miRNAs, nuclear lysates from HCT116 cells were immunoprecipitated with an antibody against PARN or control IgG followed by RT-qPCR detection of miRNAs. Interestingly, RIP assays indicate that under non-stress conditions miR125b was significantly enriched in PARN-IP (Figure 35). Both miR-30d and miR-504 also showed some association with PARN. Remarkably, all p53 3’UTR targeting miRNAs show dramatic decrease in PARN association in response to UV-induced DNA damage, suggesting that PARN might have a critical role in miRNA-mediated downregulation of p53 expression under non-stress conditions. miR320, which
Figure 34. Abundance of p53 targeting miRNAs in response to UV irradiation. Nuclear and total RNAs from UV treated HCT116 cells were isolated and miRNA abundance was assessed by qRT-PCR using a specific kit for miRNA analysis.
does not target at p53 3’UTR, was used as control for binding specificity. These results are consistent with our previous report (Devany et al., 2013) that showed that p53 mRNA and p53 protein levels are regulated by PARN under non-stress conditions and are PARN-independent after UV treatment.

To examine the role of miR-125b in PARN-mediated p53 mRNA decay HCT116 cells were transfected with miR-125b inhibitor expression plasmid, which blocks endogenous miR-125b, or control plasmid and analyzed p53 mRNA steady-state levels.

Figure 35. PARN association to different p53 targeting miRNAs is favored in non-stress conditions. RIP assays were performed as described in Material and Methods. Nuclear miRNAs immunoprecipitated were quantified by qRT-PCR.
In Figure 36, the functional knockdown of miR-125b showed an increase in p53 mRNA (upper panel) and protein levels (lower panel). A similar increase in both p53 mRNA and p53 expression was determined after PARN-knockdown (Devany et al., 2013). Importantly, as shown in Figure 37A, the functional inhibition of miR-125b elongated the poly(A) tail length of p53 mRNA (quantification is shown in Figure 37B), indicating miR-125b regulates p53 mRNA steady-state level through modulating its poly(A) tail length.

Figure 36. Functional knockdown of miR-125b increases p53 mRNA and protein levels. Samples from HCT116 cells transfected with miR-125b inhibitor expression plasmids or control vectors were analyzed for p53 mRNA levels by qRT-PCR and for p53 protein levels by Western blot.
miRNA inhibitor

-200
-100
-0
-actin exon 3-4

Rapid Amplification cDNA Ends poly A Test (RACE-PAT)

B

Extended poly(A) tail

RNA length (nt)

300 200 100

A                                                                      B

Figure 37. miR-125b regulates p53 mRNA poly(A) tail length. Nuclear RNAs from HCT116 cells transfected with miR-125b inhibitor expression/control vectors were reverse-transcribed using an oligo(dT)-anchor primer and amplified using an oligonucleotide that hybridizes within the 3’UTR of p53 mRNA. The products were separated on a non-denaturing PAGE and detected by ethidium bromide staining. An RT-PCR product from a non-PARN target gene (ACTIN exon 3–4) was used as a loading control. A representative PAGE from three independent assays is shown. Molecular weight standard (MWS, 100 base pair ladder from Promega) is also included.

Then I tested whether miR-125b is involved in PARN association with p53 mRNA. Nuclear lysates from HCT116 cells transfected with miR-125b inhibitor expression or control plasmids were used for miRNA-IP assays using PARN antibodies. The RIP assays showed that the functional expression of miR-125b increased the binding
of PARN to p53 mRNA (Figure 38). To further assess the regulatory function of miR-125b in miRNA-mediated deadenylation, NEs from HCT116 cells transfected with miR-125b inhibitor or control vectors were analyzed for deadenylation assays. These assays were performed using an *in vitro* transcribed, radiolabeled, capped and polyadenylated (A20) RNA encompassing the WT p53 3’UTR (Figures 39A) and the mutants described in Figure 29 (Figures 39B). Importantly, samples from cells with function loss of miR-125b showed a decrease in the deadenylation of WT p53 3’UTR (Figure 39A), indicating miR-125b is important to promote miRNA-mediated deadenylation of p53 mRNA. In Figure 39B, the deadenylation assays showed that the presence of the targeting sequence was important for this miR-125b-mediated deadenylation of p53.

![Graph showing fold change of p53 mRNA in PARN IP](image)

**Figure 38. Functional loss of miR-125b attenuates p53 mRNA association with PARN.** RIP assays were performed as described before, using NEs from HCT116 cells transfected with miR-125b inhibitor expression plasmids or control vectors.
Figure 39. Functional knockdown of miR-125b inhibits deadenylation of p53 mRNA. A) Deadenylation assays were performed NEs from HCT116 cells transfected with miR-125b inhibitor expression/control plasmids and a radiolabeled/polyadenylated RNA encompassing the p53 WT 3’UTR. Positions of the polyadenylated RNA (p53 3’UTR A20) and deadenylated product (p53 3’UTR) are indicated. Numbers beneath gel lanes indicate relative deadenylation (RD). RD was calculated as \([p53 \text{ 3’UTR}/(p53 \text{ 3’UTR} + p53 \text{ 3’UTR A20})] \times 100\). Quantifications were done with Image J software. B) Regulatory signals at the 3’UTR of p53 are important for deadenylation of p53 mRNA. Deadenylation assays were performed using NEs from HCT116 cells and radiolabeled/polyadenylated (A20) RNA encompassing WT or signal replaced (noARE, nomiR, and noBOTH) 3’UTRs of p53. Deadenylation reactions were analyzed as in Figure 24.
DISCUSSION

miRNA pathway was originally believed to act only in the cytoplasm. However, a growing number of evidence has shown that this pathway has also a nuclear component (Ohrt et al., 2008). Although it has been shown that nuclear miRISCs are involved in miRNA-mediated gene silencing in the nucleus (Nishi et al., 2013; Robb et al., 2005), the mechanism(s) underlying miRNA-mediated regulation of gene expression in the nucleus remain(s) to be elucidated. Two deadenylase complexes, CAF1/CCR4/NOT1 and Pan2-Pan3, have been shown to facilitate cytoplasmic miRNA-mediated deadenylation in mammalian cells (Behm-Ansmant et al., 2006; Fabian et al., 2009; Piao et al., 2010), however, little is known about miRNA-mediated deadenylation in the nucleus. Here, my studies indicate that PARN deadenylase plays a critical role not only in ARE- but also in miRNA-mediated regulation of mRNA stability and, consequently, expression levels in the nucleus of mammalian cells. Consistent with this, biochemical evidence shows that PARN is a nuclear miRISC-associated deadenylase: both PARN and elements of miRISC were present in the nuclear fractions (Figure 21), PARN physically interacted with Ago-2, a key a component of mammalian miRISC complex (Figures 22-23), and Ago-2 activated PARN deadenylase activity (Figures 24-25). Extending these studies, I showed that PARN regulated the expression of one of its targets, p53, by interacting with not only ARE sequences but also miRNA targeting sites present in the 3’UTR of p53 mRNA (Figure 31-33). Furthermore, my results indicate that Ago-2 (Figure 27) and miR125b (Figure 38) facilitated the binding of PARN to the target p53 mRNA 3’UTR resulting in p53 mRNA poly(A) tail shortening and decrease in p53 transcript and protein levels.
Taken together, these results provide new insights into the nuclear function of miRISCs in the PARN-mediated regulation of deadenylation and gene expression.

Previous work from Dr. Kleiman’s lab showed an alternative mechanism to regulate the expression levels of p53 based on the control of the steady-state levels of p53 mRNA by PARN deadenylase under non-stress conditions (Devany et al., 2013). Based on the results presented here, I propose a model in which, under non-stress conditions, miRISC targets p53 mRNA through miR-125b resulting in the recruitment of PARN deadenylase by its direct interaction with miRISC major component Ago-2. The binding of PARN to these protein complexes activates its deadenylation activity resulting in p53 mRNA decay and control of gene expression (Figure 40). The steady-state levels of other PARN target mRNAs, such as c-myc, c-fos, c-jun, and transcripts in the p53 and BARD1/BRCA1 pathways, might be regulated following a similar model. Although more work is necessary to determine the identity of the ARE-BPs involved in PARN-mediated degradation of p53 mRNA, these results indicate that ARE sequences are also involved in this process. Most of the known PARN-associated ARE-BPs, such as tristetraprolin (TTP) and KH-type splicing regulatory protein (KSRP), are involved in the deadenylation of ARE-containing mRNAs followed by the recruitment of the exosome (Cevher and Kleiman, 2010).
Figure 40. Model of multicomponent complexes required for regulation of p53 mRNA steady-state levels by nuclear PARN deadenylase in different cellular conditions. Cooperation of ARE-BPs, miRNAs, miRISC, PARN deadenylase and exosome is essential for the regulation of p53 mRNA stability in different cellular conditions. The recruitment of the ARE-BPs to the ARE sequence and/or the targeting of miR-125b-loaded miRISC complexes to the most proximal site to the ARE sequence assist in the recruitment of PARN deadenylase to the target mRNA. Changes in the ARE-BPs bound to the 3’UTR, miRNA abundance and Ago-2 cellular localization might signal the DDR. miRNA targeting sites present in p53 mRNA 3’UTR are shown in different colors (miR-25 gray, miR-30d purple, miR-125b red, miR-504 orange).
Regulation of mRNA stability by sequences in the UTR of the mRNA is not a new concept. The typical scenario is the control of mRNA stability by 3’UTR sequences and the recruiting of diverse protein regulators. The implication of both cis-acting RNA elements and trans-acting protein factors increases the complexity of the regulation of gene expression. The studies presented in this dissertation indicate that the presence of both miRNA binding sites and AREs at the 3’UTR is critical for PARN-dependent regulation of mRNA deadenylation and stability. However, further studies are necessary to determine whether the proximity of these regulatory elements is important in this PARN-associated regulatory pathway. Although the exact contribution of miRNAs, miRISC, AREs and ARE-BPs to mRNA decay has not been elucidated yet, recent studies have described a functional overlap between ARE- and miRNA-mediated mRNA turnover pathways (Zhang et al., 2010). It has been described that miRNAs can functionally interact with ARE-BPs, and that Dicer and Ago are required for ARE-mediated decay (Jing et al., 2005). For example, it has been shown that HuR can bind to AREs present in c-myc 3’UTR at a site proximal to that recognized by let-7 miRNA, facilitating the targeting of let-7-loaded miRISC and mediating the reduction of c-Myc mRNA levels (Kim et al., 2009). Another example is the functional interaction of ARE-BP TTP and miRISC that results in the recruitment of the deadenylase and the exosome for tumor necrosis factor-α mRNA degradation (Jing et al., 2005). Interestingly, 20% of PARN mRNA targets in the p53 signaling pathway showed both miRNA target sites and an ARE in their 3’UTRs (Table 1). Some of my studies work indicates that PARN regulates the stability of short-lived ARE-containing mRNAs involved in the control of cell growth, DDR and differentiation, and keeps their levels low (Cevher et al., 2010;
Devany et al., 2013). PARN deadenylase is recruited to ARE sequences by ARE-BPs KSRP (Gherzi et al., 2004), CUG-BP (Moraes et al., 2006) or TTP (Korner and Wahle, 1997; Lai et al., 2003). It is possible that miRNA-loaded miRISC might contribute with one of these ARE-BPs to the recruitment of PARN to its target mRNAs.

The results presented in this dissertation also indicate that Ago-2 shuttles from the nucleus to the cytoplasmic compartment upon DNA-damaging conditions (Figure 21), and this might contribute to the UV-induced increase in p53 expression levels. The UV-induced decrease of nuclear Ago-2, which is involved in the recruitment of PARN to p53 mRNA and activation of its deadenylase activity, might explain my observation that the UV-induced changes in p53 expression are PARN-independent (Devany et al., 2013). Furthermore, the UV-induced changes in miRNA-expression profiles (Pothof et al., 2009) might also contribute to the previously described PARN-independent changes in p53 expression levels (Devany et al., 2013), providing an additional layer of gene regulation and a new dimension to DDR. The UV-induced changes in the binding of different ARE-BPs to the 3’UTRs of the PARN target mRNAs might also influence the resulting expression levels. For example, it has been shown that after UV treatment ARE-BP Human Antigen R (HuR) binds to AREs, resulting in the dissociation of TTP and KSRP from ARE-containing mRNAs and the up-regulation of genes involved in DDR (Cevher and Kleiman, 2010).

Taken together, my characterization of PARN-dependent regulatory pathways indicates a novel nuclear function of miRISC in controlling gene expression of different genes in different cellular conditions. The study presented in this dissertation addresses the mechanism behind miRNA-dependent control of deadenylation in the nucleus,
showing the functional interplay among PARN deadenylase, the AREs in the 3’UTR, miRNA abundance and Ago-2 cellular localization. My studies also provide new insights on regulatory mechanisms in the p53 pathway. While most of the studies on the expression of genes involved in stress response pathways have traditionally focused on transcription as a major regulator, it has recently become apparent that the posttranscriptional control of mRNA steady-state levels may play an equally important role. The mechanism(s) behind the regulation of p53 mRNA levels under non-stressed as well as DNA-damaging conditions has(ve) not been well characterized. My results indicate that PARN deadenylase keeps p53 levels low in non-stress conditions by interacting with both AREs and miRNA targeting sites in the 3’UTR and destabilizing the p53 mRNA. Importantly, these studies support the functional overlap between ARE- and miRNA-mediated mRNA turnover pathways, increasing the complexity of the signaling present in the 3’UTR of different genes. This is consistent with the idea that cell-specific 3’ processing profiles, and hence gene expression patterns, depends on the complexity of the signaling in the 3’UTR of the genes and the functional/dynamic interaction of the 3’ processing machinery and the DNA damage response/tumor suppression factors, providing functional connection between mRNA processing and cancer subtypes, and proposing new approaches in the design of new cancer therapies.
CHAPTER V

NUCLEOLIN REGULATES PARN-DEPENDENT DEADENYLATION

IN DIFFERENT CONDITIONS
INTRODUCTION

Nucleolin is a highly conserved, multifunctional RNA binding protein (RBP) implicated in several cellular processes, such as chromatin remodeling, rRNA synthesis, mRNA processing, ribosome assembly, and nucleo-cytoplasmic transport (Angelov et al., 2006; Ginisty et al., 1998; Hovanessian et al., 2010; Turner et al., 2009). Elevated levels of nucleolin are found in highly proliferative cells including a variety of tumors. Nucleolin is primarily located in the nucleolus but it is also found in other nuclear regions as well as in cytoplasm and plasma membrane (Borer et al., 1989; Ginisty et al., 1999). Nucleolin has RNA- and p53-binding properties (Serin et al., 1997; Srivastava and Pollard, 1999; Tajrishi et al., 2011; Tuteja and Tuteja, 1998), and can control gene expression by regulating mRNA stability and modulating the p53 signaling pathway (Abdelmohsen and Gorospe, 2012; Abdelmohsen et al., 2011; Bhatt et al., 2012; Chen et al., 2012; Daniely et al., 2002; Saxena et al., 2006; Takagi et al., 2005; Willimott and Wagner, 2010). For example, nucleolin modulates gene expression by direct binding to the untranslated regions (UTRs) of its target mRNAs resulting in a change of mRNA stability or translation level. Nucleolin can also control gene expression by modulating the p53 signaling pathway indirectly through protein-protein interactions. For example, it has been shown that the N-terminus of nucleolin associates with p53-antagonist Hdm2 in hyperproliferative cells to stabilize p53 protein and causes p53-mediated apoptosis (Bhatt et al., 2012).

Most of the nucleolin target mRNAs are from stress-responsive genes and from genes whose expression is affected in disease, such as Alzheimer’s, cancer and inflammation. Some of nucleolin target mRNAs include antiapoptotic factor bcl-2
(Ishimaru et al., 2010; Otake et al., 2007; Willimott and Wagner, 2010), amyloid (Rajagopalan et al., 1998), JNK-mediated interleukin 2 (Chen et al., 2000), \(\beta\)-globin (Jiang et al., 2006), tumor suppressor p53 (Takagi et al., 2005), potent pro-survival protein Akt1 (Abdelmohsen et al., 2011), and the growth arrest- and DNA damage inducible 45 protein (Gadd45\(\alpha\); (Zhang et al., 2006). Most of them bear AU-rich elements (AREs) or G-rich elements at their UTRs (reviewed by (Abdelmohsen and Gorospe, 2012). Nucleolin has been demonstrated to either increase or decrease the stability of the target mRNAs. For example, during DDR, nucleolin can stabilize the antiapoptotic mRNA \textit{bcl-XL} by interacting with the AREs in their 3'UTR and protecting the poly(A) tail by association with poly(A) binding protein (PABP; (Zhang et al., 2008) and inhibit p53 mRNA translation by interacting with the 5'UTR (Chen et al., 2012; Takagi et al., 2005). Recently, transcriptome-wide analyses revealed that nucleolin enhances translation of mRNA with G-rich motif (Abdelmohsen et al., 2011). Interestingly, nucleolin’s expression, subcellular localization and post-translational modification are linked to its role as an RNA-binding stress responsive protein (Aoki et al., 2002; Daniely et al., 2002; Ginisty et al., 1998; Gorospe et al., 2011; Grinstein and Wernet, 2007; Kim et al., 2005; Masuda et al., 2009; Rickards et al., 2007). Therefore, it has been suggested a role for nucleolin in the regulation of mRNA stability of different target genes upon DNA damage, providing multiple levels of differential regulation of gene expression:

It has been shown that nucleolin binds to p53 mRNA at a 5’UTR and 3’UTR base-pairing region in unstressed cells resulting in the repression of p53 mRNA translation (Chen et al., 2012). After DNA damage and stress stimulation, RPL26 is
recruited to this double-stranded RNA structure occupied by nucleolin forming nucleolin-RPL26 heterodimers and enhancing the translation of p53 mRNA. However, whether nucleolin associates with AREs present in the 3’UTR of p53 mRNA and affects p53 mRNA stability has not been tested. A recent report has shown that nucleolin mediates microRNA (miRNA)-directed deadenylation of the colony stimulating factor-1 (CSF-1) mRNA by binding factors from the miRNA-induced silencing complex (miRISC), such as Argonaute-2 (Ago-2) and cytoplasmic poly(A) binding protein 1 (PABPC-1) (Woo et al., 2013). Interestingly, the nucleolin/Ago2/PABPC-1 complex formation requires ARE regulatory elements present on the CSF-1 mRNA 3’UTR, supporting the functional interplay between miRNA- and ARE-mediated regulation of mRNA stability discussed in Chapter IV.

Nucleolin can be phosphorylated by numerous kinases. Changes in the phosphorylation of nucleolin play a role in several critical cellular processes, such as cell growth, proliferation, cell cycle arrest, apoptosis as well as DNA damage response (DDR) (Daniely et al., 2002; De et al., 2006; Ishimaru et al., 2010; Kim et al., 2005; Nalabothula et al., 2010; Saxena et al., 2006; Storck et al., 2007; Takagi et al., 2005; Yang et al., 2009; Yang et al., 2002). Nucleolin is highly phosphorylated at the N-terminus by two major kinases: interphase casein kinase II (CKII) and mitotic cyclin-dependent kinase (Cdk/Cdc2) (Schneider et al., 1986; Warrener and Petryshyn, 1991). CKII-mediated phosphorylation of several cytosolic and nuclear substrates, including nucleolin, appears to be important for the regulation of cell growth. The extensive phosphorylation of nucleolin by CKII that occurs during interphase suggests that
phosphorylation may be a mechanism for regulating nucleolin function during the cell cycle.

Nucleolin has distinct domains: the *acidic N-terminus*, which is highly phosphorylated by CKII and Cdns and contains the bipartite nuclear localization signal (NLS); four central RNA binding Domains (RBDs) that confer RNA-binding specificity, and the C-terminal Glycine-Arginine Rich (GAR) domain that exhibits helicase activity and is involved in many protein interactions including PABP and p53 (Figure 43; Tuteja and Tuteja, 1998; Ginisty et al., 1999; Srivastava and Pollard, 1999; Daniely et al., 2002; (Bhatt et al., 2012; Daniely et al., 2002; Ginisty et al., 1999; Srivastava and Pollard, 1999; Tuteja and Tuteja, 1998; Zhang et al., 2008). Our collaborator, Dr. Saxena (Brooklyn College, CUNY), has engineered a novel system with tet-off promoter in human NARF6 cells to express 3xFlag-tagged WT-nucleolin or the phospho-variant 6/S*A-nucleolin. In the phospho-variant nucleolin, six consensus CK2 phosphorylation sites were mutated from serine to alanine (Figure 41). The Tet-off promoter system allows nucleolin variants expression after removal of doxycycline (Dx) from the culture medium. The 6/S*A-nucleolin was tested deficient in phosphorylation by Dr. Saxena Lab (unpublished data kindly provided by Dr. Saxena). They also showed that although both phospho-variants of nucleolin increase p53 protein levels in unstressed cells, a greater increase in p53 is evident with 6/S*A expression. Interestingly, 6/S*A expression inhibits cell proliferation, probably due to increased p53 levels (Dr. Saxena personal communication). These differential effects on p53 induction and cell proliferation suggest a role for nucleolin phosphorylation in regulating gene expression of different target
genes in the p53 pathway. Thus, these inducible cell lines are valuable tools to facilitate studies and testing the role of nucleolin in gene regulation during the DDR.

Figure 41. Modular structure of Nucleolin protein (WT and 6/S*A), indicating the positions of principal domains. The six consensus CK2 sites (serine residues) mutated to alanine in phospho-mutant construct-6/S*A are denoted by asterisks.

Based on these studies, in this chapter I propose to dissect nucleolin’s role in governing mRNA stability of diverse target genes involved in DDR, and also provide mechanistic insights about the role of nucleolin phosphorylation in DDR. The specific hypothesis is that nucleolin interacts with mRNA 3’ processing factors and regulates the mRNA stability of genes involved either in the DNA repair or apoptotic response during the DDR. The working model is that nucleolin regulates stability of ARE-containing mRNAs by recruiting PARN deadenylase into its target mRNAs, such as p53 mRNA,
and these functions are modified by nucleolin phosphorylation state. Preliminary studies from Dr. Saxena’s lab indicate that cells expressing 6/S*A-nucleolin show increased levels of p53 expression in non-stress conditions, suggesting that the expression of this nucleolin phospho-variant mimics stress conditions. They also showed that nucleolin/PARN interaction increases after UV treatment, and this increase is abolished in samples expressing the nucleolin phospho-variant (not shown). Interestingly, my studies show that both nucleolin-WT and 6/S*A can interact directly with N-terminal domain of PARN in *in vitro* assays. The expression of WT nucleolin is necessary for the UV-induced activation of deadenylation, and this activation is abolished by the expression of 6/S*A nucleolin, suggesting that active dephosphorylation of nucleolin might be necessary for the activation of PARN deadenylation during DDR. Nucleolin interacts with the 3’UTR of one of PARN substrates, the p53 mRNA, under non-stress conditions in an AU-rich element (ARE)-dependent manner. Nucleolin phosphorylation affects its association with p53 mRNA both before and after UV treatment. Together these results indicate that the functional interaction of nucleolin with PARN deadenylase might provide a molecular mechanism for the rapid response to DNA damage allowing changes in the stability of different mRNAs, and this response might be regulated by nucleolin phosphorylation.

**RESULTS**

Preliminary data from Dr. Saxena’s lab showed that nucleolin/PARN interaction increases after UV treatment, and this increase is abolished in samples expressing the nucleolin phospho-variant (not shown). Extending those studies, I first tested whether nucleolin interacts directly with PARN. Pull-down assays were performed using both
Flag-tagged nucleolin phospho-variants purified from NARF6 cells and different His-PARN derivatives (Figure 42). The results indicated that PARN can interact directly with both WT- and 6/S*A-nucleolin, and that the N-terminal domain, which contains the nuclease activity and is responsible for cap-binding properties of PARN (Martinez et al., 2001), is important for this interaction (Figure 42). Together these results indicate that both nucleolin phospho-variants can interact with the N-terminal domain of PARN. This suggests that the decrease in the UV-induced formation of the nucleolin/PARN complex in cells expressing 6/S*A variant observed by Dr. Saxena’s lab might be due the need of active dephosphorylation or other factors for the complex formation.

**Figure 42. Nucleolin directly interacts with N-terminal domain of PARN.** Different His-PARN derivatives (FL: full-length, CTD: carboxyl-terminal domain, NTD: amino-terminal domain) were immobilized on nickel beads and incubated with WT or 6/S*A mutant nucleolin. Bound proteins were eluted, resolved by SDS-PAGE and detected with anti-nucleolin antibodies. 5% of Flag-tagged nucleolin is shown as input.
To test the role of nucleolin in PARN-mediated deadenylation, in vitro reconstituted deadenylation assays were performed using radiolabeled L3(A$_{30}$) RNA substrate to monitor the reaction, limiting amounts of recombinant His-PARN and increasing amounts of either WT-nucleolin or 6/S*A-nucleolin. As previously shown, increasing amounts of PARN deadenylase resulted in an increase of RNA substrate deadenylation (Figure 43, lanes 2-5). Importantly, only increasing amounts of purified Flag-tagged WT nucleolin can induce deadenylation in a reaction using a limited amount

**Figure 43. WT-nucleolin but not the 6/S*A phospho-variant activates PARN deadenylase activity.** Deadenylation assays were performed in the presence of radiolabeled capped L3(A$_{30}$) RNA substrate as described (Cevher et al., 2010) using different concentrations of His-PARN and increasing amounts of either Flag-WT-NCL or Flag-6/S*A-NCL. Positions of the polyadenylated RNA L3(A$_{30}$) and the L3 deadenylated product are indicated. Numbers beneath gel lane indicate relative deadenylation (RD), which was calculated as $[\text{L3 fragment}/(\text{L3(A}_{30}) + \text{L3 fragment})] \times 100$. Quantifications were done with Image J software.
of His-PARN in a cell-free assay, suggesting that this is a transactivation-independent function of nucleolin. PARN-mediated deadenylation was enhanced up to 5.2 folds in the presence of WT-nucleolin (compare lane 2 and lane 10-13). The addition of Flag-tagged 6/S*A-nucleolin did not have an effect on the deadenylation reaction (lanes 6-9). None of the phosphor-varinats were able to deadenylate the substrate in the absence of His-PARN (not shown). These results suggested that nucleolin is an activator of PARN deadenylase activity and nucleolin phosphorylation is necessary for this activation.

Extending these studies, the role of nucleolin in deadenylation was tested using nuclear extracts (NEs) from cells expressing nucleolin-variants treated with either UV irradiation or Isopropyl β-D-1-thiogalactopyranoside (IPTG) to induce ARF/p53 expression. NEs from those cells were assayed for deadenylation activity using a radiolabeled L3(A30) RNA substrate. As described in previous studies (Cevher and Kleiman, 2010; Devany et al., 2013), Figure 4A shows that deadenylation activity in NEs of cells expressing WT-nucleolin increased significantly (up to 3.3 folds) after UV treatment (compare lanes 2 and 3). Interestingly, UV-induced activation of deadenylation was abolished in samples from cells expressing 6/S*A-nucleolin (compare lanes 5 and 6). As cells expressing 6/S*A-nucleolin show increased expression of p53 in non-stress conditions (information kindly provided by Dr. Saxena’s) and previous studies from Dr. Kleiman’s lab showed that p53 is an activator of PARN deadenylase activity (Devany et al., 2013), I decided to test whether p53 expression is sufficient to activate deadenylation. Interestingly, the IPTG-induced expression of p53 enhanced deadenylation to a similar level to that observed in samples from cells expressing WT-nucleolin treated with UV irradiation (compare lanes 1 to 3). These results suggest that deadenylation can be
activated by the induction of p53 expression in either DNA damage or oncogenic stimuli conditions. Importantly, IPTG treatment induced deadenylation in samples from cells expressing 6/S*A– nucleolin (compare lanes 1 to 4). Together, these results indicate that

Figure 44. Nucleolin phosphorylation is necessary for deadenylation activation upon UV-induced but not in the oncogenic stimuli. A) NEs prepared from cells expressing nucleolin phospho-variants treated with/without UV irradiation (40 Jm⁻²) were prepared 2 h post-treatment and analyzed for deadenylation. Alternatively, cells were treated with 1.5 mM IPTG for 24 h to induce ARF/p53 expression. Positions of the polyadenylated RNA L3(A₃₀) and the L3 deadenylated product are indicated. Numbers beneath gel lane indicate relative deadenylation: [L3 fragment/(L3(A₃₀)+ L3 fragment)] x 100. Quantifications were done with Image J software. B) The bar graph shows the ratio of deadenylation in UV- or IPTG-treated cells relative to non-stress conditions.
nucleolin phosphorylation is necessary for deadenylation activation upon UV-induced DNA damage but not in the oncogenic stimuli (Figure 44B), suggesting that (a) mechanism(s) other than p53-induced activation of the deadenylation is involved in this response.

My results indicate that the RNA binding protein nucleolin binds directly to PARN deadenylase resulting in the activation of its activity. To further analyze whether nucleolin plays a role in PARN-mediated regulation of mRNA stability I first tested the binding of nucleolin to one of PARN mRNA targets, the p53 mRNA (Devany et al., 2013). The binding of nucleolin to the p53 mRNA was determined by RNA immunoprecipitation (RIP) assays using antibodies against nucleolin. RIP assays showed that p53 mRNA can form a complex with nucleolin under non-stress conditions in samples from cross-linked HeLa cells (Figure 45A), suggesting that nucleolin might regulate p53 mRNA stability by its association to PARN deadenylase and the 3’UTR of p53. This result is consistent with a model proposed by a recent report that nucleolin homodimer binds to p53 mRNA in unstressed cells resulting in the repression p53 translation; while RPL26 disrupts the nucleolin homodimer and forms nucleolin-PRL26 heterodimers resulting in p53 induction after DNA damage, (Chen et al., 2012). As nucleolin has been shown to associate with its target mRNAs through ARE regulatory elements in the 3’UTR (Sengupta et al., 2004) and miR targeting sequences (Woo et al., 2013), I tested whether these cis-acting elements present in the p53 3’UTR are also important for nucleolin association with the mRNA. RNA pull-down assays were performed using with in vitro transcribed biotinylated RNAs, either with sequences of the p53 3’UTR or ARE/miRNA-replaced p53 3’ UTR, and NEs from RKO cells. RNA-pull
Figure 45. Nucleolin interacts with the 3’UTR of p53 mRNA under non-stress conditions in an ARE-dependent manner. A) Nucleolin can interact with endogenous p53 mRNA under non-stress conditions. HeLa cells were treated with formaldehyde to generate protein-RNA cross-links, then the samples were sheared by sonication followed by incubation with either anti-nucleolin or IgG antibodies. The endogenous nuclear RNA immunoprecipitated with the antibodies was quantified by qRT-PCR using primers specific for p53 gene. The qRT-PCR values were calculated from three independent samples. Input indicates samples before RIP. B) Nucleolin interacts with the p53 3’UTR in an ARE-dependent manner. RNA-pull down assays were performed using NEs from RKO cells and biotinylated RNA of either p53 or p53 mutant 3’UTRs [no-ARE: AU-rich element replaced; no-miR: miR504/miR125-b targeting sites replaced].

down assays showed that the replacement of an ARE regulatory sequence in the p53 3’UTR abolished nucleolin binding to p53 mRNA (Figure 45B). Interestingly, the replacement of miR-504/miR-125b targeting site also decreased the nucleolin’s association to the p53 mRNA. Together, these data indicates that nucleolin interacts with
the 3’UTR of p53 mRNA under non-stress conditions and that the presence of the cis-acting regulatory sequences ARE and miR-504/miR-125b targeting sites at the 3’UTR of p53 mRNA are important for the RNA-nucleolin interaction. Interestingly, I showed in Chapter IV that PARN regulates the stability of p53 mRNA by interacting with not only ARE sequences but also miR-504/miR-125b targeting sites present in the 3’UTR of p53 mRNA (Figures 32-33), supporting the idea that nucleolin and PARN functional interaction over the regulation of p53 mRNA stability under non-stress conditions.

Next, I tested whether phosphorylation of nucleolin affect its association with p53 mRNA. RIP assays were performed using Flag antibodies and samples from NARF cells expressing different phospho-variants of nucleolin treated with or without UV irradiation. RIP assays showed that (Figure 46). Upon UV-induced DNA damage, both WT- and 6/S*A-nucleolin showed an increase in their association with p53 mRNA. However, p53 mRNA showed stronger interaction with WT-nucleolin than with the 6/S*A-nucleolin phospho-variant, indicating that nucleolin phosphorylation affects nucleolin association with p53 mRNA.
Figure 46. Nucleolin phosphorylation affects its association to p53 mRNA. A) RIP assays of samples from NARF cells expressing the WT or 6/S*A nucleolin phospho-variants were performed as before using anti-Flag antibodies. The nuclear RNA in immunoprecipitated was quantified by qRT-PCR using primers specific for p53 gene. B) The bar graph shows the ratio of the fold change for UV-treated/non-stress RNA values in nucleolin phospho-variants.

DISCUSSION

The post-transcriptional fate of mRNAs could be affected by many factors. RNA binding proteins are believed to play a fundamental role in the recognition of mRNA transcripts and regulating the recruitment of other trans-acting factors and/or factors that modulate the poly(A) tail, such as deadenylases and poly(A) binding proteins. The specific sequences that any RNA binding protein recognizes on its target transcripts and the location relative to other elements are of vital importance for regulation of gene expression. Diverse mRNA isoforms carrying different arrangement of regulatory signals
can be generated and dramatically alter mRNA stability and/or translation, hence gene expression. The RNA binding protein nucleolin has been shown to recognize the ARE signal at the 3’UTR of several mRNA targets and regulate their stability (Sengupta et al., 2004). However, whether nucleolin plays a role in modulating p53 mRNA steady-state levels through its ARE element has not been tested. Previous studies have shown that PARN deadenylase is recruited to the p53 mRNA under non-stress conditions (Devany et al., 2013), however, the RNA-binding protein(s) necessary to recruit the active deadenylase to its mRNA substrates leading to mRNA deadenylation and degradation was not identified. Here my studies indicate that nucleolin binds the N-terminal domain of PARN deadenylase (Figure 42) and both of them bind to the same ARE element present in the p53 3’UTR (Figure 45; (Devany et al., 2013)). The PARN/nucleolin interaction resulted in the activation of PARN deadenylase activity (Figures 43-44). The results also indicate that the phosphorylation state of nucleolin is critical for the PARN/nucleolin functional interaction (Figures 43-44) and for nucleolin binding to p53 mRNA (Figure 46). Together these results indicate that nucleolin is one of the RNA binding proteins that recruit PARN deadenylase to the tumor suppressor p53 mRNA, representing a novel regulatory mechanism for p53 gene expression.

While most of the studies on the expression of genes involved in stress response pathways have traditionally focused on transcription as a major regulator, it has recently become apparent that the posttranscriptional control of mRNA steady-state levels may play an equally important role. The mechanism(s) behind the regulation of p53 mRNA levels under non-stressed as well as DNA-damaging conditions has(ve) not been well characterized. The results presented here indicate that nucleolin recruits PARN
deadenylase to p53 mRNA keeping p53 levels low in non-stress conditions by interacting with AREs in the 3’UTR and destabilizing the p53 mRNA. It is possible that a similar mechanism is used to regulate the steady-state levels of other mRNAs, hence gene expression, in different conditions. Nucleolin has been implicated in many different cellular processes through the identification of its target transcripts. It has been shown that nucleolin participates in the control of the steady-state levels of ARE-containing mRNA targets by regulating their stability and translation. It has been shown that nucleolin is involved in the stabilization of bcl2 mRNA through its association with an ARE signal present in the 3’UTR of bcl2 transcript resulting in an increase of its expression (Sengupta et al., 2004). Another target of nucleolin is bcl-XL mRNA, which is stabilized by nucleolin association to its ARE in response to UVA irradiation (Zhang et al., 2008). However, nucleolin also contributes to the selective destabilization of some other mRNA targets. For example, nucleolin associates with the 3’UTR of amyloid precursor protein (APP) mRNA and enhances its degradation in response to stress (Westmark and Malter, 2001). Consistent with this, other ARE-BPs have also been shown to increase and decrease mRNA stability in a gene-specific manner. For example, HuR-elicited stabilization of target mRNA has been extensively documented: stabilization of ARE-containing mRNAs during UV irradiation (Wang et al., 2000), heat shock (Gallouzi et al., 2000), hypoxia (Levy et al., 1998) and energy depletion (Jeyaraj et al., 2005). HuR has also been shown to recruit miRNA-induced silencing complex (miRISC) miRISC to destabilize c-myc mRNA and repress its expression (Kim et al., 2009).
It is a widespread strategy to regulate ARE-mediated mRNA decay by changing the phosphorylation state of RNA binding proteins resulting in changes in their ability to bind their target mRNAs. For example, the phosphorylation of tristetraprolin (TTP) by MAPKAP kinase 2 (MK2) reduces binding affinity of TTP for its target mRNA, such as tumor necrosis factor-α (TNF-α), Granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin 2 (IL-2) (Chrestensen et al., 2004). The p38 pathway-mediated phosphorylation of KSRP reduces its association to the ARE region at the 3’UTR leading to the stabilization of its mRNA targets (Briata et al., 2005). On the contrary, HuR phosphorylation enhances its binding to target mRNAs (Lafarga et al., 2009). Interestingly, consistent with the results shown in Figure 46, it has been reported that the RNA-binding activity of nucleolin is also affected by phosphorylation. Nucleolin is phosphorylated in response to DNA damage by stress-activated protein kinases (SAPK) p38, and this increases its association with a subset of stress responsive transcripts in a UV irradiation dose-dependent manner (Yang et al., 2002). The results presented here indicate that cells expressing the dephospho-variant of nucleolin show a decrease in nucleolin binding to p53 mRNA (Figure 46) and in PARN deadenylase activity (Figures 43-44), which might probably result in an increase in p53 mRNA levels and explain the increase in p53 protein expression observed by Dr. Saxena’s lab (personal communication).

Interestingly, Woo et al. (2013) recently described a role for nucleolin in miRNA-directed mRNA deadenylation. Nucleolin interacts with the miRISC component Ago2 indirectly via RNA and induces deadenylation of CSF-1 mRNA. Interestingly, nucleolin/Ago2-mediated degradation of CSF-1 mRNA depends on the functional
overlapping of miRNA targeting sites and AREs located at the 3’UTR of the target transcript (Woo et al., 2013). The results shown in this dissertation also indicate that both ARE sequences and miR targeting sites are involved in nucleolin binding to the p53 mRNA (Figure 45), suggesting that nucleolin might regulate mRNA deadenylation of different targets through a similar mechanism. Surprisingly, the studies presented in Chapter IV indicate that PARN can interact directly with Ago2 (Figure 23) and that regulates the stability of p53 mRNA by interacting with not only ARE sequences but also miR-504/miR-125b targeting sites present in the 3’UTR of p53 mRNA (Figures 32-33). Furthermore, my results indicate that Ago-2 (Figures 27 and 32) and miR-125b (Figure 38) facilitated the binding of PARN to the target p53 mRNA 3’UTR resulting in p53 mRNA poly(A) tail shortening and decrease in p53 transcript and protein levels. Further work is necessary to investigate whether the phosphorylation state of nucleolin, which is important for the activation of PARN deadenylase and association to p53 mRNA, has an effect on p53 mRNA decay under different cellular conditions.

Together, these studies suggest a functional interplay among PARN deadenylase, the RNA binding protein nucleolin, ARE elements, miRNA targeting sites, miRNA-loaded miRISC, and miRNA abundance; and address the molecular mechanism behind miRNA-mediated/ARE-directed deadenylation of p53 mRNA in the nucleus. The model proposed in this work also provides a molecular mechanism for the rapid response to DNA damage allowing changes in the stability of different mRNAs by regulating nucleolin phosphorylation.
CHAPTER VI

FUTURE DIRECTIONS
The modulation of mRNA stability plays important roles in the regulation of gene expression. Many mechanisms and factors are implicated in this precise control of mRNA turnover, which is more complex than it was previously thought. PARN deadenylase is one of the important factors involved in these regulatory pathways. PARN has been involved in several important biological processes, such as oocyte maturation, early development, DNA damage response (DDR) and cell-cycle progression. The results presented in this dissertation indicate that PARN deadenylase is involved in an alternative mechanism to regulate of p53 expression levels under non-stress conditions. Moreover, my studies also show that the functional interaction of PARN with microRNA (miRNA)-induced silencing complex contributes to the regulation of the stability of p53 mRNA (Figures 35-38). This data is innovative because it is the first description of PARN deadenylase function in miRNA-dependent control of mRNA decay. Importantly, this is also the first mechanistic report of miRNA-function in the nucleus, which has been traditionally believed to take place only in the cytoplasm.

Previous studies have shown that PARN is implicated in AU-rich elements (ARE)-mediated deadenylation and that several ARE-binding proteins facilitate PARN access to its targets, ARE-containing mRNAs, resulting in PARN dependent deadenylation. In this dissertation, these studies have been extended showing that PARN deadenylase has a specific effect on the steady-state levels of mRNAs containing not only AREs but also miRNA target sites (Figures 10, 12-13, Table 1). More importantly, I found that miR-125b-loaded miRNA-induced silencing complex (miRISC) contributes to the specific recruitment of nuclear PARN to one of its targets, the tumor suppressor p53 mRNA (Figure 38), and ARE-binding protein nucleolin associates to the ARE element
that overlaps miR-125b target site and regulates PARN deadenylase activity (Figures 45-48), representing a novel regulatory mechanism for p53 gene expression.

The studies presented in this dissertation are innovative because they are the first to address the mechanism behind miRNA-dependent control of deadenylation in the nucleus and the functional interplay among PARN deadenylase, the regulatory signals in the p53 3’UTR, ARE-binding proteins, miRNA abundance and Ago-2 cellular localization. Now, it is important to further elucidate the complete mechanism behind PARN-mediated regulation of its targets in different cellular responses. The following proposed studies might help to understand some aspects of the working model shown in this dissertation.

1) **Further characterization of the role of PARN deadenylase in the regulation of mRNA stability of genes containing overlapped AREs and miRNA target sites:**

   In recent years, the mechanisms underlying the miRNA-mediated repression of target mRNAs have been extensively explored. miRISCs, which deliver miRNAs to their mRNA targets, regulate gene expression through post-transcriptional events either by mRNA degradation, translation inhibition, or a combination of both. In this dissertation, it has been shown that Ago-2, the core component of the miRISCs, interacts directly and can coexist in complexes with PARN resulting in the activation of its deadenyase activity (Figures 22-25), supporting the idea that Ago-2 is critical for miRNA-mediated poly(A) removal. It is known that Ago-2 associates to the other miRISCs components, such as the GW182 factor and cytoplasmic poly(A) binding protein 1 (PABPC1), that contribute to miRNA-directed deadenylation (Fabian et al., 2009; Jinek et al., 2010; Zekri et al., 2009).
GW182 protein is considered the link between miRISCs and deadenylases, and it is important for promoting poly(A) removal (Lazzaretti et al., 2009; Takimoto et al., 2009; Zipprich et al., 2009). The other critical component of the miRISC, PABPC1, has a bimodal effect on PARN deadenylase activity: it can either activate or inhibit PARN deadenylase depending on the salt concentration (Korner and Wahle, 1997). Interestingly, PABPC1 has been traditionally considered to localize only in the cytoplasmic compartment. However, recent studies have revealed a role of PABPC1 during mRNA biogenesis in the nucleus (Lemay et al., 2010). It is also possible that the nuclear poly(A) binding protein (PABPN) might be the one associated with nuclear miRISCs and involved in recruiting PARN to the poly(A) tail and allowing miRNA-mediated deadenylation. Whether GW182 and/or PABC1 associates with PARN and affects its deadenylase activity remains to be determined. Therefore, the elucidation of the functional interactions between PARN and these factors involved might provide a dynamic picture of the molecular networks used to regulate gene expression in different cellular conditions. It is important to highlight that Ago-2 binds directly PARN deadenylase and that is sufficient to recruit PARN to its target mRNAs (Figures 23, 27, 32), suggesting that PARN interaction with any of the other components of the miRISC might be regulatory.

The results presented in this dissertation indicate that PARN regulates the stability of short-lived ARE-containing mRNAs implicated in the control of cell growth, DDR and differentiation, and keeps their levels low in non-stress conditions (Cevher et al., 2010; Devany et al., 2013). Interestingly, it has been shown that PARN deadenylase is recruited to ARE sequences by ARE-binding protein KH-type splicing regulatory protein
(KHSRP) (Gherzi et al., 2004), CUG binding protein (CUG-BP; (Moraes et al., 2006) or tristetraprolin (TTP; (Korner and Wahle, 1997; Lai et al., 2003). In this study, the possible role of RNA binding protein nucleolin in the PARN-mediated regulation of p53 expression was examined. The data presented in this dissertation showed that nucleolin associates with p53 mRNA through an ARE element at the 3’ untranslated region (3’UTR), and its phosphorylation state affects both nucleolin targeting to p53 mRNA and PARN-dependent deadenylation (Figures 43-46). However, whether nucleolin and its phosphorylation state play a role in facilitating PARN recruitment to the target mRNA and promoting p53 mRNA degradation under non-stress conditions need to be further elucidated. It is also important to determine how nucleolin functional interaction with PARN changes during the DDR. It would be also important to study the possible regulatory effect on PARN deacylase of other ARE-binding proteins, such as HuR and Wig-1, that have been shown to bind the p53 3’UTR at an ARE signal proximal to miR-125b targeting site.

Although the exact contribution of miRNAs, miRISC, AREs and ARE-BPs to mRNA decay has not been elucidated yet, recent studies have revealed a functional overlap between ARE- and miRNA-mediated mRNA turnover regulatory pathways (Zhang et al., 2010). For example, it has been shown that the functional interaction of ARE-binding protein TTP and miR-16-loaded miRISC results in the recruitment of the deacylase and the exosome to tumor necrosis factor-α mRNA resulting in its degradation (Jing et al., 2005). Another example is the binding of HuR to AREs present in c-myc 3’UTR at a site proximal to that recognized by let-7 miRNA, facilitating the targeting of miRISC and mediating the reduction of c-myc mRNA levels (Kim et al.,
The studies presented in this dissertation indicate that 20% of PARN mRNA targets in the p53 signaling pathway showed both miRNA target sites and an ARE in their 3’UTRs (Table 1). It has also been shown that the presence of both miRNA binding sites and AREs at the 3’UTR is critical for PARN-dependent regulation of mRNA deadenylation and stability (Figures 30-33 and 39). However, further studies are necessary to determine whether the proximity of these regulatory elements is important in this PARN-associated regulatory pathway.

2) Characterization of the role of mRNA 3’ processing in regulating p53 mRNA expression during DDR:

p53 is an important tumor suppressor which is implicated in various cellular processes by regulating the expression of a large number of genes involved in DNA repair, cell cycle arrest, and apoptosis through transcription activation. Besides its well established role as a transcriptional regulator, p53 has also been described to have transactivation-independent functions, such as mediating expression of certain miRNAs (reviewed in (Takwi and Li, 2009) and regulating mRNA 3’ processing (Devany et al., 2013). It is the most commonly mutated gene in human tumors, and the regulation of p53 levels under different cellular conditions is critical. It is well established that in response to cellular stress signals, p53 protein accumulates as a result of the downregulation of Mdm2 levels, which acts as an E3 ligase targeting p53 for degradation under non-stress conditions.

The results presented in this dissertation showed that p53 and p53 signaling pathway are specific targets of PARN deadenylase under non-stress conditions. However, after UV-induced DNA damage, the changes in both p53 mRNA and protein levels are
PARN independent (Figure 14), suggesting that other mechanisms are involved in the regulation of p53 expression during DDR. Interestingly, data presented in this dissertation indicate that after UV irradiation Ago-2 translocates to cytoplasm and the abundance of p53 mRNA targeting miRNAs decreases, such as miR-125b and miR-504 (Figures 21 and 34). These observations suggest that when cells are under stressed conditions, changes in Ago-2 cellular localization and miRNA abundance might signal the DDR, leading to PARN dissociation from p53 mRNA. Since the regulation of p53 expression in non-stress conditions requires the cooperation of miRISCs, ARE-binding proteins and PARN deadenylase, an intriguing possibility could be that the dynamic change in the interaction of different factors with the same region of p53 3’UTR results in the stabilization of p53 mRNA, leading to the p53 induction in response to DNA damage.

HuR, an ubiquitously expressed ARE-binding protein that belongs to the Hu (ELAV) family, has been shown to bind an ARE element at p53 mRNA 3’UTR and increase p53 mRNA stability after stress (Zou et al., 2006). Importantly, a recent study described a combinatorial regulation of mRNA stability by HuR and miRNAs (Mukherjee et al., 2011). In that study it was shown that over 75% of mRNAs with miRNA binding sites also have HuR binding sites, and most of these signals are less than 10 nt distance from each other. HuR has been shown to be involved in miRNA-mediated regulation of mRNAs stability by either a competitive or cooperative function. For example, it has been shown that HuR plays a role in recruiting let-7-loaded miRISC to repress c-myc expression (Kim et al., 2009). On the other hand, it has also been shown that upon stress conditions HuR can outcompete and relieve CAT-1 mRNA from miR-
122-induced repression, leading to the stabilization of the mRNA in human liver cells (Bhattacharyya et al., 2006). As HuR recognizes the ARE sequence that overlaps with miR-125b targeting site in p53 mRNA upon DNA-damaging conditions, it is important to further investigate the potential functional overlapping of PARN/nucleolin-mediated inhibition of p53 expression under non-stress conditions and HuR-mediated induction of p53 expression upon DNA damage response.

3) **Determination of the mechanisms behind the PARN-mediated regulation of the steady-state levels of other target genes:**

PARN is one of the major mammalian deadenylases and is associated with a variety of important cellular processes through mediating the deadenylation of a specific subset of mRNAs. The data presented in this dissertation indicate that the mRNA levels of both housekeeping genes and ARE-containing genes are regulated by PARN deadenylase under different cellular conditions (Figure 10). As the tumor suppressors BARD1 (Cevher et al., 2010) and p53 (Devany et al., 2013) can activate PARN deadenylase, it is possible that malignant cells display altered levels of polyadenylation of specific mRNAs. In fact, increased expression of PARN has been detected in acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML; (Maragozidis et al., 2012), suggesting that the alteration of PARN deadenylase expression might be used as a potential biomarker for cancer cells. It has been shown that PARN is involved in the regulation of the stability of several cancer-related mRNAs, such as IL-8, Vascular endothelial growth factor (VEGF), c-myc, c-fos, c-jun, urokinase-type plasminogen activator (uPA) and TNF-α (Chou et al., 2006; Lai et al., 2003; Moraes et al., 2006; Suswam et al., 2008). This is consistent with the microarray results presented here: the
most affected genes by PARN depletion are p53-related genes that have been shown linked with cancerous growth and DNA repair activities. Interestingly, 20% of these PARN mRNA targets in the p53 signaling pathway showed both miRNA target sites and ARE in their 3’UTRs. The studies presented in this dissertation were carried out using p53 mRNA as a model to investigate the mechanism underlying PARN-dependent regulation of mRNA stability. Interestingly, like p53, both fos and myc mRNAs have proximal AREs and miRNAs targeting sites in their 3’UTRs. Besides the increase in c-fos and c-myc mRNA levels in PARN-depleted cells in non-stress conditions were confirmed by qRT-PCR (Figures 10 and 13). More importantly, functional overlap between ARE-and miRNA-mediated regulatory pathways has been described to regulate c-myc mRNA stability and translation levels (Kim et al., 2009). As mentioned before, c-myc expression is repressed by both miRNA let-7 and ARE-binding protein HuR, which binds to ARE present in c-myc 3’UTR at a site proximal to that recognized by let-7 miRNA (Kim et al., 2009). However, whether PARN is the deadenylase mediating c-myc mRNA reduction through association with either let-7-loaded miRISC or HuR is not known. Together, these studies and the results presented in this dissertation suggest a possible role of PARN in regulating genes containing a conserved arrangement of signals in their 3’UTR, a miRNA targeting site overlapping or in close proximity to ARE regulatory signal. Together, future studies designed to test this hypothesis and the studies presented in this dissertation will serve as a valuable framework both for understanding these critical biological processes and for developing appropriate therapeutic approaches to a variety of disorders, including cancer.
CHAPTER VII

EXPERIMENTAL PROCEDURES
Tissue culture methods - HeLa, HCT116, HCT116 p53/- cell lines were cultured in Dulbecco’s modified Eagles medium (DMEM)-10% fetal bovine serum (FBS) and 1% penicillin/streptomycin antibiotic. RKO, RKO-E6 cell lines were cultured in Eagle's minimal essential medium (EMEM)-10% FBS and 1% penicillin/streptomycin antibiotic supplemented with 2 mM glutamine.

DNA-damaging agents - 90% confluent cultures were exposed to UV and harvested at the indicated times. UV doses (40 J/m²) were delivered in two pulses using a stratlinker (Stratgene). Prior to pulsing, medium was removed and replaced immediately after treatment.

Knockdown expression of PARN or Ago-2 by siRNA - siRNAs specific for either human PARN or Ago-2 and the control siRNA used as non-silencing were obtained from Dharmacon RNA technologies. Cells were grown in a 10-cm plate in complete DMEM/EMEM. At 50-60% confluence, the cells were transfected with 100 nM of PARN, Ago-2 or control siRNA and 60 µl of Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. After culturing the cells in antibiotic/FBS free medium for 8 h, medium was changed to complete medium. After additional 16 h, cells were transfected again and harvested for analysis 48 h after the initial transfection. To determine the specificity of siRNAs used protein levels were monitored.

miRNA inhibitor expression plasmid transfection – Either miR-125b inhibitor expression plasmid (HmiR-AN0096-AM03, GeneCopoeia) or control plasmid (AM03, GeneCopoeia) were were transfected into HCT116 cells. Cells were grown in a 10-cm plate in complete DMEM. At 50-60% confluence, the cells were transfected with 24 µg
of either miR-152b inhibitor expression plasmid or control plasmid and 60 µl of Lipofectamine TM 2000 (Invitrogen) according to the manufacturer’s protocol. After culturing the cells in antibiotic/FBS free medium for 8 h, medium was changed to complete medium. After additional 16 h (24 h after the initial transfection), cells were harvested for analysis.

**Nuclear extracts (NEs) preparation** - After UV treatment, NEs were prepared from harvested cells essentially as described (Cevher et al., 2010; Nazeer et al., 2011). Cells were lysed by douncing in 4 ml of 10 mM Tris pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). Lysates were centrifuged for 10 min at 6000 g, and pellets were resuspended in 20 mM Tris pH 7.9, 1.5 mM MgCl₂, 25% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, and 0.3 M NaCl. Preparations were rocked for 30 min at 4°C and centrifuged for 30 min at 6000 g. Supernatants were quickly frozen and stored at −80°C.

**Fractionation assays** - The fractionation of HCT116 cells was performed using Subcellular Protein Fractionation kit (Thermo Scientific) following the manufacturer’s protocol. Equivalent amounts of cytoplasmic and nuclear component were subjected to SDS-PAGE and proteins were detected by immunoblotting using antibodies against Ago-2 (H-300, Santa Cruz Biotechnology), PARN (kindly provided by Dr. A. Virtanen, Uppsala University), Topoisomerase II (H-8, Santa Cruz) and actin (A2066, SIGMA).

**Immunoprecipitation assays** – 100 µg of total protein from NEs prepared from different cell lines was pre-cleared with 50 µl of protein-A-Sepharose and immunoprecipitated with polyclonal antibody against either PARN (H-105 Santa Cruz Biotechnology) or...
Ago-2 (Santa Cruz Biotechnology) bound to protein A-agarose beads. Antibodies were coupled to protein A-agarose beads for 3 h at room temperature in buffer IPP (50 mM Tris pH 7.4, 50 mM NaCl and 0.1% Nonidet P-40). Immunoprecipitations were carried out for 3 h at 4°C in 200 μl of buffer A (1× phosphate-buffered saline (PBS): 137 mM NaCl, 3 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 0.01% Nonidet P-40, 0.5 mM PMSF, and 0.04% bovine serum albumin). The beads were recovered by centrifugation and treated at 4°C with 50 μg of RNase A/ml for 10 min. Finally, washing was performed with buffer A plus increasing amounts of NaCl. Aliquots of pellets and supernatants were analyzed by SDS-PAGE and immunoblotting. Results from three independent samples were analyzed and quantified using Image J program.

**Purification of recombinant proteins** - A plasmid encoding the full-length Ago-2 (kindly provided by Dr. Novina, Harvard Medical School) was transformed into Rosetta cells and GST-fusion proteins were purified by binding to and elution from glutathione–agarose beads as described in (Wang et al., 2009). The plasmid encoding His-PARN and its derivatives were transformed into BL21 cells, His fusion proteins were expressed and purified by binding to and elution from Ni-Agarose column (Qiagen) as described (Nilsson and Virtanen, 2006).

**Protein-protein interaction assays** - 2 μg of GST-Ago-2 or GST was incubated with glutathione-agarose beads for 2 h at 4°C in 300 μl final volume of binding buffer (1xPBS, 0.04% bovine serum albumin, 0.5 mM PMSF, 0.001% NP40). Beads were washed extensively six times with binding buffer. 2 μg of His-PARN derivatives (full-length, N-terminal domain (NTD) and C-terminal domain) were added to the GST-/GST-Ago-2-
bound beads and incubated for 2 h at 4°C in 300 μl final volume of binding buffer. The beads were washed six times with binding buffer plus 300 mM NaCl, resuspended in loading buffer, and proteins were fractionated by 12% SDS-PAGE. Equivalent amounts of pellets and supernatants were analyzed by immunoblotting. 2 μg His-PARN was incubated with Ni-Magnetic beads for 2 h at 4°C in 300 μl final volume of binding buffer (20 mM HEPES pH 7.9, 0.5 M KCl, 0.5% NP-40, 10% glycerol, 2 mM-mercaptoethanol and 2.5 mM imidazole). Beads were washed extensively six times with binding buffer. 2 μg of GST-Ago-2 was added to the His-PARN-bound beads and incubated for 2 h at 4°C in 300 μl final volume of binding buffer. The beads were washed six times with binding buffer plus 400 mM NaCl, resuspended in loading buffer, and proteins were fractionated by 9% SDS PAGE. Equivalent amounts of pellets and supernatants were analyzed by immunoblotting.

**In vitro deadenylation assays** - Conditions for *in vitro* deadenylation assays were as described (Martinez et al., 2001). Deadenylation assays with His-PARN, derivatives of PARN and different concentrations of GST-Ago-2 or Flag-nucleolin phospho-variants (kindly provided by Dr. Saxena, Brooklyn College) were carried out in reaction mixtures containing 25 mM Hepes pH 7, 100 mM NaCl, 0.1 mM EDTA, 1.5 mM MgCl₂, 0.5 mM DTT, 2.5% polyvinyl alcohol, 10% glycerol, 0.25 U RNasin, and 10 nM ⁷MeGpppG capped *in vitro* transcribed L₅(A₃₀) RNA substrate, radioactively labeled by the inclusion of ³²P-α-UTP during *in vitro* transcription. Incubations were performed at 30°C for 30 min; the reactions were terminated and analyzed by electrophoresis in 10% polyacrylamide/7 M urea gels. Results from independent samples were quantified by using image J program.
**32P-labelled p53 3’UTR substrate preparation** - WT and mutant p53 3’UTRs were amplified from luciferase constructs by PCR using a forward primer including a T3 promoter and a reverse primer with 20 adenines to create poly(A) tail at the 3’ end of the transcript (Forward 5’-ATGGATTCAATTAACCCTCACTAAAGGAACATTCTCCA CTTCTTGTTCCTCCACTAC-3’ and Reverse 5’-GGATGATCCATAAGCTT(A)20TGG GATATAAAAAGGG-3’). The PCR fragments were digested with Hind III to generate the poly(A) tail. Then polyadenylated radiolabeled RNA substrates were synthetized by *in vitro* transcription with T3 polymerase as described (Cevher et al., 2010).

**NEs Deadenylation assays** - Conditions for NEs deadenylation assays were as described (Cevher et al., 2010). Deadenylation assays using equivalent amounts of total proteins from NEs from different cell lines with/without UV treatment or siRNA mediated knockdown of Ago-2 were carried out in reaction mixtures containing 25 mM Hepes pH 7, 100 mM NaCl, 0.1 mM EDTA, 1.5 mM MgCl₂, 0.5 mM DTT, 2.5% polyvinyl alcohol, 10% glycerol, 0.25 U RNasin, and 10 nM 7MeGpppG capped *in vitro* transcribed L₅(A₃₀) or p53 3’UTR RNA substrate, radioactively labeled by the inclusion of 32P-α-UTP during *in vitro* transcription. Protein concentrations of the NEs were equalized by Bradford assays (Bio-Rad) before used in deadenylation reactions. Incubations were performed at 30°C for 2 h; the reactions were terminated and analyzed by electrophoresis in 10% polyacrylamide/7 M urea gels. Results from independent samples were quantified by using image J program.

**RNA purification and microarray analysis** - Nuclear RNA was purified from HeLa cells using the RNeasy kit (Qiagen) following manufacturer’s protocol. The RNA
concentrations of the RNA samples obtained under different conditions were equalized. Equivalent amounts of purified RNA were used in microarray analysis. The GeneChip Human Gene 1.0 ST (Affymetrix) expression array was used. Microarray data were normalized using the Robust Multichip Average (RMA) method. Significant genes determined by t-test (P < 0.05) were subsequently subjected to pathway analysis using the Ingenuity Pathway Analysis database.

**Analysis of endogenous mRNAs or miRNA abundance by qRT-PCR** – Total RNA (nuclear or total) was purified from different cell lines using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s directions. The RNA concentration of the total RNA samples obtained under different conditions was equalized. Equivalent amounts of purified RNA (2 μg) were used as a template to synthesize cDNA using random hexamer primers or oligo-d(T) primers and MMLV reverse transcriptase (Promega) according to the manufacturer’s protocol. qPCR was performed using the reverse transcriptase products and Taqman master mix (Applied Biosystem). Commercially available primers (GAPDH, ACTIN, c-fos, c-myc, TP53, BCL-XL, HDAC1 and RB1CC1) were used in the qRT-PCR reactions (Applied Biosystems). miRNAs abundance was assessed by qRT-PCR using All-in-One™ miRNA qRT-PCR Reagent Kits and Validated Primers (GeneCopoeia). Relative levels were calculated using ΔCt method.

**RNA isolation and qRT-PCR analysis of mRNA half-lives** - Control and PARN knockdown RKO cells (see above) were treated with Act-D (8 μg/ml) for 30 min before the beginning of the time course. Nuclear RNA was purified at different time points and
analyzed by qRT-PCR. Total RNA was isolated at different time points using RNeasy Mini Kit (QIAGEN) according to the manufacturer’s directions. p53 mRNA abundance was assessed at each time point by qRT-PCR and normalized the non-PARN target gene actin.

**Constructs of Luciferase Reporter Vectors** - Luciferase vector pEZX-MT01 with TP53 miTarget™ miRNA 3' UTR target clones (product ID: HmiT054283) was purchased from GeneCopoeiaTM. Mutations in the miRNA targeting sites, ARE sequence or both signals of p53 3’UTR were introduced with the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) and the following primers 5’-GGGTCAATTTCCGTTCGCAATTCTGTTCTGATCTGCTTTTTCTTTTGAGACTGGG -3’ and 5’-CCCAGTCTCAAAAGAAAAAGCAGATCAGAACAGAATTCGCGA-ACGGAAATTGACCC-3’ for ARE sequence replacement, primers 5’- CTGGAGATCCACCAAGACTTGTTTATGATTTTTTTTTTTTTTTTTTTT-3’ and 5’-AAAAAAAGAAAAAGAAATCATAAAAACAAGTCTTGGGTGGATCCAG-3’ for miRNA targeting sites replacement and primers 5’-CCAAGACTTTTGTTATGATCTCCGTTCGCG- AATTCTGCTGTGATCTGCTTTTTCTTTTGAGACTGGG -3’ and 5’-CCAGTCTCA- AAGAAAAGCAGATCAGAACAGAATTCGCGAACGGACATGCATAAAACAG TCTTGG -3’ for both signals replacement following the manufacturer’s instructions. Plasmids were sequenced to confirm the presence of the mutation. 24 µg of the different luciferase constructs were transfected into cells using Lipofectamine™ 2000 reagent (Invitrogen).
**Luciferase assay** - Cells were co-transfected with 24 µg of different luciferase constructs (Lipofectamine TM 2000 reagent, Invitrogen) and either siRNA-targeting PARN or control siRNA. 48 h after transfection cells were harvested and dual luciferase assay was performed using Luc-pair miR Luciferase kit from GeneCopoeia following manufacturer’s instructions.

**RNA immunoprecipitation (RIP) assays** - Immunoprecipitation of nuclear RNA-protein complexes was performed as described (Selth et al., 2011). Briefly, cells were treated with 1% formaldehyde, then NEs were prepared followed by sonication. Extracts were treated with DNase (TURBO DNA-free Kit, Ambion), and the resulting material was immunoprecipitated with antibodies against PARN (H-105) or control rabbit IgG (Sigma). Protein-RNA complexes were treated with proteinase K and reversal of cross-linking. RNA was extracted from the IPs with phenol-chloroform and analyzed by RT-qPCR assays.

**RT-qPCR Assays** - As described before (Cevher et al., 2010; Nazeer et al., 2011), equivalent amounts (2 µg) of purified RNA were used as a template to synthesize cDNA using random hexamer primers, oligo-d(T) primers, and GoScript Reverse Transcriptase (Promega). Relative levels were calculated using the ΔCt method.

**RNA Pull-down** - Biotin-labeled RNAs were *in vitro* transcribed with the biotin RNA labeling mix (Roche) and T7 RNA polymerase (Promega) following manufacturer’s instructions. 3 µg of biotinylated RNA treated with RNase free DNase I (Promega) was heated to 90°C in RNA structure buffer (10 mM Tris pH7, 0.1 M KCl, 10 mM MgCl₂) and shifted to room temperature for 20 min for proper folding. Folded RNA was then mixed
with 1 mg of NEs in RIP buffer (150 mM KCl, 25 mM Tris pH 7.4, 0.5 mM DTT, 0.5% NP40, 1 mM PMSF and protease inhibitor) and incubated at room temperature for 1 h. 60 µl of washed Streptavidin magnetic beads were added to each reaction and incubated at room temperature for another 1 h. Beads were washed 3 times with RIP buffer and eluted in SDS buffer and analyzed by immunoblotting.

**RACE-poly(A) test (PAT) assays** - Nuclear RNA from RKO cells treated with PARN/control siRNA for 48 h (see above) was isolated using Rneasy Mini Kit (QIAGEN) according to the manufacturer’s directions. 100 ng of RNA was reverse-transcribed using oligo (dT)-anchor primer (5’- GGGGATCCGCGGTTTTT TT TT TT TT -3’) and GoScript Reverse Transcriptase (Promega). 1 µl of each cDNA was used for PCR amplification by GoTaq PCR mix (Promega) using p53 3’UTR specific primer (5’-CTGCATTTTCAC-CCCACCCTCC -3’ located 90 bp upstream of poly(A) site) and oligo(dT)-anchor. PCR products were separated in 8% PAGE and the gels were stained with 0.2 mg/ml Ethidium Bromide for 20 min as previously described (Kleiman et al., 1998).
CHAPTER VIII

REFERENCES


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