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Mechanisms of regulation of mRNA 3' processing by p53 pathway

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Mechanisms of regulation of mRNA 3' processing by p53 pathway

by

Emral Cakmak Devany

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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ABSTRACT

Mechanisms of regulation of mRNA 3’ processing by p53 pathway

by

Emral Cakmak Devany

Adviser: Professor Frida Esther Kleiman

Although the p53 network has been intensively studied, genetic analyses long hinted at the existence of components that remained elusive. This dissertation focuses on the study of the regulation of mRNA 3’ processing during DNA damage response (DDR) by the p53 pathway and the regulation of p53 expression by the mRNA 3’ processing machinery. The results in this dissertation revealed new roles of tumor suppressor p53 in mRNA 3’ processing. In Chapter II, I showed that p53 inhibits the cleavage step of polyadenylation reaction and that cells with different levels of p53 expression have different mRNA processing profiles. As part of the same response to DNA damage, my results indicate that p53 also activates PARN-dependent deadenylation in the nucleus (Chapter III). In Chapter IV, I demonstrated that p53 mRNA is one of the biological targets of nuclear PARN under non-stress conditions. Extending these studies, in Chapter V, I established that both AU-rich element (ARE) and miR-125b binding site are important for the binding of PARN to the p53 mRNA and activation of p53 pathway. Together these results show a feedback loop between PARN deadenylase and one of its targets, the tumor suppressor p53: While PARN keeps p53 levels low by destabilizing p53 mRNA through ARE- and microRNA-binding sites in non-stress conditions; the increase in p53 levels after UV treatment results in the activation of PARN deadenylase in a transcription-independent manner.
As the levels of p53 expression levels increase after DNA damage, the PARN-mediated down-regulation of p53 mRNA should be reverted during the progression of DDR. In Chapter VI, I found that under DNA damaging conditions HuR, a ubiquitously expressed ARE-binding protein, can compete for binding to the p53 3’UTR with both PARN and Ago-2, resulting in the release of PARN and Ago-2 from p53 mRNA and the increase of p53 expression levels.

Finally in Chapter VII, I analyzed the usage of alternative polyadenylation signals (APA) during DDR. My results indicate that increase in intronic-polyadenylated isoforms of genes involved in DDR occurred after UV treatment, indicating that APA might represent another potential mechanism of controlling gene expression during the response to DNA damage.

Together this dissertation provides new insights into p53 function and the mechanisms behind the regulation of mRNA 3’ end processing and hence gene expression in different cellular conditions.
SIGNIFICANCE

Every cell in human body is constantly exposed to stress caused by environmental stimuli resulting in average 74,000 damages per day. The cellular response to DNA damage is a protective mechanism against disease. This cellular response could either be in survival mode, where DNA repair occurs and gene expression is controlled along with cell cycle arrest, or in the cell death mode, where apoptosis is induced. Although the mechanistic connection between DNA repair/cell survival and apoptosis has not been well established, several proteins are involved in both pathways. One of these factors is the tumor suppressor p53, which is mutated in more than 50% of known human cancers. p53 is a well-known transcription factor that binds to p53-response elements in the promoter of genes in p53 pathway and regulates transcription. This dissertation reveals some of transcription-independent roles of this key protein and how it contributes to DNA damages response (DDR), showing that p53 regulates not only transcription but also post-transcriptional events. My studies indicate that p53 plays an important role regulating mRNA 3’ processing either by inhibiting the cleavage step of the polyadenylation reaction or activating PARN-mediated deadenylation. These findings lead to broader understanding of the interplay between tumor suppression and mRNA processing pathways following DNA damage. Importantly, these studies indicate that different cell lines exhibit different 3’ processing profiles depending on p53 expression levels, consistent with the idea that the interaction of the 3’ processing machinery and factors involved in the DDR/tumor suppression might result in cell-specific 3’ processing, hence gene expression, profiles.

The dynamic nature of mRNA 3’ 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. As the tumor suppressor p53 lies at the center of many signaling
pathways that are essential for regulation of cell growth and apoptosis induced by genotoxic and non-genotoxic stresses (Melino et al., 2002; Vogelstein et al., 2000; Vousden and Lu, 2002), the regulation of p53 expression is extremely important and tightly controlled under normal conditions and after stress. While most of the studies on the expression of genes involved in stress response pathways have traditionally focused on transcription as the major regulator, it has recently become apparent that post-transcriptional control of mRNA steady-state levels may play an equally important role. This dissertation shows a novel mechanism of control of p53 expression under non-stress conditions via destabilization of p53 mRNA by PARN deadenylase through AU-rich elements (AREs) and microRNA (miRNA)-binding sites in its 3’UTR.

However, p53 levels should increase after UV-induced DNA damage for the progression of DDR. While a functional overlapping between miRNA-induced silencing complex (miRISC) and PARN deadenylase is important for destabilization of p53 mRNA under normal conditions; a competition between a ubiquitously expressed ARE-binding protein HuR and PARN-associated miRISC component Ago-2 leads to higher p53 expression upon UV treatment. These findings describe a novel mechanism by which p53 expression can be controlled and regulated under different cellular conditions. Importantly, these studies are one of the first to demonstrate the functional overlapping between ARE- and miRNA-mediated mRNA turnover pathways, increasing the complexity of the signaling present in 3’UTR of different genes. Understanding the complexity of the signaling in the 3’UTR of genes and functional interaction of 3’ processing machinery and factors involved in DDR/tumor suppression might help us to understand cell-specific
profiles, improving the developing of new therapies and the identification of cancer subtypes.

Finally, evidence presented in this dissertation shows that transcripts of genes involved in DDR, such as RNAP II, p21 and Ephrin B2, undergo alternative intronic polyadenylation upon UV-induced DNA damage. Alternative polyadenylation appears to be an additional mechanism to control expression of these mRNAs by producing a shorter isoform of their mRNAs. These intronic polyadenylation events change the transcriptome of the cell, providing alternative ways to regulate gene expression during DDR. Importantly, these events may also lead to changes in the cell’s proteome, leading to shorter protein isoforms with either different or similar functions to the native protein. Although more work is necessary to identify the function of these shorter mRNA isoforms, this dissertation provides valuable information for understanding how alternative polyadenylation events contribute to cells’ rapid response to DNA damage.
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CHAPTER I

BACKGROUND
p53 pathway

The tumor suppressor p53 lies at the center of many signaling pathways that are essential for regulation of cell growth and apoptosis induced by genotoxic and non-genotoxic stresses (Melino et al., 2002; Vogelstein et al., 2000; Vousden and Lu, 2002). It has been shown that p53 expression can be regulated by different mechanisms, such as factors that promote p53 degradation (Mazan-Mamczarz et al., 2003); posttranslational modifications on p53 that change protein stability (Bode and Dong, 2004; Brooks and Gu, 2003); binding of transcription factors to p53 promoter that change gene expression (Liu et al., 2007; Raman et al., 2000); regulatory factors that change translation of p53 mRNA (Vilborg et al., 2010).

In normal cells under non-stress conditions, the level of p53 protein is very low due to the short half-life of the protein and the binding of other proteins, such as MDM2, COP1, PIRH2 or JNK, that promote p53 degradation via the ubiquitin/proteasome pathway (Vilborg et al., 2010). The expression of most of these genes is induced by p53, representing a feedback regulatory loop (Figure 1). After stress, p53 protein levels increase by inhibition of its interaction with MDM2 and other negative regulators (Mazan-Mamczarz et al., 2003), increase in p53 gene expression by transcription (Okoshi et al., 2008), induction of p53 mRNA translation (Vilborg et al., 2010), change in p53 activity by posttranslational modifications (Bode and Dong, 2004; Brooks and Gu, 2003). Upon activation, p53 can regulate many genes that are part of DNA damage response (DDR), cell cycle arrest and/or apoptosis through different mechanisms.

p53 protein can be divided into five domains, each corresponding to specific functions (Figure 2) (Dai and Gu, 2010): 1) The amino-terminus part (aa 1-42) contains
Figure 1. Activation of p53 upon cellular stress. Cellular stress, such as DNA damage or hypoxia, is recognized by mediators in the cell, such as ATM, CHK2 or p19Arf. These mediators activate p53 either through direct modification of p53 protein (phosphorylation, acetylation, sumoylation, etc.) or through inhibiting MDM2 or other negative regulators of p53. Active p53 induces MDM2, which in turn leads to a negative feedback loop to decrease p53 levels after cells recover from stress.

The acidic transactivation domain and the MDM2 protein-binding site. II) Region aa 40-92 contains series repeated proline residues that are conserved in the majority of p53 in different species. It also contains a second transactivation domain. III) The central region (aa 101-306) contains the DNA binding domain and it is important for transactivating properties of p53. It is the target of 90% of p53 mutations found in human cancers. IV) The oligomerization domain (aa 307-355) consists of a beta-strand followed by an alpha-helix, which are necessary for p53 dimerization. p53 is composed of a dimer of two dimers. A nuclear export signal (NES) is also localized in this oligomerization domain. V) The
carboxy-terminus of p53 (aa 356-393) contains 3 nuclear localization signals (NLS) and a non-specific DNA binding domain. This region can bind to single stranded RNA, DNA and damaged DNA with high affinity. (Liu and Kulesz-Martin, 2006). This region is also involved in downregulation of DNA binding of the central domain. It has been shown that p53 can specifically bind to 5’UTR region of Cdk4, FGF-2 and p53 mRNAs and regulate their expression (Galy et al., 2001; Miller et al., 2000; Mosner et al., 1995). It has also been suggested that p53 shows sequence-nonspecific RNA binding, with slight preference to certain mRNAs, regulating the expression of a wide range of mRNAs (Riley et al., 2006).

Posttranslational modifications regulate p53 activity in different conditions. At least 20 sites in the human p53 protein are modified depending on the type of damage and stress signaling pathways activated. Phosphorylation has been studied most intensively and has been found to play a critical role in the stabilization and activation of p53. Following DNA damage, p53 is phosphorylated at various sites (Bode and Dong, 2004). These phosphorylation events selectively activate various functions of p53 in response to DNA damage. For instance, while p53 phosphorylation at Ser 15, 20 and 37 has been associated with the rapid response to DNA damage and leads to reduced interaction of p53 with its negative regulator, MDM2 (Shieh et al., 1997), phosphorylation at Ser 46 has been associated with activation of apoptosis (Taira et al., 2007). The induced phosphorylation of p53 is mediated by DDR kinases, such as ATM following ionizing radiation-induced damage and ATR or DNA-PK following ionizing/UV irradiation (Bode and Dong, 2004). BRCA associated Ring Domain 1 (BARD1), independently of BRCA1, directs phosphorylation of p53 at Ser15 by binding to Ku-70, the regulatory subunit of DNA-PK
(Feki et al., 2005). BARD1 can bind to both unphosphorylated and Ser15 phosphorylated p53 (Feki et al., 2005). This is an important regulatory event because phosphorylation at Ser15 results in the dissociation of p53 from MDM2 and the initiation of DDR (Shieh et al., 1997).

Figure 2. Overview of p53 domain structure and post-translational modifications. The major sites for p53 phosphorylation, ubiquitination, neddylation, sumoylation, acetylation and methylation are plotted. The enzymes responsible for each type of modification are shown on the right. Abbreviations: TAD, transactivation domain; PRD, proline rich domain; DBD, DNA-binding domain; TD, tetramerization domain; CRD, C-terminal regulatory domain. Taken from (Dai and Gu, 2010).
One of the key regulators of p53 expression is the MDM2 protein. As MDM2 is a E3 ubiquitin ligase, its binding to p53 leads to inactivation and degradation of p53 protein via ubiquitin/proteasome pathway (Honda et al., 1997). This binding occurs within the p53 transactivation domain, interfering with recruitment of basal transcription machinery components (Thut et al., 1997). On the other hand, p53 binds specifically to the mdm2 gene and stimulates its transcription (Barak et al., 1993). This negative feedback loop serves to keep p53 expression controlled during DDR, inducing p53 expression after the damage and inhibiting the p53 pathway after the damage is repaired (Oren, 1999).

Although the role of p53 posttranslational modifications in controlling stability and accumulation upon stress has been studied extensively, it is becoming increasingly clear that regulation of p53 gene transcription and p53 mRNA translation is also important in the p53-mediated response to stress. Transcriptional regulation of p53 gene is partly mediated by the p53 promoter. p53 transcription is enhanced by Protein kinase C delta (PKCδ) and Homeobox A5 (HOXA5), which are involved in the regulation of growth, apoptosis, morphogenesis and differentiation of a variety of cell types in response to genotoxic stress (Liu et al., 2007; Raman et al., 2000). p53 transcription is repressed by the proto-oncogene BCL6 in germinal-center B cells (Phan and Dalla-Favera, 2004). Moreover, it has been shown that p53 regulates its own mRNA expression (Lynch and Milner, 2006).

In addition to p53 promoter regulation, a growing number of reports have addressed post-transcriptional regulation of the p53 mRNA (Figure 3). Using protein synthesis inhibitors, it was shown that the regulation of translation is necessary for efficient up-regulation of p53 expression after DNA damage (Fritsche et al., 1993). While the protein
RPL26, which is a ribosomal protein that is also targeted for degradation by binding to MDM2, acts on the 5’ untranslated region (UTR) to enhance p53 mRNA translation, nucleolin, a protein involved in synthesis and maturation of ribosomes, can prevent p53

![Image of regulatory proteins and miRNAs](image)

**Figure 3. Proteins and miRNAs that regulate p53 mRNA degradation-associated translation efficiency.** While RPL26 and Wrap53 bind to 5’ UTR and increase p53 protein levels, nucleolin inhibits translation. Mdm2 binds to coding region and stimulates p53 translation. Several miRNAs and an unidentified 40 kDa protein bind to 3’ UTR and decrease p53 expression. HuR and Wig-1 stabilizes p53 mRNA through their binding to AREs in the 3’ UTR. While RNPC1a destabilizes p53 mRNA under both normal and damaging conditions, Pdcd4 destabilizes p53 mRNA only under normal conditions through the ARE sequence in the 3’UTR. Gld-1 has been shown to inhibit translation of yeast p53 homolog CEP-1. Modified from (Vilborg et al., 2010).
translation through 5’UTR (Takagi et al., 2005). Furthermore it has been reported that p53 translation is regulated by base pairing interactions between 5’- and 3’UTR sequences and these interactions are important for both RPL26- and nucleolin-mediated regulation of p53 expression (Chen and Kastan, 2010). Besides, it has been shown that the Wrap53, a conserved natural antisense transcript of p53, targets p53 mRNA via the 5’UTR, increasing translational efficiency and p53 protein levels (Mahmoudi et al., 2009). p53 protein binds tightly to the 5’-UTR region and inhibits the translation of its own mRNA, most likely mediated by the p53-intrinsic RNA re-annealing activity (Mosner et al., 1995). MDM2 has been shown to bind to a sequence in the open reading frame (ORF) of p53 mRNA and stimulate p53 mRNA translation (Candeias et al., 2008). The binding of MDM2 to p53 mRNA also reduces the level of MDM2 available for ubiquitination of p53 protein, resulting in an increase of p53 protein levels.

Interestingly, the 3’UTR of p53 mRNA has several regulatory elements that affect p53 gene expression (Figure 3). In recent years, miRNA-induced silencing complexes (miRISC) that act on the 3’UTR of p53 mRNA regulating the p53 mRNA degradation-associated translation and, consequently, expression levels have been described. Several miRNAs are known to bind to p53 3’UTR and reduce p53 mRNA stability, such as miR-125a (Zhang et al., 2009), miR-125b (Le et al., 2009), miR504 (Hu et al., 2010) and the recently discovered miR25 and miR30d (Kumar et al., 2011). In addition to the miRNA binding sites, the p53 mRNA 3’UTR contains two AU-rich elements (AREs). Wild-type p53-induced gene 1 (Wig-1) (Vilborg et al., 2009) and HuR (Mazan-Mamczarz et al., 2003; Zou et al., 2006) stabilize p53 mRNA via their binding to the AREs present in the 3’UTR, resulting in an increase in p53 protein expression. While RNPC1a protein binds to the
proximal ARE and destabilizes p53 mRNA under both normal conditions and after DNA damage (Zhang et al., 2011); Pdcd4 binds to the proximal ARE and destabilizes the p53 transcript only under normal conditions (Wedeken et al., 2011). Gld1, a translational repressor implicated in multiple C. elegans germ cell fate decisions, binds to the 3’UTR of C. elegans p53 homolog CEP-1 mRNA and destabilizes it (Schumacher et al., 2005). A G-quadruplex structure, which is located downstream of p53 mRNA 3’ cleavage site, has also been described (Decorsiere et al., 2011). This structure protects p53 mRNA from degradation upon stress by binding to hnRNP H/F, which can bind to the G-rich sequences and play a role in 3’-end processing. Interestingly, the mRNA levels of p53 in the cell are very low and do not change after UV treatment (Lakin and Jackson, 1999). The mechanism(s) behind the regulation of the steady-state levels of p53 mRNA has(ve) not been elucidated yet.

It has been described that the induction of p53 expression upon ultraviolet (UV) treatment is associated with changes in the levels of total poly(A) mRNA (Ljungman et al., 1999; McKay and Ljungman, 1999). Interestingly, this p53-associated changes in poly(A) RNA levels might also be functionally related to the UV-induced inhibition of mRNA polyadenylation (Kleiman and Manley, 2001). As 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.

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 (p53 RE) that is found either in promoters or introns of target genes (el-Deiry et al., 1992; Tokino and Nakamura, 2000). For instance, activation of transcription targets, such as growth arrest and DNA damage inducible 45 (Gadd45), promotes DNA repair by Gadd45 association with the DNA replication and repair factor PCNA (Smith et al., 1994). The most important transcriptional target for p53-mediated cell-cycle arrest is p21, a potent inhibitor of several cyclin-dependent kinase (CDK) complexes (el-Deiry et al., 1993). p53 transcriptional control of apoptosis is mediated mainly by target genes such as PUMA and Noxa (Jeffers et al., 2003). Although it is well established that p53 is a transcriptional regulator, other transactivating functions of p53 have also been described (He et al., 2007; Takwi and Li, 2009). For example, certain miRNAs are activated 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).

*mRNA 3’ end formation: polyadenylation/deadenylation machineries.*

Almost all eukaryotic mRNA precursors 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 cleaved product (Figure 4) (Shatkin and Manley, 2000; Zhao et al., 1999). 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.
Figure 4. 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 (77 kD) 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 (Russell 2010; Zhao et al., 1999).
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; Takagaki and Manley, 1998; Takagaki et al., 1996) and in diseases especially in cancer (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 specificity 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), RNA polymerase II (RNAP II) and poly(A) polymerase (PAP) play a role in the cleavage reaction; CPSF, PAP, symplekin and poly(A) binding protein (PABP) are involved in the polyadenylation step.

CstF is one of the essential 3’ processing factors required for the endonucleolytic cleavage step and helps to specify the site of processing. CstF is a heterotrimeric protein with subunits CstF1 (50 kD), CstF2 (64 kD) and CstF3 (77 kD). CstF2 is largely responsible for RNA binding (MacDonald et al., 1994; Takagaki and Manley, 1997), while CstF3 is for interactions with other protein factors (Takagaki and Manley, 1994). Another subunit, CstF1, plays important roles in regulation of mRNA processing by interacting with other factors. It contains seven WD-40 repeats, which are characteristic of regulatory proteins (Neer et al., 1994) and are involved in protein-protein interactions (Takagaki et al., 1992). CstF1 has been shown to interact with the carboxy-terminal domain of RNAP II
largest subunit (RNAP II LS), likely facilitating the RNAP II-mediated activation of processing (Hirose and Manley, 1998; McCracken et al., 1997). It also has been shown that CstF1 interacts with BARD1 (Kleiman and Manley, 1999), and with the DNA replication/repair factor PCNA (Kleiman and Manley, 1999).

The turnover rates of mRNAs can vary in response to changes in the cellular environment and the mRNA poly(A) tail is one of the fundamental cis-acting elements required for proper mRNA degradation (Colgan and Manley, 1997; Zhao et al., 1999). In mammalian cells, the initiation and rate-limiting step in mRNA degradation is the removal of poly(A) tail (Wilusz et al., 2001). Although the polyadenylation reaction is relatively well understood and most of the polyadenylation factors have been identified, the mechanisms behind poly(A) removal have not yet been clearly elucidated. Poly(A) specific ribonuclease (PARN) is one of the major poly(A) specific 3’ exoribonucleases identified in mammalian cells (Chen and Shyu, 1995; Mitchell and Tollervey, 2000; Wu et al., 2005). PARN is expressed ubiquitously in all tissues of most eukaryotic organisms (Copeland and Wormington, 2001). 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 has been identified as an oligomeric, highly
processive, metal-ion dependent and cap-interacting poly(A) specific 3’ exonuclease (Grishin, 1998).

Gene expression and mRNA levels are accurately regulated under different cellular conditions. For example following UV-induced DNA damage, cellular levels of polyadenylated transcripts are transiently decreased and normal recovery depends on transcription-coupled repair (TCR) (Ljungman et al., 1999). This decrease is partly due to UV-induced inhibition of transcription (Donahue et al., 1994). However, UV-treatment has also an effect on posttranscriptional control points of gene expression. mRNA 3’ cleavage is inhibited as a result of the association of the tumor suppressors BARD1/BRCA1 with CstF (Kleiman and Manley, 2001) and the proteasome-mediated degradation of RNAP II (Kleiman et al., 2005), suggesting the existence of alternative mechanisms to regulate gene expression under different cellular conditions. BARD1 is a 97 kDa nuclear protein that associates with the breast cancer susceptibility gene product BRCA1. Both proteins possess N-terminal RING finger motifs and two BRCA1 C-terminal (BRCT) domains, which are responsible for the BRCA1/BARD1 interaction and are involved in DNA repair and cell cycle regulation. BRCA1/BARD1 stabilizes each other and their association enhances their functions (Wu et al., 1996). 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), and is abolished by siRNA-mediated depletion of BARD1 and BRCA1 (Kleiman et al., 2005). A Thr734 mutation in BARD1 abolishes not only the UV-induced phosphorylation of BARD1 but also the DNA-damage functions of BARD1, such as its interaction with CstF1, inhibition of mRNA 3’ cleavage and degradation of RNAP II (Kim et al., 2006). Another mutation in BARD1, Gln564His,
reduces the binding to not only CstF1, interfering with the role of BARD1 in mRNA 3’ processing (Kleiman and Manley, 2001), but also p53, interfering with the role of BARD1 in apoptosis (Irminger-Finger et al., 2001).

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. Recent studies from Dr. Kleiman’s lab have shown that following UV-induced DNA damage, the polyadenylation factor CstF1 directly interacts with C-terminal domain of the deadenylation factor PARN, and this is accompanied with an increase in PARN expression levels (Cevher et al., 2010). The formation of CstF1/PARN complex has a role in the activation of PARN-mediated deadenylation and in the inhibition of mRNA 3’ cleavage as a result of repressed CstF activity. BARD1 further activates deadenylation by PARN in the presence of CstF1. Under DNA-damaging conditions, the formation of BARD1/CstF/PARN complex plays a role in downregulating the mRNA levels of some housekeeping genes, such as GAPDH and β-actin. Interestingly, the BARD1/CstF/PARN complex also 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 (Cevher et al., 2010). This is the first report describing a mechanism of gene expression regulation in response to DNA damage involving the deadenylation/polyadenylation machinery (Figure 5). In untreated cells, the cap binding factor CBP80 binds to nuclear PARN and inhibits its deadenylase activity. The cap binding complex (CBC) also enhances the polyadenylation of pre-mRNAs by increasing the stability the RNA/CstF complex
Figure 5. 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).

(Flaherty et al., 1997). As a result of these functional interactions polyadenylation takes place and normal levels of total mRNA are observed. After UV, RNAP II-CstF holoenzyme stalls at the sites of DNA damage. BRCA1/BARD1 complex is recruited to the sites of DNA repair, inhibiting RNAP II and the associated polyadenylation machinery by ubiquitination followed by degradation of RNAP II. Under these conditions CBP80 dissociates from PARN and PARN binds to CstF/BARD1 complex. As a result of these functional interactions polyadenylation is inhibited and deadenylation decay pathway is activated, generating the observed decrease in total mRNA levels.
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. Interestingly, 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’UTRs are responsible for the selection among these alternative polyadenylation (APA) sites. Two major types of APA have been identified: APA in either 3’UTRs (3’UTR-APA) or introns (intron-APA). The selection between the distal or proximal alternative polyadenylation signals regulates 3’UTR length and causes the inclusion or exclusion of *cis*-acting RNA elements, such as AREs and miRNA target sequences, which are involved in polyadenylation/deadenylation processes (Figure 6). Hence, 3’UTR-APA can significantly impact mRNA stability and metabolism, resulting in gene expression regulation. 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 3’UTR-APA 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., 2009b; Sandberg et al., 2008; Zlotorynski and Agami, 2008).

Intron-APA events can result in change of ORF of mRNA due to the incorporation of intronic regions in the transcript or the elimination of number of exons, leading to a significant change in the mRNA length. These abnormal mRNAs can lead to a change in proteome where different isoforms of proteins may emerge and accumulate in the cell with potentially deleterious effects (Spraggon and Cartegni, 2013) (Figure 6).
Figure 6. Regulation of APA leads to changes in gene expression or proteome.

Intron-APA that often occurs within the first intron leads to shorter mRNA isoform, which in turn cause changes in cell’s transcriptome and proteome. 3’UTR-APA leads to the exclusion or inclusion of cis-acting RNA sequences, which might mediate polyadenylation/deadenylation processes and hence cause changes in gene expression.

**ARE-mediated deadenylation**

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;
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 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 the stability of ARE-containing mRNAs. ARE-BPs either recruit deadenylases to the target mRNAs promoting mRNA degradation or block the recruitment of deadenylases and exosome stabilizing the mRNA (Figure 7). 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). 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.
Figure 7. 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. Taken from Zhang et al. (2010).

So far, 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). HuR is a ubiquitously expressed ARE-BP that belongs to the Hu (ELAV) family of RNA binding proteins, which also includes the neuro-specific proteins HuB, HuC, and HuD. It is mainly localized in the nucleus. It has reported roles in splicing, mRNA processing and translation (Mukherjee et al., 2011). HuR binds its substrates in the nucleus as early as co-transcriptionally, and escorts them to cytoplasmic polysomes to stabilize and enhance translation (Fan and Steitz, 1998), Mukherjee et al., 2011). It has been shown that HuR predominantly targets U-rich stretches of single stranded RNA (Uren et al., 2011).
Many HuR targets encode proteins important for cell growth, proliferation, cell death, and immune response such as FOXO1 (Li et al., 2013), p21 (Wang et al., 2000) and IL-17 (Chen et al., 2013). Network analysis of identified HuR targets show that those factors are highly interconnected, suggesting that HuR might regulate specific pathways (Uren et al. 2011). Importantly, HuR has also been shown to bind the AREs in p53 3’UTR and increase p53 mRNA stability (Zou et al., 2006) and translation (Mazan-Mamczarz et al., 2003) after stress.

**miRNA-mediated deadenylation**

miRNAs comprise a large family of small single-stranded non-coding RNAs (~21nt in length), which 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. miRNAs act at the post-transcriptional level to regulate 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. 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). 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. It has also been described that knockdown of Ago-2 in human HEK293 cells lead to a more profound effect on miRNA-mediated repression than the knockdown of the other three Ago proteins, suggesting that in mammals Ago-2 may have some specific functions that other Ago proteins cannot complement (Schmitter et al., 2006).

miRNAs can destabilize target mRNAs through deadenylation and subsequent decay (Figure 8). Several studies have shown that the levels of specific miRNAs, or the activity of the miRNA machinery, have severe effects on the level of miRNA-targets. 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). Both the knockdown of human Ago-2 and disruption of GW182-Ago interaction abrogate miRNA-mediated deadenylation (Eulalio et al., 2008; Till et al., 2007). The cytoplasmic
poly(A) binding protein 1 (PABPC-1) is also critical for 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 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. 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, knockdown of CAF1 or NOT1 expression and the over-expression of CCR4 or CAF1 mutants significantly reduce miRNA-mediated deadenylation and mRNA decay, but not translational repression (Behm-Ansmant et al., 2006; Fabian et al., 2009; Piao et al., 2010).

Functional Overlapping of ARE- and miR-mediated mRNA regulation.

Interestingly, some of the seed signals recognized by miRNA overlap with AREs in the 3’UTR of a number of mRNAs (Bhattacharyya et al., 2006a; 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).
Figure 8. Model of miRNA-mediated deadenylation. miRISCs, which contain Ago, GW182, PABPC1 and CAF1-CCR4-NOT deadenylase, deliver miRNAs to the target mRNAs and mediate deadenylation, which leads subsequently to mRNA degradation. Taken from Zhang et al. (2010).

It has been shown that ARE-binding protein TTP and miR-16 cooperates 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. A recent study has shown that HuR sites in 3'UTRs of mRNAs overlap extensively with predicted miRNA target sites (Uren et al., 2011), suggesting interplay between the functions of HuR and miRNAs. Consistent with this, it has been reported that over 75% of mRNAs with Ago-2 binding sites also have HuR binding sites (Mukherjee et al., 2011). Most of these Ago-2 and HuR binding site pairs are in less than 10 nt distance, suggesting a competitive or cooperative regulation of target mRNAs (Mukherjee et al., 2011). In fact, earlier studies have shown both cooperation and competition between miRISC- and HuR-dependent regulations of target mRNAs. For instance, HuR recruits let-7/RISC to repress c-Myc
Figure 9. Model of overlapping in the ARE - and miRNA-regulated deadenylation. A) Cooperative regulation of mRNAs by ARE s-BPs and miRNAs. The recruitment of the ARE -BPs HuR to the ARE sequence assists the targeting of let-7-loaded miRISC complexes to the most proximal site to the ARE sequence of c-myc mRNA. Taken from Zhang et al. (2010). B) Competitive regulation of mRNAs by ARE-BPs and miRNAs. HuR can outcompete and relieve CAT-1 mRNA from miR122-mediated repression upon environmental stimuli. 

expression (Kim et al., 2009). However, HuR can also outcompete and relieve CAT-1 mRNA from miR122-mediated repression upon environmental stimuli, which resulted in changes of target mRNAs stability in the cytoplasm (Bhattacharyya et al., 2006).
CHAPTER II

EFFECTS OF TUMOR SUPPRESSOR p53 ON mRNA 3’ END PROCESSING
INTRODUCTION

The p53 gene is the most commonly mutated target in human tumors. Activation of p53 affects the expression level of a large set of genes and mediates several cellular responses, such as DNA repair, cell cycle arrest and/or apoptosis (Vogelstein et al., 2000; Levine et al., 2006). Although it is well established that p53 is a transcriptional regulator, transactivation-independent functions of p53 have been described (reviewed by He et al., 2007; Takwi and Li, 2009). For example, certain microRNAs are transactivated by p53, and these microRNAs cause dramatic changes in gene expression, offering an indirect p53-mediated control of gene expression at the posttranscriptional level (Chang et al., 2007). It has been described that the induction of p53 expression upon ultraviolet (UV) treatment is associated with changes in the levels of total poly(A) mRNA (Ljungman et al., 1999; McKay and Ljungman, 1999). Interestingly, this p53-associated changes in poly(A) RNA levels might also be functionally related to the UV-induced inhibition of mRNA polyadenylation (Kleiman and Manley, 2001). As 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.

The 3’ end of the mRNA is processed by the cleavage of the mRNA followed by the addition of a non-template polyadenylated tail, which in mammalian cells is of approximately 200–300 adenosines. The assembly of the cleavage/polyadenylation machinery requires specific signal sequences in the mRNA precursor as well as interactions of a large number of protein factors (reviewed by Mandel et al., 2008; Zhao et al., 1999). It has been shown that the regulation of mRNA 3’ end formation can have significant roles in cancer (Kleiman and Manley, 2001; Topalian et al., 2001; Scorilas, 2002; Rozenblatt-
Rosen et al., 2009). Most importantly, alternative mRNA cleavage and polyadenylation changes the length of the 3’-untranslated region (3’UTR) and regulates gene expression of different mRNAs in cancer cells (Mayr and Bartel 2009; Singh et al., 2009) and during cell differentiation (Sandberg et al., 2008; Zlotorynski and Agami, 2008; Ji et al., 2009). Cleavage stimulation factor (CstF) is one of the essential 3’ processing factors and is most likely active as a dimer of an heterotrimer, consisting of three protein factors called CstF3, CstF2 and CstF1. CstF2 interacts directly with the mRNA, and cells deficient in CstF2 undergo cell cycle arrest and apoptotic death (Takagaki and Manley, 1998). Both the CstF1 and CstF3 subunits interact specifically with the C-terminal domain of RNA polymerase II (RNAP II), likely facilitating the RNAP II-mediated activation of 3’ end processing (McCracken et al., 1997; Hirose and Manley, 1998). After DNA damage, mRNA 3’ processing is inhibited as a result of CstF/BARD1/BRCA1 complex formation (Kleiman and Manley, 1999) and of the proteasome-mediated degradation of RNAP II (Kleiman et al., 2005), suggesting the existence of possibly redundant mechanisms to explain the inhibitory effect of UV irradiation.

Dr. Nazeer and colleagues (2011) found that p53 functionally interact with BARD1 and CstF1, having an effect on mRNA 3’ processing as well as on the polyadenylation of cellular RNAs. As part of that study, they showed the direct interaction of the C-terminal domain of p53 with CstF1 and BARD1, and the existence of protein complexes of these factors in extracts of different cell lines. Importantly, p53 inhibits mRNA 3’ cleavage in vitro. Supporting these results, a tumor-associated mutation in p53 not only decreases the interaction with BARD1 and CstF, but also decreases the UV-induced inhibition of 3’ processing, all of which is restored by wild-type-p53 expression. Nazeer and colleagues
also found that p53 expression levels affect the polyadenylation levels of housekeeping genes, but not of p21 and c-fos genes, which are involved in DDR. As part of those studies, I determined that there is a reverse correlation between the levels of p53 expression and the levels of mRNA 3’ cleavage under different conditions. Taken together, these results suggest a novel function of p53 as an inhibitor of the mRNA 3’ processing machinery.

RESULTS

To further investigate the functional overlapping of BARD1, CstF1 and p53, Nazeer and colleagues (2011) examined the physical association of p53 with BARD1 and CstF1 by pull-down assays using recombinant proteins. These studies showed a direct interaction of the C-terminal domain of p53 with CstF1 and BARD1, both of which are involved in the UV-induced inhibition of mRNA 3’ processing. Interestingly, the C-terminal domain of p53 has been described to have regulatory functions in DDR (Figure 2) (Sauer et al., 2008). Coimmunoprecipitation assays indicated the existence of protein complexes of these factors in nuclear extracts (NEs) of different cell lines (Nazeer et al., 2011). Although these results do not show how many complexes p53 can form with CstF and BARD1, they clearly show that UV treatment induced the interaction between those three factors, supporting the idea that p53 and CstF might be simultaneous binding partners of BARD1. In vitro RNA cleavage assays were performed with limiting amount of NE of HeLa cells and addition of increasing amounts of different His-tagged p53 derivatives. Interestingly, the same region of p53 required for binding CstF1 and BARD1 is necessary for inhibiting mRNA 3’ cleavage.
To further characterize the role of p53 in mRNA 3’ processing, I performed siRNA-mediated knockdown of p53 in cells expressing different levels of p53 and then analyzed the UV-induced inhibition of 3’ cleavage. The cell lines included in this study were colon carcinoma RKO cells that express normal levels of p53; the isogenic cell line RKO-E6 and HeLa cells that express low levels of p53 due to a stably integrated papilloma virus; and lung cancer H-1299 cells that carry an homozygous partial deletion of p53 and lack expression of the protein. Figure 10C shows that a 48 h siRNA treatment resulted in a substantial depletion of p53 (~90%) in NEs of RKO cells independently of the UV treatment. Importantly, the expression levels of p53 in all the RKO cells samples were much higher than that in the other cell lines analyzed. NEs were prepared from cells treated with UV irradiation and allowed to recover for 2 h. Consistent with previous results (Noda et al., 2000), UV treatment significantly increased accumulation of p53 in RKO cells and this was not affected by control siRNAs (Figure 10C). NEs from those cells were analyzed for L3 pre-mRNA 3’ cleavage assay. My results indicate that NEs from cells expressing normal levels of p53 showed no significant levels of 3’ cleavage using this assay (Figure 10A). The lack of detectable levels of 3’ processing in NEs of RKO cells was irrespective of control siRNA and UV treatments. These results are consistent with the possibility that high levels of p53 inhibit 3’ mRNA processing. Supporting this idea, siRNA-mediated knockdown of p53 in RKO cells resulted in extracts exhibiting significant levels of 3’ cleavage. It is important to highlight that p53 siRNA treatment did not completely deplete p53 in UV-treated RKO cells. Interestingly, the samples from p53 siRNA-treated RKO cells showed similar levels of both p53 expression and 3’ cleavage to samples from non-
treated HeLa cells. These results indicate that p53 has an inhibitory effect on mRNA 3’ cleavage and that this effect is dependent on the cellular levels of p53.

Interestingly, samples of RKO-E6 cells behaved similarly to those of HeLa cells, showing detectable levels of 3’ cleavage in UV-treated. Samples from lung cancer H-1299 cells showed high levels of 3’ processing independently of UV treatment. To further test the role of p53 in mRNA 3’ processing I performed in vitro RNA cleavage assays with limiting amount of NE of RKO-E6 and H-1299 cells and addition of increasing amounts of full-length His-p53. RKO-E6 and H-1299 cells provided a good system to study exogenously added p53 as these cells have very low levels of p53. Interestingly, the addition of increasing amounts of full-length His-p53 to limiting amounts of NE from RKO-E6 and H-1299 cells significantly reduced the 3’ cleavage of the radiolabeled adenoviral L3 pre-mRNA (Figure 10B).

Taken together, these results indicate that this p53 function in RNA 3’ processing is not a cell type-specific effect and that p53 can inhibit the 3’ cleavage step of the polyadenylation reaction in an expression level-dependent manner. Besides, the addition of recombinant His-p53 to a cell-free assay is sufficient to induce inhibition of mRNA 3’ end processing, suggesting that the p53-mediated inhibition of 3’ processing is independent of transactivational functions of p53.

DISCUSSION

This is the first study showing a 3’ RNA processing inhibitory function of p53, adding a new level of complexity to the DDR by linking RNA processing to the p53
Figure 10. The levels of 3’ cleavage inversely correlate with expression levels of p53. A) Samples from cells expressing different levels of p53 show different levels of mRNA 3’ processing. NEs from RKO, RKO-E6 and H-1299 cells were treated with UV irradiation, allowed to recover for 2 h and then were analyzed for L3 pre-mRNA 3’ cleavage. NEs from RKO cells treated with p53/control siRNA and UV irradiation were also analyzed. HeLa cell samples were also included as a control. B) p53 can inhibit 3’ cleavage in samples from RKO-E6 and H-1299 cells. NEs from RKO-E6 and H-1299 cells were preincubated with no addition or increasing amounts of recombinant full-length His-p53 derivative (40, 80 and 120 ng). After 15 min, L3 pre-mRNA was added and incubation continued for 90 min. C) Comparison of p53 expression levels in NEs from RKO, RKO-E6, H-1299 and HeLa cells. Protein concentrations from NEs from UV-treated/untreated and siRNA-treated/untreated cells were normalized by immunostaining with antibodies against Topoisomerase II (Topo II).
network. First, Nazeer and colleagues (2011) showed the direct interaction of the C-terminal domain of p53 with CstF1 and BARD1, both of which are involved in the UV-induced inhibition of mRNA 3’ processing, and the existence of protein complexes of these factors in extracts of different cell lines. Second, p53 can inhibit the 3’ cleavage reaction in vitro and p53 expression levels inversely correlate with levels of mRNA 3’ cleavage (Figure 10 and (Nazeer et al., 2011). Supporting these results, a tumor-associated mutation in p53 not only decreases the interaction with BARD1 and CstF, but also decreases the UV-induced inhibition of 3’ processing, all of which is restored by wild-type-p53 expression. Nazeer and colleagues (2011) also found that p53 expression levels affect the polyadenylation levels of housekeeping genes but not of genes involved in DDR. Taken together, these results suggest a novel function of p53 as an inhibitor of the mRNA 3’ processing machinery.

It has been proposed in previous studies from Dr. Kleiman’s lab that there is a general effect of DNA-damaging conditions on mRNA levels, and that the UV-induced inhibition of 3’ end processing plays an important role in decreasing the total cellular mRNA levels as part of this response (Cevher and Kleiman, 2010). Consistent with this, the levels of poly(A) mRNA of genes not involved in DDR decrease after DNA damage (Ljungman et al., 1999; Dheda et al., 2004; Akeo et al., 2007; Maccoux et al., 2007; Mirkin et al., 2008). It has been shown that full recovery of total mRNA levels within 6 h after the DNA-damaging exposure correlates with cellular protection against apoptosis in a p53-dependent manner (McKay et al., 2001). On the other hand, there is also a gene-specific effect of DNA-damaging conditions on the levels of poly(A) mRNAs of genes involved in the DDR, those genes are either down- or up-regulated at different time points after DNA
damage (reviewed by Cevher and Kleiman, 2010). Nazeer and colleagues (2011) discovered that under DNA-damaging conditions, p53 in association with the 3’ processing factor CstF and the tumor suppressor BARD1 can control mRNA 3’ processing of housekeeping genes, but not of p21 and c-fos genes, which are involved in DDR. On the basis of these results, it has been proposed that p53, a protein with compromised expression in most cancers, plays a role in the general response to DNA damage by stabilizing the CstF/BARD1 complex and inhibiting 3’ processing of aborted nascent RNA products, allowing the elimination of prematurely terminated transcripts to avoid the expression of deleterious proteins and facilitating DNA repair. As DNA repair proceeds, the levels of p53 expression decrease allowing the recovery of total mRNA levels. My results (Figure 10) indicate that different cell lines exhibit different 3’ processing profiles depending on p53 expression levels, consistent with the idea proposed by 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.

Supporting the idea that the 3’ processing machinery is interconnected with the p53 pathway, it has been shown that Rbbp6, a p53-binding protein, and Ku-70 subunit of DNA-PK, which is involved in the BARD1-mediated phosphorylation of p53 Ser15 upon DNA damage (Fabbro et al., 2004), are part of the pre-mRNA 3’ processing complex (Shi et al., 2009). Other tumor suppressors, such as CSR1 and Cdc73, have also been shown to functionally associate with 3’ processing factors, such as CPSF3 (Zhu et al., 2009) and CPSF/CstF (Rozenblatt-Rosen et al., 2009). Recently, it has been shown that the use of alternative mRNA 3’ cleavage and polyadenylation sites can control the expression of certain genes by eliminating or including several cis-acting elements, such as microRNA
target sites and AU-rich elements, in cancer cells and during development (Sandberg et al., 2008; Zlotorynski and Agami, 2008; Ji et al., 2009; Mayr and Bartel, 2009; Singh et al., 2009). Although more work is necessary to determine the functional relevance of the CstF/BARD1/p53 interaction in the regulation of expression of specific genes involved in DDR, it is possible that the p53-mediated inhibition of mRNA 3’ processing might also play a role in the selection of different alternative mRNA 3’ cleavage sites and, consequently, in the regulation of the mRNA levels of genes involved in DDR. Considering that the p53 pathway is tightly controlled in cells following DNA damage (reviewed by Vousden, 2006), the p53-associated control of mRNA 3’ processing could be an effective mechanism employed to control gene expression in cells upon DNA-damaging conditions.

Taken together, these studies identified a novel 3’ RNA processing inhibitory function of p53 and suggest that the CstF/BARD1/p53 interaction contributes to UV-induced inhibition of pre-mRNA 3’ processing, providing evidence of another link between mRNA 3’ processing and tumor suppression.
CHAPTER III

EFFECTS OF TUMOR SUPPRESSOR p53 ON NUCLEAR mRNA DEADENYLATION
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 principal structures required for correct regulation of mRNA degradation. The poly(A) tails are also crucial for regulation of mRNA processing, translation and subcellular localization, such as nuclear export (Colgan and Manley, 1997; Zhao et al, 1999; Mandel et al, 2008). 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. In mammalian cells, the earliest and rate-limiting step in mRNA decay is the removal of the mRNA poly(A) tail (Wilusz et al, 2001; Chen and Shyu, 2003). Poly(A)-specific ribonuclease (PARN) is one of the major nuclear deadenylase in mammalian cells. It is reported to bind to both 5’cap and poly(A) tail concurrently increasing its processivity and enhancing the degradation rate (Dehlin et al., 2000; Martinez et al., 2001). It has been reported that the cap binding factor CBP80 binds PARN and inhibits its deadenylase activity, representing a mechanism by which PARN is recruited to the nascent pre-mRNA and ensuring that PARN does not degrade the poly(A) tail of pre-mRNAs (Balatsos et al., 2006). PARN also interacts with mRNA 3’ processing factor CstF1 and the BRCA1-associated RING domain protein (BARD1) upon DNA damage conditions, reverting PARN inhibition by CBP80 (Cevher et al., 2010). Another PARN interactor is the peripheral exosome subunit Mpp6. This interaction mediates the recruitment of the exosome to deadenylated mRNAs for subsequent 3’→5’ degradation (Lehner and Sanderson, 2004).
Recent studies have shown that PARN expression and phosphorylation state are altered in cancer (Maragozidis et al., 2012), indicating that PARN deadenylase regulation is critical in tumorigenesis. Consistent with this, PARN is involved in the degradation of several cancer-related genes (Cevher et al., 2010; Lai et al., 2003; Moraes et al., 2006) and can functionally interact with the tumor suppressor BARD1 (Cevher et al., 2010). PARN also participates in cytoplasmic functions as part of the dendritic cytoplasmic polyadenylation element binding protein (CPEB)-associated polyadenylation apparatus that is involved in hippocampal synaptic plasticity during learning and memory processes (Udagawa et al., 2012). Synaptic stimulation induces phosphorylation of CPEB, PARN expulsion from the ribonucleoprotein complex, and polyadenylation of mRNAs in dendrites.

Mechanisms controlling deadenylation are highly regulated and play key roles in cellular responses, such as mRNA surveillance, DDR, and tumor progression, as well as cell development and differentiation (Cevher and Kleiman, 2010; Cevher et al., 2010; Ji et al., 2009b; Mayr and Bartel, 2009; Singh et al., 2009). Interestingly, 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 (Cevher et al., 2010). As described in Chapter II, CstF1 can also form a complex with another tumor suppressor, p53 (Nazeer et al., 2011). The functional interaction between p53/CstF/BARD1 leads to inhibition of mRNA 3’ cleavage step of polyadenylation reaction.
Extending those studies, I explored the possibility that p53 might regulate not only mRNA 3′ cleavage (Nazeer et al., 2011) but also PARN-dependent deadenylation in different cellular conditions.

RESULTS

To investigate the role of p53 in deadenylation, I used a group of isogenic cell lines that express different levels of p53 (Figure 1A): the colon cancer HCT116 and p53-null HCT116 cell lines, the colon carcinoma RKO and RKO-E6 cell lines, and the mouse embryonic fibroblasts (MEFs) and p53-null MEFs. Nuclear extracts (NEs) from those cells were assayed for deadenylation activity using a radiolabeled L3(A30) RNA substrate as described (Cevher et al., 2010). As described in previous studies (Cevher et al., 2010), deadenylation activity in NEs of RKO cells treated with control siRNA increased significantly after UV treatment (Figure 1A). Interestingly, siRNA-mediated knockdown of p53 in RKO cells abolished the UV-induced activation of deadenylation, suggesting that p53 might activate deadenylation. Consistent with this, RKO-E6, p53-null HCT116 and p53-null MEFs cells did not show UV-induced activation of deadenylation, which was observed in RKO, HCT116 and MEFs cells. Levels of p53 and PARN proteins in the cells treated with control, UV and/or p53 siRNAs are shown in Figure 11B. Together, these results indicate that p53 expression levels correlate with levels of mRNA deadenylation, suggesting that p53 might be an activator of deadenylation during DDR.

To further determine whether PARN is the target of p53 activation, *in vitro* deadenylation assays were performed using limited amounts of His-PARN and increasing amounts of different His-p53 derivatives (Figure 12). Importantly, only increasing amounts
Figure 11. The levels of deadenylation correlate with expression levels of p53.

A) Samples from cells expressing different levels of p53 show different levels of deadenylation. A representative deadenylation reaction from three independent assays is shown. siRNA-mediated knockdown of p53 abolishes UV-induced activation of deadenylation in RKO cells. NEs of the indicated cells treated with UV irradiation and allowed to recover for 2 h were analyzed for radiolabeled L3(A30) deadenylation. NEs from RKO cells treated with p53/control siRNA and UV irradiation were also analyzed. Positions of the polyadenylated RNA L3(A30) and the L3 deadenylated product are indicated. Numbers beneath gel lanes indicate relative deadenylation (RD). RD was calculated as [L3 fragment/(L3 fragment + L3 (A30))] × 100. Quantifications were done with ImageJ software (http://rsb.info.nih.gov/ij/). B) Levels of p53 and PARN proteins in the cells treated with control, UV and/or p53 siRNAs are shown.
of either full-length His-p53 (Figure 12A-B) or the C-terminal fragment of p53 (Figure 12B) can induce deadenylation in a reaction using His-PARN in a cell-free assay, suggesting that this is a transactivation-independent function of p53. However, neither the two p53 derivatives that lacked the C-terminal region of p53 nor GST alone had an effect

Figure 12. p53 activated PARN deadenylase in vitro. A) p53 can activate PARN-dependent deadenylation in vitro. Deadenylation assays using different concentrations of His-PARN were performed in the presence of capped L3(A30) RNA substrate as described and increasing amounts of His-p53. Reactions were analyzed as in Figure 11A. B) The C-terminal domain of p53 activates PARN in vitro. Deadenylation assays were performed as in A with the addition of either full-length p53 (FL) or His-p53 derivatives. The truncated forms of p53 used in this assay were described before (Nazeer et al., 2011) and include p53 amino acids 1-293, p53 amino acids 94-293 (DNA binding domain) and p53 amino acids 94-393.
on the deadenylation reaction (Figure 12B). None of the His-p53 derivatives was able to
deadenylate the substrate in the absence of His-PARN (Figure 12B). Taken together, these
results indicate that this p53 function in deadenylation is not a cell type-specific effect and
that p53 can activate PARN deadenylation in an expression level-dependent manner.

To further analyze the role of p53 in PARN-mediated deadenylation, I examined the
physical association of p53 with PARN deadenylase. Pull-down (Figure 13A) and
coimmunoprecipitation (co-IP) assays (Figure 13D) indicate that p53 can form (a) protein
complex(es) with PARN in NEs from RKO cells in non-stress conditions and after UV
treatment. My results showed that the C-terminal domain of PARN (Figure 13B), which
has been described to interact with CstF1 and CBP80, and the C-terminal domain of p53
(Figure 13C), which has been described to have regulatory functions in DDR (Sauer et al.,
2008), are important for the complex formation. As samples were treated with RNase A,
the observed interactions were probably not due to an RNA tethering effect. Together these
results indicate that p53 can interact with PARN to form (a) complex(es) and that p53
expression levels can activate PARN deadenylase and, therefore, might regulate gene
expression.
Figure 13. p53 interacts with PARN to form a protein complex. A) Immobilized His-PARN (left panel) or His-p53 (right panel) was incubated with NEs from untreated or UV-treated RKO cells. Equivalent amounts of the pull-downs (PD) and supernatants (SN) were analyzed by immunoblotting with the indicated antibodies. 20% of the NE used in the PD reactions is shown as input. 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. B) C-terminal domain of PARN interacts with p53. PD assays were performed as in (A) using full-length, N-terminal domain (NTD) or C-terminal domain (CTD) of His-PARN C) C-terminal domain of p53 interacts with PARN. Pull-down assays were performed as in (A) with full-length or the His-p53 derivatives described in Figure 12B. D) PARN and p53 co-IP from NEs of HeLa cells. The NEs were IPed with anti-p53 and anti-PARN. Equivalent amounts of the SN and the pellets (IP) were analyzed by immunoblotting using the indicated antibodies 20% of the NE used in the IP reaction is shown as input.
DISCUSSION

Here I described a novel function of p53 in nuclear mRNA 3′ processing and deadenylation. First, I showed that 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, I showed the 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). Previous work from Dr. Kleiman’s lab has shown the formation of the PARN/CstF/BARD1 complex after UV-induced DNA damage (Cevher et al., 2010). The results I presented here indicate that p53 protein is a part of this complex and activates PARN-dependent deadenylation after UV treatment. Although these results indicate that this complex formation and activation of deadenylation might be part of the general response to UV-induced DNA damage, further studies are needed to determine whether p53-mediated activation of PARN plays a role in the gene specific response to UV treatment.

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). The change in the levels of total poly(A) mRNA levels might represent an inhibition in their formation or an activation in their deadenylation degradation. Consistent with this, my previous studies (Chapter II, Nazeer et al. 2011) and this chapter 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 10). 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.

As mRNA poly(A) tails are important for the regulation of mRNA stability, it is possible that these changes of poly(A) mRNA levels by PARN deadenylase might represent another mechanism of p53-mediated control of gene expression. It has been shown that control of deadenylation represents 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).

The characterization of the PARN/p53 targets and the factors involved in this PARN-dependent regulatory pathway may allow us to better understand the p53 pathway and to find alternative strategies for treating tumorigenesis and metastasis in various cancers.
CHAPTER IV
IDENTIFICATION OF GENES REGULATED BY PARN DEADENYLASE
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., 2009b; 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). It has been reported that knockdown of PARN does not lead to global increase of mRNAs under non-stress conditions indicating that it has specific targets (Weißbach et al., 2013). 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). Previous studies from Dr. Kleiman’s lab 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 (Cevher et al., 2010).
Deadenylation of mRNA is regulated by cis-acting elements, such as microRNAs (miRNAs) and adenylate-uridylate–rich element (ARE), and trans-acting elements, such as ARE-binding proteins, polyadenylation factors, and RNA binding 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 BRCA1-associated RING domain protein (BARD1; Cevher et al., 2010) and p53 (Chapter III; Devany et al., 2013), associated to the polyadenylation factor CstF1 have been shown to regulate deadenylation by functional interactions with PARN deadenylase.

Extending those studies, the mRNA targets of PARN in non-stress conditions were determined. The results shown in Chapter III and IV 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 (Cevher et al., 2010), Dr. Kleiman’s lab decided to extend these studies and determine which mRNAs might be regulated by PARN using microarray assays. These assays were performed by Dr. Xiaokan Zhang 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, pathway analysis of regulated genes was performed 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 14). In 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.

The microarray analysis showed a 1.25 fold increase in p53 mRNA levels in PARN siRNA-treated samples compared to control siRNA-treated samples (Devany et al. 2013). Importantly, I validated these microarray results and showed that PARN knockdown resulted in a significant increase not only of p53 mRNA steady-state levels (Figure 15A) but also of p53 protein levels (compare lanes 1 and 2 in Figure 15B), 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 that 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. 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).
The results in Figure 15A indicate that PARN knockdown significantly increase the levels of p53 transcript. 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 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).
The biological relevance of the PARN-mediated regulation of p53 expression is supported by the fact that PARN knockdown induces an apoptotic response similar to that observed after UV treatment in a p53-dependent manner (Figure 17). The cell death assays were performed with colon carcinoma cells expressing different levels of p53: RKO, RKO-E6 (low p53 levels), and the isogenic cell lines HCT116 and p53-null HCT116. Cells were treated with either control/PARN siRNAs and/or UV irradiation. Cell death was determined using Cell Death Detection ELISA PLUS assay from Roche Applied Science according to the manufacturer’s instructions. Briefly, fragmentation of DNA after apoptosis induction was determined by photometric enzyme immunoassay. Cytoplasmic fractions from different cells were transferred to streptavidin-coated plates incubated with biotinylated anti-histone antibody. The amount of fragmented DNA of nucleosomes bound to the antibody was evaluated by peroxidase-conjugated monoclonal anti-DNA antibody.

UV-treated cells were harvested 12 h after the exposure as recommended by the manufacturer.

The results in Figure 17 indicate that UV treatment and PARN knockdown have a similar effect on cell death in cells expressing normal levels of p53 (HCT116 and RKO), indicating increased p53 levels upon PARN knockdown is enough to induce apoptotic response. RKO-E6 cells showed levels of apoptosis similar to those in RKO cells because the disruption of wild-type p53 function by E6 expression results in loss of p53-dependent DNA repair but not UV-induced apoptosis (Ford et al., 1998). p53-null HCT116 cells did not show an induction of apoptosis after either UV treatment (consistent with (Ford and Hanawalt, 1995) or PARN knockdown, suggesting that the effect of PARN expression on cell death is p53-dependent. Together, these results indicate that PARN plays a role in
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).
**Figure 17.** PARN knockdown and UV-treatment induce similar apoptotic responses in a p53-dependent manner. Fragmentation of DNA after induction of apoptosis was determined by photometric enzyme immunoassay. RKO/ RKO-E6 and HCT116/HCT116 p53 -/- cells were transfected with siRNAs targeting PARN/control and harvested for analysis 48 h after the transfection. Control siRNA-treated cells were exposed to UV treatment and harvested after 12 h. The DNA fragmentation was calculated from three independent samples. Errors represent the standard deviation derived from three independent experiments.

controlling p53 mRNA steady-state levels and p53 expression in non-stress conditions. 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). My results provide new insights into a mechanism behind the control of p53 expression in different conditions.
DISCUSSION

The studies presented in this dissertation (Chapters III and IV) 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 (Figure 18). 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 (Chapter III, Figures 11-12). Second, my results 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 NEs (Figure 13). Third, PARN significantly affects the cellular expression of genes in the p53 pathway under non-stress conditions (Figures 14-15) and the stability and poly(A) length of the p53 mRNA (Figure 16). Fourth, PARN knockdown and UV treatment induce a similar increase in p53 expression (Figure 15B) and apoptotic responses (Figure 17). Taken together, the results in this dissertation 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 18A). Supporting this, previous studies from Dr. Kleiman’s lab 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 (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). As suggested in Chapter III, it is possible
that these changes of poly(A) mRNA levels might represent another mechanism of p53-
mediated control of gene expression. In fact, my studies indicate that an increase in the
expression of p53 inhibits the mRNA 3′ cleavage step of polyadenylation (Chapter II,
Figure 10, Nazeer et al., 2011) and induces PARN deadenylase activity (Chapter III,
Figures 11-12, 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 18B). 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.

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). Consistent with this, the microarray data presented
here also revealed significant down-regulation of genes involved in similar pathways, such
as structure morphogenesis, cell adhesion, cell migration, and so on. More detailed
information can be found in Devany et al. (2013). PARN knock-down cells 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 collagen alpha-2(I)
chain. However, the p53 signaling pathway was not reported by Lee et al. (2012), suggesting cell-specific functions of PARN. Like Lee et al. (2012), we 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.

As RNA binding factors recognize cis-acting sequences present in the 3’UTR of deadenylases target genes, 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.
Figure 18. 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).
CHAPTER V

MECHANISMS OF PARN-MEDIATED REGULATION OF p53 mRNA STEADY-STATE LEVELS UNDER NON-STRESS CONDITIONS
INTRODUCTION

Regulation of poly(A) tail length of mRNAs 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 cells rapid response to stress. Nuclear poly(A) specific ribonuclease (PARN), a poly(A) specific 3’ exoribonuclease, has been shown to play a role in DNA damage response (DDR) (Cevher et al., 2010). 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 (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 AU-rich elements (AREs) and microRNA (miRNA) targeting sites. These cis-acting 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. Several studies support the idea that miRNAs destabilize mRNA through deadenylation pathways or inhibition of translation, while AREs regulate mRNA stability by either preventing
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). 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 (Cevher and Kleiman, 2010, Figure 7). 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). miRNAs function through ribonucleoprotein complexes called miRISC, which deliver miRNAs to their mRNA targets. 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. Members of the Argonaute (Ago) protein family are central to miRISC function. They bind the mature miRNA and orient it for interaction with a target mRNA. Ago-2 is one of the major components of miRISC. It has also been described that knockdown of Ago-2 in mammalian cells lead to a much more profound effect on miRNA-mediated repression than the knockdown of the other Ago proteins, suggesting that in mammals Ago-2 may have some specific functions that other Ago proteins cannot complement (Schmitter et al., 2006). Ago-2 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 connection between miRNA pathway and PARN deadenylase in the nucleus has not been elucidated.

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 show 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 remain(s) to be elucidated.

Interestingly, some of the seed signals recognized by miRNA overlap with AREs in the 3′UTR of many mRNAs. 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. 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 facilitates 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., 2006a).

My studies 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). 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 evidence that PARN deadenylase regulates the mRNA stability of one of its targets, the p53 mRNA, (Devany et al., 2013) via ARE- and a nuclear miRNA-mediated pathway. The binding of PARN to the p53 3’UTR and downregulation of p53 expression depends on the presence of both AREs and miRNA target sites. These results reveal a novel regulatory pathway where PARN deadenylase plays a role in both ARE- and miRNA-mediated regulation of p53 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

Previously, I have shown that PARN plays a role regulating p53 mRNA steady-state level through destabilizing p53 transcript by shortening the polyA tail length (Figures 13-15; (Devany et al., 2013). Extending those studies, I investigated the mechanisms underlying regulation of p53 mRNA stability by PARN deadenylase.

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 which 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 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). Interestingly, the increase in luciferase activity upon PARN knockdown (Figure 19B) was of similar magnitude to the increase observed in p53 protein levels upon PARN knockdown (Figure 15). The firefly/Renilla ratios for the construct with the p53 3’UTR relative to the control construct were similar in cells expressing different levels of p53.
(compare RKO, RKO-E6, HCT116 and HCT116 p53<sup>−/−</sup>), indicating that PARN-mediated regulation of p53 expression by the 3’UTR is p53-independent.

Interestingly, the 3’UTR of p53 mRNA also contains ARE that associates with ARE-binding proteins, such as wild-type p53-induced gene 1 and HuR and regulates p53 mRNA steady-state levels. Importantly, I showed that the replacement of the

![Diagram of firefly luciferase reporter constructs with the vector or 3’ UTR sequence from the p53 gene. Polyadenylation signals (PAS) are indicated. 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).](image)

**Figure 19. PARN regulates p53 expression through 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).
ARE sequence from the p53 3’UTR (Figure 22: noARE construct) significantly increases the firefly/Renilla ratio compared with the WT p53 3’ UTR construct (Figure 20A), 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 (Figure 20B), indicating that the AREs in the p53 3’UTR are necessary for PARN-mediated regulation of p53 expression. Together, these results indicate that the AREs in the p53 3’UTR are important for the PARN-mediated regulation of p53 mRNA steady-state levels and that this regulation is p53-independent.

It is known that PARN is involved in ARE-mediated deadenylation (Korner and Wahle, 1997; Lai et al., 2003; Lin et al., 2007). Studies from Dr. Kleiman’s lab have shown that Ago-2 activates PARN deadenylase through direct protein interaction and participates in recruiting PARN to some of its mRNA targets, suggesting that PARN might play a role not only in ARE- but also miRNA-mediated deadenylation. Consistent with this idea, others have shown the functional overlapping of ARE- and miRNA-mediated regulatory pathways (Bhattacharyya et al., 2006a; 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 as shown in Figure 21 some of these signals overlap. 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
Figure 20. PARN regulates p53 expression through ARE sequence present in the 3’UTR of p53 mRNA. A) Constructs carrying the p53 3’UTR (p53) or ARE-replaced p53 3’UTR (noARE) were transfected in RKO/RKO-E6 and HCT116/HCT116 p53 -/- cells. The ratios of the firefly/renilla values for each construct relative to the vector construct are shown. B) Luciferase assay done as in A) using 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. Taken from Devany et al. (2013).
non-stress conditions, constructs were generated that have the luciferase gene under the control of WT p53 3’UTR or replacement mutants in AREs, miRNA targeting sequences that are in close proximity in the p53 3’UTR (Figure 22). The experiments presented here focused on miR-125b (Le et al., 2009) and miR504 (Hu et al., 2010; Le et al., 2009). Both miRNA binding sites are adjacent to or overlapping with the ARE in the p53 3’UTR (Vilborg et al., 2009; Zou et al., 2006) that is important for PARN binding to p53 3’UTR and PARN-mediated regulation of p53 mRNA steady-state levels (Figure 20; (Devany et al., 2013).

**Figure 21. p53 mRNA 3’UTR sequence.** 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), which is important for PARN binding to p53 3’UTR and PARN-mediated regulation of p53 mRNA steady-state levels.
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 23). Together, these results indicate that both regulatory signals at the p53 3’UTR are necessary for PARN-mediated regulation of p53 expression.

Next, I examined whether PARN physically associates with p53 mRNA through ARE and/or miRNA targeting sites in the 3’UTR. RNA pull-down (RNA-PD) assays were performed using in vitro transcribed biotinylated RNAs encompassing WT or the mutant variants of p53 3’UTRs and NEs from HCT116 cells. RNA-PD assays showed that PARN interaction with p53 3’UTR depended on the presence of both the ARE sequence and miRNA targeting site (Figure 24A). As expected, the interaction of Ago-2 with p53 3’UTR was not decreased by the replacement of the ARE. Furthermore, RNA-PD assays were

Figure 22. Diagram of firefly luciferase reporter constructs with different derivatives of 3’UTR sequences from the p53 gene. Polyadenylation signals (PAS) are indicated.
Figure 23. 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.

performed with biotinylated WT p53 3’UTR and NEs from HCT116 cells treated with either control or Ago-2 siRNAs. Interestingly, the depletion of Ago-2 from the NEs samples resulted in a decrease of PARN pulled-down by biotinylated p53 3’UTR RNA (Figure 24B), and this effect was reversed when I added increasing amounts of recombinant GST-Ago-2 to the reaction (Figure 24C). Together, these results indicate that Ago-2 has a role in recruitment of PARN deadenylase to the p53 3’UTR. As RNA-PD assays are a cell-free system, these results also 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.

Figure 24. Ago-2 protein, ARE and miR-125b targeting signal are necessary for PARN binding to p53 3’UTR. A) 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. B) RNA pull-down assays were performed using biotinylated WT p53 3’UTR and NEs from Hela cells treated with control or Ago-2 siRNAs. C) Ago-2 facilitates PARN binding to p53 mRNA 3’UTR. RNA pull-down assays were performed using biotinylated WT p53 3’UTR, NEs from HCT116 cells treated with Ago-2 siRNA, and increasing amounts of recombinant GST-Ago-2 protein.
Then I tested whether miRNAs play a role in the PARN-mediated regulation of p53 expression in the nucleus of human cells. My studies focused on miR-125b which was 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). To examine the role of miR-125b in PARN-mediated p53 mRNA decay HCT116 cells were transfected with either control or miR-125b inhibitor expression plasmid, which blocks endogenous miR-125b, and analyzed p53 mRNA steady-state levels. As expected, the functional knockdown of miR-125b showed an increase p53 protein levels (Figure 25). Interestingly, a similar increase in both p53 mRNA and p53 expression was determined after PARN-knockdown (Devany et al., 2013). Importantly, as shown in Figure 26A, the functional inhibition of miR-125b elongated the poly(A) tail length of p53 mRNA (quantification is shown in Figure 26B), indicating that miR-125b regulates p53 mRNA steady-state level by modulating its poly(A) tail length.

To further assess the regulatory function of ARE and miR-125b binding site PARN-dependent deadenylation of p53 mRNA I performed deadenylation assays using NEs from HCT116 cells and different derivatives of p53 3’UTR RNA (Figure 27). These assays were

![Figure 25. Functional knockdown of miR-125b increases p53 protein levels.](image)

Samples from HCT116 cells transfected with either control or miR-125b inhibitor expression plasmids were analyzed by Western blot.
Figure 26. miR-125b regulates p53 mRNA poly(A) tail length. A) 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. B) Quantification of polyA tail length done using Image J software.

performed using an in vitro transcribed, radiolabeled, capped and polyadenylated (A$_{20}$) RNA encompassing the the WT p53 3’UTR or the mutants described in Figure 22. Interestingly, these deadenylation assays confirmed that the presence of the ARE and miR125b site was important for deadenylation of p53 3’UTR. Importantly, the RNA substrate specificity of the deadenylation activity was determined using the p53 3’UTR-G15 RNA substrate, which was radioactively labeled in its RNA body. This RNA substrate
was unaffected by addition of HCT116 NEs (Figure 27). Although these results indicate that both ARE and miR-125b binding site are necessary for deadenylation, I cannot discard the possibility that other nuclear deadenylases might be involved in this deadenylation assay. Future studies are needed to investigate the possible role of other deadenylases, such as CCR4-NOT and Pan2-Pan3, in the regulation of p53 mRNA in the nucleus under non-stress conditions.

Figure 27. Both ARE and miR125b binding site are involved in p53 mRNA deadenylation. Deadenylation assays were performed using NEs from HCT116 cells and radiolabeled/polyadenylated (A20) RNA encompassing WT or signal replaced (noARE, nomiR, and noBOTH) or polyG 3’UTRs of p53.

DISCUSSION

My previous work 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). The studies presented in this chapter further investigate the mechanisms involved in PARN-mediated regulation of p53 mRNA steady-state levels. My results 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 mammalian cells. Consistent with this, 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 (Figures 19-24). Consistent with this, studies from Dr. Kleiman’s lab have shown the interaction of the miRISC component Ago-2 with nuclear PARN and activation of PARN deadenylase by Ago-2 (Dr. Zhang personal communication). Furthermore, my results indicate that Ago-2 (Figure 24) and miR125b target site (Figure 24-25) facilitated the binding of PARN to the target p53 mRNA 3’UTR, resulting in p53 mRNA poly(A) tail shortening (Figure 26) and decrease in p53 protein levels (Figure 25). Taken together, these results provide new insights into the nuclear function of ARE and miRISCs in the PARN-mediated regulation of deadenylation and gene expression. This study also allows us to better understand the mechanisms involved in the regulation of p53 expression.

Based on the results presented in this dissertation and work from Dr. Kleiman’s lab, 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 28). 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, my results indicate that ARE sequences are also important for regulation of p53 mRNA by PARN. 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). Other ARE-BPs, such as programmed cell death protein 4 (Pcdp4) and RNA-binding region (RNP1, RRM) containing 1a (RNPC1a), have been shown to bind to the AREs in p53 3’UTR and destabilize the p53 transcript (Wedeken et al., 2011; Zhang et al., 2011). Studies from Dr. Kleiman’s lab have also shown the direct interaction between PARN and nucleolin, a eukaryotic nucleolar phosphoprotein, and that nucleolin binding to p53 3’UTR is decreased significantly in the absence of ARE sequence (Dr. Zhang personal communication). Future studies are necessary to investigate PARN functional binding to other miRISC components, such as other members of the Ago family and GW182, and to ARE-BPs.

These studies 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). Previous studies from Dr. Kleiman’s lab indicate 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.

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. These studies also provide new insights in the p53 pathway, proposing new approaches in the design of new cancer therapies.
Non-stress condition

Under DNA damage condition
Figure 28. 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).

CHAPTER VI
MECHANISMS OF PARN-MEDIATED REGULATION OF p53 mRNA LEVELS DURING THE PROGRESSION OF DNA DAMAGE RESPONSE
INTRODUCTION

Recent reports suggest that microRNA (miRNA)-mediated repression of mRNAs can be effectively reversed upon stress and other developmental cues, resulting in a more dynamic regulation of gene expression in response to specific cellular needs (Bhattacharyya et al., 2006a, b). p53 is an important tumor suppressor that regulates gene expression by activation of transcription of a number of genes (el-Deiry et al., 1992, Tokino and Nakamura, 2000), upregulation of certain miRNAs (Chang et al., 2007) and regulation of mRNA 3’ processing (Nazeer et al., 2011; Devany et al., 2013). It is the most mutated gene in cancer and regulation of p53 expression under different cellular conditions is critical. p53 can be regulated at transcriptional, post-transcriptional and post-translational levels. It is well established that p53 protein accumulates in the cell upon DNA damage via the degradation of Mdm2, which ubiquitinates and targets p53 protein for degradation under normal conditions (Honda et al, 1997). As part of the DNA damage response (DDR), p53 mRNA should be rapidly relieved from repression, contributing to rapid response to stress by inducing p53 expression.

Consistent with this, growing evidence suggests that regulation of p53 at the mRNA level is also important for DDR. A number of proteins have been shown to regulate p53 mRNA translation by functional interaction with 5’ untranslated region (UTR), coding region and 3’UTR (reviewed in Vilborg et al., 2010 and Candeias et al., 2011). Recently, we have shown that poly(A) specific ribonuclease (PARN) regulates p53 mRNA under normal conditions (Chapter IV, Devany et al, 2013) through AU-rich element (ARE)-mediated deadenylation (Chapter V, Devany et al, 2013) and miRNA-mediated deadenylation (Chapter V). PARN is one of the major nuclear deadenylases in mammalian cells and is activated upon UV treatment by the polyadenylation factor CstF1 bound to
tumor suppressors, such as BARD1 (Cevher et al. 2010) and p53 (Devany et al. 2013). Interestingly, after UV treatment, the changes in the levels of p53 mRNA and p53 protein were PARN-independent (Figure 15), indicating there is(are) other mechanism(s) involved in the regulation of p53 expression during DDR.

As shown in Chapter V, PARN is also involved in miRNA-mediated regulation of mRNA stability and, consequently, expression levels in the nucleus of mammalian cells. Consistent with this, studies from Dr. Kleiman lab shows that PARN is a nuclear miRNA-induced silencing complex (miRISC)-associated deadenylase: both PARN and elements of miRISC are present in nuclear fractions; PARN physically interacts with Argonaute (Ago)-2, a major component of mammalian miRISC complex, and Ago-2 activates PARN deadenylase activity (Dr. Zhang personal communication). 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 (Chapter V). Furthermore, my results indicate that Ago-2 and miR125b contribute to p53 mRNA poly(A) tail shortening (Figure 26) and decrease in p53 transcript and protein levels (Figure 25). Interestingly, during DDR, Ago-2 translocates to cytoplasm and the abundance of miRNAs that are known to target p53 3’UTR, such as miR125b and miR504, decrease in the nuclear fractions (Dr. Zhang personal communication).

HuR is an AREs-binding protein (BP) that has been shown to increase p53 mRNA stability (Zou et al., 2006) and translation (Mazan-Mamczarz et al., 2003) after stress. HuR is an ubiquitously expressed ARE-BP that belongs to the Hu (ELAV) family of RNA-BPs, which also includes the neuro-specific proteins HuB, HuC, and HuD. It is mainly localized in the nucleus. It has reported roles in splicing, mRNA processing and translation.
HuR has been suggested to bind its substrates in the nucleus, as early as co-transcriptionally, and escort them to the cytoplasm and polysomes to stabilize and enhance translation (Fan et al., 1998, Mukherjee et al., 2011). In a recent study, it has been reported that over 75% of mRNAs with Ago-2 binding sites also have HuR binding sites (Mukherjee et al., 2011). Most of these Ago-2 and HuR binding site pairs are in less than 10 nt distance, suggesting a competitive or cooperative regulation of target mRNAs (Mukherjee et al., 2011). Besides, HuR sites in 3'UTRs of mRNAs overlap extensively with predicted miRNA target sites (Uren et al., 2011), suggesting interplay between the functions of HuR and miRNAs.

Here I show that HuR is involved in PARN-mediated regulation of p53 mRNA through 3’UTR. I studied the dynamic interactions of PARN deadenylase and p53 3’UTR with the trans-acting elements HuR and Ago-2. The results indicate that HuR can revert the PARN-associated Ago-2-mediated destabilization of p53 mRNA after UV treatment, providing a mechanism to regulate p53 expression during the progression of DDR.

RESULTS

To investigate whether HuR is involved in PARN-mediated regulation of p53 mRNA stability, I performed luciferase assays with samples from cells expressing different levels of PARN and HuR and transfected with a dual luciferase reporter construct that encodes firefly luciferase under the control of p53 3’UTR. Details of the luciferase construct are described in Figure 19. While the siRNA-mediated knockdown of PARN resulted in an increase in luciferase activity under normal conditions, the siRNA-mediated
knockdown of HuR resulted in a decrease in luciferase activity upon UV treatment (Figure 29A). These results suggest that both PARN and HuR are involved in the regulation of p53 mRNA stability under non-stress and stress conditions, respectively.

My previous studies and other studies from Dr. Kleiman’s lab have shown that PARN deadenylase is recruited to the p53 mRNA by the miRISC complex and that Ago-2 nuclear levels have an effect on PARN-p53 mRNA complex formation. Extending those studies, I looked at the effect of levels of these proteins and HuR on p53 expression. Nuclear extracts (NEs) that were treated with control, PARN, Ago-2 or HuR siRNAs and treated with UV were analyzed by Western Blot for p53 expression. Consistent with my previous results, PARN knockdown increased p53 protein levels under normal conditions but did not have an effect on p53 protein levels after UV treatment (Figures 15 and 29B; Devany et al., 2013). Under non-stress conditions, Ago-2 knockdown had similar effects on p53 protein levels as PARN knockdown. However, UV-treatment increased p53 levels when Ago-2 but not PARN was knocked-down. In contrast, HuR knockdown caused a decrease in p53 protein levels in a UV-dependent manner (Figure 29B). My results indicate that PARN and Ago-2 keep p53 protein levels low under non-stress conditions, suggesting a destabilizing effect of these proteins on p53 mRNA. On the contrary, HuR expression induces p53 expression, suggesting a stabilizing effect of HuR on p53 mRNA.

Next, I examined how PARN, Ago-2 and HuR physically associate with p53 mRNA through ARE and/or miRNA targeting sites in the 3’UTR. RNA pull-down (RNA-PD) assays were performed as in Chapter V (Figure 24) using in vitro transcribed biotinylated RNAs encompassing WT or the mutant variants of p53 3’UTRs and NEs from HCT116 cells treated with UV irradiation. RNA-PD assays showed that PARN and Ago-2
interacted with p53 3’UTR RNA under non-stress conditions (Figure 30). After UV treatment, the levels of PARN and Ago-2 pulled-down by p53 3’UTR RNA decreased, whereas the levels of HuR bound to p53 3’UTR RNA increased. While the interaction of PARN and HuR with p53 3’UTR RNA was

![Graph A](image)

**Figure 29.** p53 expression is regulated through the 3’UTR of p53 mRNA by PARN, Ago-2 and HuR expression. A) Constructs carrying the luciferase-p53 3’UTR and the indicated siRNAs were transfected in HCT116 cells. The values for firefly/renilla ratio obtained for each siRNA are shown. Errors represent the SD derived from three independent experiments. B) Western blot analysis of p53 expression was performed using NEs from cells treated with UV irradiation and control, PARN, HuR and Ago-2 siRNA. A representative Western blot from three independent assays is shown. Topo II was used as loading control.
disrupted by the ARE sequence replacement, the interaction of Ago-2 with noARE-p53 3’UTR was slightly favored. Besides, HuR bound the nomiR mutant p53 3’UTR better than p53 3’UTR, whereas the opposite was observed for PARN and Ago-2. Together, these

Figure 30. Binding of PARN, Ago-2 and HuR to the p53 3’UTR during the progression of DDR. PARN, Ago-2 and HuR interaction with p53 3’UTR RNA was analyzed by RNA-pull down (RNA-PD) assays. RNA-PD assays were performed using NEs from HCT116 cells treated with UV and biotinylated p53 3’UTR RNA (p53), ARE-replaced p53 3’UTR RNA (noARE), miRNA-seed region replaced p53 3’UTR RNA (nomiR) or both sequences replaced p53 3’UTR RNA (noBoth). Quantification of three independent RNA-pull down experiments is done using Image J software.

results indicate a competition between PARN-associated Ago-2 and HuR in binding the p53 3’UTR, suggesting that HuR might replace PARN/Ago-2 after UV treatment resulting in the increase of the steady-state levels of p53 mRNA.

To determine the physiological relevance of HuR- and PARN-mediated regulation of p53 expression during the progression of DDR, I performed DNA fragmentation assays using HCT116 cells either overexpressing HuR or depleted in PARN expression after UV
Cells were treated with control/PARN siRNA or transfected with control/HuR encoding plasmids. Plasmids were kindly provided by Dr. Bhattacharyya, Friedrich Miescher Institute Basel, Switzerland. As shown previously, UV treatment and PARN knockdown have a similar effect on cell death in cells expressing normal levels of p53 (HCT116, Figures 17 and 31). Interestingly, the overexpression of HuR was sufficient to induce p53-mediated apoptosis (Figure 31). Consistent with Ford and Hanawalt (1995), p53-null HCT116 cells did not show an induction of apoptosis after UV treatment, HuR overexpression or PARN knockdown, suggesting that the effect of PARN or HuR expression on cell death is p53-dependent. These results indicate that either the decrease of PARN expression or increased of HuR expression are sufficient to induce apoptotic response in a p53-dependent manner. Together, these results indicate that PARN and HuR play a role in controlling p53 expression in non-stress and stress conditions, respectively.

My results indicate that HuR and PARN bind to the same region in p53 3’UTR (Figure 30). Besides, PARN (Devany et al. 2013) and HuR (Zou et al., 2006) have opposite effects on p53 mRNA stability and p53 expression levels (Figure 29 and 30) under normal and stress conditions, respectively. Based on these results, it is possible that p53 mRNA stability is competitively regulated by PARN and HuR under different cellular conditions, representing a model for the change of p53 expression during the progression of DDR. To test this idea binding competition assays were performed using RNA-PD assays. These assays were performed using biotinylated p53 3’UTR RNA, NEs from HCT116 cells and increasing concentrations of either His-PARN or His-HuR. As Ago-2 functionally interacts with PARN helping to recruit the deadenylase to p53 3’UTR under non-stress conditions (Figure 24), GST-Ago-2 was also included in this study. As shown in Figure 32A and
Figure 31. HuR overexpression is sufficient for the progression of DDR via p53-induced apoptosis. Both PARN knockdown and HuR overexpression induced apoptosis to similar levels to that observed after UV treatment. Fragmentation of DNA after induction of apoptosis was determined by photometric enzyme immunoassay. HCT116/HCT116 p53-/- cells were transfected with either siRNAs targeting PARN/control or HuR/control encoding plasmid and harvested for analysis 48 h after the transfection. Control siRNA-treated cells were exposed to UV treatment and harvested after 12 h. The DNA fragmentation was calculated from three independent samples. Errors represent the standard deviation derived from three independent experiments.

32B, increasing amounts of either PARN or Ago-2 decrease the binding of HuR to p53 mRNA 3’UTR. Besides, increasing amounts of Ago-2 resulted in an increase of PARN binding to p53 3’UTR, supporting my previous results that Ago-2 recruits PARN to p53 mRNA (Figure 24). Supporting the PARN/HuR competitive binding to p53 3’UTR, increasing concentrations of HuR decrease the binding of both PARN and Ago2 (Figure 32C). Extending those studies, I performed RNA-PD assays with NEs from HCT116 cells.
treated with control, HuR, PARN or Ago2 siRNAs (Figure 33). The binding of PARN and Ago-2 to p53 3’UTR RNA increased when HuR expression was depleted (Figure 33A). Binding of HuR to p53 3’UTR increased when PARN expression was knocked down (Figure 33B). While the binding of PARN to p53 3’UTR RNA decreased when Ago-2 expression decreased, the binding of HuR to p53 3’UTR RNA increased in the same conditions (Figure 33C). Together, these results indicate that PARN-associated Ago-2 bind

Figure 32. HuR competes with PARN and Ago-2 for binding to p53 3’UTR RNA. Addition of PARN (A) and Ago-2 (B) decreased the binding of HuR to p53 3’UTR RNA, whereas increasing amounts of HuR (C) decreased the binding of both PARN and Ago-2 to the p53 3’UTR RNA. Competition RNA-pull down assays were performed as in Figure 29 using biotinylated p53 3’UTR RNA, NEs from HCT116 cells and increasing amounts of the indicated recombinant protein.

p53 mRNA resulting in a decrease in the messenger steady-state levels under non-stress conditions. HuR binds to the same region of p53 3’UTR displacing PARN-associated Ago-2 and increasing p53 expression levels during the progression of DDR. The competitive
Figure 33. HuR competes with PARN and Ago-2 for binding to p53 3’UTR RNA. A) In the absence of HuR binding of PARN and Ago-2 to p53 3’UTR RNA increased. B) PARN knockdown increased HuR binding to p53 3’UTR RNA. C) In the absence of Ago-2 binding of PARN to p53 3’UTR RNA decreased and binding of HuR to p53 3’UTR RNA increased. RNA-PD assays were performed using biotinylated RNA of the p53 3’UTR and NEs of HCT116 cells treated with control, HuR, PARN or Ago-2 siRNAs. 20% of the NE used in the pull-down reactions is shown as input. Knock-down of HuR, PARN and Ago-2 proteins confirmed by Western Blot are shown below each RNA-PD assay.
binding of PARN-associated Ago-2 and HuR to p53 mRNA under different cellular conditions can explain the changes in p53 expression observed during the progression of DDR.

To analyze the association of PARN and HuR with endogenous p53 mRNA in different cellular conditions I performed RNA immunoprecipitation (RIP) assays (Figure 34). Antibodies against PARN, HuR or IgG were used to immunoprecipitate protein-RNA complexes from UV-treated HCT116 cells. Samples from crosslinked cells were immunoprecipitated with the indicated antibodies. Bound RNA was used for reverse

![Figure 34](image)

**Figure 34.** While PARN associates with p53 mRNA under normal conditions, HuR association with p53 mRNA is favored after UV treatment. A) PARN binds to p53 mRNA under non-stress conditions. After UV treatment levels of PARN associated with p53 mRNA decreases. The extracts were immunoprecipitated with anti-PARN, anti-HuR or IgG antibodies. Nuclear RNA immunoprecipitated with the antibodies was quantified by qRT-PCR using primers specific for p53 mRNA. B) HuR binds to p53 mRNA in a UV-dependent manner. C) qRT-PCR products from RNA-immunoprecipitation reaction were separated in 2% agarose gel.
transcription reaction with random primers and the resulting cDNA was further analyzed by qRT-PCR using primers specific for p53 mRNA. Supporting the competitive binding of PARN and HuR to p53 mRNA observed in the RNA-PD assays, my RIP assays indicated that while PARN dissociated from p53 mRNA after UV treatment (Figure 34A and 34C), HuR associated to p53 mRNA after UV treatment (Figure 34B-C).

To further investigate the competitive binding of PARN/Ago-2 and HuR to p53 mRNA I performed RNA-IP assays using HCT116 cells that were treated with control,

**Figure 35. HuR competes with PARN-associated Ago-2 for binding p53 3’UTR.** A) Either PARN or Ago-2 knockdown increased the association of p53 mRNA with HuR under non-stress conditions. The extracts from cells treated with the indicated siRNAs were IPed with the indicated antibodies. The endogenous nuclear RNA-IPed with the antibodies was quantified by qRT-PCR using primers specific for p53 mRNA. The qRT-PCR values were calculated from three independent samples. B) While HuR knockdown favored the association of PARN with p53 mRNA, Ago-2 knockdown decreased PARN/p53 mRNA interaction. C) HuR knockdown increased the association of Ago-2 with p53 mRNA, while PARN knockdown did not affect this interaction.
HuR, PARN or Ago-2 siRNAs (Figure 35). Either Ago-2 or PARN knockdown increased the association of HuR with the p53 mRNA in samples from HCT116 cells under non-stress conditions. In the absence of HuR, the association of PARN with p53 mRNA was favored. Consistent with the RNA-PD assays that show that Ago-2 facilitates the binding of PARN to p53 mRNA (Figures 24 and 33), Ago-2 knockdown caused a decrease in PARN-p53 mRNA complexes (Figure 35). Cellular levels of PARN did not affect complex formation between Ago-2 and p53 mRNA, supporting the idea that PARN does not bind directly p53 mRNA and that Ago-2 facilitates PARN recruitment to p53 3’UTR. Importantly, HuR knockdown increased Ago-2-p53 mRNA complex formation. These results confirm that HuR can compete with PARN-associated Ago-2 for binding to p53 3’UTR.

Previous studies have shown that HuR promotes dissociation of miRISC complex from target mRNAs and inhibits miRNA-dependent deadenylation of mRNAs (Kundu et al., 2012). Oligomerization of HuR is important for these functions. HuR and related proteins, such as HuB, HuD and Drosophila ELAV, are known to oligomerize along RNA substrates. Although HuR is a monomer in the solution, three or more HuR molecules cooperatively oligomerize along 18 nt or longer ARE regions of mRNAs (Fialcowitz-White et al., 2007), contributing to the formation of HuR–RNA complexes. To investigate if the oligomerization of HuR is also important for the dissociation of PARN from p53 mRNA, RIP assays were performed with samples from HCT116 cells transfected with vectors encoding different derivatives of HuR. Vectors expressing different HuR variants were kindly provided by Dr. Bhattacharyya (Friedrich Miescher Institute, Basel, Switzerland). A scheme of the HuR derivatives used in this study is shown in Figure 36A: full length HuR
(FL-HuR), a deletion variant that lost the C-terminal RNA recognition motif (RRM) but that can still oligomerize (HuRΔ3), and a deletion variant that lost the C-terminal RRM and the hinge region and cannot oligomerize (HuRΔH3). The complex formation of PARN and p53 mRNA is abolished when either FL-HuR or HuRΔ3 mutant were overexpressed in HCT116 cells (Figure 36B). Interestingly, HuRΔH3 overexpression did not affect the

Figure 36. Oligomerization of HuR is important for dissociation of PARN-associated Ago-2 from p53 mRNA. A) Schematic representation of HuR oligomeric derivatives. B) The complex formation of PARN and p53 mRNA was abolished when either FL-HuR or HuRΔ3 mutant are overexpressed in HCT116 cells. Oligomerization deficient form of HuR did not affect PARN/p53 mRNA complex. NEs from HCT116 cells expressing HuR oligomeric derivatives were used in RNA-IP assays. The extracts were IPed with either with anti-PARN or IgG antibodies. C) While recombinant FL and HuRΔ3 decreased the binding of PARN and Ago-2 to p53 3’UTR RNA, recombinant HuRΔH3 did not have an effect on the complex formation of p53 3’UTR RNA with either PARN or Ago-2. RNA-PD assays were performed using biotinylated p53 3’UTR RNA, NEs from HCT116 cells and increasing concentrations of different recombinant HuR derivatives.
PARN-p53 mRNA association (Figure 36B), indicating that HuR oligomerization is important for the competitive binding to p53 mRNA. In addition, RNA-PD assays were performed with biotinylated p53 3’UTR, NEs from HCT116 cells and with the addition of increasing concentrations of His-tagged HuR derivatives. While recombinant FL and HuRΔ3 decreased the binding of PARN and Ago-2 to p53 3’UTR, recombinant HuRΔH3 did not have an effect on the PARN/Ago-2/p53 mRNA complex formation (Figure 36C), suggesting that the oligomerization of HuR and the consequent miRISC dissociation are important for this dynamic mechanism. Together, these results support the idea that PARN-associated Ago-2 bind to p53 mRNA under non-stress conditions, then the PARN-Ago-2 complex dissociate from p53 mRNA after UV treatment allowing the oligomerized HuR to bind the p53 mRNA.

I have previously shown that PARN deadenylase affects p53 expression by regulating the messenger poly(A) length (Devany et al., 2013). To investigate whether HuR-mediated release of PARN-associated Ago-2 from p53 mRNA affects the p53 mRNA poly(A) tail length, I performed RACE-poly(A) Test (RACE-PAT) assays with RNA extracted from cells transfected with control/HuR encoding plasmid. Importantly, poly(A) tail of p53 mRNA was significantly longer in cells transfected with HuR encoding plasmid (Figure 37A). Although most of the p53 mRNA was polyadenylated after UV-treatment, this was reverted by HuR knockdown that resulted in the increase of the levels of deadenylated forms of p53 mRNA. These results suggest that UV-induced increase in poly(A) tail length of p53 mRNA might be partly due to HuR binding and release of PARN-associated Ago-2 (Figure 37A). Quantification of poly(A) tail lengths are shown in Figure 37B.
Figure 37. HuR is involved in the UV-induced increase in the poly(A) tail length of p53 mRNA. A) HuR overexpression increased the length of p53 mRNA poly(A) tail. RACE-poly(A) Test assays were performed to measure poly(A) tail length of p53 mRNA under different conditions. Nuclear RNA from cells transfected with control/HuR encoding vectors 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 and non-HuR 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 HuR overexpression lanes using ImageJ software.
To further assess the regulatory function of HuR in the changes in the poly(A) length of p53 mRNA in different cellular conditions, NEs from UV-treated HCT116 cells were analyzed for deadenylation assays using an in vitro transcribed, radiolabeled, capped and polyadenylated (A$_{20}$) RNA encompassing the WT p53 3’UTR. Approximately half of the p53 3’UTR substrate was deadenylated using NEs from non-UV treated cells. Deadenylation of p53 3’UTR substrate was inhibited when NEs from UV-treated cells were used (Figure 38A). As a substrate encompassing the p53 3’UTR with a poly(G$_{15}$) was

Figure 38. UV treatment inhibits deadenylation of p53 mRNA. A) UV treatment inhibits deadenylation of p53 3’UTR substrate. Deadenylation assays were performed using capped, polyadenylated (A$_{20}$), radioactively labeled p53 3’UTR and NEs from HCT116 cells treated with UV irradiation. Positions of polyadenylated RNA and deadenylated product are indicated. Numbers beneath gel lanes indicate relative deadenylation (RD). RD was calculated as [p53 3’UTR (A$_{20}$)/( p53 3’UTR + p53 3’UTR (A$_{20}$))] × 100. Quantifications were done with ImageJ software (http://rsb.info.nih.gov/ij/). B) p53 3’UTR poly(G$_{15}$) substrate is not modified by addition of NEs from HCT116 cells either non-treated or UV-treated.
not affected by addition of non-treated or UV-treated HCT116 NEs (Figure 38B), I can conclude that the changes observed were due to deadenylation and not endonucleolytic cleavage. Interestingly, addition of increasing amounts of recombinant HuR to the reaction

**Figure 39. HuR inhibits deadenylation of p53 3’UTR.** **A)** Recombinant HuR inhibited the deadenylation of p53 3’UTR RNA. NEs from HCT116 cells were analyzed for deadenylation of p53 3’UTR poly(A)$^+$ RNA in the presence of increasing amounts of recombinant HuR. **B)** HuR knockdown increased deadenylation of p53 3’UTR poly(A)$^+$ RNA. NEs from HCT116 cells treated with control or HuR siRNAs were analyzed for changes in p53 3’UTR RNA deadenylation. Relative deadenylation values are shown. **C)** HuR levels do not affect deadenylation of L3A$_{30}$ substrate. NEs treated with UV or not were incubated with capped, polyadenylated, radioactively labeled L3(A$_{30}$) RNA substrate.
mix decreased the deadenylation levels observed using NEs from non-UV treated cells (Figure 39A). Deadenylation of the p53 3’UTR probe increased dramatically using NEs from HCT116 cells treated with siRNA targeting HuR (Figure 39B), indicating that binding of HuR inhibits the deadenylation of p53 mRNA possibly by promoting dissociation of PARN (Figure 39B). Interestingly, the HuR knockdown in HCT116 cells did not have any effect on the deadenylation of L3(A30) probe, which does not have AREs or miRNA binding sites, indicating that the presence of cis-acting elements are necessary for the effect HuR on deadenylation (Figure 39C).

The results described in this dissertation are consistent with the previously described inhibitory effect of HuR on miRNA-dependent deadenylation of mRNAs and with HuR–dependent dissociation of miRISC complex from target mRNAs (Kundu et al., 2012). Kundu et al. (2012) reported that there is no interaction between miRISC components and HuR. My coimmunoprecipitation assays indicate that PARN and HuR did not associate to form complex in HCT116 NEs (Figure 40), supporting the idea that HuR inhibits PARN-mediated deadenylation by promoting dissociation of miRISC and PARN from the p53 3’UTR rather than through protein-protein interactions.

![Figure 40](image)

**Figure 40. PARN and HuR do not form a complex.** Coimmunoprecipitation assays were performed using HCT116 NEs and either PARN or HuR antibodies. Co-immunoprecipitated proteins were analyzed by Western Blot using antibodies against Topo II, PARN and HuR.
p53 is a key transcription factor that regulates expression of a vast number of genes to determine cell’s fate upon genotoxic stresses. The response to stress can be either in survival mode where cells normal functions are restored after DNA damage is repaired or in apoptosis where the tumor suppressor p53 induces apoptosis through its transcriptional targets when the damage is beyond repair. Because of its pivotal role during DNA damage and tumorigenesis, p53 expression levels under normal conditions and after DDR are tightly regulated. In Chapters IV-V of this dissertation I have shown that PARN regulates p53 mRNA through ARE sequences present in its 3’UTR under non-stress conditions (Devany et al., 2013). Furthermore, studies from Dr. Kleiman’s lab have also shown that PARN interacts with the miRISC component Ago-2 and that Ago-2 can activate PARN deadenylase under non-stress conditions (Dr Zhang personal communication). Interestingly, as shown in Chapter V, these two cis-acting elements functionally overlap to regulate p53 steady-state levels and the binding of PARN deadenylase to p53 3’UTR.

As the levels of p53 expression increase after DNA damage treatment, the PARN-mediated down-regulation of p53 mRNA should be reverted during the progression of the DDR. The ARE sequence in p53 3’UTR is also a known target of HuR upon DNA damage. HuR binds to p53 mRNA in a UV-dependent manner and translocates it to the polysomes where translation of p53 mRNA is increased (Mazan-Mamczarz et al., 2003). Here I show that PARN-associated Ago-2 binds to and regulates p53 mRNA stability under normal conditions, and that HuR binds to and regulates p53 mRNA stability upon UV treatment (Figures 29-30 and 35). HuR can compete for binding to the p53 3’UTR with both PARN and Ago-2 both in in vitro and in vivo assays (Figures 32-33 and 35-36), and that
oligomerization of HuR is important for this function (Figure 36). Furthermore, HuR affects the poly(A) tail length and PARN-mediated deadenylation of p53 mRNA (Figures 37 and 39).

Based on my previous findings and the results described in this Chapter, I propose a working model where the dynamic binding of PARN-associated Ago-2 and HuR to the p53 mRNA plays a role in the progression of DDR (Figure 41). My results suggest a competitive regulation of p53 mRNA by PARN-associated Ago-2 and HuR. Under non-stress conditions, PARN-associated Ago-2 outcompetes HuR and destabilizes p53 mRNA by inducing ARE- and miRNA-mediated deadenylation. After UV-treatment, PARN-associated Ago-2 detaches from p53 mRNA, probably due to changes in abundance of miRNAs involved in this regulation and translocation of Ago-2 from nucleus to cytoplasm (Dr. Zhang personal communication). This allows binding of HuR to the AREs in the p53 3’UTR in the nucleus and increases translation by escorting p53 mRNA to the polysomes.

Reports by others have also shown that HuR is involved in miRNA-mediated regulation of mRNA stability. In a recent study, it has been reported that over 75% of mRNAs with Ago-2 binding sites also have HuR binding sites (Mukherjee et al., 2011). Most of these Ago-2 and HuR binding site pairs are in less than 10 nt distance, suggesting a competitive or cooperative regulation of target mRNAs (Mukherjee et al., 2011). In fact, earlier studies have shown both cooperation and competition between miRISC- and HuR-dependent regulation of target mRNAs stability. For instance, HuR recruits let-7/RISC to repress c-Myc expression (Kim et al., 2009). However, HuR can also outcompete and relieve miRNA-mediated depression upon environmental stimuli, which resulted in changes of target mRNAs stability (Bhattacharyya et al., 2006b).
Consistent with previous reports (Mazan-Mamczarz et al., 2003), I did not detect a significant change in nuclear HuR levels after UV treatment (Figure 28B). However, it has been reported that post-translational modifications in the HuR protein, such as the phosphorylation or ubiquitination, occurs upon DNA damage, and these modifications change binding affinity of HuR to its mRNA substrates and its cellular localization (Kim et al., 2008; Meisner and Filipowicz, 2011). Currently, it is not known which DNA damage-regulated kinase or ubiquitin ligase is linked to the regulation of HuR function. Nevertheless, it is possible that changes in the phosphorylation or ubiquitination state of HuR could lead to enhanced binding of HuR to p53 mRNA, hence dissociation of PARN/Ago-2 complex upon UV treatment. A good candidate for this regulatory function is kinase MK2 from the p38MAPK/MK2 pathway. Interestingly, MK2 phosphorylates not only HuR but also Ago-2 and PARN (Reinhardt et al., 2011). Importantly, MK2-mediated phosphorylation of PARN at Ser577 interferes with its deadenylase activity upon UV-treatment (Reinhardt et al., 2011). On the other hand, other deadenylases, such as CCR4-NOT complex and Pan2-Pan3, are not components of p38MAPK/MK2 pathway, suggesting that this regulatory mechanism might be PARN specific.

As shown in Figure 38, knockdown of HuR affects deadenylation of p53 mRNA but not deadenylation of L3(A30) RNA, which lacks cis-acting elements. However, PARN/Ago-2 complex can deadenylate the L3(A30) RNA substrate (Dr. Zhang personal communication). Together, these results support the idea that HuR’s inhibitory effect on deadenylation of p53 mRNA might be due to the displacement PARN/Ago-2 from p53 messenger RNA and not due to direct inhibition of PARN-associated Ago-2 deadenylase.
activity. Consistent with this, no interaction has been shown between HuR and the components of the miRISC complex (Kundu et al., 2012).

![Diagram showing the regulation of p53 mRNA steady-state levels by PARN-associated Ago-2 and HuR during the progression of DDR.](image-url)

**Figure 41.** Model of regulation of p53 mRNA steady-state levels by PARN-associated Ago-2 and HuR during the progression of DDR. Under normal conditions, PARN-associated Ago-2 regulates p53 expression through miRNA- and ARE-targeting sites. After UV treatment, PARN-associated Ago-2 dissociates from p53 mRNA due to changes in abundance of miRNAs involved in this regulation and translocation of Ago-2 from nucleus to cytoplasm. Once PARN-associated Ago-2 releases p53 messenger, HuR binds to and oligomerizes along p53 mRNA and then escorts it to cytoplasm where it increases the p53 translation.
Together, my results show dynamic interactions of PARN/Ago-2 and HuR with p53 3’UTR under different cellular conditions, indicating that changes of these factors binding to p53 mRNA is important for regulating p53 protein levels during the progression of DDR.
CHAPTER VII

REGULATION OF ALTERNATIVE POLYADENYLATION

DURING DNA DAMAGE RESPONSE
INTRODUCTION

Messenger RNAs (mRNAs) in eukaryotic cells are produced from primary transcripts (pre-mRNAs) by extensive post-transcriptional processing, including 5' end capping, removal of introns by splicing, and 3' end cleavage and polyadenylation. The polyadenylation reaction occurs in the nucleus through a two-step reaction: an initial cleavage step, which specifies the 3’ end of the mRNA, followed by synthesis of a 200-adenosine residue tail to the 3’ end of the upstream cleavage product (reviewed by Zhao et al, 1999; Shatkin and Manley, 2000). First, the cleavage and polyadenylation specificity factor (CPSF) binds to the polyadenylation signal, which is often the conserved 6 nucleotide sequence AAUAAA, and catalyzes RNA cleavage. The cleavage occurs 10–30 nucleotides downstream of the polyadenylation site. The cleavage stimulation factor (CstF) recognizes a G/U- and U-rich region located downstream from the polyadenylation signal (Takagaki and Manley, 1997) and also participates in the RNA cleavage. The cleavage occurs 10–30 nucleotides downstream of the polyadenylation signal and other factors are involved in this step, such as cleavage factors 1 and 2 (CF I and CF II), RNA polymerase II (RNAP II) and poly(A) polymerase (PAP). CstF interacts with RNAP II likely facilitating the RNAP II-mediated activation of 3’-end processing (McCracken et al, 1997; Hirose and Manley, 1998) and the co-transcriptional cleavage of the RNA. After the RNA is cleaved, the polyadenylation reaction starts, catalyzed by PAP and other factors, such as CPSF, symplekin and poly(A) binding protein (PABP). When the poly(A) tail is approximately 200 nucleotides long the enzyme can no longer bind to CPSF and polyadenylation stops, determining the length of the poly(A) tail.
The polyadenylation machinery is also physically and functionally linked to splicing factors. U1 small ribonucleoprotein particle (U1 snRNP) has been implicated in inhibition of mRNA 3’ processing via PAP (Ashe et al., 2000; Gunderson et al., 1998; Vagner et al., 2000). This mechanism has recently been suggested to play a key role in controlling transcript length (Kaida et al., 2010; Berg et al., 2012). U1 snRNP includes the 164 nt U1 snRNA, seven Sm proteins, and three U1-specific proteins (U1-70K, U1-A, and U1-C). The large ribonucleoprotein complex is responsible for the removal of intronic sequences and subsequent rejoining of exons. The spliceosome assembles through the sequential binding of five snRNPs (U1, U2, U4, U5, and U6) and multiple auxiliary RNA-binding proteins to form the large, active spliceosome (Wahl et al., 2009). The splicing mechanism requires 1:1 stoichiometric ratios of all the snRNPs in the actual removal of each intron. U1 snRNP plays an essential role in this process by driving the initial steps of spliceosome assembly onto the pre-mRNA at the exon-intron boundary, through definition of the 5’ splice site (ss) by RNA-RNA base-pairing with the 5’ end of U1 snRNA, which can occur in multiple registers. Multiple splicing and cleavage/polyadenylation factors, including U1 snRNP and CstF, are directly associated with the RNAP II c-terminal domain (CTD) from the onset of transcription and are then deposited on their cognate-binding sites along the pre-mRNA determining splice-site and poly(A) signal (PAS) selection. Surprisingly, despite the observation that for splicing reaction all snRNPs come together in 1:1 stoichiometric ratio, the cellular levels of U1 snRNP exceed those of other snRNPs by 2-3-fold (Beserga et al., 1993), suggesting that U1 snRNP may carry out additional roles besides its canonical role in splicing. Consistent with this, as mentioned before, U1 snRNP
is involved in different aspects of pre-mRNA 3’ end formation (Gunderson et al., 1998; Ashe et al., 2000; Kaida et al., 2010; Vagner et al., 2000).

Well over half of the mammalian genes contain more than one cleavage and polyadenylation site (pA), leading to alternative polyadenylation (APA; (Hoque et al., 2013; Tian et al., 2005). APA is highly dynamic across tissue types (Wang et al., 2008; Zhang et al., 2005), in cell proliferation and differentiation (Ji et al., 2009a; Sandberg et al., 2008), and in response to extracellular cues (Flavell et al., 2008). Two major types of APA have been identified. APA in 3’UTR regulates 3’UTR length. Messenger 3’UTRs contain various cis-elements for post-transcriptional control, such as microRNA (miRNA) target sites and AU-rich elements (AREs). As 3’UTR-APA creates 3’UTRs of different length lacking or containing these cis-elements, 3’UTR-APA can significantly impact mRNA stability and metabolism and regulate gene expression. Another type of APA involves cleavage and polyadenylation in introns. Polyadenylation sites are highly abundant but seldom used in intronic regions (Tian et al., 2005). APA events of this type (named intron-APA) can result in change of open reading frame (ORF) of mRNA. As a result of intronic-APA, certain intronic regions may be incorporated in the transcript and/or number of exons might be eliminated leading to significantly shorter mRNAs. These mRNAs may be unable to express functional protein or may cause a change in proteome where different isoforms of proteins may emerge and accumulate in the cell with potentially deleterious effects (Spraggon and Carstegni, 2013). The core mammalian mRNA polyadenylation machinery and additional cis-acting elements within the 3’UTR, which function to alter the efficiency of polyadenylation and have also been identified as auxiliary RNA elements, are responsible for the selection among these APA sites (Hall-Pogar et al., 2007; Lutz, 2008;
Interestingly, recent studies showed that U1 snRNP has an inhibitory role in intronic-APA (Kaida et al., 2010). Shorter polyadenylated mRNAs and inhibition of splicing are observed upon functional depletion of U1 using antisense morpholino nucleotides. In those shorter transcripts, cleavage and polyadenylation occur prematurely usually within the first intron (Kaida et al., 2010).

DNA damage occurs upon a number of environmental exposures, including UV irradiation. The DNA damage response (DDR) involves functional and structural changes in a number of nuclear proteins, resulting in a coordinated control of gene expression and DNA repair. One key aspect is the transient decrease of the cellular levels of mRNA following UV-irradiation and its recovery (Hanawalt et al., 1994; Ljungman et al. 1999). Although the mechanism involved in this response is still not completely resolved, it has been suggested that the UV-induced inhibition of transcription is responsible for the decrease in the mRNA levels (Donahue et al. 1994). However, studies from Dr. Kleiman lab and others have shown that 3’ end processing also plays an important role in DDR. First, 3’ end formation is affected in a similar time frame to transcriptional control after DNA damage, resulting in a general, transient decrease of the cellular levels of polyadenylated transcripts. Second, mRNA levels of genes involved in DDR (gene specific response, Cevher et al., 2010, Devany et al. 2013) appear to be specifically regulated at the 3’-end processing step. Third, the 3’ end processing factor CstF1 and PARN deadenylase have functional interaction with tumor suppressors and DNA repair factors whose expression is commonly compromised in most cancers, such as BARD1 and p53 (Kleiman and Manley 2001, Kleiman et al. 2005, (Mirkin et al., 2008), Cevher et al. 2010, Cevher and Kleiman 2010, Nazeer et al. 2011, Devany et al. 2013). We have found that, as part of
the gene-specific response, the BARD1, CstF, and PARN complex has a role in decreasing the levels of short-lived mRNAs involved in the regulation of cell growth, differentiation and DDR, and keeping their expression levels low under non-stress conditions (Cevher et al. 2010, Devany et al. 2013).

In this chapter, I explore the mechanisms and consequences of APA upon UV-induced DNA damage. In collaboration with Dr. Bin Tian, Rutgers University, we determined that intronic-APA was induced upon UV treatment using 3' region extraction and deep sequencing (3'READS). Intronic-APA was confirmed in three of the mRNAs detected by using semi-quantitative PCR as well as qRT-PCR. Importantly, these three mRNAs, cyclin-dependent kinase inhibitor 1A (CDKN1A, p21), and polymerase (RNA) II (DNA directed) polypeptide A (POLR2A) and Ephrin B2, are involved in DDR. Furthermore, a decrease in U1 RNA levels were observed after UV treatment, which could explain the activation of intronic-APA based on previous reports. Supporting this, the functional depletion of U1 RNA by antisense morpholino caused a similar UV-mediated activation of intronic-APA, and this was rescued by over expression of U1 RNA. These studies establish a new paradigm in DDR research, providing insights into the mechanisms behind the regulation of gene expression during DDR.

RESULTS

In collaboration with Dr. Bin Tian, Rutgers University, we examined APA in DDR using 3'READS, a recently developed deep sequencing method to analyze APA isoforms expression genome-wide (Hoque et al., 2013). Nuclear RNA from colon carcinoma RKO cells treated or not with UV irradiation and allowed to recover for 2 h were analyzed by
3’READS (Figure 4). 3’READS accurately maps APAs and quantitatively measure APA isoform expression overcoming the problem of priming of oligo(dT) at internal A-rich sequences and minimizing the complications of oligo(A) tail. This is achieved by using conditions that distinguishes RNAs with long A-tails from those with short A-tails, and by avoiding the use of oligo(dT) in the generation of cDNAs by reverse transcription and in the sequencing step. Briefly, after RNA fragmentation, poly(A)-containing RNA fragments were captured onto magnetic beads coated with a chimeric oligonucleotide (oligo CU₅T₄₅), which contains 45 thymidines (Ts) at the 5’ portion and 5 uridines (Us) at the 3’ portion. Experimental condition which enriched RNAs with 60 terminal As by ~12-fold as compared to those with 15 As were developed. RNase H treatment released the RNA from the beads and eliminated most of the As of the poly(A) tail. Eluted RNA was ligated to 5’ and 3’ adapters, followed by reverse transcription, PCR amplification, and deep sequencing. The resulting reads were aligned to the genome, and those with at least 2 non-genomic As at the 3’ end were considered as PolyA Site Supporting (PASS) reads, and were used for pAs analysis.

Using 3’READS, APA isoforms in over 1300 mRNA genes were found. Interestingly, over 700 genes displayed regulated APA in 3’UTRsvafter UV treatment. Strikingly, over 600 genes displayed activated intronic-APAs after UV treatment (Figure 42B). Genes subjected to 3’UTR-APA and intron-APA fall into different functional groups (Figure 42C). Interestingly, genes involved in the response to UV treatment and nucleotide-excision repair are more susceptible to 3’UTR regulation under UV treatment.

To validate our genomic analysis, I examined APA for three key genes that are highly relevant to DDR (Figure 43): cyclin-dependent kinase inhibitor 1A (CDKN1A,
p21), polymerase (RNA) II (DNA directed) polypeptide A (POLR2A) and Ephrin B2. Using RT-qPCR, I validated their APA events during different recovery time points after UV treatment. After recovery from UV treatment, nuclear RNA was isolated and cDNA was synthesized by reverse transcription using oligo(dT) primers. I performed PCR using these cDNA as template and three primers for detection of products of intronic-APA (short isoform) and full-length mRNAs (long isoform, Figure 43B. The forward primers used in the reactions were located in exon 1 of the studied genes. Two reverse primers were added to the reaction, one corresponding to intron 1 for detection of short isoform and one

**Figure 42. Regulation of APA after UV-treatment.** A) Top, usage of 3’UTR-APA; bottom, usage of intron-APA. B) Summary of 3’UTR-APA and intron-APA events. C) Gene ontology (GO) analysis associated with genes having APA regulation. P-values are based on the Fisher’s exact test.
Figure 43. Intron-APA is up-regulated upon UV-induced DNA damage.

HCT116 cells were treated with UV irradiation. UV treated cells were allowed to recover for indicated times and then harvested. Nuclear RNA was isolated using QIAgen RNA isolation kit. cDNA was prepared using oligo(dT) primers and used for PCR reaction with primers specific for intronic-APA (short) or full length-mRNA (long) of POLR2A, CDKN1A and Ephrin B2.
corresponding to exon 2 for detection of long isoform. The ratio of short isoform to long isoform is shown in Figure 43B. My results confirmed the 3’READS results that indicated that UV treatment induced intronic-APAs. Interestingly, the increase in intronic-APA isoforms was observed from 2-6 hr after UV-treatment, but these shorter isoforms disappear after 10 hr, reaching the levels of untreated cells. The transient nature of these intronic-APA isoforms is consistent with previously characterized responses to DNA damage (Kleiman and Manley, 2001; Cevher et al., 2010). These results were also performed by qRT-PCR (Figure 44). Together, these results indicate the UV treatment induce the usage of polyadenylation signals in the first intron of genes involved in DDR, suggesting a possible role for these intronic-APA events in controlling gene expression during the response.

Earlier studies have shown a decrease in U1 and U2 small RNA levels in HeLa cells upon UV-treatment (Eliceiri and Smith, 1983). Interestingly, the functional depletion of U1 RNA shortens mRNAs due to the usage of proximal polyadenylation signals in 3’UTR and introns (Berg et al., 2012). Based on these earlier reports, I decided to examine whether intronic polyadenylation is triggered by U1 RNA reduction in response to UV irradiation. First, I examined the effect of UV treatment on the levels of U1 RNA using HCT116 and RKO cells (Figure 45). Cells were treated with UV irradiation and allowed to recover for the indicated time points. Consistent with the studies of (Eliceiri and Smith, 1983), qRT-PCR analysis of nuclear RNA samples from these cells showed a transient decrease in U1 RNA levels upon UV-treatment (Figure 45). Although a decrease in U1 RNA was detected as early as 30 min after UV treatment, the lowest level of U1 RNA was observed 6 h after UV treatment for both cell lines. The levels of U1 RNA increased 24 hr after UV treatment,
Figure 4. Intron-APA is transiently up-regulated upon UV-induced DNA damage. Samples were prepared as in Figure 42 but then were analyzed by qRT-PCR reaction with primers specific for intronic-APA or full length mRNA (FL) of POLR2A, CDKN1A and Ephrin B2 and SYBR green master mix. The ratio of intronic/full length of each mRNA is shown.

reaching the levels of untreated cells. Extending those studies, the levels of other components of the U1 and U2 snRNPs were also analyzed by qRT-PCR as described above. A decrease was also observed in U2 RNA, U1A, and U1-70K after UV-treatment (Figure 46). No significant change was observed in U1C levels. Importantly, functional depletion of U1 RNA using morpholino oligonucleotides increased significantly the ratio of intronic/full-length polyadenylated isoforms for POLR2A, CDKN1A and Ephrin B2
The change in the intronic/full-length ratio by U1 RNA depletion (Figure 47) was similar in magnitude to that observed after UV-treatment (Figure 44). Together, these results indicate that there is a correlation between decrease in U1 RNA levels and increase in the usage of intronic polyadenylation signals. Furthermore, the decrease of U1 RNA was sufficient to increase intronic-APA isoforms of three genes involved in DDR, suggesting that the UV-induced decrease in U1 RNA levels might be responsible for activation of intronic-APA for these mRNAs upon UV treatment. Although more work is needed, this UV-induced activation of intronic-APA might represent a new mechanism of control of gene expression.

**Figure 45. U1 RNA levels transiently decrease upon UV-treatment.** HCT116 and RKO cells were treated with UV irradiation. UV-treated cells were allowed to recover for the indicated times and then harvested. Nuclear RNA was isolated and cDNA was prepared using random primers. qRT-PCR reaction was performed with primers specific for U1 RNA.
**Figure 46. Levels of components of spliceosome complex upon UV-treatment.**

HCT116 cells were treated with UV irradiation and analyzed as in Figure 45 by qRT-PCR reaction with primers specific for U2 RNA, U1A, U1C and U1-70K.

**Figure 47. Functional depletion of U1 RNA causes up-regulation of intronic-APA.** HCT116 cells were transfected with control or anti-sense morpholino targeting U1 RNA (U1 AMO). Cells were transfected using scrape delivery method. Briefly, 10 nmoles of control oligo or U1 snRNP targeting morpholino oligonucleotides were added to the cell medium in 6-well plates. Cells were scraped using a cell scraper and transferred to a new 6-well plate. Cells harvested 48 h after transfection and nuclear RNA was isolated. cDNA was prepared and used in qRT-PCR reactions as in Figure 43.
DISCUSSION

Regulation of APA is an important mechanism to control gene expression as well as proteome of the cell. One of the major components of spliceosome complex, U1 snRNP, plays an important role not only in splicing and regulation of alternative splicing but also in regulation of APA. Others have previously demonstrated inhibitory functions of U1 snRNP in mRNA 3’ processing (Gunderson et al., 1998; Ashe et al., 2000; Vagner et al., 2000). Importantly, activation of intronic polyadenylation has been observed by Kaida et al. (2010) and others (Vorlova et al., 2011; Berg et al., 2012) upon functional depletion of U1 RNA using antisense morpholinos. Changes in U1 snRNP expression levels resulted in mRNA isoforms with different lengths (Kaida et al. 2010; Berg et al. 2012). These studies indicate that while a moderate decrease in the levels of U1 snRNP expression results in the usage of proximal APAs and generates shorter mRNA isoforms (Berg et al. 2012), a more pronounce decrease in U1 snRNP expression results in the usage premature/cryptic APAs (Kaida et al. 2010). It has been suggested that this is a co-transcriptional mechanism in which U1 snRNP binds to the nascent mRNA avoiding the premature 3’ end processing by interacting with the pre-mRNA and inhibiting polyadenylation (Gunderson et al. 1998; Vagner et al. 2000). Significantly, the formation of truncated mRNA isoforms or shorter protein products as a result of intronic-APA has been shown to contribute to tumorigenesis (Gschwind et al., 2004). In that scenario, U1 snRNP has been proposed to be part of a cellular surveillance pathway that inhibits usage of cryptic polyadenylation sites within the introns.

The results in this chapter describe the activation of intronic-APA upon UV treatment (Figure 42). Three of the mRNAs detected to undergo intronic-APA were
confirmed using semi-quantitative PCR (Figure 43) as well as qRT-PCR (Figure 44). Furthermore, a decrease in U1 RNA levels was observed after UV treatment, which could explain the activation of the usage of intronic polyadenylation signals based on previous reports (Figures 45-46). Supporting this, the functional depletion of U1 RNA by antisense morpholino caused a similar activation of APA (Figure 47).

Intronic-APA is an important event since it leads to changes in transcriptome and may change the proteome of the cell. My results show that the shorter forms of the regulated mRNAs are stable to be detected in cells after UV treatment. So far, the function of these shorter forms is unknown. Whether they act as a sponge to functionally knockdown particular proteins and/or RNAs in the cell, or they encode different isoforms of their own protein need to be further investigated. We found that UV treatment induced intronic-APA isoforms of three factors involved in DDR; POLR2A, CDKN1A and Ephrin B2. Although the UV-induced changes in transcription and protein levels of these factors have been extensively studied, the UV-induced effect on the usage of different APAs and steady-state levels of their transcripts has not been elucidated. It has been shown that UV damage switches the usage of poly(A) site from the proximal to the distal one for the yeast largest subunit of RNAP II gene (Yu and Volkert, 2013). My results indicate that UV treatment induces the usage of an intronic-APA in a not conserved region (Figure 43). These discrepancies might be explained by the fact that factors involved in the regulation of mRNA 3’ processing during DDR, such as CstF1 and BARD1, are not expressed in yeast.

Besides, it is well documented that a fraction of the largest subunit of RNAP II decreases by DNA damage-induced ubiquitination in mammalian cells (Bregman et al.,
This modification can be detected within 15 min after exposing HeLa cells to UV irradiation, persists for about 8–12 hr (Bregman et al., 1996), and appears to require CTD phosphorylation, characteristic of elongating RNAP II (Ratner et al., 1998; Mitsui and Sharp, 1999). Importantly, ubiquitination is accompanied by transient reductions of RNAP II levels, likely mediated by proteasomal degradation (Ratner et al., 1998). Interestingly, the polyadenylation factor CstF associated to the tumor suppressors BRCA1/BARD1 play a role in the proteasome-mediated degradation of RNAP II during DDR (Kleiman et al., 2005; Starita et al., 2005). Both the interaction of CstF and BRCA1/BARD1 complex (Kleiman and Manley, 2001; Kleiman et al., 2005; Nazeer et al., 2011) and the proteasome-mediated degradation of RNAP II (Kleiman et al., 2005; Starita et al., 2005) contribute the 3’ end cleavage inhibition that occurs after DNA damage. However, it is also possible that the UV-induced decrease in RNAP II might be due to the UV-induced increase in intronic-APA, which results in a decrease in full-length mRNA (Figure 43) and probably in RNAP II protein levels. As the mRNA of the intronic-APA isoforms is stable, there is also a possibility that intronic-APA mRNAs are translated and that this isoform has a dominant-negative effect on the full-length protein and it can out-compete the full-length protein in some way.

It has been shown that while UV treatment induces p53 binding and histone acetylation at the p21 promoter, it does not induce a strong increase in p21 mRNA levels (Donner et al., 2007) indicating that other mechanisms contribute to the regulation of p21 mRNA levels upon UV-treatment. Activation of intronic-APA and accumulation of shorter mRNA isoforms might provide an alternative explanation of p21 regulation during DDR.
Together, these results indicate that gene-specific regulatory mechanisms are activated after UV-treatment and that the usage of APA might control gene expression and be involved in the progression of DDR.
CHAPTER VIII
FUTURE DIRECTIONS
The p53 pathway has long been studied. The tumor suppressor p53 is a key transcription factor that regulates expression of a vast number of genes to determine cell’s fate upon different genotoxic stresses. The response to stress could be either in a survival mode, where cellular functions are restored after DNA damage is repaired, or in a death mode, where p53 induces apoptosis through its transcriptional targets when DNA damage is beyond repair. Because of the vast number of regulated targets and its pivotal role during DNA damage and tumorigenesis, the expression levels of p53 under normal conditions and during DNA damage response (DDR) are tightly regulated.

The findings presented in this dissertation show two novel critical transcriptional-independent roles of p53. First, p53 inhibits the 3’ cleavage step of the polyadenylation reaction through its interaction with the mRNA processing factor CstF1 and the tumor suppressors BARD1/BRCA1 (Chapter II). Second, p53 activates PARN-mediated deadenylation as part of DDR (Chapter III). Furthermore, as a part of this dissertation, I described that p53 mRNA is a biological target of the major nuclear deadenylase PARN under non-stress conditions (Chapter IV). PARN destabilizes p53 mRNA by shortening the poly(A) tail under non-stress conditions. Extending those studies, I also described that PARN regulates p53 expression through AREs and miRNA binding sites that are present in the 3’UTR of p53 mRNA (Chapter V). Importantly, HuR binding and PARN/Ago-2 dissociation from the p53 3’UTR is a crucial step for p53 induction and progression of DDR (Chapter VI).

Now, it is important to further elucidate the complete mechanism behind PARN-mediated regulation of p53 in different cellular conditions. The following proposed studies might help to understand some aspects of the working model shown in this dissertation.
Identification of ARE-binding proteins (ARE-BPs) that are involved in PARN-mediated regulation of p53 mRNA steady-state levels under non-stress conditions.

The results presented in this dissertation indicate that PARN regulates the stability of ARE-containing p53 mRNA and keeps its levels low in non-stress conditions (Cevher et al., 2010; Devany et al., 2013). It has been shown that PARN deadenylase is recruited to ARE sequences by ARE-BP 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). However, these ARE-BPs have shown to be not interacting with p53 mRNA AREs (Mazan-Mamczarz et al., 2003).

The results presented here lay the groundwork for further studies on how PARN is recruited to ARE-containing mRNAs and which ARE-BPs are involved in this process. The identification of the particular ARE-BP that contributes to PARN recruitment to p53 mRNA is important to understand the mechanisms involved in the regulation of p53 expression under different cellular conditions. The identification of these ARE-BPs will also help to further characterize the functional overlap of ARE-mediated and miRNA-mediated deadenylation pathways. My results show that both cis-acting elements in p53 3’UTR are important for PARN binding and p53 mRNA regulation. Therefore, the identification of the ARE-BPs that are involved in this regulatory process might help us to further understand the complexity ARE-BP and miRISC pathways.

Post-transcriptional regulation of p53 mRNA has been studied extensively in recent years. It is known that p53 mRNA is regulated by several RNA-BPs. A recent study has shown that nucleolin binds to p53 mRNA at the 5’UTR and 3’UTR base-pairing region in
unstressed cells resulting in the repression of p53 mRNA translation (Chen et al., 2012). Nucleolin is a eukaryotic nucleolar phosphoprotein involved in synthesis and maturation of ribosomes. After DNA damage and stress stimulation, RPL26, which is a ribosomal protein that is also targeted for degradation by binding to MDM2, is recruited to this double-stranded RNA structure occupied by nucleolin forming nucleolin-RPL26 heterodimers and enhancing the translation of p53 mRNA. Studies from Dr. Kleiman’s lab have also shown the direct interaction between PARN and nucleolin and that nucleolin binding to p53 3’UTR is decreased significantly in the absence of ARE sequence (Dr. Zhang personal communication). Future studies are necessary to investigate the physiological relevance of the PARN/nucleolin interaction. Besides it will be important to determine the role of PARN/nucleolin complex in the miRISC pathway.

In addition, wild-type p53-induced gene 1 (Wig-1) (Vilborg et al., 2009) and HuR (Mazan-Mamczarz et al., 2003; Zou et al., 2006) proteins have been shown to bind AREs in p53 3’UTR and stabilize the transcript in a UV-dependent manner. Recent studies have shown that other proteins, including RNPC1a (Zhang et al., 2011) and Pdcd4 (Wedeken et al., 2011), can bind the AREs in p53 mRNA 3’UTR and destabilize it. Additionally, Rosenstierne et al. (Rosenstierne et al., 2008) described that heterogenous nuclear ribonucleoprotein H1 (hnRNPH1) as the main nuclear protein that binds differentially a WT and ARE-deletion mutant of p53 mRNA by using mass spectroscopy. Understanding the critical function of these ARE-BPs on the regulation of p53 mRNA function is relevant for developing appropriate therapeutic approaches to a variety of disorders, including cancer.
Further characterization of the regulation of p53 mRNA expression during the progression of DDR.

HuR is a ubiquitously expressed ARE-BP that targets mRNAs that encode proteins important for cell growth, proliferation, cell death, and immune response. It is mainly localized in the nucleus and has shown to play roles in splicing and alternative polyadenylation (Mukherjee et al., 2011). It has been shown that HuR binds p53 mRNA in the cytoplasm in a UV-dependent manner and translocates it to the polysomes where translation is activated (Mazan-Mamczarz et al., 2003). The results presented in this dissertation showed that HuR regulates p53 expression in a UV-dependent manner not only in the cytoplasm but also in the nucleus (Chapter VI). I have shown a competitive regulation of p53 mRNA by HuR and miRISC-associated PARN deadenylase (Figures 31-34); where HuR inhibits deadenylation of p53 mRNA in the nucleus probably by promoting dissociation of miRISC complex and PARN deadenylase (Figures 38-39). These studies should be extended once the identity of the ARE-BP involved in PARN-mediated p53 mRNA regulation under non-stress conditions is known.

Future studies are important to reveal the details of this mechanism. These studies will help us to understand how the regulation of p53 mRNA changes between non-stress conditions and after DNA damage. Other studies have shown that miR125b levels decrease in the nucleus and cytoplasm resulting in an increase in p53 levels under stress conditions (Le et al., 2009); Dr. Zhang personal communication). As the ARE- and miRNA-mediated pathways are functionally connected (Chapter V), the change in miRNA levels may explain the exchange of RNA-BPs upon UV-induced DNA damage. However, it is also possible
that HuR post-translational modifications may play a regulatory role. Further studies on the effects of post-translational modifications of HuR protein on this regulation are crucial for understanding this important mechanism. HuR protein has shown to be post-translationally modified under different cellular conditions. Phosphorylation is the most well studied modification of HuR. A recent report showed that ubiquitination of HuR is also important for HuR binding to its target mRNAs (Zhou et al., 2013). This study shows that HuR is di-ubiquinitated under normal conditions and this modification leads to evacuation of HuR from the RNA-protein complexes. After UV treatment, it has been shown that HuR is de-ubiquinated leading to enhanced binding of HuR to its substrates (Zhou et al., 2013). As mentioned before, understanding how p53 mRNA is regulated under different cellular conditions may help to improve therapeutic approaches to diseases such as cancer.

**Determination of the mechanisms for p53-activated PARN-mediated regulation of other mRNA targets.**

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. Recent studies, including the data presented in this dissertation (Chapter IV), indicate that the mRNA levels of both housekeeping genes and ARE-containing genes are regulated by PARN deadenylase under different cellular conditions (Cevher et al., 2010; Devany et al., 2013). 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 results presented in this dissertation that indicate that the most affected genes by PARN depletion are p53-related genes that have been shown linked with cancerous growth and DNA repair activities (Chapter IV). Future studies are necessary to investigate how the mechanisms that regulate PARN activity described in this dissertation are affected in cancer cells.

In addition, my results indicate that the UV-induced increase in p53 levels resulted in activation of PARN deadenylase. Cevher and colleagues (2010) suggested that UV-induced increase in PARN activity is related to the general response to DNA damage, where a decrease in mRNAs from housekeeping genes occurs. Consistent with this, my results indicate that p53 mRNA is not a target of PARN deadenylase after UV treatment (Figure 15). It is known that induction of p53 is crucial for DDR. UV-induced transcriptional functions of p53 include the regulation of specific target genes by binding to a p53 response element that is found either in promoters or introns of target genes (el-Deiry et al., 1992; Tokino and Nakamura, 2000). The data presented in this dissertation show transcriptional-independent roles of p53. p53 plays a role in the UV-induced inhibition of polyadenylation (Chapter II) and UV-induced activation of PARN deadenylase (Chapter III) in the nucleus. Future studies should focus on the identification of mRNAs that are
regulated by p53-activated PARN upon UV-treatment. The presence of possible common regulatory elements present in the 3’UTR of these mRNAs should be determined and analyzed to better understand the mechanisms behind p53-activated PARN.
CHAPTER IX

EXPERIMENTAL PROCEDURES
**Tissue culture methods** - HeLa, HCT116, p53-null HCT116, MEFs, p-53-null MEFs and H-1299 cell lines were cultured in Dulbecco’s modified Eagles medium (DMEM)-10% fetal bovine serum (FBS) and 1% penicillin/streptomycin antibiotic. RKO and 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 Stratalinker (Stratagene). Prior to pulsing, medium was removed and replaced immediately after treatment.

**Knockdown expression of PARN, p53, Ago-2 and HuR by siRNA** - siRNAs specific for human PARN, p53, Ago-2, HuR and the control siRNA used as non-silencing were obtained from Dhharmacon 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, p53, Ago-2, HuR 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.

**HuR overexpression and miRNA inhibitor expression plasmids transfection** – HuR full-length or derivatives (kindly provided by Dr Bhattacharyya, Friedrich Miescher Institute Basel, Switzerland), miR-125b inhibitor expression plasmid (HmiR-AN0096-AM03, GeneCopoeia) or control plasmid (AM03, GeneCopoeia) 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 the plasmid of interest 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); (Lee et al., 1988) 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. Equivalent amounts of nuclear proteins were subjected to SDS-PAGE and proteins were detected by immunoblotting using antibodies against HuR (3A2, 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), p53 (SC-126, Santa Cruz Biotechnology), HuR (3A2, Santa Cruz Biotechnology) or Ago-2 (4F2, Santa Cruz Biotechnology) bound to protein A-magnetic 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 Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, 0.01% Nonidet P-40, 0.5 mM PMSF, and 0.04% bovine serum albumin). The beads were recovered 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** - The plasmid encoding His-PARN (kindly provided by Dr. A. Virtanen, Uppsala University) 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). Plasmid encoding His-p53 and derivatives (kindly provided by Dr. Prives, Columbia University) and plasmids encoding His-HuR and derivatives (kindly provided by Dr Bhattacharyya, Friedrich Miescher Institute Basel, Switzerland) were expressed in BL21 cells, and purified by binding to and elution from Ni-agarose columns as described (Kleiman and Manley, 2001). The plasmid encoding the full-length GST-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 (Wang et al., 2009).

**Protein-protein interaction assays** - 2 µg of His-PARN derivatives (full-length, N-terminal domain and C-terminal domain) or His-p53 derivatives (full-length, p53 aa 1-293,
p53 aa 94-293 and p53 aa 94-393) was incubated with Ni-Magnetic beads. The truncated forms of His-PARN and His-p53 used in this assay were described before (Cevher et al., 2010; Nazeer et al., 2011). His-tagged proteins were 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. 200 µg of NEs from untreated or UV-treated RKO cells was added to the His-tagged proteins-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, p53 and derivatives of p53 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 L3(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 (product ID: HmiT054283, Genecopecia) 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’-ATGGATTCAATTAACCCTCACTAAAGGGAACATTCTCCACTTCTTGTTCCTCCACTAC-3’ and Reverse 5’-GGATGATCCATAAGCTT(A)_{20}TGGGATATAAAAAGGG-3’). The whole p53 3’UTR, including polyadenylation and cleavage signals, was amplified. The PCR fragments were digested with Hind III to generate the poly(A) tail. Then polyadenylated radiolabeled RNA substrates were synthesized 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 p53 or HuR 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 \(^7\)MeGpppG capped *in vitro* transcribed L₃(A₃₀) or p53 3’UTR RNA substrate, radioactively labeled by the inclusion of \(^32\)P-\(\alpha\)-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 APA isoforms 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 GoScript 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, TP53) were used in the qRT-PCR reactions (Applied Biosystems). **POLR2A:** forward primer 5’-TGCGCACCATCAAGAG AGTCCA-3’, intron-APA reverse primer 5’-CCTCCTTCTCACCCCTCCAGCCA-3’, full-length mRNA reverse primer 5’-GCGGCCA GTCCGCTCAATCA-3’. **p21:** forward primer 5’-GGCGGAGAGCGGGATTACAAGT-3’, intron-APA reverse primer 5’-AGGTGGTAGCAGCTGGCGTA-3’, full-length mRNA reverse primers 5’-TGAGAGGTTCCTAAGAGTGCTGGGC-3’ and 5’-TGACAG CGATGGGAAGAGCCA-3’. **Ephrin B2:** forward primer 5’-CGTGTGGAGTAC TGCTGGGCT-3’, intron-APA reverse primer 5’-AAAGGCAGAGACCGTGCTCGGT-3’, full-length mRNA reverse primers 5’-CAGTTTTAGATCCACTTGAGGGCA-3’. Relative levels were calculated using ΔCt method.

**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’-GGGTCAATTTCGTTGTCTCTGCTGTGATCTGCTTTTTCTTTGAGACTGGG-3’ and 5’-CCCAGTCTCAAGACTTGATCTGCTTTTTCTTTGAGACTGGG-3’ for ARE sequence replacement, primers 5’-CTGGATCCACCAAGACTTTTTATGATTCTCTTTTTCTTTTTTTTTTTT3’ and 5’-AAAAAAAGAAAAAGAAATCATAAAAACAAGTCTTGGTGGATCCAG-3’ for miRNA targeting sites replacement and primers 5’-CCAGTCTCAAAAGAAAAAGCAGATCAGAATTCGCGAACGGAAAATTGACCC-3’ and 5’-CCAGTCTCAAAAGAAAAAGCAGATCAGAATTCGCGAACGGAAAATTGACCC-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 siRNA-targeting PARN, HuR 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, Santa Cruz Biotechnology), HuR (3A2, Santa Cruz Biotechnology), Ago-2 (4F2, Santa Cruz Biotechnology) 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 qRT-PCR assays.

**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’-GGGGATCCGCGGTTTTTTTTTTTTTTTTTTTT-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’-CTGCATTTCACCCCCACCCCTCC-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).

**RNA preparation and 3' region extraction and deep sequencing (3'READS) analysis:**

Nuclear RNA from colon carcinoma RKO cells treated or not with UV irradiation and allowed to recover for 2 h was purified using the RNeasy kit (Qiagen) following manufacturer’s protocol. The RNA concentrations of the RNA samples obtained under different conditions were equalized and used in 3'READS analysis by Dr. Tian, Rutgers University as described (Hoque et al., 2013). Briefly, after RNA fragmentation, poly(A)-containing RNA fragments were captured onto magnetic beads coated with a chimeric oligonucleotide (oligo CU₅T₄₅), which contains 45 thymidines (Ts) at the 5’ portion and 5 uridines (Us) at the 3’ portion. Experimental condition which enriched RNAs with 60 terminal As by ~12-fold as compared to those with 15 As were developed. The RNA was released from the beads by RNase H treatment, which also eliminated most of the As of the poly(A) tail. Eluted RNA was ligated to 5’ and 3’ adapters, followed by reverse transcription, PCR amplification, and deep sequencing. The resulting reads were aligned to the genome, and those with at least 2 non-genomic As at the 3’ end were considered as PolyA Site Supporting (PASS) reads, and were used for pAs analysis.
CHAPTER X

REFERENCES


