Polyadenylation/Deadenylation/Tumor Suppressor Factors Regulate 3' End Processing Under Different Cellular Conditions

by

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ABSTRACT

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Under DNA damaging conditions the steady-state levels of cellular mRNAs change as a result of regulation of either or both their biosynthesis and turnover. mRNA 3’ end cleavage, involved in the regulation of mRNA stability, is strongly but transiently inhibited upon UV treatment. This inhibition is mediated by both the formation of the BRCA1/BARD1/CstF complex and the proteasomal-mediated degradation of RNA polymerase II (RNAP II). As CstF-50 interacts with the tumor suppressor BARD1 to inhibit 3’ processing and with RNAP II to activate 3’ cleavage, it has been proposed that this cleavage factor plays a coordinating role in the DNA damage response.

BARD1 is modified by ATM kinase-dependent phosphorylation at the consensus site T734 upon UV treatment. Here we show that the T734A mutation abrogates the UV-induced BARD1/CstF complex formation; the UV-induced degradation of RNAP II and the UV-induced inhibition of 3’ cleavage. Chromatin immunoprecipitation reactions revealed that BARD1, CstF and RNAP II, involved in the UV-induced inhibition of 3’ cleavage, associate at sites of DNA damage. Together these results indicate that BARD1 with the 3’ processing factor CstF play a role in the DNA damage response.

To further understand the role of CstF-50 in the DNA damage response; we analyzed other CstF-50 interactors and found that DNA damage not only induces the formation of the BARD1/CstF-50/poly(A) specific ribonuclease (PARN) complex but also the expression levels of PARN. Based on the nature of the factors, it is possible to hypothesize that the PARN/CstF-50/BARD1 interaction regulates mRNA turnover in different cellular responses. Consistent with this, the CstF-50/PARN/BARD1 complex plays a role in inhibition of 3' cleavage and activation of deadenylation upon DNA damage. CstF-50/BARD1 can revert the cap binding protein 80-mediated inhibition of PARN activity. Importantly, it is shown that PARN affects both polyadenylation and stability of different mRNA precursors, such as housekeeping genes and some clinically significant genes, under different cellular conditions. These studies indicate that the PARN/CstF/BARD1 complex plays a role in the regulation of gene expression upon DNA damage, representing an alternative mechanism to prevent the processing of premature terminated messengers and to control the expression of oncogenes and DNA repair factors.

SIGNIFICANCE

Cells are constantly exposed to stress caused by environmental factors such as oxidation, hydrolysis, alkylation, radiation and toxic chemicals. Depending on the cellular stress, the expression levels of certain genes are enhanced or suppressed. For example, the expression levels of some proteins involved in DNA repair and cell cycle are enhanced, while the expression levels of most proteins are transiently suppressed to avoid the formation of deleterious proteins that may be harmful to the cell. The regulation of gene expression is a fundamental cellular process that is controlled at multiple levels.

Determination of the mechanisms by which mRNA turnover is regulated constitutes a major challenge in understanding control of gene expression, especially during the DNA repair process. Although most of the studies linked translation to mRNA degradation during the DNA damage response, the associations of mRNA processing to the mRNA degradation have yet to be determined. Following ultraviolet (UV)-irradiation, the cellular levels of mRNA are transiently decreased. The cellular mechanisms involved in this response are unknown but it probably implies a functional interaction of the DNA repair, transcription, and RNA processing machineries. Supporting this model, it has been described that mRNA 3’ end processing is also transiently inhibited after UV-treatment. As mRNA poly(A) tail is important in the regulation of mRNA stability; changes in the polyadenylation levels either by activation/inhibition of the reaction or by controlling the balance between polyadenylation and deadenylation could account for the observed changes in mRNA levels. The cleavage stimulation factor 50 (CstF-50) is a good candidate to bridge the 3’ processing reaction to the DNA damage response because it can functionally interact

with the tumor suppressors BARD1 and p53, with RNAP II, and with PARN under different cellular conditions. These interactions suggest a central role for CstF-50 in this response because it can regulate various nuclear functions, such as regulation of mRNA 3' end cleavage and deadenylation. The working model in this dissertation is that the regulation of mRNA levels occurs by a functional interaction of CstF-50 with the UV-induced phosphorylated C-terminal domain of BARD1 and with PARN. Interestingly, DNA damage was also shown to induce BARD1/p53 association through C-terminal domain of BARD1, resulting in the activation of p53 apoptotic pathway. All together, these studies suggest a central role for a polyadenylation factor and an innovative interplay between 3'end processing, tumor suppression and apoptosis. Any mutations or functional defects in those proteins are prone to result in malfunctions that may lead to deregulation of proper gene expression, which may cause cancer. In fact, mutations at the C-terminal domain of BARD1 have been shown to cause various cancers, such as breast and ovarian cancers (Thai et al. 1998, Karppinen et al. 2004 and Ishitobi et al. 2003). Moreover, defect in the polyadenylation machinery have also been shown to cause the formation of truncated polyadenylated deleterious mRNAs, which might also affect cell function (Mirkin et al. 2008).

Abnormal regulation of gene expression has been directly implicated in the pathogenesis of some diseases and may contribute to the disease process in unrecognized ways in many others. Furthermore, novel treatment strategies for a number of different diseases may hinge upon our ability to exploit mechanisms that normally alter the expression of endogenous genes. While the study of gene regulation has traditionally focused on transcription as a major regulator of gene expression, it has recently become

apparent that the post-transcriptional control of gene expression may play an equally important role. In particular, rapid, context-specific regulation of the stability of mRNA transcripts encoding highly active proteins, such as cytokines, growth factors, oncogenes and cell-cycle regulators, appears to play a key role in the control of these molecules and the processes they mediate. In fact, over expression of the clinically significant genes such as c-fos and c-myc have been shown to cause cancers and participate in metastasis of various tumors. (Zajchowski et al. 2001, Andersen et al. 2002, Milde-Langosch 2005). The results provided in the thesis suggest an alternative mechanism to regulate the expression levels of those genes based on the control of the polyadenylation/deadenylation machineries under different cellular conditions. It is possible that the new proposed regulatory mechanism may allow us to find alternative treatment strategies for the tumorogenesis and metastasis of the observed tumors and thus may play a role in finding therapies for various cancers.

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I would probably need to compile another thesis space if I were to express my gratitude, appreciation and thanks to my father Hayrettin Cevher, my mother Seher Sevda Cevher, my sisters Esra Cevher and Zehra Cevher and my cousin Oguz Alp Cevher, whom I consider as a brother. I would like to give my special thanks to these family members for years of support in many ways I could not possibly think. I again consider myself as one of the luckiest people to happen to have such a wonderful family. I would also like to give special thanks to my uncle Fahrettin Cevher, my aunt Sema Cevher and my cousin Yavuz Cevher for their external unforgettable help from across the seas. The support from my other relatives Fevziye Cevher, Sevki Cevher, Nizamettin Cevher, Ikbal Cevher, Fatma Unal, Ibrahim Unal, Suleyman Saritas, Muzeyyen Saritas, Saime Dogan, Ercan Dogan, Ozer Dogan, Faruk Aktas, Zehra Aktas and Osman Aktas also made me realize how family is more important than anything else in life.

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Therefore I dedicate my thesis work to all my relatives, my friends and my mentor.

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CHAPTER I

INTRODUCTION

POLYADENYLATION:

Although the replication and transcription of DNA has high processivity and fidelity, there is still a moderate chance that cellular DNA gets mutations under regular cellular conditions. The cells are also constantly exposed to stress caused by environmental factors that damage the DNA, such as oxidation, hydrolysis, alkylation, radiation and toxic chemicals. The cellular response to DNA damage involves changes in the functional and structural properties of a number of nuclear proteins, resulting in a coordinated control of gene expression and DNA repair. One example is provided by the transient decrease of the cellular levels of mRNA following UV-irradiation and its normal recovery as part of the DNA repair response (Hanawalt 1994; Ljungman et al. 1999). Although the mechanism involved in this response is not completely understood, it has been suggested that the UV-induced inhibition of transcription is responsible for the decrease in the mRNA levels (Donahue et al. 1994). This indeed may well be a significant part of the mechanism, however those studies have not considered the important effect of RNA processing on the levels of cellular mRNA. Interestingly, it has been described that 3' end formation of mRNA precursors is also affected in a similar time frame after DNA damage. mRNA 3' processing is strongly but transiently inhibited following treatment of cells with DNA damage inducing agents (Kleiman and Manley 2001).

Polyadenylation is important in the regulation of mRNA stability, translation and RNA transport from the nucleus (Colgan and Manley 1997, Zhao et al. 1999), therefore, it is fundamental for the control of the mRNA levels and of gene expression in

eukaryotes. The polyadenylation reaction consists of an endonucleolytic cleavage of the pre-mRNA followed by synthesis of the poly(A) tail towards the 3' end (reviewed in Zhao et al. 1999, Shatkin and Manley 2000). While a relatively simple signal sequence in the mRNA precursor is required for the reaction, many interactions between a large number of protein factors are necessary for the correct formation of the polyadenylation complex, and the diversity and specificity of these interactions may be an important aspect in 3' end formation. The polyadenylation reaction is regulated for some specific mRNAs, in different tissues and in different developmental stages. Regulation of 3' end formation can play significant roles in cell growth control (e.g., Takagaki et al. 1996, Takagaki and Manley 1998, Chupvilo et al. 1999) and perhaps in disease, especially in tumor cells (reviewed by Scorilas 2002). The basic mechanism of 3' processing involves the recognition of the highly conserved hexamer AAUAAA located at 10 to 30 nucleotide upstream of the cleavage site by the cleavage and polyadenylation specificity factor (CPSF) and the G/U- and U-rich element located downstream of the cleavage site by CstF (Takagaki and Manley 1997). While CPSF, CstF, cleavage factors 1 and 2 (CFI and CFII), RNAP II and poly(A) polymerase (PAP) play a role in the cleavage reaction (Figure 1 A); CPSF, PAP and poly(A) binding protein (PABP) are involved in polyadenylation step (Figure 1B). While the CPSF complex has been shown to perform endonuclease activity for the cleavage reaction and direct PAP to the correct polyadenylation site, the CF I and CF II have been shown to enhance recognition of the conserved AAUAAA signal in the pre-mRNA substrate for proper polyadenylation and interact with RNA, CstF, RNAP II, PAP and CPSF (reviewed in Mandel et al. 2008). In

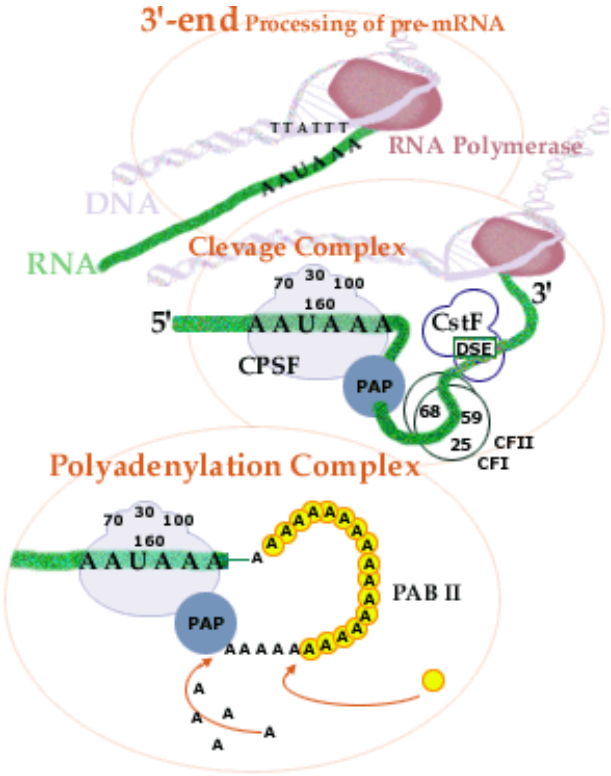

Figure 1: 3' end Processing of pre-mRNA. A) RNAPII transcribes the DNA strand and the transcribed AAUAAA and GU/U rich region at the 3'end of the pre-mRNA is occupied by CPSF and CstF complex respectively. These proteins recruit the CFI and II and the PAP to the cleavage site. **B)** First, CFI and II endonucleolytically cleave the RNA substrate in the presence of PAP, then PAP adds the poly(A) tail followed by the binding of PABII to the poly(A)tail to protect it from degradation.

http://nobelprize.org/educational_games/medicine/dna/a/splicing/splicing_endformation.html

mammalian cells, the poly(A) tail length is approximately 200-250 nucleotides long (Brawerman 1981).

As it was mentioned before, CstF is one of the essential 3' processing factors. CstF is active most likely as a dimer of two heterotrimers, each consisting of three protein subunits called CstF-77, CstF-64, and CstF-50, according to their molecular weights in kDa. CstF-64 is an RNA binding protein (MacDonald et al. 1994). It binds to a GU/U rich element present downstream of the cleavage site. Genetically modified chicken B cells deficient in CstF-64, one of the CstF subunits, undergo cell cycle arrest and apoptotic death (Takagaki and Manley 1998). CstF-77, another subunit of the CstF complex, plays a role bridging the CstF-64 and the CstF-50 proteins. It interacts with CPSF-160, contributing to the stability of the CPSF-CstF-RNA complex. CstF-77 also binds to the C-terminal domain of RNAP II but with much less affinity than CstF-50 (McCracken et al. 1997). CstF-77 is required for proper 3' end cleavage and may function as a dimer at a crucial stage in pre-mRNA 3' processing (Mandel et al. 2008). Another subunit, CstF-50, plays important roles in regulation of mRNA processing by interacting with other factors (Figure 2). It contains six WD-40 repeats. The WD-40 repeats are approximately 40 amino acid motifs that terminate with tryptophan-aspartic acid (W-D) dipeptide (Neer et al. 1994). The WD-proteins are a large family of regulatory proteins, whose functions range from signal transduction and transcription regulation to cell cycle control and apoptosis. The WD-40 repeats are involved in protein-protein interactions and are scaffolds for the assembly of other proteins. G-protein, TAFII transcription factor and E3 ubiquitin ligases are examples of proteins that contain WD-40 repeats (Li and Roberts 2001, Smith et al. 1999).

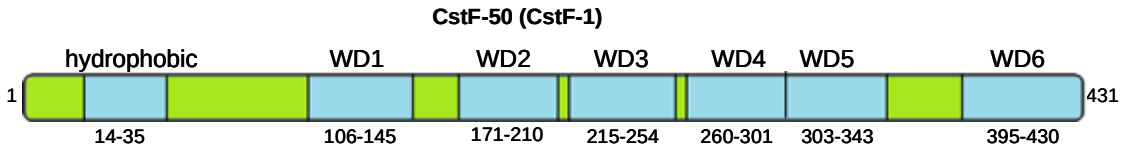

Figure 2: The 3' processing factor CstF-50. The CstF-50 subunit of the CstF trimer complex contains 6 WD-40 (tryptophan-aspartic acid) repeats and an N-terminal hydrophobic region.

Both the CstF-50 and CstF-77 subunits interact specifically with the carboxy-terminal domain (CTD) of RNA polymerase II largest subunit (RNAP II LS), likely facilitating the RNAP II-mediated activation of 3' end processing (McCracken et al. 1997, Hirose and Manley 1998). The stimulatory role of RNAP II in polyadenylation highlights the link between transcription and RNA processing.

The 3' processing reaction can be inhibited by the direct interaction of CstF-50 with BRCA1-associated RING domain 1 (BARD1, Kleiman and Manley 1999). BARD1 is a nuclear protein that associates with the breast cancer susceptibility gene product BRCA1 (Baer and Ludwig 2002). The DNA damage-induced inhibition of polyadenylation mentioned above correlates with increasing amounts of a BRCA1/BARD1/CstF-containing complex (Kleiman and Manley 2001) and is prevented by siRNA mediated depletion of BARD1 and BRCA1 (Kleiman et al. 2005), indicating the involvement of BRCA1/BARD1 in the inhibition of 3' RNA processing following DNA damage. Moreover, 3' end processing can also be repressed following DNA damage as a result of the proteasome-mediated degradation of RNAP II, representing another,

possible redundant, mechanism to explain the inhibitory effect of UV irradiation on 3' processing (Kleiman et al. 2005). Currently, other interactors of CstF-50, such as p53, ubiquitin and PARN, and their effect on 3' end processing are being investigated in Dr. Kleiman's laboratory (Figure 3), suggesting a central role for CstF-50 in regulating 3' end processing in different cellular conditions.

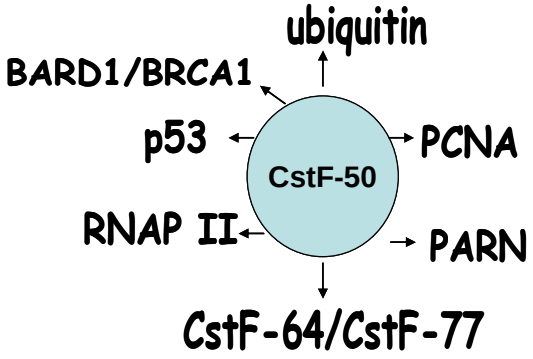

Figure 3: Interactors of the CstF-50 factor. The yeast two-hybrid assay performed previously identified several interactors of CstF-50. The CstF-50/BARD1 interaction is involved in the UV-induced inhibition of 3' end processing. The CstF-50/ubiquitin interaction might be involved in the ubiquitination by BARD1/BRCA1 of different substrates. The CstF-50/RNAP II interaction is involved in the activation of 3' end processing activation. The CstF-50/PARN interaction is involved in the UV-induced activation of deadenylation and UV induced inhibition of 3' end processing. The CstF-50/p53 interaction is involved in the regulation of 3' end cleavage. The CstF-50 also interacts with the DNA replication and repair factor PCNA.

TUMOR SUPPRESSION:

The statistical analysis shows that more than one out of nine women gets breast cancer over their life time. 5-10% of all the breast cancer is thought to be caused by mutations in BRCA1 and BRCA2 genes (Miki et al. 1994). BRCA1 is involved in DNA repair, transcription, RNA processing, and check point control (Deng and Brodie 2000, Venkitaraman 2002, Baer and Ludwig 2002, Rosen et al. 2003). The tumor suppressor BRCA1 and BARD1 form a heterodimer and play important roles in the DNA damage response (Gudmundsdottir and Ashworth 2006). BRCA1 is an Ataxia-telangiectasia mutated (ATM)/ATM and Rad 3-related (ATR) kinases substrate. ATM/ATR kinases are PIKK (phosphoinositide-3-kinase) related protein kinases and they mediate cellular responses upon DNA damage (Kastan and Lim 2001). The phosphorylation by ATM of Ser 1423 and Ser 1524 of BRCA1 is essential for its DNA repair function (Kastan and Lim 2001). When these two sites are mutated, the cells are deficient in the DNA repair process and do not survive, indicating that the tumor suppression function of the protein is lost. BARD1 is a 97 kDa protein that was originally identified by its association with BRCA1 (Figure 4, Wu et al. 1996). Both proteins share structural features, they possess N-terminal RING finger motifs, which are responsible for the BRCA1/BARD1 interaction, three ankyrin repeats that are involved in protein-protein interaction, and two BRCA1 C-terminal (BRCT) domains that are involved in DNA repair and cell cycle regulation. BRCA1/BARD1 stabilizes each other and this enhances their function (Baer and Ludwig 2001).

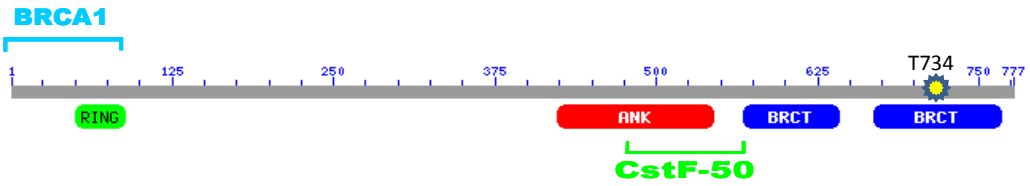

Figure 4: The tumor suppressor BARD1. BARD1 is a 97 kDa protein. It contains an N-terminal RING finger motif, which constitutes the BRCA1 interaction domain, three ankyrin repeats, which constitutes the CstF-50 binding domain, and two BRCT domains at the C-terminal end. The figure also shows the consensus phosphorylation site of the BARD1 protein (T734).

The function and subcellular location of BARD1 and BRCA1 are regulated by nuclear-cytoplasmic shuttling (Henderson 2005). The BARD1/BRCA1 heterodimerization masks the nuclear export signals within each protein, causing nuclear retention of the duplex and allowing its functions in DNA repair, RNA processing, and centrosome duplication (Jefford et al. 2004). Given that BRCA1 and BARD1 are not consistently co-expressed in different tissues, there may also be independent functions for them (Figure 5). The transforming effect of BARD1 inhibition in cultured cells (Irminger-Finger et al. 1998) and the existence of tumorigenic mutations in BARD1 (Thai et al. 1998, Ghimenti et al. 2002) support the view of BARD1 as a tumor suppressor by itself. It has been described that BARD1 deficient cells are defective for the apoptotic response to genotoxic stress; this proapoptotic activity of BARD1 is stimulated by nuclear export (Rodriguez et al. 2004) and it involves binding to p53 (Irminger-Finger et al. 2001).

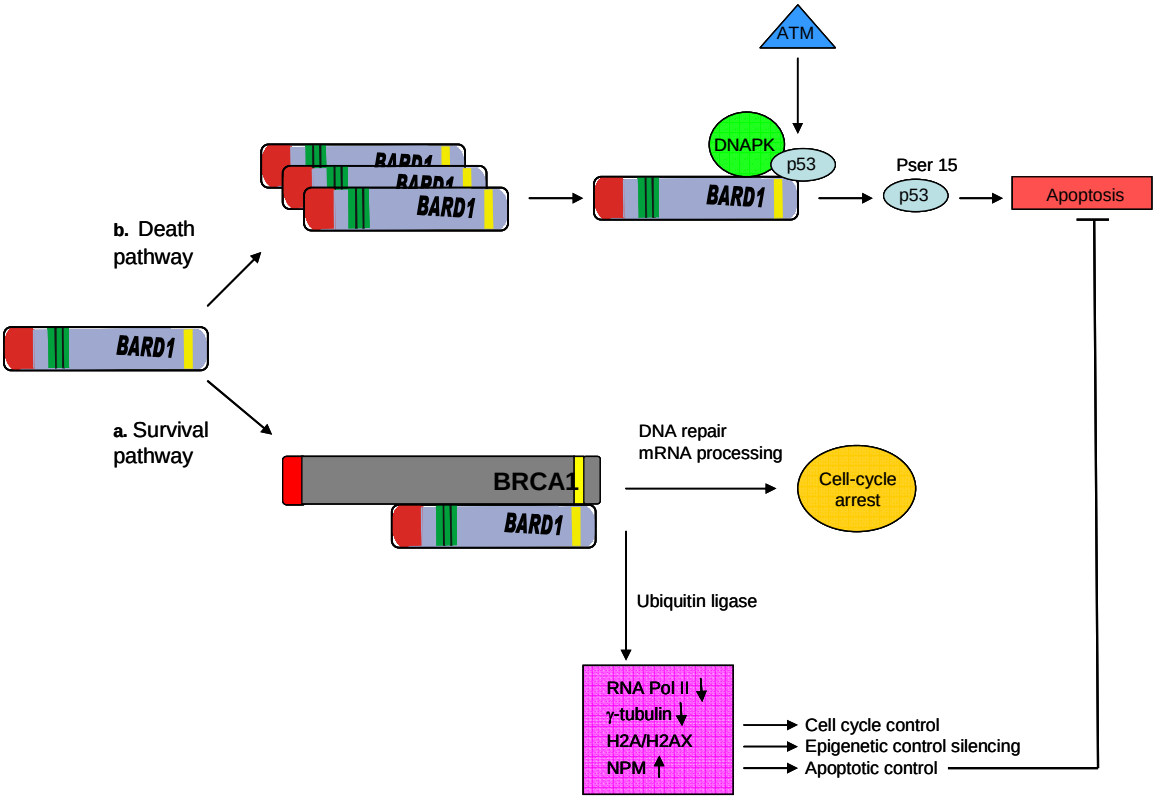

Figure 5: Role of BARD1 in cell fate. BARD1 functions both dependent (A) and independent (B) of BRCA1 (Modified from Irminger-Finger and Jefford 2006). **A)** Upon DNA damage, in the survival pathway, BRCA1/BARD1 performs E3 ubiquitin ligase activity and leads to RNAP II ubiquitination inducing cell cycle arrest, gamma-tubulin ubiquitination preventing centrosome duplication, stabilizes H2A/H2AX in tumors silencing epigenetic control, and stabilizes NPM inhibiting apoptosis. **B)** In the death pathway, BARD1 acts independently from BRCA1. BARD1 phosphorylates p53 at serine 15 and activates the apoptotic pathway.

The BRCA1/BARD1 heterodimer exhibits E3 ubiquitin ligase activity and ubiquitinates some substrates involved in DNA damage repair, such as CtIP, RNAP II and topoisomerase IIα, and some others involved in genomic stability, such as γ-tubulin and Npm1 (Sato et al. 2004; Starita et al. 2004; Starita and Parvin 2006, Yu et al. 2006). After DNA damage, the BARD1/BRCA1 heterodimer relocate to sites of DNA damage (Celeste et al. 2003), ubiquitinates RNAPO II LS, the hyperphosphorylated form of the enzyme that functions in transcription elongation, but not RNAP IIA LS, the hypophosphorylated form that engages promoters, and leads to its proteosomal degradation (Kleiman et al. 2005, Starita et al. 2005). As part of those studies, it was also shown that siRNA-mediated knockdown of BRCA1/BARD1 resulted in stabilization of RNAP II after DNA damage. As it was mentioned before, it has also been shown that upon genotoxic stress, the heterodimer forms a complex with the cleavage factor CstF-50 and prevents its function in mRNA 3' end processing (Kleiman and Manley 1999, 2001). It has been suggested that the degradation of RNAP II and the inhibition of 3' processing after DNA damage might help to clear the region for the repair factors, improving the efficiency of the repair process.

While the regulation of BRCA1 function by phosphorylation in response to DNA damage has been extensively studied (Teng et al. 2008), very little is known about the regulation of BARD1 activity. Recent studies from Dr. Lee and colleagues (Kim et al. 2006) have shown that the threonine 734 of BARD1 is also phosphorylated by ATM kinase dependent pathway after DNA damage. The T734 lies within the second BRCT domain of BARD1 (Figure 4). As discussed in more detail in Chapter III, I have functionally extended those studies showing that the DNA damage-induced BARD1

phosphorylation at the T734 is important not only for the BARD1/CstF-50 complex formation but also for the UV-induced inhibition of both mRNA 3’ end processing and RNAP II ubiquitination and degradation (Kim et al. 2006).

DEADENYLATION:

The steady state levels of cellular mRNA are determined by the balance between their biosynthesis and turnover. Depending on the type of gene and the cellular conditions, the stabilities of different mRNAs range from less than 10 minutes to many hours (Khodursky and Bernstein 2003). In addition, the half life of mRNAs can change throughout the cell cycle or in response to cell signaling, suggesting that mRNA degradation is regulated in different cellular environments. Very little is known about the effect of UV treatment on mRNA turnover and its consequences on the decrease of mRNA levels. Several mRNA decay pathways exist in eukaryotic cells, which are mostly dependent on deadenylation (reviewed by Parker and Song 2004). PARN is involved in two general mRNA degradation pathways (Figure 6). Upon cellular stress conditions, such as serum deprivation, the cytoplasmic translation initiation factor eIF4E gets dephosphorylated and loses its affinity to the 5' cap. Furthermore, the PABP protein affinity to the poly(A) is also reduced. The dephosphorylation of eIF4E gives the deadenylase PARN access to the 5'cap and increases its fidelity and processivity for deadenylation (Seal et al. 2005). These changes expose both the 5' cap and the poly(A) tail to PARN for deadenylation and signal that the removal of the poly (A) comes first in the degradation of mRNA (Fraser et al. 1999; Seal et al. 2005). However, after the initial deadenylation, PARN is not bound to the mRNA anymore, giving access to the

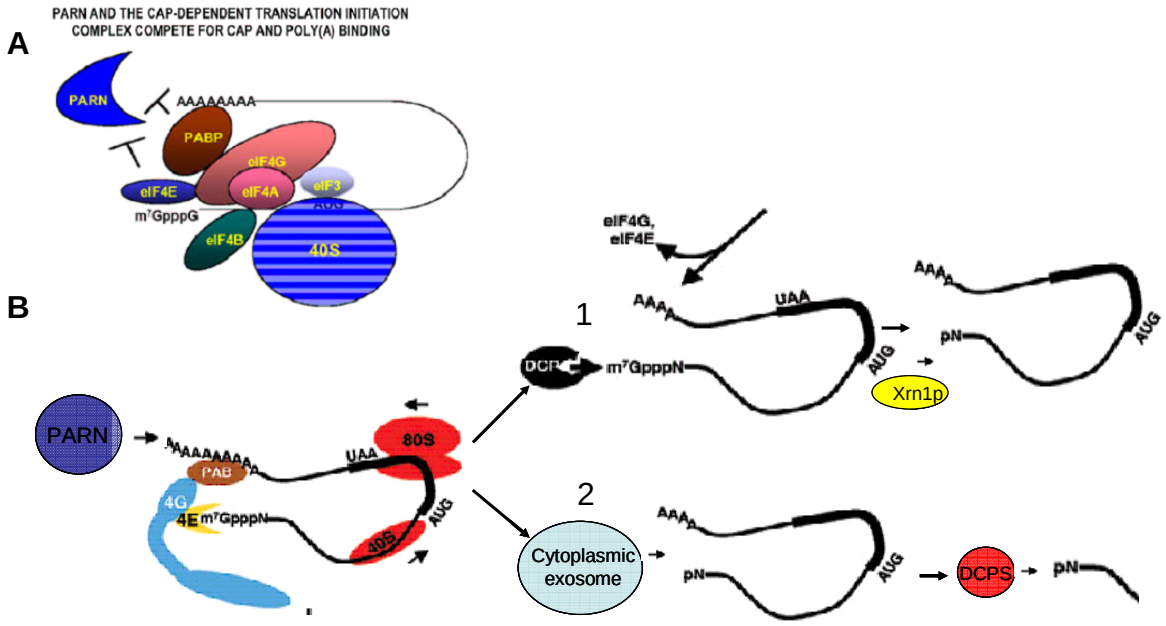

Figure 6: PARN-mediated degradation of mRNA in the cytoplasm. A) The cytoplasmic translation initiation factor eIF4E is phosphorylated under regular cellular conditions and shields the 5' end of mRNA from PARN. **B)** Upon cellular stress eIF4E gets dephosphorylated and dissociate from 5' end exposing that region to PARN. The PARN-dependent deadenylation is activated when exposed to the 5' cap and the 3' poly(A) tail, and this results in the two general mRNA degradation pathways. **1)** Upon deadenylation, the mRNA gets decapped by DCP that results in exonuclease Xrn1p-dependent degradation in the 5'→3' direction. **2)** Upon deadenylation, the mRNA gets degraded by the exosome in the 3'→5' direction followed by decapping of the 5' end. Modified from Haar et al. 2004.

DCP1:DCP2 decapping complex to remove the 5' cap of the nascent mRNA, exposing the 5' end, which is then recognized by the exonuclease Xrn1p and degraded in the 5'→3' direction. Alternatively, after deadenylation mRNA degradation can occur in the cytoplasmic exosome in the 3'→5' direction (Mukherjee et al. 2002) and the resulting cap structure is hydrolyzed by DCPS decapping enzyme (Liu et al. 2002). Thus the binding of PARN to the mRNA could regulate its degradation either in 3'→5' or in 5'→3' direction.

In addition to these general mRNA decay pathways, other degradation pathways exist. mRNAs encoding premature translational stop codon are degraded in a pathway known as nonsense-mediated decay (NMD, Mitchell and Tollervey 2003, Takahashi et al. 2003, Lejeune et al. 2003). In this pathway, the mRNA is degraded either by deadenylation followed by 3'→5' exonucleolytic degradation or by decapping without deadenylation (Mitchell and Tollervey 2003, Takahashi et al. 2003, Lejeune et al. 2003). NMD is believed to occur not only in the cytoplasm but also in the nucleus since some ribosomes and translation activity have been also detected (Ishigaki et al. 2001). Finally, it is known that AU-rich elements (AREs) within the 3' untranslated region decrease the stability of the mRNAs (Chen and Shyu 1995). AREs range in size from 50 to 150 nucleotides. The destabilizing functions of AREs are important because in their absence proto-oncogenes, such as c-fos, c-myc, jun, could become oncogenes (Schiavi et al. 1992). Other mRNAs, such as IL-3, need AREs in order to inhibit the growth of autocrine tumor mast cells by an immunosuppressant cyclosporin A (Nair et al. 1994).

Unlike the polyadenylation reaction, the mechanisms behind poly(A) removal has proven more difficult to define. In mammalian cells, the earliest and rate limiting step in mRNA decay is thought to be removal of the poly(A) tail deadenylation, (Wilusz et al.

2001). The major deadenylase required for mRNA decay is PARN (Mitchell and Tollervey 2000, Wu et al. 2005). There are two isoforms of PARN that differ in their nuclear-cytoplasmic distribution; while the 74 kDa form is exclusively nuclear, the 62 kDa form is cytoplasmic (Korner et al. 1998). PARN interacts simultaneously with the 5'-end-located cap structure, shielding the 5' from decapping enzymes, and the 3' end located poly(A) tail, initiating the deadenylation process. Interestingly, the communication between both the 3' and 5' ends of mRNA is very important because it integrates the initiation of translation, translation and mRNA turnover (Martinez et al. 2001).

PARN is composed of an R3H domain, which is constituted by an invariant arginine that is separated by three residues from a highly conserved histidine, that binds single stranded nucleotides and a nuclease domain that performs the deadenylation reaction (Wu et al. 2005). It is present in most of the eukaryotic cells and is the deadenylase that silences maternal mRNAs of Xenopus oocytes during maturation (Copeland and Wormington 2001, Kim and Richter 2006). While the cytoplasmic PARN's activity has been extensively studied, the nuclear functions of PARN are still a mystery. Although most deadenylases shuttle out of the nucleus to the cytoplasm, PARN is found mostly in the nucleus (Yamashita et al. 2005). In fact, Yamashita et al. (2005) have described that PARN does not play a key role in cytoplasmic mRNA decay.

As mentioned before, PARN is the only deadenylase that interacts both with 5' end and 3'end of the mRNA integrating the initiation of translation and mRNA turnover (Martinez et al. 2001). PARN activity and its access to the 5' cap region and the poly (A) region are regulated by its phosphorylation (Seal et al. 2005). Inside the nucleus, as the

pre-mRNA is being transcribed, the 5' cap region is occupied by the nuclear cap binding complex (CBC). CBC is composed of two proteins, the cap binding protein 80 (CBP-80) and the cap binding protein 20 (CBP-20). CBP-80 binds to the carboxy-terminal domain of PARN and inactivates its deadenylase activity in the nucleus (Balatsos et al. 2006). Once the mRNA leaves the nucleus, the CBP-80 shuttles in association with mRNA to the cytoplasm (Visa et al. 1996; Ishigaki et al. 2001) and then the CBP-80 is replaced by the translation initiation factor eIF4E (reviewed in Gingras et al. 1999). Under cellular stress, like serum deprivation, the phosphorylation of eIF4E diminishes and it dissociates from the 5' cap. The eIF4E dissociation gives PARN the access to the 5' cap and increases its processivity for deadenylation. The poly(A) binding protein PABPN1 also inhibits PARN's activity via binding to the 3' poly(A) region and blocking PARN access to the 3' end (Gao et al. 2001). These interactions point out the importance of the communication between the two extreme ends of the mRNA.

It has been suggested that the CBC-PARN interaction could represent a mechanism by which PARN is recruited to the nascent pre-mRNA and the CBC-mediated inhibition ensures that PARN does not degrade the RNA unless PARN is activated, which could occur as part of the NMD mechanism. Interestingly, PARN copurifies with essential NMD factors (Maquat 2004) and PARN down-regulation abrogated nonsense-mediated decay (Lejeune et al. 2003). Besides, PARN can promote deadenylation of ARE-containing mRNAs in the presence of tristetraprolin (TTP, Lai et al. 2003). Interestingly, it has been described that UV induces stabilization of ARE-containing mRNAS; such as c-fos, kin17, c-jun, IκB and c-myc (Blattner et al. 2000). It has also been shown that the UV-induced transcript stabilization and enhanced protein levels of

short basal half–life, such as oncogenes, apoptosis and cell-cycle related genes, growth factors and cytokines, is due to the inhibition of cytoplasmic mRNA deadenylation and degradation (Gowrishankar et al. 2005).

CHAPTER II

DNA damage-induced BARD1 phosphorylation is critical for the function of BRCA1/BARD1 complex in 3’ end processing

INTRODUCTION

Protein phosphorylation is critical in the cellular response to DNA damage, acting as a molecular switch that regulates many important DNA damage checkpoint responses. The principal kinases involved in this signaling process are members of the PIKK-family, such as ATM (ataxia-telangiectasia mutated) and ATR (ATM and Rad3-related) (Abraham 2001; Shiloh 2003). . Any defects on the ATM gene causes ataxia telangiectasia, a complex autosomal recessive disorder that includes growth retardation and premature ageing (Paterson et al. 1979). Many of the ATM/ATR kinase substrates, such as BRCA1, CHK2, NBS1, MRE11, p53 and SMC1, signal cell cycle checkpoint responses to DNA damage, playing important roles in cell-cycle arrest, apoptosis, and DNA repair (Kastan and Lim 2003).

The tumor suppressor BRCA1 is phosphorylated by ATM upon DNA damage, and together with BARD1, performs multiple functions in the DNA damage responses, including in DNA repair, in transcription, and in RNA processing (Deng and Wang 2003; Baer and Ludwig 2002). BRCA1/BARD1 forms a complex through their respective N-terminal RING domains and exhibits significant E3 ubiquitin (Ub) ligase activity (Baer and Ludwig 2002). Through its Ub ligase activity, the BRCA1/BARD1 complex can undergo autoubiquitination (Ruffner et al. 2001; Chen et al. 2002), and can ubiquitinate substrates such as p53 (Dong et al. 2003), Nucleophosmin/B2 (Safo et al. 2004), γ-tubulin (Starita et al. 2004) and RNAP II (Kleiman et al. 2005; Starita et al. 2005). BRCA1/BARD1-mediated ubiquitination of RNAP II targets it for proteasome-mediated degradation and subsequent inhibition of transcription and RNA processing in response to genotoxic stress (Kleiman et al. 2005). In contrast to BRCA1 whose function is

regulated by phosphorylation in response to genotoxic stress, how BARD1 activity is regulated and whether BARD1 is phosphorylated during DNA damage have not been examined.

Dr. Sean Lee and colleagues (Genetics of Development and Disease Branch, National Institute of Diabetes & Digestive & Kidney Diseases, NIH) studied the possible phosphorylation of BARD1 in response to DNA damage. As part of their studies, they found that BARD1 undergoes phosphorylation upon ionizing radiation (IR) or UV radiation (Figure 7) and that ATM is responsible for BARD1 UV-induced phosphorylation (Kim et al. 2006). ATM kinase phosporylates Ser or Thr residues which are immediately followed by a Gln residue (SQ/TQ) (Kastan and Lim 2000). Examination of primary sequence revealed that there are four potential ATM phosphorylation sites (SQ/TQ) in the human BARD1 (Thr^{165}, Ser^{244}, Thr^{714} and Thr^{734}). Sequence comparison with other BARD1 orthologs revealed that the last two TQ motifs located in the second BRCT domain (Thr^{714} and Thr^{734}) are evolutionarily conserved (Figure 8A). To determine which of these ATM phosphorylation sites are modified in response to genotoxic stress, simultaneous or individual mutations of the four Thr/Ser to Ala were analyzed. When cells were transfected with the BARD1 mutant that contains AQ substitutions at all four SQ/TQ sites (Quad), the BARD1 mutant was not phosphorylated after IR treatment (Figure 8B). To define the phosphorylation sites more precisely, U2OS cells were transfected with FLAG-tagged BARD1 expression vectors containing individual substitutions at each phosphorylation site and labeled with ^{32}P-orthophosphate after IR treatment. As shown in Figure 8B, transient overexpression of the different BARD1 mutants in cells led to in vivo phosphorylation of the WT, T165A,

S244A, and T714A versions of BARD1. Interestingly, the observed phosphorylation was irrespective of DNA damage, probably because overexpression of BARD1 triggers maximal phosphorylation. More importantly, mutation of Thr^{734} to Ala almost completely abolished BARD1 phosphorylation to a similar extent to Quad BARD1, even after IR treatment (Figure 8B). This result demonstrates the specificity of Thr^{734} phosphorylation of BARD1 in the cellular response to DNA damage.

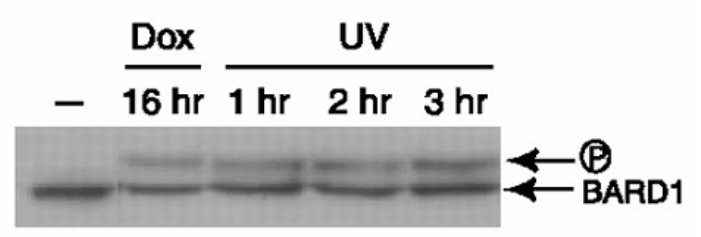

Figure 7. BARD1 phosphorylation in response to DNA damage. U2OS cells were either treated with 1μg/ml of doxorubicin or with UV (100J/m^2) and nuclear extracts prepared at indicated times were immunoblotted with anti-BARD1 antibody.

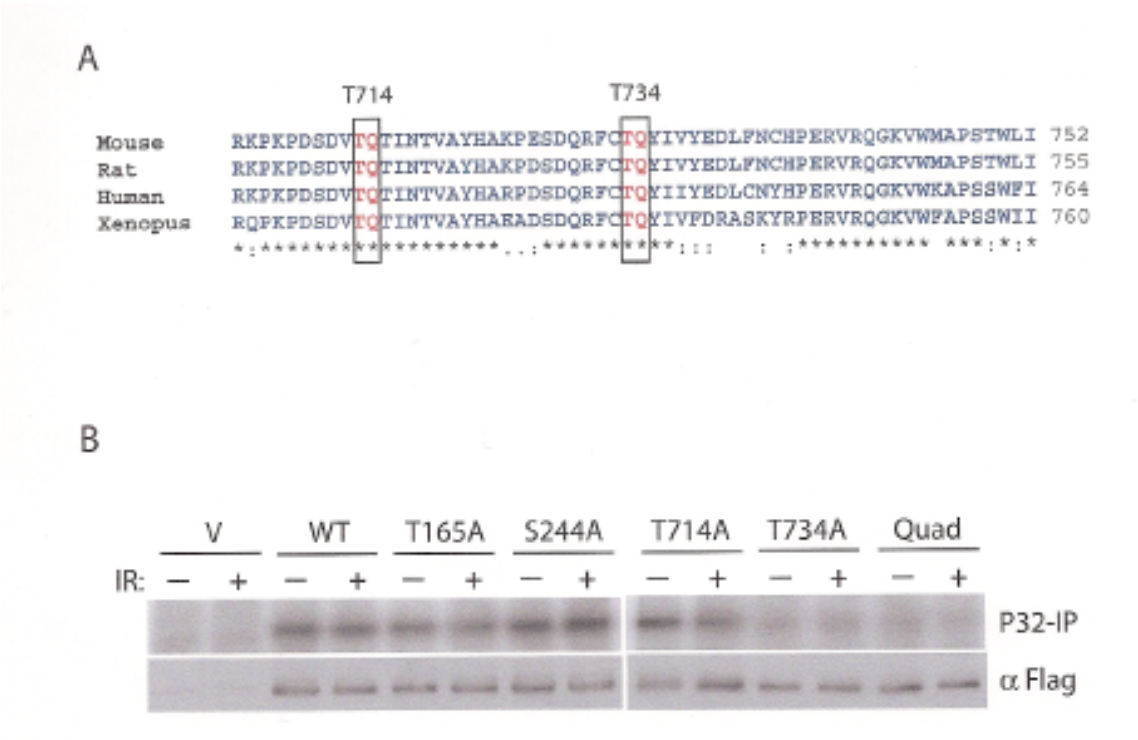

Figure 8. BARD1 Thr^{734} is evolutionarily conserved and phosphorylated in vivo. (**A**) Amino acid sequence alignment near the C-terminal BRCT domain of different BARD1 orthologs. Boxes indicate conserved PIKK-phosphorylation (TQ) sites. T714 and T734 refer to the human BARD1 residues. (**B**) U2OS cells stably transfected with different BARD1 expression constructs were either untreated or IR-treated (10Gy) and then metabolically labeled with ^{32}P-orthophosphate. Cell extracts were immunoprecipitated with anti-FLAG antibody, resolved by SDS-PAGE, and transferred to nitrocellulose membrane followed by autoradiography (P32-IP). Subsequently, membrane was immunoblotted with anti-FLAG M2 antibody (α-FLAG).

As part of those studies, I determined whether the DNA damage-induced BARD1 phosphorylation is important for the degradation of RNAP II and the inhibition of 3' processing after DNA damage. As mentioned before, the tumor suppressors BRCA1-associated BARD1 can interact with CstF-50 to inhibit the 3' end processing reaction upon DNA damage (Kleiman and Manley 1999). CstF-50 can also interact with the CTD of RNAP II to activate mRNA 3' end processing (McCracken et al. 1997; Hirose and Manley 1998), and RNAP II is a specific target of the BRCA1/BARD1 E3 Ub ligase activity (Kleiman et al. 2005, Starita et al. 2005). It has also been shown that 3' end processing can also be repressed following DNA damage as a result of the proteasome-mediated degradation of RNAP II, representing another, possible redundant, mechanism to explain the inhibitory effect of UV irradiation on 3' processing (Kleiman et al. 2005). My results indicate that DNA damage-associated functions of BARD1, such as inhibition of pre-mRNA 3' processing and degradation of RNAP II, are abrogated in T714A and T734A mutants. Taken together all these findings suggest that phosphorylation of T734 BARD1 is critical for the DNA damage functions of BRCA1/BARD1 complex.

RESULTS

It has been shown that in response to DNA damage BRCA1/BARD1 complex ubiquitinates RNAP II (Kleiman et al. 2005; Starita et al. 2005) and subsequently leads to a rapid degradation of RNAP II by the proteasome. Interestingly, BARD1/BRCA1 heterodimer was shown to ubiquitinate RNAP IIO LS, which is the hyperphosphorylated form that functions in transcription elongation, but not RNAP IIA LS, which is the hypophosphorylated form that engages promoters. Thus, I next examined whether the DNA damage-induced BARD1 phosphorylation is important for the degradation of RNAP II after DNA damage. To this end, the U20S cells transformed with several BARD1 threonine mutants were used. These stable cell lines were established in Dr. Lee's laboratory. Cells were treated or non-treated with UV (20 Jm^{-2}), and after 2 hours of recovery, NEs were prepared as described in Experimental Procedures. NEs were analyzed by Western blot with antibodies against BRCA1, RNAP II, RNAP IIO, CstF-64 and actin.

Consistent with previous results, UV treatment reduced the accumulation of both hypo- (RNAP IIA) and hyperphosphorylated (RNAP IIO) forms of RNAP II in cells stably transfected with empty vector or WT BARD1 (Figure 10). In contrast, cells stably transformed with the T714A or the T734A versions of BARD1 showed a stabilization of both RNAP II isoforms following UV treatment, especially of the RNAP IIO isoform. The level of proteins like actin, BRCA1 and CstF-64 did not show changes in their expression levels. This result suggests that phosphorylation of BARD1 at Thr^{714} and Thr^{734} is important for the preferential degradation of RNAP IIO mediated by the BRCA1/BARD1 complex in response to DNA damage.

In addition to RNAP II, BARD1 also interacts with CstF-50, a component of the mRNA 3' processing complex, and as a result, the 3' processing machinery is inhibited in *in vitro* functional assays (Kleiman and Manley 1999, 2001). To examine the role of BARD1 phosphorylation in the DNA damage-induced inhibition of mRNA 3' end formation, we performed *in vitro* RNA cleavage assays with nuclear extracts isolated from different cell lines expressing various BARD1 mutants. Radiolabeled SV40 late precursor RNA (SVL) was used as substrate as described in Experimental Procedures. As shown in Figure 11, the inhibition of 3' cleavage after DNA damage was completely abolished in cells expressing the T714A and T734A BARD1 mutants, whereas the vector and WT BARD1 expressing cells exhibited normal inhibition of RNA cleavage after UV treatment.

The transient inhibition of 3' RNA processing following DNA damage reflects the formation of the BRCA1/BARD1/CstF complex (Kleiman and Manley 2001). To test the effect of BARD1 phosphorylation on the BRCA1/BARD1/CstF complex formation, we analyzed the complex in nuclear extracts from UV-treated cells expressing different mutants of BARD1. As the BARD1-CstF-50 interaction involves the intact CstF complex (Kleiman and Manley 2001), we used monoclonal antibodies against CstF-64, another CstF subunit, to immunoprecipitate the complex. As shown in Figure 12, T734A BARD1 mutant did not form a complex with CstF irrespective of UV treatment, whereas wild-type BARD1 was able to form a complex which increased significantly after the UV treatment. Unexpectedly, T714A version of BARD1 still formed a complex with CstF even in the absence of genotoxic stress; however, unlike the wild-type BARD1, this interaction did not increase with DNA damage (Figure 12). The results indicate that the

phosphorylation of the Thr^{734} plays an important role in the BARD1/CstF interaction. In contrast, BARD1/BRCA1 interaction was still retained in the T714A, T734A and Quad BARD1 mutants (Kim et al. 2006), indicating that the failure of T734A to form a complex with CstF is not due to a gross alteration in protein conformation.

CONCLUSIONS

DNA damage leads to different cellular responses such as cell-cycle arrest, inhibition of transcription and of RNA processing, DNA repair and apoptosis. ATM/ATR kinases are able to control many aspects of the DNA damage response by phosphorylating specific substrates important in different cellular pathways (Kastan and Lim 2000, Shiloh 2003). As Dr. Lee and colleagues described, of the four potential ATM phosphorylation sites in human BARD1, only two TQ sites near the tandem BRCT motifs are evolutionarily conserved (Figure 8A), suggesting the importance of BARD1 phosphorylation at these residues. Interestingly, mutation of the single TQ site (T734A) almost completely abrogates DNA damage-induced BARD1 phosphorylation (Figure 8B) and results in a dysfunctional BARD1 in mediating inhibition of 3' RNA processing and degradation of RNAP II after DNA damage (Figures 10-11). Loss of UV-induced inhibition of RNA processing in T734A mutant is likely due to its inability to form a complex with CstF (Figure 12), suggesting that phosphorylation of Thr^{734} is critical for the DNA damage-induced BARD1-CstF interaction. Although the half-life of both T734A and Quad BARD1 mutants was reduced compared to WT or other BARD1 mutants (Kim et al. 2006), it is unlikely that T734A and Quad mutants are grossly misfolded since these BARD1 mutants retained the ability to interact with BRCA1 (Kim et al. 2006). Degradation of the mutant BARD1 was delayed with proteasome inhibitor MG132, suggesting that the observed instability of BARD1 was due to proteasome-mediated degradation (Kim et al. 2006). These results suggest that phosphorylation of T734 may also be an important determinant of BARD1 stability.

In contrast, the substitutiton of Thr^{714} for Ala did not significantly affect BARD1 phosphorylation (Figure 8B), suggesting that Thr^{714} is not, per se, the major site of the DNA damage-induced modification of BARD1. Nevertheless, it is a critical residue for the BARD1 activity as the mutation of Thr^{714} to Ala resulted in a dysfunctional BARD1 in the degradation of RNAP II and 3' processing assays (Figures 10-11). Surprisingly, unlike the T734A substitution, T714A mutation did not abolish BARD1/CstF interaction (Figure 12). This observation suggests that the DNA damage-induced inhibition of 3' processing by BARD1 may not simply be due to sequestration of CstF but implicates a more direct role in the inhibition of CstF complex. Our study thus provides mechanistic insights by which BARD1 activity can be regulated by PIKK-mediated phosphorylation.

It is likely that BARD1 has additional DNA damage-induced phosphorylation sites other than Thr^{714} and Thr^{734}, and that additional phosphorylation may also regulate different aspects of BARD1 function. Consistent with this view, it has been shown that BARD1 can be phosphorylated by a CDK-Cyclin complex in a cell-cycle dependent manner (Choudhury et al. 2005, Hayami et al. 2005) and mutations in the CDK2-Cyclin E1/A1 phosphorylation sites of BARD1 confer increased sensitivity to mitomycin C treatment (Choudhury et al. 2005). The precise mechanisms by which CDK/Cyclin- or PIKK-mediated BARD1 phosphorylation regulate its activity is not known. One possibility is that the phosphorylation sites of BARD1 may directly be involved in the binding of other proteins (as in Thr^{734} phosphorylation leading to formation of BARD1-CstF complex) or may indirectly influence protein-protein interaction by inducing conformational changes. Since the BRCT domain serves as a

phospho-peptide binding module (Manke et al. 2003; Yu et al. 2003), DNA damage-induced phosphorylation of Thr^{714} and Thr^{734} residues of BARD1 (which are located in the second BRCT domain) may convert the BRCT domain from a phospho-peptide binding module into a phospho-protein docking site for other phosphorylation-specific binding proteins. Additionally, though not mutually exclusively, Thr^{714} and Thr^{734} phosphorylation, as well as CDK-Cyclin-mediated phosphorylation, may also serve to activate or enhance the activity of BARD1 complex, such as its E3 ubiquitin ligase activity or homology-directed DNA repair (Westermark et al. 2003). Elucidation of BARD1 structure with interacting peptides or a phosphorylated BARD1 may provide further insight into the mechanisms of BARD1 regulation and action during cellular response to DNA damage.

CHAPTER III

The 3' processing factor CstF, RNA polymerase II and BARD1 associates at sites of DNA repair.

INTRODUCTION

The cellular response to DNA damage involves changes in the properties of a number of nuclear proteins, resulting in coordinated control of gene expression and DNA repair. One example is provided by the transient decrease in mRNA levels following UV irradiation (Hanawalt 1994, Ljungman et al. 1999). Although the mechanism underlying this response is still unresolved, it has been suggested that the UV-induced inhibition of transcription, reflecting turnover of the RNAP II largest subunit, is responsible for the decrease (Donahue et al. 1994). This indeed is likely a significant part of the mechanism. However, those studies have not considered the important effect of RNA processing on mRNA levels. Indeed, it has been shown that processing of mRNA precursors, and specifically 3' end formation, is also affected by DNA damage. The mRNA 3' end processing in cell extracts is strongly but transiently inhibited following treatment of cells with DNA damage-inducing agents (Kleiman and Manley 2001). These results suggested a functional interaction between RNA processing and DNA repair.

The poly(A) tail found on almost all eukaryotic mRNAs plays important roles in regulation of mRNA stability, translation and RNA transport from the nucleus (Neugebauer 2002, Mangus et al. 2003, Anderson 2005). While a relatively simple signal sequence in the mRNA precursor is required for the reaction, a surprisingly large number of protein factors are necessary for 3' processing. CstF is one of the essential 3' processing factors. Genetically modified chicken B cells deficient in CstF-64, a CstF subunit, undergo cell cycle arrest and apoptotic death (Takagaki and Manley 1998). Another subunit, CstF-50, has been shown to interact with the CTD of the RNAP II LS,

likely facilitating the RNAP II-mediated activation of 3' processing (McCracken et al. 1997, Hirose and Manley 1998). The stimulatory role of RNAP II in 3' processing highlights the link between RNA processing and transcription. This link is supported by a variety of chromatin immunoprecipiation experiments documenting the association of 3' mRNA processing factors with transcribed genes (e.g., Calvo and Manley 2005, Kim et al. 2004, Venkataraman et al. 2005).

As part of the efforts to characterize links between mRNA 3' processing and other nuclear events, an association between CstF and the BRCA1/BARD1 tumor suppressor complex was uncovered and characterized. It was determined that this association was mediated by a direct interaction between CstF-50 and BARD1, and inhibits 3' processing in vitro (Kleiman and Manley 1999). The complex is increased transiently in concentration following DNA damage-inducing treatments, and results in inhibition of 3' processing in extracts from the treated cells (Kleiman and Manley 2001). It has also been shown that DNA damage-induced BARD1 phosphorylation is critical for inhibition of 3' end processing and RNAP II LS degradation (Kim et al. 2006). After UV treatment, a fraction of RNAP II LS is phosphorylated, ubiquitinated and degraded by the proteasome (reviewed by van den Boom et al. 2002, Muratani and Tansey 2003). Dr. Kleiman and colleagues have shown that degradation of RNAP IIO in fact contributes to inhibition of 3' processing in response to DNA damage (Kleiman et al. 2005), suggesting the existence of another, possibly redundant, mechanism to explain the inhibitory effect of UV irradiation. Significantly, both BRCA1 and BARD1 are necessary for ubiquitination of RNAP II LS and its turnover in response to UV treatment (Kleiman et al. 2005, Starita et al. 2005).

UV-induced turnover of RNAP II is part of the transcription-coupled repair (TCR) response (reviewed by van den Boom et al. 2002, Muratani and Tansey 2003). TCR is a pathway that operates on certain types of DNA damage found in the transcribed strand of expressed genes. Accumulated evidence suggests that the blockage of elongating RNAP II at sites of DNA damage is an early event that initiates TCR. Levels of mRNA are transiently decreased, and normal recovery depends on TCR (Hanawalt 1994, Ljungman et al. 1999, Derheimer et al. 2005, Mullenders 1998). One of the earliest indications of the existence of TCR was the key observation that when mammalian cells are exposed to UV light, RNA synthesis resumes before any significant amount of UV-induced damage is removed from the bulk of the genome by global genome repair (Mellon et al. 1987). One reason for this may be that TCR serves to repair transcription-blocking lesions and, therefore, to facilitate a rapid recovery of transcription. Transcription complexes can be extremely stable when they are stalled at endogenous pause sites or at sites of damage (Svejstrup 2002). It has been suggested that RNAP II stalled at sites of DNA damage could respond in either of two ways. If the lesion is repaired rapidly, RNAP II reengages and continues transcription, but if the lesion persists, RNAP II is ubiquitinated and degraded (Woudstra et al. 2002, Tornaletti et al. 2003, Brueckner et al. 2007). Stalling and/or degradation of RNAP II has another potential function: to prevent transcription across sites of DNA repair and thereby prevent formation of potentially deleterious proteins. However, this could result in release of prematurely terminated transcripts, and inhibition of the 3' processing machinery would then function to prevent polyadenylation and stabilization of such RNAs.

Dr. Mirkin and colleagues (2008) described in their work new links between 3' processing and DNA repair. They provided evidence that UV treatment in fact affects both transcription and polyadenylation of nascent mRNAs in vivo. They also showed that depletion of CstF in DT40 cells enhances sensitivity to UV treatment, reduces UV-induced ubiquitination of RNAP II and, significantly, causes a delay in TCR. As part of those studies, I determined that following UV treatment BRCA1/BARD1, RNAP II and CstF associate at sites of repaired DNA. Taken together, these results indicate that CstF plays active roles not only in 3' processing but also in DNA repair, providing a link between transcription-coupled RNA processing and DNA repair.

RESULTS

RNAP II, CstF and BARD1 associate at sites of DNA repair.

The data presented above provides evidence that CstF participates in ubiquitination of RNAP II in response to DNA damage, and in the TCR response itself. Based on this and on previous data from Dr. Kleiman's lab establishing an interaction between CstF and BRCA1/BARD1, I next determined whether RNAP II, CstF and BRCA1/BARD1 all associate at sites of DNA damage. To this end, a variation of the chromatin immunoprecipitation (ChIP) assay (Orlando 2000, Takahashi et al. 2000) was employed. This method has been used largely to study chromatin associated factors, but has also been valuable in analysis of proteins apparently associated with elongating RNAP II (e.g., Komarnitsky et al. 2000, Schroeder et al. 2000, Fousteri et al. 2006). In my experimental design, BrdU was added to HeLa cells immediately after exposure to UV light to label repaired DNA. Cells were crosslinked with formaldehyde at different times after UV exposure. As ubiquitination of RNAP II occurs within 15 min of exposing cells to UV and persists for about 8-12 hr (Bregman et al. 1996), the analysis was performed in a period between 0-5 hours after UV treatment. Extracts of these cells were prepared and following sonication DNA-protein complexes were IPed by incubation with an anti-BrdU monoclonal antibody. Following reversal of crosslinks, rather than analyzing DNA by PCR, it was determined whether specific proteins were associated with the BrdU-containing DNA by Western blot. Samples from cells not treated with UV were used as a control.

The data shows that RNAP II, CstF and BARD1 all associated with repaired/BrdU-containing DNA (Figure 13). These findings corroborate earlier

observations that part of RNAP IIO does not dissociate from the damaged DNA during the assembly of the TCR complex (Fousteri et al. 2006). The presence of RNAP II supports the hypothesis proposed in yeast that RNAP II is not always degraded at sites of DNA damage and might reengage and continue transcription (Woudstra et al. 2002, Fousteri et al. 2006, Brueckner et al. 2007). RNAP II associated with the repaired DNA was detected at the earliest time after UV irradiation (0.4 hr), suggesting that the arrest of the RNAP II is an early event in TCR. Consistent with previous results, the Western blot analysis also revealed that UV treatment decreased accumulation of RNAP II at later times (Figure 13, 2 hrs after UV treatment; Kleiman et al. 2005, Fousteri et al. 2006), likely reflecting the turnover of stalled RNAP II (Mirkin et al. 2008). Significantly, we also detected CstF-64 and BARD1 associated with the BrdU-containing DNA, with a time course very similar to that displayed by RNAP II. Together, this data supports the idea that RNAP II, CstF and BARD1 associate at sites of DNA damage and play a direct role in the DNA repair response.

DISCUSSION

Previous work from Dr. Kleiman ‘s laboratory showed that 3’ mRNA end processing is inhibited after DNA damage as a result of both BRCA1/BARD1/CstF complex formation (Kleiman and Manley 2001) and proteasome-mediated degradation of RNAP II (Kleiman et al. 2005). As CstF-50 can interact with BARD1 to inhibit the 3’ processing reaction (Kleiman and Manley 1999) and with the CTD of RNAP II to activate the 3’ processing reaction (McCracken et al. 1997, Hirose and Manley 1998), we proposed that CstF plays an important role in the response to DNA damage. Mirkin and colleagues (2008) provided evidence that prematurely terminated polyadenylated transcripts can be detected in vivo following DNA damage, especially under conditions when the CstF/BARD1/BRCA1 checkpoint is not activated. They also determined that cells with reduced levels of CstF displayed enhanced sensitivity to UV treatment. The depletion of CstF was found to correlate with decreases in both ubiquitination and turnover of RNAP IIO and repair of the transcribed DNA strand, which are events in the TCR response (Bregman et al. 1996, Ratner et al. 1998, Luo et al. 2001, McKay et al. 2001, Fousteri et al. 2006). Consistent with the model proposed for CstF function, my results also showed that RNAP IIO, BARD1 and CstF were all transiently associated with sites of repaired DNA. This finding also suggests that a fraction of RNAP II elongation complexes arrested at sites of DNA damage are stable and remain associated with the DNA. Taken together, our results suggest that the polyadenylation machinery, specifically CstF, plays an important role in the response to DNA damage.

Based on the results presented in the work of Mirkin and colleagues (2008), the model proposed in previous work can be confirmed and extended (Figure 9; Kleiman and

Manley 1999, 2001, Fousteri et al. 2006). All together the data has provided evidence that DNA damage can induce premature transcription termination and polyadenylation, likely at sites of DNA damage, and that accumulation of such species is blocked by activation of the CstF/BRDA1/BRCA1 checkpoint. How might the checkpoint prevent such RNAs from accumulating? Milligan et al. (2005) observed not only reduction in the levels of different mRNA species but also of truncated RNAs in yeast strains with a defective poly(A) polymerase. Defective polyadenylation of prematurely terminated transcripts is known to activate a nuclear surveillance pathway, eliminating those mRNAs by deadenylation and exosome-mediated degradation (Milligan et al. 2005, Wyers et al. 2005, Thiebaut et al. 2006). Extending this idea, the work presented in Chapter V of this thesis (unpublished data) indicates that another CstF-50-interacting protein is the poly(A) specific ribonuclease (PARN; Mitchell and Tollervey 2000, Wilusz et al. 2001, Wu et al. 2005). PARN has been shown to copurify with essential nonsense-mediated decay factors (Maquat 2004) and PARN down-regulation abrogates nonsense-mediated decay (Lejeune et al. 2003). Although more work is necessary to determine the functional relevance of the CstF/PARN interaction, the association of a polyadenylation factor and a deadenylation factor is mechanistically intriguing, and could contribute to the turnover of different RNA species by a nuclear quality control pathway after UV treatment.

Taken together, the data from Mirkin's work (2008) have indicated that CstF plays a role in the DNA repair response. As just discussed, CstF could affect DNA repair by inhibiting the erroneous processing of nascent, truncated RNAs, by inducing RNAP II ubiquitination, and/or by reengaging and continuing transcription with stalled RNAP II

complexes. It is also possible that CstF plays a more direct role in the repair process. Several observations support this hypothesis. First, CstF interacts with the DNA replication and repair factor PCNA (Kleiman and Manley 1999). It has been shown that PCNA colocalizes with BRCA1/BARD1 at sites of DNA repair (Scully et al. 1997, Wang et al. 2000) and associates with DNA repair proteins as part of the TCR response (Balajee et al. 1998). It is possible that PCNA is the repair factor that links the stalled RNAP II complex to the repair machinery during TCR. Second, several polyadenylation factors have been shown to interact with DNA repair factors. For example, cleavage factor $CFII_m$ copurifies with the BRCA1-associated protein hMre11 (de Vries et al. 2000), which has been implicated in DNA repair and cancer predisposition (reviewed by Petrini 2000). Additionally, the transcriptional coactivator PC4 interacts not only with CstF-64 (Calvo and Manley 2001) but also with the DNA repair protein XPG (Wang et al. 2004). XPG is known to function in multiple DNA repair pathways. XPG recruits PC4 to the bubble-containing DNA substrate, PC4 displaces XPG and forms a DNA-PC4 complex (Wang et al. 2004). PC4 can also interact with the elongating RNAP IIO through CstF-64, preventing premature termination during the elongating phase (Calvo and Manley 2001). It is thus possible that the interaction of PC4 with CstF-64 mediates the damage-induced association of the stalled RNAP II and the DNA repair machinery.

In any case, the data from Mirkin's work (2008) have provided evidence that CstF plays a role in TCR, reinforcing the functional interaction between components of the transcription, 3' processing and DNA repair machineries.

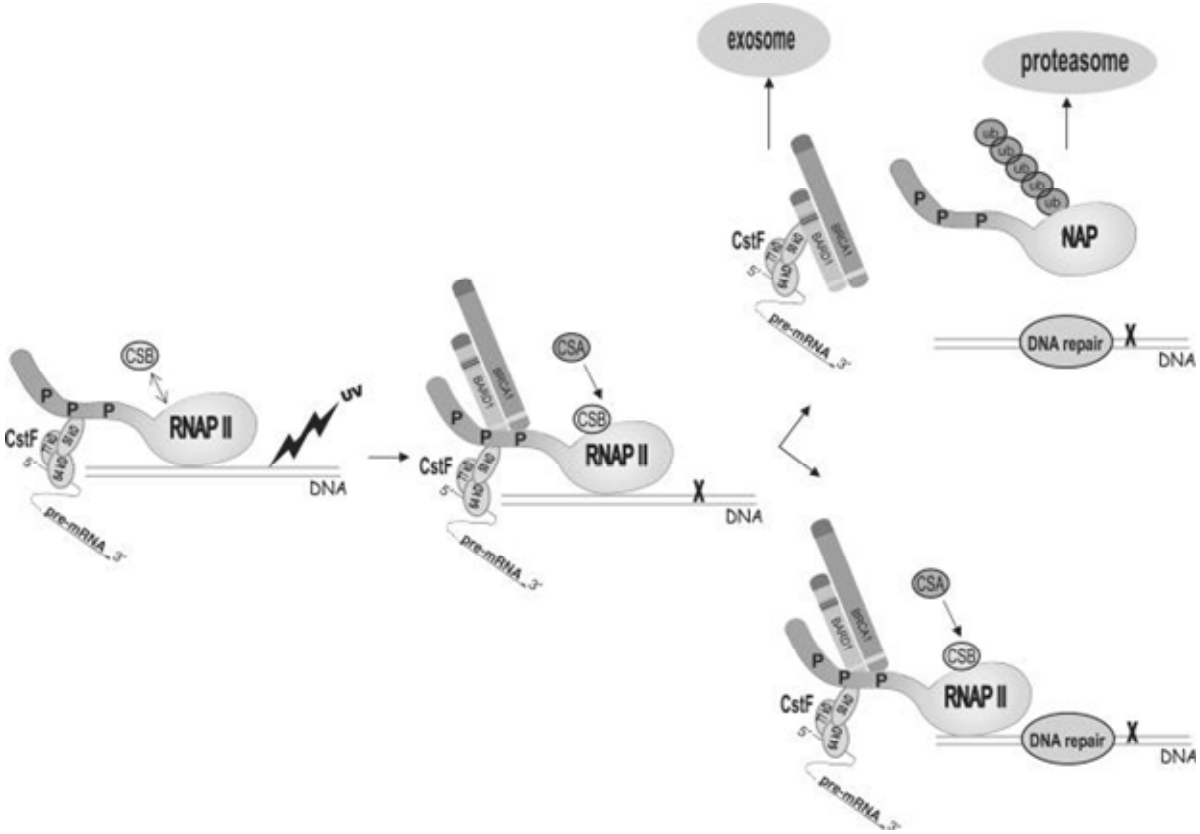

Figure 9: Model for the role of CstF in the DNA damage response. Coupling polyadenylation, transcription and DNA repair. After exposure to DNA damage-inducing agents, the elongating RNAP II-CstF holoenzyme complex stalls at sites of damage. A BRCA1/BARD1-containing complex is activated and recruited to sites of repair, inhibiting RNAP II and the associated polyadenylation machinery by ubiquitination followed by degradation of the RNAP IIO. This process facilitates repair by allowing access to the repair machinery, while simultaneously preventing polyadenylation of aborted nascent mRNAs, which are eliminated by exosome-mediated degradation in a nuclear surveillance pathway. Alternatively, the RNAP II complexes arrested at certain DNA lesions is not degraded, and reengages and continues transcription once repair is completed. Given that CstF-50 can functionally interact with all the elements of this model, we propose an important role for this protein in the transcription-coupled DNA damage response.

CHAPTER IV

Nuclear deadenylation/polyadenylation factors regulate 3’ processing in response to DNA damage.

INTRODUCTION

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

The polyadenylation reaction requires the assembly of a rather large number of interacting protein factors that recognize a relatively simple set of cis-acting signal sequence elements in the mRNA precursor. Cleavage stimulation factor (CstF) is one of the essential polyadenylation factors. CstF is active most likely as a dimer with each subunit consisting of three protein factors called CstF-77, CstF-64, and CstF-50. CstF-64

interacts directly with the down-stream located GU-rich cis-acting element. Both the CstF-50 and CstF-77 subunits interact specifically with the carboxy-terminal domain (CTD) of RNA polymerase II largest subunit (RNAP II LS), likely facilitating the RNAP II-mediated activation of 3' end processing (McCracken et al. 1997, Hirose and Manley 1998). Moreover, 3' end processing can be repressed following DNA damage as a result of the interaction between CstF-50 and BRCA1-associated RING domain protein (BARD1, Kleiman and Manley 1999) and of the proteasome-mediated degradation of RNAP II (Kleiman et al. 2005). We have recently shown that cells with reduced levels of CstF display decreased viability following UV treatment, reduced ability to ubiquitinate RNAP II, and defects in repair of DNA damage (Mirkin et al. 2008), supporting the idea that CstF plays a direct role in the DNA damage response.

Although most of the polyadenylation factors have been described and the reaction is now relatively well understood, the mechanisms behind poly(A) removal are much less defined. In mammalian cells, the earliest and rate limiting step in mRNA decay is the removal of the mRNA poly(A) tail (Wilusz et al. 2001, Chen and Shyu 2003). PARN is one of the three major poly(A) specific 3' exoribonuclease identified in mammalian cells and characterized thus far (Mitchell and Tollervey 2000, Parker and Song 2004, Wu et al. 2005). It is expressed ubiquitously in all tissues of most eukaryotic organisms (Copeland and Wormington 2001) and localizes both to the nucleus and the cytoplasm. PARN shows high specificity for single stranded poly(A) (Korner and Wahle 1997, Martinez et al. 2001) and its deadenylating activity is stimulated by the mRNA 5' end located cap structure (Dehlin et al. 2000, Gao et al. 2000, Martinez et al. 2001, Nilsson et al 2007, Wu et al. 2009). Although the exact function of PARN in the nucleus

is unknown, it has been established that the CBP80 (Balatsos et al. 2006) and the poly(A) binding protein 1 (PABPN1, Gao et al. 2001) both inhibit PARN activity. Interestingly, the cap binding complex (CBC) has also been shown to play a role in polyadenylation by stabilizing the RNA/CstF complex formed in the nucleus and the depletion of CBC reduces the mRNA cleavage reaction (Flaherty et al. 1997).

In the current study, we have found that the polyadenylation factor CstF-50 interacts strongly with the same region (C-terminal domain) of PARN as its inhibitor CBP80. Like the previously described CstF/BARD1/BRCA1 complex, the CstF/PARN complex formation is stimulated upon UV light–treatment and participates in the inhibition of the 3' cleavage reaction of polyadenylation upon DNA damaging conditions. More importantly, here we also show that the CstF-50/PARN interaction activates deadenylation *in vitro* and that UV treatment can activate nuclear PARN deadenylase activity. We also show that the tumor suppressor BARD1 strongly activates deadenylation by PARN in the presence of CstF-50 and that the siRNA-mediated knockdown of BARD1 decreases the UV-induced activation of deadenylation. In addition, our data show that CBP80 and CstF-50 could compete for binding to PARN, providing a mechanism to regulate PARN deadenylase activity in different cellular conditions. Consistent with this, we show that these functional interactions correlate with changes in both stability and polyadenylation of different mRNA precursors, such as housekeeping genes and some clinically significant genes, upon UV treatment and that reduced expression of PARN is sufficient to revert the observed changes. Based on our study we propose that the CstF/PARN complex plays a role in the inhibition of 3' cleavage of polyadenylation and the activation of deadenylation in the nucleus upon

DNA damaging conditions, suggesting the existence of alternative mechanisms to regulate gene expression in different cellular conditions.

RESULTS

The polyadenylation factor CstF-50 binds to the deadenylation factor PARN upon DNA damaging conditions and this is accompanied with an increase in PARN expression levels.

To identify proteins that interact with the polyadenylation factor CstF-50, we performed some time ago a yeast two-hybrid screen and identified BRCA1-associated BARD1 as a prominent interactor (Kleiman and Manley 1999). We have recently revisited the primary data of this screen and realized that one of the most abundant interactors of CstF-50 corresponded to the C-terminal fragment of PARN. This new finding suggests a functional interaction between the two components as well as between the deadenylation and polyadenylation machineries. To further investigate this possibility, we performed "pull down" assays using GST-tagged full-length CstF-50 (GST-CstF-50) and full-length PARN (His-PARN), the C-terminal fragment of PARN (amino acids 443 to 639, His-CTD-PARN) and the N-terminal fragment of PARN (amino acids 1 to 470, His-NTD-PARN). The results showed that both His-PARN (Figures 14A, lane 2, and 1B, left panel, lane 4) and His-CTD-PARN (Figure 14B, lane 6) interacted directly and strongly *in vitro* with GST-CstF-50. However, GST-CstF-50 did not bind either to the derivative encompassing the N-terminal region of PARN (Figure 14B, lane 5) or to the GST alone (lane 7). As samples were treated with RNase A, the observed CstF/PARN interaction was not due to RNA tethering effect. All together, these results indicate that the C-terminal domain of PARN, which has been described to interact with CBP80 (Balatsos et al. 2006), constitute the PARN/CstF-50 interaction domain.

To examine the status of the CstF-50/PARN complex in nuclear extracts (NE), we analyzed extracts from HeLa cells by coimmunoprecipitation assays. For these studies, we used an antibody directed against either PARN or the CstF subunit CstF-64, to ensure that any detected interactions were between PARN and intact CstF, and samples were treated with RNase A. Figure 15 (left panel) shows that only a very small fraction of nuclear PARN coprecipitated with CstF-64. Similar results were obtained in the reciprocal coimmunoprecipitation analysis (Figure 15, right panel). As the results of the coimmunoprecipitation assays did not reflect the strong interaction observed in the GST "pull down" assays, we decided to analyze the complex formation under conditions of DNA damage since earlier studies had revealed a link between CstF and the UV-induced DNA damage response (Kleiman and Manley 2001). Thus, the coimmunoprecipitation assays were repeated with NE of cells exposed or not to UVC light (20 Jm^{-2}) and allowed to recover for 2 hrs as described before (Kleiman and Manley 2001). Surprisingly, we observed in this case a significant increase in the detected amount of CstF/PARN complexes as well as an increase in PARN expression (Figures 15). Extraneous antibodies did not immunoprecipitate either protein (lanes 3 and 4), nor did either antibody cross-react with other proteins (not shown). The increased amounts of PARN in extracts of UV-irradiated cells can not solely explain the increased association between the two proteins. Although similar amounts of PARN and CstF are immunoprecipitated by their own antibody in samples exposed or not to UV, a complex formation with CstF is detected only in the UV-treated samples, even in darker exposures of the Western blot analysis (not shown).

To further investigate the increased expression levels of PARN, NE of HeLa cells treated with UV irradiation and allowed to recover for the times indicated in Figure 16 were analyzed. As described before (Kleiman and Manley 2001), Western blots revealed no significant changes in either components of CstF (CstF-64) and CPSF (CPSF-160, not shown) or BRCA1/BARD1 (not shown) in response to UV. No changes were detected in the Topoisomerase II (Topo II) levels as well. However, a transient increase in the expression of PARN was observed from 2-10 hr after UV treatment, but normal levels were restored after 15 hrs, reaching the levels of untreated cells (not shown).

As UV treatment was known to induce CstF/BARD1/BRCA1 complex formation (Kleiman and Manley 2001), we decided to test whether the tumor suppressor BARD1 might also interact with PARN. Interestingly, a significant amount of BARD1 co-precipitated with PARN in NEs from UV-treated cells (Figure 15). Similarly, we could detect PARN in the reciprocal coimmunoprecipitation experiment where we used antibodies directed against BARD1 (data not shown). Although these results do not demonstrate how many complexes CstF can form with PARN and BARD1, they clearly show that UV treatment induced the interaction between those factors. Interestingly, the appearance of this/these complex/es coincided with the observed inhibition of 3' cleavage upon DNA damaging conditions (Kleiman and Manley 2001).

To test whether BARD1 might also interact directly with PARN, we performed "pull down" assays using recombinant His-PARN, GST-CstF-50 and GST-BARD1 (Kleiman and Manley 1999) polypeptides (Figure 17). In the left panel, the "pull-downs" were done with nickel beads. While only a small amount of BARD1 is pulled down by His-PARN (lane 3), this amount increases in the presence of CstF-50 (lane 4). In the right

panel, the “pull-downs” were done with glutathione beads and only GST-CstF-50 associated with His-PARN. These results indicate that BARD1/CstF/PARN complex could be formed in the presence of CstF-50, suggesting that CstF-50 acts as a scaffold to bring PARN and BARD1 into the same complex. Taken together, these sets of experiments revealed several lines of evidence that PARN and CstF-50 interact with each other and that their association, including the CstF-associated protein BARD1, was accentuated after UV treatment.

PARN is necessary for the UV-induced inhibition of the 3’ cleavage reaction.

To study the significance of the interaction between PARN and CstF-50, we performed siRNA mediated knockdown of PARN in HeLa cells. Figure 18A shows that a 24 hrs siRNA treatment resulted in a substantial depletion of PARN (≈90%) in NEs, independently of the UV treatment. We next investigated the effect of siRNA mediated knockdown of PARN on the UV-induced inhibition of 3’ cleavage described earlier (Kleiman and Manley 2001). Surprisingly, the depletion of PARN abolished the UV-induced inhibition of mRNA 3’ end cleavage (Figure 18A, compare lanes 2 and 4), indicating that PARN has an inhibitory effect on mRNA 3’ cleavage under DNA damaging conditions. In consistence with former work of our lab, NEs from control siRNA-treated cells disclosed the UV-induced inhibition of 3’ processing. As observed before (Kleiman and Manley 2001, Kleiman et al. 2005), no significant changes were detected for CstF, BRCA1, Topo II or BARD1 levels in response to UV, whereas the levels of RNAP II were reduced after UV treatment.

Extending these results, we followed during a time course experiment the 3' cleavage activity after UV treatment in the presence or absence of siRNA targeting PARN (Figure 18B). From this analysis it is evident that the absence of PARN abolished the UV-induced inhibition of 3' cleavage. To further document the involvement of PARN in the response, we added back recombinant His-PARN (Figure 19, upper left), His-CTD-PARN (lower left), His-NTD-PARN (lower right) and GST protein as control (upper right) into 3' cleavage reactions performed with PARN siRNA knockdown and UV-treated extracts. Only the increasing concentrations of His-PARN and His-CTD-PARN recovered the inhibition of 3' cleavage reaction observed after UV-treatment. All together, these results indicate that PARN has an inhibitory effect on mRNA 3' cleavage under DNA damaging conditions. Initially, this inhibition was ascribed to the CstF/BARD1/BRCA1 complex formation (Kleiman and Manley 2001) and the proteasome-mediated degradation of RNAP II (Kleiman et al. 2005). However, these results indicated that other factors, such as PARN, might also be involved in the response. Although our data does not reveal the mechanism involved in this cellular response, it is possible that the UV-induced inhibition of 3' cleavage by PARN could be the result of several alternative mechanisms, such as a direct interaction between PARN and the essential polyadenylation factor CstF-50, by a destabilization effect of the essential CstF/RNA complex, and/or by the formation of any other inhibitory complex.

CstF-50 plays a role in the UV-induced activation of nuclear PARN deadenylase activity.

The results presented above showed that PARN was induced upon UV treatment and that complex formation between PARN and CstF-50 paralleled the inhibitory effect of UV treatment on the 3' end cleavage reaction. PARN has previously been shown to be present both in the nucleus as well as in the cytoplasm (Korner et al. 1998). As CstF-50 is primarily located in the nucleus (Zhao et al. 1999, Shatkin and Manley 2000), we asked what happened to nuclear PARN deadenylation activity during the response to DNA damage. HeLa cells were first exposed to UV light and then allowed to recover for 2 hrs before NEs were prepared, and assayed for the presence of deadenylation activity of a radiolabeled $L_3(A_{30})$ RNA substrate. Figure 20A shows that the deadenylation activity detected in NEs of cells non-exposed to UV treatment was very weak and this activity was increased significantly after UV treatment. siRNA mediated knockdown of PARN showed an effect not only in the UV-induced inhibition of 3'cleavage (Figure 20B, left panel) but also in UV-induced activation of deadenylation (right panel, compare lanes 6 and 8). Western blot analysis confirmed the knockdown of PARN (lower panel). Besides, these results confirm that PARN is the deadenylase under regulation in this DNA damage response.

Further analysis showed that the activation of deadenylation in NEs was UV-dose dependent and transient, increasing between 2 and 5 hrs after UV treatment and disappearing after 10 hrs (Figure 21, upper panel). Interestingly, this transient pattern of deadenylation activation was reflected by a concomitant inhibition of the 3' end cleavage reaction (lower panel). Thus, UV treatment not only induced the association of CstF-50,

PARN and BARD1 (Figure 22 and 15) but also activated PARN dependent deadenylation activity in NEs (Figure 20-22), suggesting that CstF-50 could participate in the UV-induced activation of PARN mediated deadenylation.

To directly test if CstF-50 could influence PARN activity we performed *in vitro* reconstituted deadenylation reactions, where we monitored deadenylation of L3(A_{30}) RNA substrate in a reaction with limiting amount of His-PARN and in the absence or presence of increasing amounts of GST-CstF-50. The addition of GST-CstF-50 enhanced the deadenylation activity of PARN up to ten folds (Figure 23A, compare lanes 4 and 9), suggesting that CstF-50 is an activator of PARN activity. Importantly, we did not detect any deadenylation activity when using CstF-50 alone (lanes 10-11) or in combination with the deadenylase-deficient PARN fragment (CTD-PARN, Figure 23B), which interacts strongly with CstF-50 (Figure 14B). However, CstF-50 failed to increase the deadenylase activity of the NTD-PARN derivative, which lacks the CstF-50 interacting domain (Figure 24), indicating that the CstF-50/PARN interaction is necessary for the activation of PARN activity. Taken together, we conclude that CstF-50 activates PARN deadenylation activity in the absence of any other factors, suggesting that the CstF-50/PARN complex that we have identified could play a role both in the UV-induced inhibition of the polyadenylation 3' end cleavage reaction as well as in the concomitant activation of nuclear deadenylation.

The CstF-50/BARD1 complex can rescue PARN deadenylase activity from the CBP80 induced inhibition.

The UV-induced association of the CstF, PARN and BARD1 (Figures 15 and 22) raises the possibility that BARD1 plays a role not only in the UV-induced inhibition of

polyadenylation but also in the UV-induced activation of deadenylation in the presence of CstF-50. To address this possibility, we monitored deadenylation of $L_3(A_{30})$ RNA substrate in a reaction mix with limiting amount of His-PARN, limiting concentration of GST-CstF-50, and in the absence or presence of increasing amounts of GST-BARD1 (Figure 25A). GST-BARD1 significantly enhanced deadenylation by PARN only in the presence of CstF-50 (compare Figures 25A and B), implying that the CstF-50/BARD1 complex is a stronger activator of PARN deadenylase activity than CstF-50 alone. Using a limited amount of PARN and the lowest concentration of CstF-50 tested (Figure 23A, lane 5), complete deadenylation was observed after the addition of BARD1 (Figure 25A, lane 7), reaching deadenylation levels that are similar to the ones observed with twenty times more PARN (lane 4). Importantly, we did not detect any deadenylation activity when using either the CstF-50/BARD1 complex alone (Figure 25A, lane 8) or limiting amounts of PARN and increasing amounts of BARD1 (Figure 25B).

To further characterize the role of BARD1 in the activation of deadenylation under DNA damaging conditions, we performed siRNA mediated knockdown of BARD1/BRCA1 in HeLa cells as described before (Kleiman et al. 2005). As BRCA1 and BARD1 stabilize each other (Hashizume et al. 2001), treating the cells with both BRCA1 and BARD1 siRNA simultaneously was necessary to obtain a substantial depletion of BARD1 (≈90%, Figure 25C, lower panel) in NEs. Interestingly, samples from the BARD1/BRCA1 siRNA-treated cells showed a decrease in the UV-induced activation of deadenylation (Figure 25C). Thus, our results indicate that the tumor suppressor BARD1, which is involved in the UV-induced inhibition of polyadenylation, in the presence of

CstF-50 activates PARN-mediated deadenylation both *in vitro* and in samples from UV-exposed cells.

It has been described that the CBC complex, through its CBP80 subunit, binds the C-terminal domain of PARN and inhibits PARN deadenylase activity (Balatsos et al. 2006) and also enhances the stability of the RNA/CstF complex and thereby activates the 3' end cleavage reaction (Flaherty et al. 1997). Interestingly, our results showed that CstF-50 (Figure 14B), like CBP80, binds the C-terminal domain of PARN, raising the possibility that both proteins compete for binding to the same region of PARN. While the formation of the PARN/CBP80 complex has been described in non-damaged cells (Balatsos et al. 2006), our current results indicate that the formation of the CstF/PARN complex is induced after UV-treatment (Figure 15). Given the nature of CBP80 and CstF-50 as inhibitor and activator of PARN deadenylase activity, respectively, it could be possible that these proteins play a regulatory role in mRNA turnover under different cellular conditions.

To address this possibility, we analyzed the formation of the CstF/PARN and PARN/CBP80 complexes by coimmunoprecipitation assays with antibodies against PARN in NEs from untreated and UV-treated cells followed by Western blot analysis with antibodies against either CstF or CBP80. Figure 26A shows, in keeping with earlier results, that PARN coprecipitated a small fraction of CstF (Figure 15 and 22) and a significant amount of CBP80 (Balatsos et al. 2006) in extracts of untreated cells. However, and most importantly, a significant decrease in the amount of coprecipitated CBP80 was observed after UV exposure (Figure 26A, compare lanes 1 and 3).

To characterize the interaction of CstF-50 and CBP80 to the same region of PARN, competition assays were carried out by incubating His-PARN immobilized on nickel beads with either limiting amounts of GST-CBP80 or GST-CstF-50, and with increasing amounts of either GST-CstF-50 or GST-CBP80, respectively. Protein samples were treated with RNase A prior the binding assays. Increasing amounts of CstF-50 significantly diminished the binding of CBP80 to immobilized PARN (Figure 26B, lanes 4-7), indicating that CstF-50 and CBP80 compete on binding to the same region of PARN. Similar conclusions were reached when increasing amounts of CBP80 were used in the "pull-down" assay (lanes 8-11).

To examine the possible regulatory relationship between the two different PARN associated complexes, we performed *in vitro* deadenylation assays with PARN in the presence and/ or absence of recombinant CstF-50 and CBP80. As seen in Figure 27, addition of CstF-50 could partially suppress the CBP80-induced inhibition of PARN activity (compare lanes 4 to 5-6), and thereby activate PARN deadenylase activity. The release of the CBP80-induced inhibition of PARN activity was even more pronounced when BARD1 was also included into the reactions (compare lane 9 to 11). Taken together, these results indicate that CBP80 and CstF-50 could compete for binding to PARN in different cellular conditions, inhibiting deadenylase activity through the PARN/CBP80 complex under normal conditions and activating deadenylase activity under DNA damaging conditions through CstF-50/PARN complex formation.

PARN is involved in the degradation of different endogenous transcripts under different cellular conditions.

The data presented above provided evidence that DNA damage induces the activation of PARN mediated mRNA deadenylation in the nucleus. To further investigate this, we determined the expression levels of different endogenous mRNAs in cells treated with siRNAs targeting PARN and UV irradiation. Briefly, twenty-four hours after transfection with the indicated siRNAs, cells were exposed to UV light and total nuclear RNA was purified at different times after UV treatment. Gene expression was analyzed by RT-PCR, PCR poly(A) test (PAT, Sallés and Strickland 1995) and qRT-PCR. Random or oligo(dT) primers were used for the RT reaction, and qPCR reactions were done using commercially available primers and PCR reactions were done with a transcript specific forward primer together with either a transcript specific reverse primer or a non-specific oligo(dT) primer adapter (Figure 28). When the oligo(dT) primer adapter was used, the size of the amplification products would be heterogeneous, reflecting the length of the poly(A) tail (Kim and Richter 2006).

First, we analyzed the expression levels of two housekeeping genes, GAPDH and β-actin, in different cellular conditions. Our RT-PCR (Figure 28, lanes 1-3 and 7-9) and qRT-PCR (Figure 29A) analysis showed that the mRNA levels of these genes decreased upon DNA damaging conditions in cells treated with control siRNA. A similar decrease was observed when oligo(dT) primer adapter was used in the PCR reactions (Figure 28, lanes 4-6 and 10-12). This data is consistent with our earlier studies (Mirkin et al. 2008) and others previous observations (Kartasova et al. 1987, Dheda et al. 2004, Maccoux et al. 2007, Akeo et al. 2007) that showed that GAPDH RNA expression can change

significantly in different biological systems and under different conditions, and that these variations can lead to experimental error between analyzed samples when GAPDH is used as a control. Interestingly, the UV-induced decrease in the mRNA levels of endogenous housekeeping genes was lost when we treated the cells with siRNAs targeting PARN (Figure 28, 29A). Similar results were observed using either the specific reverse primer (lanes 1-3 and 7-9) or the oligo(dT) primer adapter (lanes 4-6 and 10-12). Furthermore, samples from the PARN depleted cells showed an accumulation of heterogeneous PCR products after UV treatment, suggesting an increase in the stability of polyadenylated mRNAs. These results indicate that PARN is required to decrease mRNA polyadenylation levels and mRNA stability of housekeeping genes upon DNA damaging conditions, and thereby might contribute to the UV-induced decrease in the cellular levels of total mRNA.

As it has also been shown that PARN can promote deadenylation of AU-rich elements (ARE)-containing mRNAs (Lai et al. 2003, Moraes et al. 2006), we also analyzed two ARE-containing mRNAs, *i.e.* c-fos and c-myc, by qRT-PCR. Both mRNAs increased transiently upon DNA damaging conditions in cells treated with control siRNA (Figure 29), in keeping with earlier studies that indicate that ARE elements within the 3'UTR can control mRNA stability under different cellular conditions. For example, ARE elements can decrease mRNA stability under non-stress conditions and can increase mRNA stability after UV treatment in mammalian cells (Blattner et al. 2000, Wang et al. 2000, Bollig et al. 2002, Gowrishankar et al. 2006). Supporting our results, Blattner and colleagues (2000) showed that c-fos mRNA expression increased 45 min to 1 hr after UV treatment and then dramatically decreased 2 hr after UV treatment. Strikingly, PARN

knockdown cells showed increase in the stability of both c-fos and c-myc in samples from non-UV treated cells (Figure 29A). These results suggest that PARN plays a role decreasing the stability of short lived mRNAs involved in control of cell growth and differentiation, keeping their expression levels low in non-stress conditions. The reduced expression of PARN has a slight effect on the UV-induced increase in the expression levels of these genes, suggesting that other mechanism(s) might be involved in determining the stability of these genes upon DNA damage conditions.

Finally, we tested for enrichment of different mRNAs in the poly(A)$^+$ RNA population after PARN siRNA- and UV-treatment (Figure 29B). qRT-PCR quantification reveals that the studied mRNAs, c-fos, c-myc, actin and GAPDH, showed a slight decrease in the enrichment in the poly(A)$^+$ preparation over the total RNA fraction in samples from control siRNA- and UV-treated cells. This is consistent with our initial finding that UV treatment inhibits 3' processing (Kleiman and Manley 2001). Interestingly, our results showed that those mRNAs were enriched ~2 to 4 fold in the poly(A)$^+$ preparation over the total RNA fraction in samples from PARN depleted cells. Interestingly, such enrichment in the poly(A)$^+$ preparation showed a stronger increase in samples from UV-treated cells. These results indicate that PARN is involved in the UV-induced decrease of polyadenylated mRNAs either by inhibition of 3' cleavage or by activation of deadenylation.

Taken together, these results provide evidence that PARN is required to regulate the levels of different endogenous mRNAs in different cellular conditions. As we proposed before, it is possible that the competition of CBP80 and CstF-50 for binding to

PARN in different cellular conditions could play a role regulating PARN activity and, therefore, mRNA levels of different genes.

DISCUSSION

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

We have proposed in previous work that the polyadenylation factor CstF-50 plays a coordinating role in the nuclear response to UV-induced DNA damage through its interaction with different factors in different cellular environments (Kleiman and Manley 1999, 2001; Kleiman et al. 2005, Mirkin et al. 2008). In the current work, we have discovered that CstF-50 interacts with the deadenylation factor PARN (Figure 14-15) and that this interaction plays a role in inhibition of 3' cleavage of the polyadenylation reaction (Figures 18-19 and 29) and activation of deadenylation upon DNA damage treatment (Figures 20-25). Here we also found that BARD1 is not only involved in the

UV-induced inhibition of 3' cleavage (Kleiman and Manley 1999, 2001) but also in the UV-induced activation of deadenylation in the presence of CstF-50 (Figure 25 and 27). Furthermore, we found that the previously identified nuclear CBP80/PARN deadenylation inhibitory complex decreased significantly in abundance upon DNA damaging conditions, whereas the complex containing CstF-50/PARN increased and that CstF-50 compete with CBP80 on binding to the same region of PARN (Figure 26). In fact, addition of CstF-50 and BARD1 to *in vitro* deadenylation reactions reverted the CBP80 inhibition effect on PARN activity (Figure 27). Finally, we determined that PARN knockdown had an effect on the stability and polyadenylation of different genes in different cellular conditions (Figure 28-29). Taken together, our results suggest that an interplay between these factors might control gene expression under DNA damaging conditions by regulating polyadenylation/deadenylation.

Based on our studies we propose the following regulatory scenario (summarized in Figure 30). In the absence of DNA damage treatment, CBP80 binds to the C-terminal domain of nuclear PARN and inhibits its hydrolytic activity to ensure that PARN does not degrade the mRNA (Balatsos et al. 2006). In this situation, CBC is also known to enhance polyadenylation of pre-mRNAs by increasing the stability of the RNA/CstF complex (Flaherty et al. 1997). As a result of these functional interactions, polyadenylation takes place and normal levels of total mRNA are observed. After DNA damage the BRCA1/BARD1-containing complex is recruited to sites of DNA repair to inhibit mRNA processing by RNAP II ubiquitination followed by degradation of the large subunit of RNAP II, or by covalent modification of other element/s of the complex. This facilitates DNA repair and/or prevent polyadenylation of aborted nascent mRNAs. If

the UV-induced inhibition of mRNA 3' cleavage is bypassed, the CstF/PARN interaction may provide a fall-back mechanism to ensure that erroneously polyadenylated mRNAs are eliminated by the activation of deadenylation. In this situation we propose that CBP80 dissociates from PARN, allowing PARN to interact with the CstF-50/BARD1 complex. This reorganization will result in an activation of deadenylation and contribute to the inhibition of polyadenylation. The final outcome will therefore be that polyadenylation is inhibited and deadenylation activated, contributing to the observed decrease in the levels of total mRNA under DNA damaging conditions. A similar mechanism for control of gene expression has been described by Kim and Richter (2006). They have shown that cytoplasmic poly(A) tail length is regulated by polyadenylation/deadenylation under different cellular conditions by the direct interaction of PARN with the polyadenylation factor CPEB.

It has been shown that PARN co-purifies with essential nonsense-mediated decay factors (NMD, Lejeune et al. 2003, Maquat 2004) and that siRNA mediated down-regulation of PARN abrogates NMD (Lejeune et al. 2003). Although those reports focused on cytoplasmic PARN, it is possible that the activation of deadenylation by the CstF/PARN/BARD1 complex formation in the nucleus might signal the degradation of those erroneously polyadenylated prematurely terminated mRNAs, providing a mechanism of nuclear mRNA decay. Consistent with this our previous work showed that prematurely terminated polyadenylated mRNA transcripts can be detected *in vivo* following DNA damage, especially under conditions when the CstF/BARD1/BRCA1 checkpoint is not activated (Mirkin et al. 2008).

Control of polyadenylation/deadenylation in the nucleus could represent a mechanism to regulate gene expression, which could be important to allow a rapid response during development or after stress treatment. For example, UV-treatment induces a decrease in the cellular levels of total mRNA to avoid the expression of deleterious proteins that may be harmful to the cell (Hanawalt 1994, Ljungman et al. 1999). UV-treatment also induces stabilization of ARE-containing mRNAs; such as c-fos, kin17, c-jun, IκB and c-myc (Blattner et al. 2000), to induce the expression of some proteins involved in DNA repair and cell cycle. Our results indicate that PARN can decrease the stability of housekeeping genes upon DNA damaging conditions and of ARE-containing genes upon non-stress conditions (Figure 28-29). Consistent with our results, it has been shown that ARE-dependent deadenylation plays an important role in the mRNA decay of several oncogenes involved in regulation of cell growth and differentiation (Blattner et al. 2000, Lai et al. 2003, Moraes et al. 2006). Here we propose that gene expression of different genes, such as housekeeping and ARE-containing genes, might be regulated in the nucleus by the functional interaction between CstF/BARD1, CBP80 and PARN under different cellular conditions. As the tumor suppressor BARD1 is involved in this response, it is possible that malignant cells display altered levels of polyadenylation of certain mRNAs. Supporting this idea, enhanced polyadenylation has been detected in certain tumor cells (Kumar et al. 1995, Scorilas et al. 2002), polyadenylation is inactivated in M phase (Colgan et al. 1996, 1998), expression levels of poly(A) polymerase can interfere with cell growth (Zhao and Manley 1998). Moreover, antiproliferative transcription factors, such as BTG2 and TOB, have been shown to enhance deadenylation, and the subsequent mRNA decay (Ezzeddine et al. 2007,

Mauxion et al. 2008). Furthermore, the expression levels of certain ARE-containing genes, such as c-jun and c-fos, increase significantly in cancer cells (Zajchowski et al. 2001, Andersen et al. 2002, Milde-Langosch 2005). Interestingly, microRNAs, which have been either directly involved in human cancers or described as oncogenes or tumor suppressors, can direct rapid deadenylation of mRNAs and subsequent decay (Wu et al. 2006, Zhang et al. 2007).

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

CHAPTER V

FUTURE DIRECTIONS

Based on the results presented in this dissertation and previously, we have suggested an alternative pathway to explain the observed decrease in the levels of total mRNA upon DNA damage. Initially, it was described that this decrease was due to DNA damage-induced inhibition of transcription; however, the data presented here and the work of others have shown that regulation of RNA 3' processing also plays an important role in the UV-induced decrease in the total mRNA levels. Previously, it was shown that UV damage inhibits 3' end processing due to the degradation of RNAP II and BRCA1/BARD1/CstF-50 complex formation. In this dissertation, these studies have been extended and showed that UV-induced ATM kinase dependent phosphorylation of BARD1 at T734 is required for those responses. It has also been shown that PARN is involved in the UV-induced inhibition of 3' cleavage as part of the PARN/CstF-50/BARD1 complex. Moreover, the PARN/CstF-50 complex is also involved in activation of deadenylation upon DNA damage. Although the mechanism behind this UV-induced response is not known, it was found that BARD1, which is involved in the UV-induced inhibition of 3' cleavage, and CBP80 play a role in this response. Importantly, it is shown that PARN affects both polyadenylation and stability of different mRNA precursors, such as housekeeping genes and some clinically significant genes, under different cellular conditions.

Since all the data presented here shows for first time the association and interplay of factors involved in different cellular pathways, such as CBP80, PARN, BARD1 and CstF-50, it is important to further analyze their functional interaction under different cellular conditions. The following proposed studies might provide more information that would help to understand some aspects proposed in this dissertation.

1) A possible human nuclear exosome/TRAMP complex formation:

Owing to the gene regulatory characteristic of a polyadenylation and a deadenylation factor, it is critical to further analyze and understand the essence of CstF-50 and PARN association in more detail. Interestingly, it has also been described that RNA degradation by the exosome is promoted not only by active deadenylation but also by nuclear polyadenylation complex (TRAMP complex; LaCava et al. 2005, Wyers et al. 2005, Thiebaut et al. 2006, Wang et al. 2008). The TRAMP complex, which has been characterized in yeast, promotes the addition of short poly(A) tails to nuclear-retained RNAs that are subsequently degraded by the exosome. The stimulation of the surveillance pathway by TRAMP is apparently in competition with addition of long poly(A) tails leading to export, suggesting that part of the functional differences between both polyadenylation processes arises from the speed and processivity of the polyadenylation reactions. LaCava et al. (2005) have suggested that the mRNA cleavage and polyadenylation complex is highly processive, rapidly adding long poly(A) tails, preventing the access of the exosome to the 3' end of the RNA until polymerization is complete and then the transcript is protected by the poly(A) binding protein PABPN1, which also inhibits PARN activity in the nucleus (Gao et al. 2001). In contrast, the yeast TRAMP complex shows a slow polyadenylation rate and low processivity, promoting the association of the exosome to RNA.

The results presented in this dissertation show certain degree of similarity to this pathway: the deadenylation factor PARN interacts with the polyadenylation complex factor CstF upon DNA damaging conditions, and this interaction not only stimulates

mRNA deadenylation and inhibits polyadenylation but also decreases the levels of poly(A) mRNA. Whether a similar situation occurs in mammalian cells is unclear, and there are significant differences in the response to DNA damage in the two systems. Most importantly in this dissertation, true orthologs of BARD1 and CstF-50 have not been identified in yeast (Baer and Ludwig 2002, Mandel et al. 2008). Moreover, yeast strains that carry rna 14-1 mutations show not only defective pre-mRNA cleavage and polyadenylation but also the formation of long 3'-extended transcripts that are rapidly degraded by the exosome (Torchet et al. 2002). It has also been shown that in rna 14-1 strains that also lack a nuclear specific exosome component, a posttranscriptional polyadenylation activity generates functional mRNAs, suggesting the existence of alternative polyadenylation mechanisms (Torchet et al. 2002). In mammals, as we described before, DNA damage inhibits 3' processing generating prematurely terminated polyadenylated mRNA transcripts (Kleiman and Manley 2001, Kleiman et al. 2005, Mirkin et al. 2008). It is possible that a mammalian homologue of the TRAMP complex is activated in DNA damage conditions, adding short poly(A) tails and activating exosome degradation.

Supporting the role of nuclear PARN in the exosome, PARN has been detected in mammalian cytoplasmic exosome granules, and PARN down regulation abrogated AU-rich-mediated mRNA decay (AMD, Lin et al. 2007). Similar to the yeast system, a possible exosome activity has been detected in the nucleus of mammalian cells. More importantly, the nuclear and cytoplasmic exosomes are composed of similar subunits (Rrp4, Rrp40, Csl-4, Rrp41, PM/Scl-75, Rrp46, Dis3p and PM/Scl-100), most of which

are ribonuclease complexes (Allmong et al. 1999; Brouwer et al. 2001; Mitchell and Tollervey 2000; Raijmakers et al. 2002; Schilders et al. 2006).

It is therefore important to determine if any exosome core elements might be functionally associated with the PARN/CstF/BARD1 complex inside the nucleus upon UV damage. It is possible that a mammalian homologue of the TRAMP complex is activated upon DNA damaging conditions and includes factors, such as CstF and PARN that could add short poly(A) tails and activate exosome degradation, respectively. To test this hypothesis it will first be determined whether CstF-50 and nuclear PARN can also interact with elements of the exosome after UV treatment. Briefly, NE from non-treated and UV-treated HeLa cells will be prepared and analyzed by coimmunoprecipitation assays with antibodies against CstF-50, PARN and different subunits of the nuclear exosomes mentioned before. As BARD1 plays a role in both activation of deadenylation and inhibiton of 3' cleavage, it will also be included in these determinations. If any component of the exosome is coimmunoprecipitated with CstF or PARN, GST pull-down assays will be performed to confirm the direct interaction of PARN or CstF with the exosome subunits. To determine the effect of exosome factors that interact with PARN or CstF on the UV-induced inhibition of polyadenylation and activation of deadenylation, NEs from exosome siRNA-depleted and UV-treated HeLa cells will be used in 3' cleavage and deadenylation reactions as described before. All together, these experiments will indicate if any factor from the exosome is part of the DNA damage-induced BARD1/CstF50/PARN complex, and whether they are involved in the DNA damage-induced regulation of polyadenylation and deadenylation.

2) Characterization of the functional interaction of poly(A) binding protein (PABP) with PARN, CstF and CBP80 upon DNA damaging conditions.

In mammalians, the poly(A) tail is approximately 200-250 nucleotides long (Brawerman 1981). Several poly(A) binding proteins (PABP) bind to every 20-30 adenosine residues at the 3' end, stabilizing the poly(A) tail by preventing its degradation (Deo et al. 1999, Korner and Wahle 1997, Tucker et al. 2002). PABP exists both in the cytoplasm and in the nucleus. In the cytoplasm, PABP plays a role in the poly (A) length control by interacting and regulating poly(A) nuclease (PAN, Mangus et al. 2004), which is a PABP-depending exoribonuclease (Brown et al. 1996). While yeast PAN can exist both in the cytoplasm and the nucleus, mammalian PAN is only found in the cytoplasm and thus only function in cytoplasmic deadenylation (Uchida et al. 2004). It has been shown that PAN mutations cause poly(A) length increase inside cytoplasm, suggesting that the PABP-PAN complex may be necessary for inhibition or displacement of poly(A) polymerase from the transcript (Siddiqui et al. 2007). If poly(A) polymerase is not inhibited or displaced the mRNA transcripts result in longer poly(A) tail lengths.

Whether a similar situation occurs in mammalian cells is unclear, and there are significant differences in the response to DNA damage in the two systems. For example, yeast does not express orthologs of PARN, and the human PAN deadenylase exclusively exists inside the cytoplasm (Uchida et al. 2004). Therefore, another deadenylase might replace PAN's activity in mammalian cells and regulate poly(A) tail by interacting with PABP. One of the candidates for this role seems to be PARN, as it has been shown that PARN directly binds to PABP (Siddiqui et al. 2007) and its deadenylase activity can either be stimulated or inhibited by PABP under different cellular condition, such as salt

concentration (Korner and Wahle 1997), suggesting that a change in the cellular conditions might affect PARN deadenylase activity. Although the nuclear function of mammalian PABP is not clear, it has been proposed that the nuclear protein is involved in mRNA polyadenylation but not 3' cleavage, stability and quality control (Mangus et al. 2004). Interestingly, the results presented in this dissertation show that nuclear PARN activity is regulated by its interaction with the BARD1-associated 3' processing factor CstF-50 and with the cap binding protein CBP80. The results show that the previously identified nuclear CBP80/PARN deadenylation inhibitory complex decreased significantly in abundance upon DNA damaging conditions, whereas the complex containing CstF-50/PARN increased, and that CstF-50 can compete with CBP80 on binding to the same region of PARN. In fact, addition of CstF-50 and BARD1 to *in vitro* deadenylation reactions reverted the CBP80 inhibition effect on PARN activity. Since it has been shown that PARN can be inhibited or activated by PABP under different cellular conditions, it is possible that PABP also participate in the regulation of PARN activity upon DNA damage conditions. Therefore, it is critical to analyze the functional interaction of PABP/PARN inside the nucleus under different cellular conditions

To test if the interaction of nuclear PARN with PABP has any effect on the UV-induced regulation of polyadenylation/deadenylation, it will first be determined whether PARN, CstF-50 and BARD1 can also interact with PABP after UV treatment. Briefly, NE of non-treated and UV-treated cells will be analyzed by coimmunoprecipitation assays using PARN, CstF-50 or PABP antibodies as described above. Then the PABP binding domain of PARN will be determined using truncated variants of PARN and performing GST pull-down assays as described before. If PABP binds to a similar

domain of PARN as CBP80 and CstF-50, competition assays will be performed. Those studies will be extended by analyzing how the PARN/PABP interaction is affected by the other interacting proteins. Then, the effect of the PARN/PABP complex on 3' cleavage and deadenylation will be determined as described before. Briefly, NE of cells treated with PABP siRNA and UV light will be prepared and analyzed for RNA 3' cleavage and deadenylation. These experiments will reveal any possible functional connection between PABP and the nuclear DNA damage response.

3) Microarray analysis of PARN/CstF/BARD1-regulated genes:

With accumulating evidence revealing the importance of regulation of gene expression in cell function and in disease, such as in tumorigenesis, Alzheimer and kidney diseases, it is important to study the role of 3' end processing in the gene regulation under different cellular conditions. Indeed, the malfunction in gene regulation has been the target for pharmacological and clinical investigations for a long time (Sager 1997). The results presented in this dissertation indicate that the PARN/CstF/BARD1 complex can decrease the stability of housekeeping genes upon DNA damaging conditions and of ARE-containing genes, such as c-fos and c-myc, upon non-stress conditions. It is important to extend these studies to other genes that might be regulated by the PARN/CstF/BARD1 complex under different cellular conditions as DNA damage. Furthermore, it would be important to determine if the regulation of the expression of some of those genes are disease-associated.

DNA microarray is a technique extensively used in molecular biology and in medicine that allows analyzing the expression of up to 40,000 genes (Kulesh et al. 1987;

Schena et al. 1995, Lashkari et al. 1997). It consists of an arrayed series of thousands of microscopic spots of DNA oligonucleotides, called features, each containing picomoles of a specific DNA sequence. This can be a short section of a gene or other DNA element that are used as probes to hybridize a cDNA or cRNA sample (called target) under high-stringency conditions. Probe-target hybridization is usually detected and quantified by detection of fluorophore-, silver-, or chemiluminescence-labeled targets to determine relative abundance of nucleic acid sequences in the target.

To analyze the effect of the PARN/CstF/BARD1 complex on the expression levels of a wide range of genes, total nuclear RNA will be purified from cells treated with siRNAs targeting PARN or CstF-50 and UV irradiation. Gene expression will be analyzed by DNA microarray using cDNA. Random or oligo(dT) primers will be used for the preparation of the cDNA sample. Given the fact that we can analyze a large number of genes at the same time, this technique will be useful in detecting what genes are up-regulated or down-regulated and how this regulation will change by PARN, CstF-50 or BARD1 expression. The list of PARN/CstF50/BARD1 target genes will be analyzed and compared to the list of disease-associated genes, whose expression changes in different conditions. New treatment strategies for different diseases will be suggested by either trying to control PARN expression and activity via the inhibitor PARN/CBP80 complex formation or the activator PARN/CstF-50/BARD1 complex formation.

4) Functional analysis of the polyadenylation/deadenylation complex in tumor cells.

Many mRNAs encoding oncoproteins, cytokines and inflammatory genes are regulated by AU-rich elements (ARE) that are A and U rich sequences grouped in various orders (Chen and Shyu 1995, Wilusz et al. 2001) and located within the 3' untranslated region of transcripts (Chen and Shyu 1995; Guhaniyogi and Brever 2001). The AREs mediate the rapid turnover of mRNAs encoding proteins that regulate cellular growth in response to exogenous agents, such as microbes, inflammatory and environmental stimuli, suggesting that any change in the regulation of ARE-containing genes expression could dramatically influence oncogenic phenotypes. In fact, ARE-containing genes, such as c-jun, c-myc and c-fos, increase significantly in proliferating and cancer cells (Zajchowski et al. 2001, Andersen et al. 2002, Milde-Langosch 2005). It is possible that the increase in gene expression in those cancer cells might be not only due to alterations in transcriptional but also due to posttranscriptional quality control. One mechanism to regulate the turnover of those ARE-containing mRNAs is via tristetraprolin (TTP) (Blackshear 2002; Brever et al. 2004). TTP is a mRNA binding protein that recognizes the UAUU-containing sequences and mediate mRNA degradation. Interestingly, lung, breast, ovary, uterus and many other cancer patients show down regulation of TTP and thus overexpression of ARE (Brennan et al. 2009). Moreover, restoring TTP levels in aggressive tumor cell line suppress three main tumorgenic phenotypes including cell proliferation, resistance to apoptosis and expression of vascular endothelial growth factor (VEGF) mRNA, which is a proangiogenic protein (Brennan et al. 2009). TTP is shown to target ARE mRNA degradation via recruiting PARN to those mRNAs and thus initiating deadenylation that precedes mRNA degradation (Lai et al. 2003). Intriguingly,

the results presented in this dissertation describe another pathway to regulate mRNA levels via PARN/CstF-50/BARD1 complex formation. In fact, it is revealed here that PARN can decrease the stability of ARE-containing genes upon non-stress conditions, specifically for c-fos and c-myc (Figure 29). Consistent with this, it has been shown that ARE-dependent deadenylation plays an important role in the mRNA decay of several oncogenes involved in regulation of cell growth and differentiation (Blattner et al. 2000, Lai et al. 2003, Moraes et al. 2006). The results presented here indicate that oncogenes, like c-fos and c-myc, might be regulated in the nucleus by the functional interaction between CstF/BARD1, CBP80 and PARN under different cellular conditions. As the tumor suppressor BARD1 is involved in this response, it is possible that malignant cells display altered levels of polyadenylation of certain mRNAs. Supporting this idea, enhanced polyadenylation has been detected in certain tumor cells (Kumar et al. 1995, Scorilas et al. 2002), polyadenylation is inactivated in M phase (Colgan et al. 1996, 1998), expression levels of poly(A) polymerase can interfere with cell growth (Zhao and Manley 1998). It is possible that a functional failure in the PARN/CstF/BARD1 complex might increase the mRNA stability of these oncogenes, explaining the increase in gene expression in cancer cells. As the PARN/CstF/BARD1 complex is found in all tissues of mammalian systems and its function is regulated under DNA damage conditions, it is critical to analyze this new mechanism of control of mRNA stability by polyadenylation/deadenylation factors in cancer cells.

Using several approaches, the expression levels of PARN, CstF and BARD1 will be evaluated in a variety of human neoplastic syndromes. It will be determined if the expression of any of these factors change significantly in different tumor types. This is

particularly innovative for the 3' processing factors PARN and CstF. The effect of PARN and CstF-50 knockdown on three key tumorgenic phenotypes will be evaluated: cell proliferation will be analyzed quantitatively by determining the cell numbers; resistance to proapoptotic stimuli will be analyzed by treating the cells with apoptotic stimulus, such as cisplatin or staurosporine, and visually analyzing the cells; and expression of VEGF will be determined by analyzing the levels of VEGF by real time RT-PCR (Brennan et al. 2009). The analysis of gene array proposed before will provide information of possible target genes that might be regulated by the PARN/CstF-50/BARD1 complex. More importantly, various cancer cells overexpressing those target-genes will be analyzed for any mutations or deregulation that may occur in the PARN/CstF-50/BARD1 complex formation and its function in ploydenylation-deadenylation. Once the deficiency in those cancer cells is determined, the expression levels of the wild-type PARN, CstF-50 or BARD1 will be restored by stably transfecting those cells with constructs expressing FLAG-tag of the desired proteins from a tetracycline-responsive cassette. Then, as proposed before, the three key tumorgenic phenotypes will be evaluated: cell proliferation, resistance to proapoptotic stimuli, and expression of VEGF mRNA. Taken together, these studies would contribute to determine the role of the functional interaction of polyadenylation/deadenylation factors in proliferating and cancer cells, and possibly help in the development of alternative treatment strategies.

CHAPTER VI

EXPERIMENTAL PROCEDURES

Tissue culture methods and DNA damaging agents - HeLa cells were cultured in Dulbecco's modified Eagles medium (DMEM)-10% fetal bovine serum (FBS). 90% confluent cultures were exposed to UV and harvested at the indicated times. UV doses (20 or 40 Jm^{-2}) were delivered in two pulses using a stratlinker (Stratgene). Prior to pulsing, medium was removed and replaced immediately after treatment. U2OS cells transfected with different BARD1 mutant constructs were cultured in DMEM-10% FBS supplemented with 0.3 μg/μl glutamine and 110 ng/μl hygromycin B.

NE preparation and immunoblotting analysis – After UV treatment, NEs were prepared from harvested cells essentially as described (Kleiman and Manley 2001). Cells were lysed by douncing in 4 ml of 10 mM Tris pH 7.9, 1.5 mM $MgCl_2$, 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 $MgCl_2$, 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 quick frozen and stored at −80°C. Sixty μg of each NE was analyzed by immunoblotting with the indicated antibodies.

Knockdown expression of PARN and BARD1/BRCA1 in HeLa cells by siRNA - Both the siRNA specific for human PARN, BARD1/BRCA1 and the control siRNA used as non-silencing were obtained from Dharmacon. HeLa cells were grown in a 10-cm plate in complete DMEM. At 50-60% confluence, the cells were transfected with 20 nM of the PARN and 50 nM of the control siRNA and 1 ml of Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. siRNA knockdown of BRCA1/BARD1 was performed as described (Kleiman et al. 2005). After culturing the

cells for additional 24 hrs, some plates were harvested and others were transfected again and harvested for analysis 48 hrs after the initial transfection. A fraction of the cells were exposed to UV and harvested after the indicated times. NEs were prepared as described above and analyzed by Western blot and used in 3' cleavage reactions. To ascertain the specificity of siRNAs used the protein levels were monitored.

Immunoprecipitation analysis – One hundred μg of total protein from each NE from different cell lines were pre-cleared with 50 μl of protein A-Sepharose, immunoprecipitated with the anti-CstF-64 mAb (generously provided by Dr. Manley, Columbia University), anti-PARN pAb (generously provided by Dr. Wormington, University of Virginia; Korner et al. 1998), anti-α-H2A pAb (Millipore) or preimmune sera bound to protein A-agarose beads. The antibodies were coupled to the protein A-agarose beads for 3 hrs at RT in buffer IPP (50 mM Tris pH 7.4, 50 mM NaCl and 0.1% Nonidet P-40). Immunoprecipitations were carried out for 3 hrs at 4°C in 200 μl of buffer A (1× phosphate-buffered saline (PBS): 137 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.01% Nonidet P-40, 0.5 mM PMSF, and 0.04% bovine serum albumin). The beads were recovered by centrifugation and treated at 4 °C with 50 μg of RNase A/ml for 10 min. Finally, washing was performed with buffer A plus increasing amounts of NaCl. Aliquots of pellets and supernatants were analyzed by SDS-PAGE and immunoblotting. Results from three independent samples were analyzed and quantified using Image J program.

Purification of recombinant proteins – cDNA's encoding the full-length CstF-50 and BARD1 were inserted into pGEX-2TK and expressed in E. coli., and GST fusion proteins were purified by binding to and elution from glutathione-agarose beads as described

(Kleiman and Manley 1999). The plasmid encoding His-PARN and its derivatives were transformed into BL21 cells, His fusion proteins were expressed and purified by binding to and elution from Ni-Agarose column (Qiagen) as described (Nilsson and Virtanen 2006). Protein samples were treated at 4 °C with 50 μg of RNase A/ml for 10 min during the binding assays.

Protein-protein interaction assays- One μg of GST-CstF50 was incubated with glutathione-agarose beads for 2 hrs at 4°C in 300 μl final volume of binding buffer (1xPBS, 0.04% bovine serum albumin, 0.5 mM PMSF, 0.001% NP40). Beads were washed extensively six times with binding buffer. One μg of His-PARN was added to the GST-CstF50-bound beads and incubated for 2 hrs at 4°C in 300 μl final volume of binding buffer. The beads were washed six times with binding buffer plus 300 mM NaCl, resuspended in loading buffer, and proteins were fractionated by 9% SDS PAGE. Equivalent amounts of pellets and supernatants were analyzed by immunoblotting. The competition assays using His-PARN were done similarly as above. The binding buffer used in these experiments was (20 mM HEPES pH 7.9, 0.5 M KCl, 0.5% NP-40, 10% glycerol, 2 mM-mercaptoethanol and 2.5 mM imidazole) and the protein concentrations are indicated in the figures.

3′ cleavage assays - ^{32}P-labeled L3 pre-mRNA substrates were prepared as described (Kleiman and Manley 1999). Protein concentrations of the extracts were equalized by Bradford assays (BioRad) and/or by Coomassie blue staining before use in processing reactions. Cleavage assays with equivalent amounts of total protein were carried out in reaction mixtures containing 0.2–0.5 ng labeled RNA, 250 ng tRNA, 0.25 U RNasin (Promega), 8 mM Tris pH 7.9, 10% glycerol, 20 mM creatine phosphate, 1 mM $MgCl_2$, 1

mM 3′ dATP, 120 mM NaCl, 0.2 mM DTT, 2.5% polyvinyl alcohol, and 0.2 mM PMSF. NE, added proteins and pre-mRNA was added and incubated for 90 min at 30°C. RNA products were isolated and fractionated on 5% polyacrylamide, 8.3 M urea gels. Results from independent samples were analyzed by autoradiography and quantified using image J program.

Deadenylation assays –Conditions for *in vitro* deadenylation assays were as described (Martinez et al. 2001). Briefly, the deadenylation assays with His-PARN, derivatives of PARN and different concentrations of GST-CstF-50, GST-BARD1 and GST-CBP80 were carried out in reaction mixtures containing 25 mM Hepes pH 7, 100 mM NaCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM DTT, 2.5% polyvinyl alcohol, 10% glycerol, 0.25 U RNasin, and 10 nM ^{7}MeGpppG capped *in vitro* transcribed $L_3(A_{30})$ (adenylated) and $L_3(A_0)$ (control) RNA substrate, radioactively labeled by the inclusion of ^{32}P-α-ATP during *in vitro* transcription. Incubations were performed at 30°C for different times; 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.

Analysis of endogenous mRNAs by RT-PCR, PAT assay and qRT-PCR –Total nuclear RNA was purified from HeLa cells using the RNeasy (Qiagen). Equivalent amounts of purified RNA were used as a template to synthesize cDNA using random hexamer primers or oligo d(T) primers and MMLV reverse transcriptase (Promega) according to the manufacturer's protocol. PCR was performed using the RT products and Taq Polymerase (Promega) and the following primers: forward primer GAPDH (5'-CAC ATG GCC TCC AAG GAG TAAG-3'), reverse primer GAPDH (5'-TAC ATG ACA AGG TGC GGC TCCC-3'), forward primer β-actin (5'-GGT GAT AGC ATT GCT TTC

GTGT-3‘), reverse primer β-actin (5’-AAG TCA GTG TAC AGG TAA GCCC-3’) and a modified oligo (dT) primer/adapter PAT test (Sallés et al. 1992, Kleiman et al. 1998). Equal volumes of the PCR products were run on a 5% acrylamide gel and visualized by ethidium bromide staining. Commercially available primers were used in the qRT-PCR reactions (Applied Biosystems). Equal amounts of total or poly(A)$^+$ cDNAs were used in the qRT-PCR reactions to observe the poly(A) enrichment as described (Gomes et al. 2006).

Chromatin immunoprecipitation-type assays - We performed ChIP assays using a modification of previously published methods (Takahashi et al. 2000). Ninety percent con- fluent cultures of HeLa cells were exposed to UV (two pulses of 50 J/m2) using a Stratalinker (Stratagene), incubated with BrdU (10 mM) and FrdU (fluorodeoxyuridine, 1 mM) to label the repaired DNA, and then the cells were cross linked with formaldehyde at the stated times. After formaldehyde treatment, NEs were prepared from HeLa cells as described (Kleiman and Manley 2001) and samples were sonicated to produce soluble chromatin in the presence of proteinase inhibitors (Sigma, P2714). To obtain DNA fragmentation of average length of 2000 base pairs, sonications were done two times for 20 s each. Samples were then pre-cleared by treatment with protein-G Sepharose 4B beads (Sigma). DNA-protein complexes were immunoprecipitated by incubation with BrdU monoclonal antibody (Covance) coupled to blocked protein-G Sepharose 4B beads. Immunoprecipitations were carried out for 3 h at 48C in 150 ml of sonication buffer (10mM Tris pH 8.0, 1mM EDTA pH 8.0, 0.5mM EGTA pH 8.0, 0.5mM PMSF, 1 X protease inhibitor cocktail). Washing was with sonication buffer. Crosslinks were

reversed by boiling the samples for 30 min. The protein complex bound to repair DNA was analyzed by western blot.

CHAPTER VII

FIGURES AND FIGURE LEGENDS

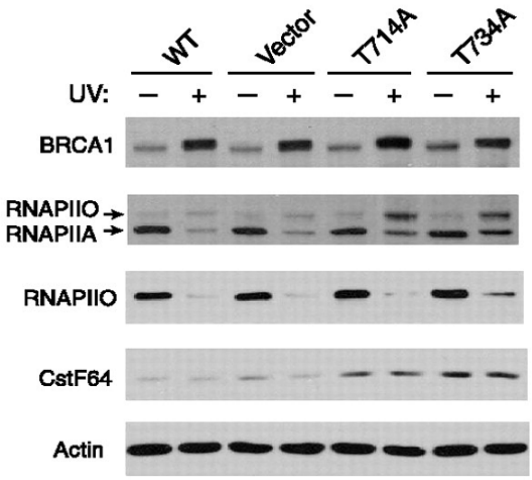

Figure 10. BARD1 T714A and T734A mutants are defective in RNAP II degradation U2OS cells stably transformed with various BARD1 mutants were untreated or treated with UV (20 J/m^2). After 2 hours, cell extracts were immunoblotted with indicated antibodies: BRCA1 (C-20, Santa Cruz Biotechnology, Santa Cruz, CA), RNAP II (8WG16, Covance, Berkeley, CA), and RNAP IIO (H5, Covance, Berkeley, CA). Anti-actin (A2066, Sigma) blot shows relatively equal loading in all samples.

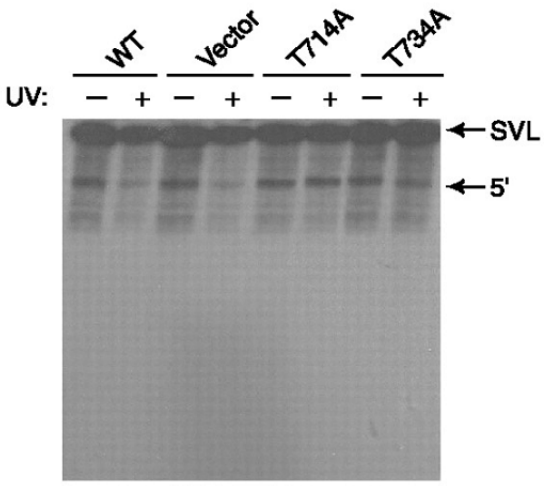

Figure 11. BARD1 T714A and T734A mutants are defective in RNAP mRNA cleavage inhibitory activity in response to DNA damage. Nuclear extracts were prepared from cells stably transformed with various BARD1 mutants, either untreated or treated with UV ($20/m^2$). *In vitro* RNA cleavage assay was done as previously described (Kleiman and Manley 2001) using SV40 late precursor RNA (SVL). 5' cleaved product and SVL precursor RNA are denoted.

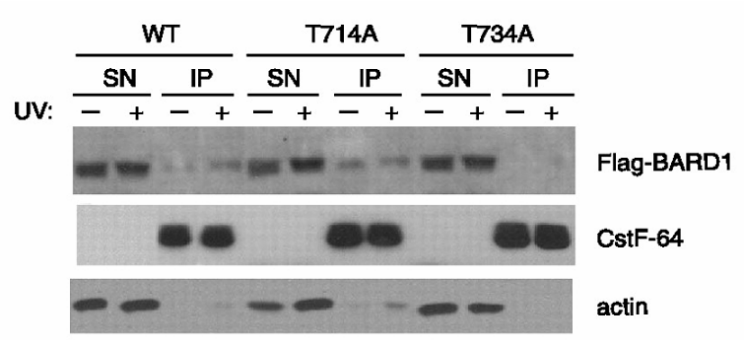

Figure 12. BARD1 T714A and T734A mutants are defective in the UV-induced CstF/BARD1 complex formation. Nuclear extracts were prepared from cells stably transformed with various BARD1 mutants, either untreated or treated with UV ($20/m^2$). The NEs were immunoprecipitated with anti-CstF64 antibody. Supernatants and the immunoprecipitated pellets were resolved by SDS-PAGE and immunoblotted with anti-FLAG M2, anti-CstF64, or anti-actin antibodies.

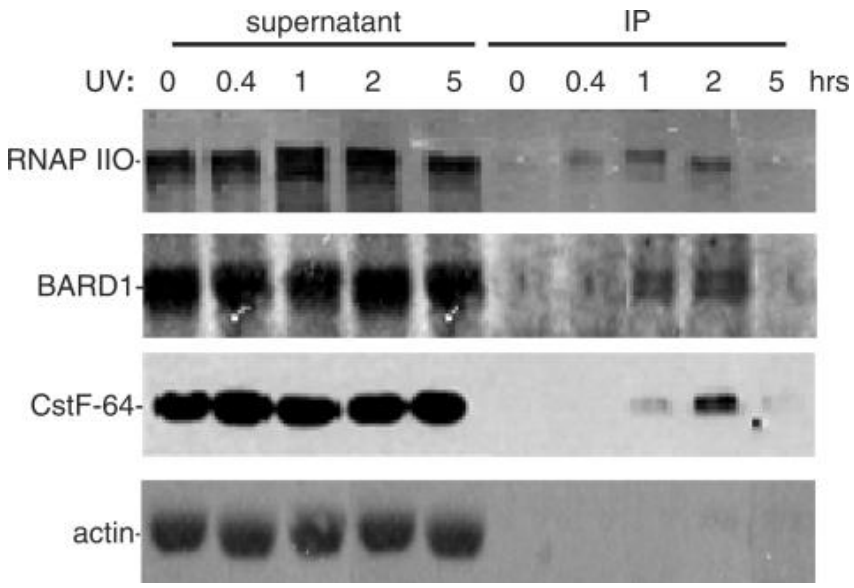

Figure 13: RNAP II, CstF and BARD1 localize to sites of repaired DNA. Analysis of protein complexes associated with BrdU-labeled DNA after UV treatment and the indicated recovery times. BrdU was added to HeLa cells immediately after exposure to UV light. Cells were cross-linked with formaldehyde after UV exposure at the times indicated. Sonicated cell extracts were IPed with anti-BrdU antibody. Equivalent amounts of the pellets (IP) and normalized amounts of the supernatants, which represents 7% of the input, were analyzed by immunoblotting with anti-RNAP II (H5), anti-CstF-64, anti-BARD1 and anti-actin antibodies.

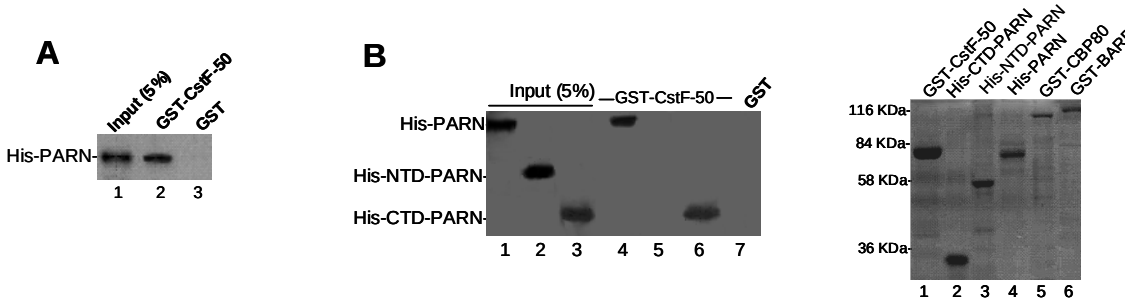

Figure 14: CstF-50 interacts directly with C-terminal domain of PARN A) Interaction of GST-CstF-50 and His-PARN. Immobilized GST-CstF-50 or GST on glutathione beads were incubated with 1 μg of His-PARN. Bound proteins were eluted, resolved by SDS-PAGE and detected with anti-PARN antibodies. 5% of PARN used in the reaction is shown as input. **B)** Requirement of PARN C-terminal domain for CstF-50 interaction. GST and the indicated His-PARN derivatives were used in "pull-down" assays with GST-CstF-50. The samples were analyzed as in (A). Coomassie blue staining of the purified recombinant proteins following SDS-PAGE is shown. Positions of size markers are indicated.

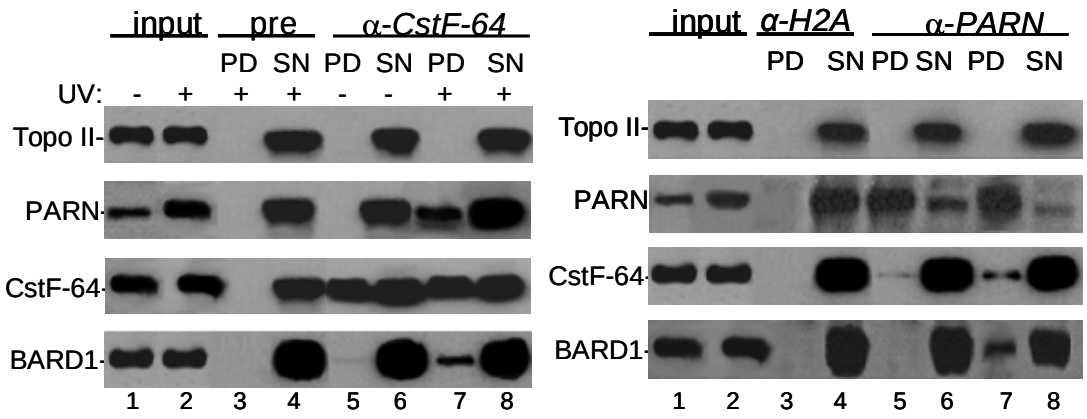

Figure 15: CstF-50 interacts with PARN and BARD1 upon DNA damage. CstF, BARD1 and PARN coimmunoprecipitate from NE of HeLa cells treated with UV irradiation. NEs were immunoprecipitated with anti-CstF-64, anti-PARN, anti-H2A or preimmune antibodies. Equivalent amounts of the pellets (PD) and supernatants (SN) were resolved by SDS-PAGE and proteins were detected by immunoblotting with antibodies against PARN and CstF-64. Antibodies against Topo II were used as a control of specificity. Positions of Topo II, CstF-64 and PARN are indicated. 20% of the NE used in the immunoprecipitation reaction is shown as input. The relative density of each band was determined by Image J program. Data shown are the mean ± SEM from three independent experiments.

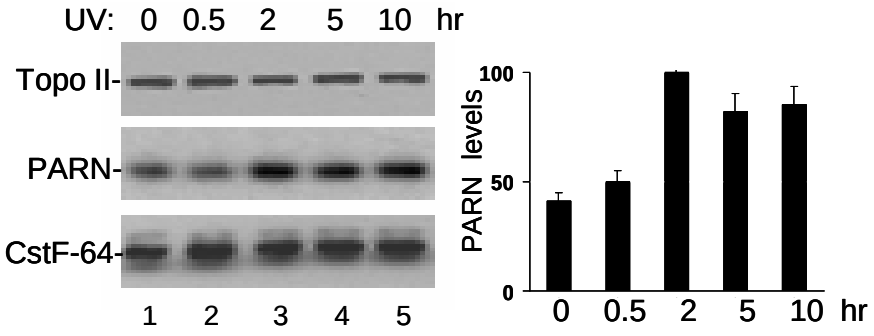

Figure 16: DNA damage induces PARN expression levels. CstF-64, Topo II and PARN protein levels in NEs from UV-treated HeLa cells were monitored by Western blotting. The relative density of each band was determined by Image J program. Data shown are the mean ± SEM from three independent experiments.

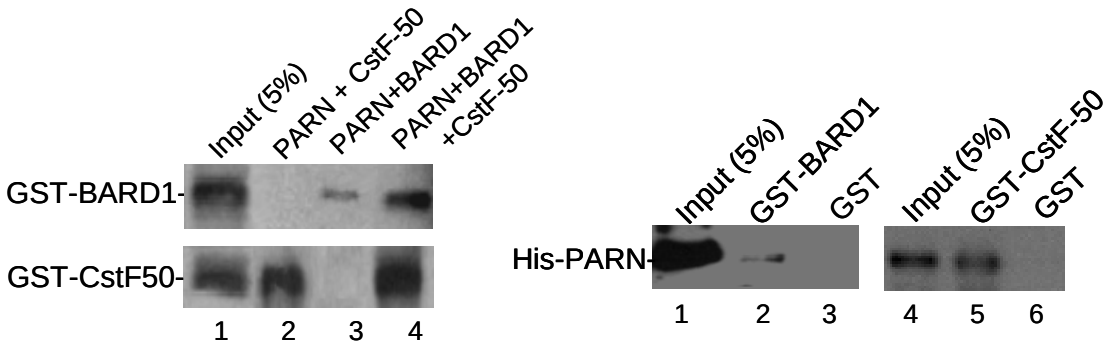

Figure 17: CstF-50 is reqired for the PARN/CstF-50/BARD1 complex formation. BARD1 does not interact strongly with PARN. In the top panel, immobilized His-PARN on nickel beads was incubated with 1 μg of GST-CstF-50, GST-BARD1 or GST. In the bottom panels, immobilized GST-CstF-50 or GST-BARD1 on glutathione beads were incubated with 1 μg of His-PARN. Bound proteins were eluted, resolved by SDS-PAGE detected with anti-PARN, anti-BARD1 and anti-GST antibodies. 5% of the proteins used in the reaction are shown as input.

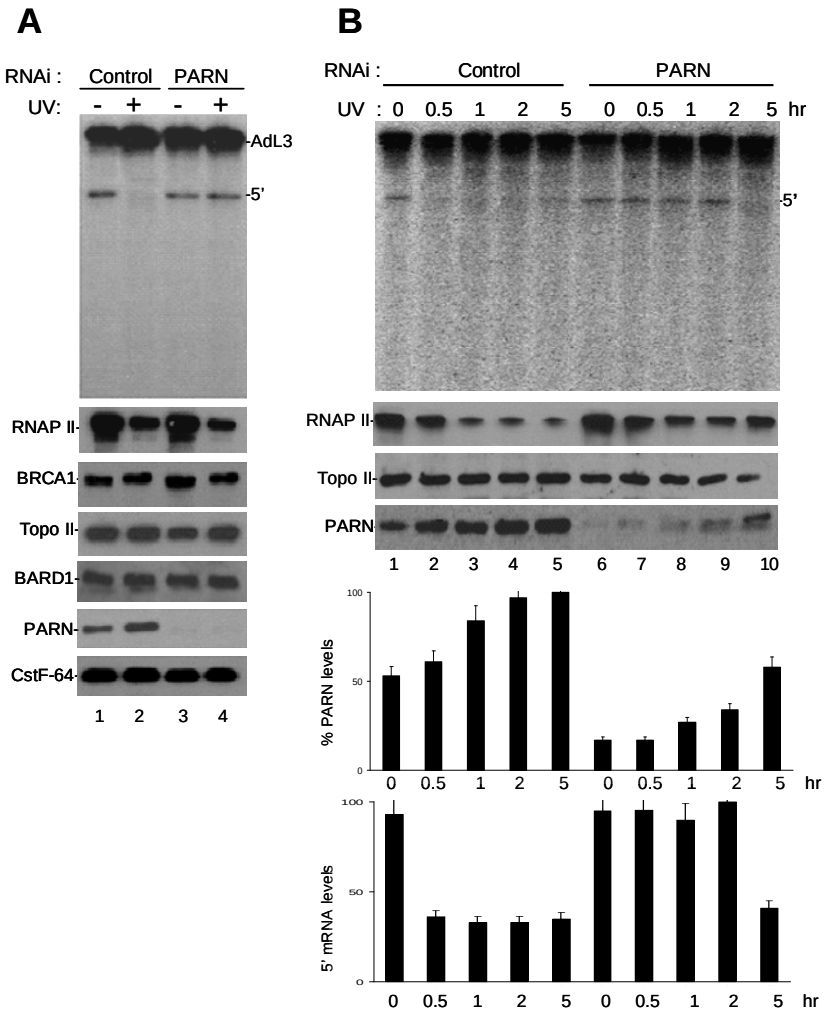

Figure 18: PARN is necessary for the UV-induced inhibition of 3' cleavage. A) NEs from cells treated with PARN/control siRNA and UV irradiation, and allowed to recover for 2 hours were analyzed for pre-mRNA 3' cleavage. NEs were incubated in a reaction mix containing L3 pre-mRNA. Positions of pre-mRNA and the 5' cleavage product are indicated. Protein levels of RNAP II, BRCA1, Topo II, BARD1, PARN and CstF-64 were analyzed by Western blot. **B)** NEs from cells treated with PARN/control siRNA and different UV irradiation recovery times were analyzed as described in (A). The levels of PARN expression and of 5' cleavage product were quantified. The relative density of each band was determined by Image J program. Data shown are the mean ± SEM from three independent experiments.

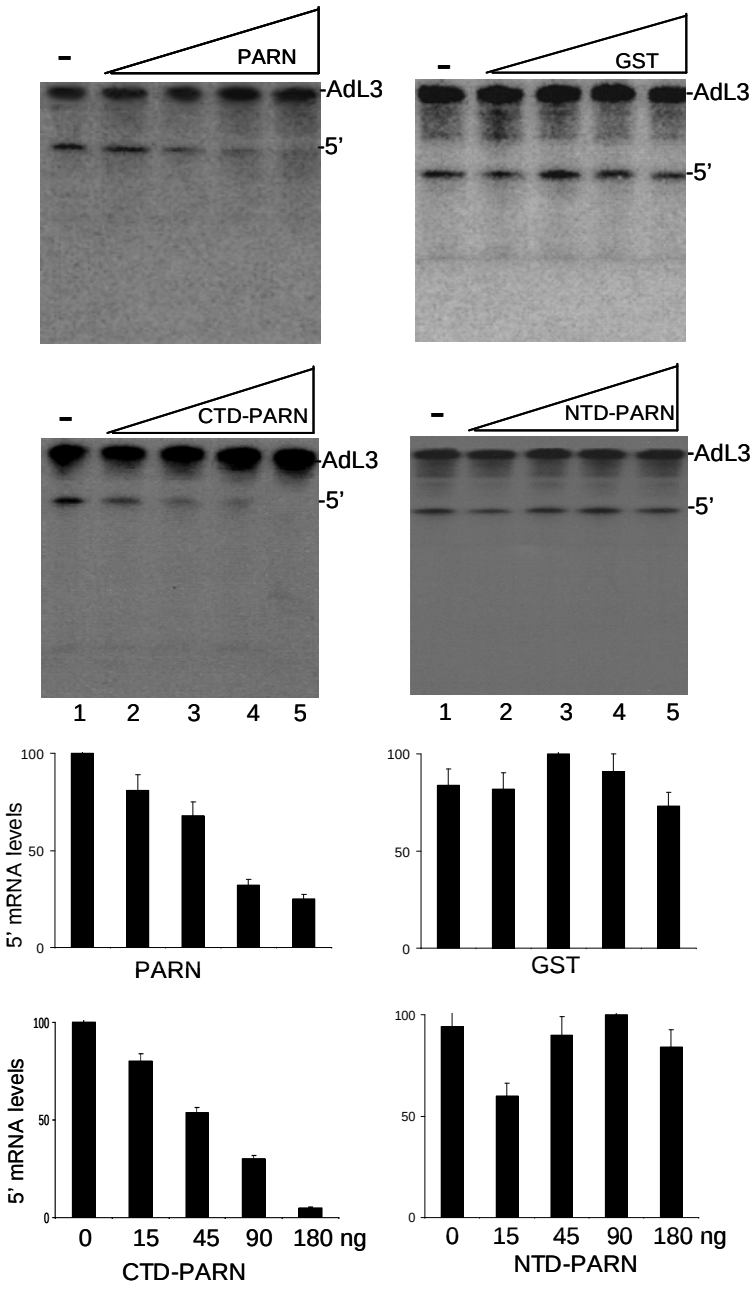

Figure 19: PARN is necessary for the UV-induced inhibition of 3' cleavage. NEs from cells treated with PARN siRNA and UV irradiation, and allowed to recover for 2 hours were incubated in a reaction mixture containing L3 pre-mRNA and increasing amounts of His-PARN, His-CTD-PARN, His-NTD-PARN and GST proteins (15, 45, 90 and 150 ng). The levels of 5' cleavage product were quantified as in Figure 9B.

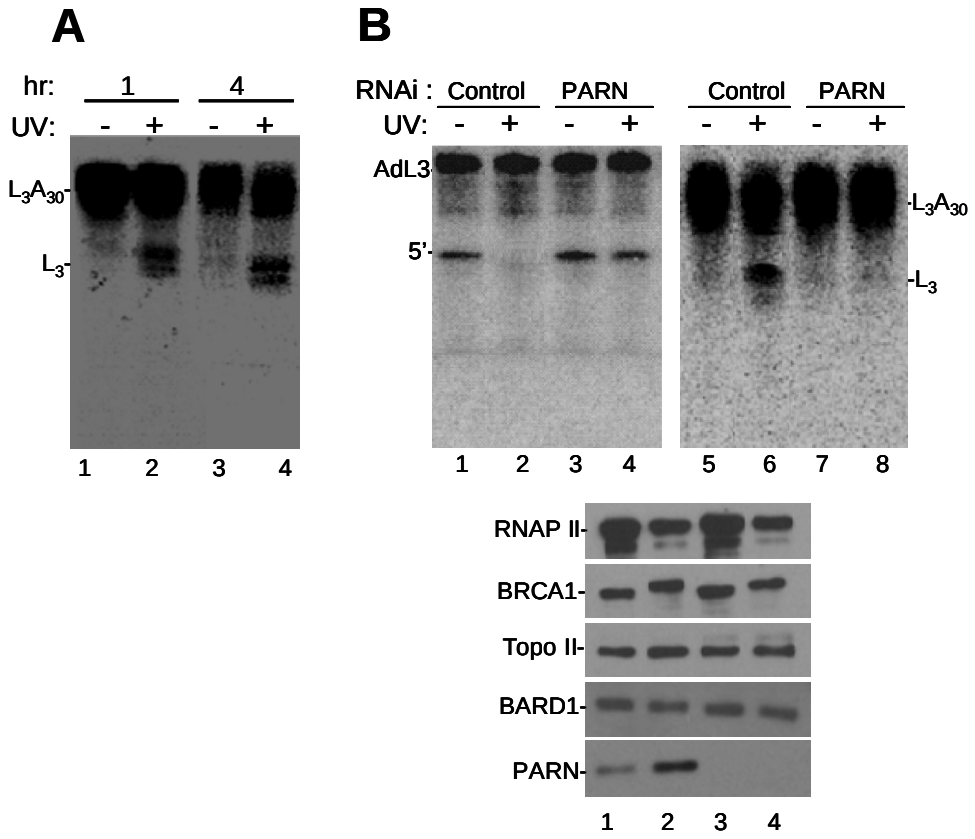

Figure 20: DNA damage induces nuclear PARN deadenylase activity. A) Effect of UV irradiation on $L_3(A_{30})$ deadenylation. NEs from cells treated with UV (20 Jm^{-2}) irradiation and allowed to recover for 2 hrs were analyzed for deadenylation. The incubation times for the deadenylation assay are indicated in the figure. **B)** The UV-induced activation of $L_3(A_{30})$ deadenylation is PARN dependent. NEs from cells treated with UV irradiation and treated with control/PARN siRNA were allowed to recover for 2 hours and were analyzed for deadenylation as well as 3' cleavage reactions as described in (A) and Figure 9A, respectively. The deadenylation assays were incubated for 1 hour. Protein levels of RNAP II, BRCA1, Topo II, BARD1 and PARN were analyzed by Western blot.

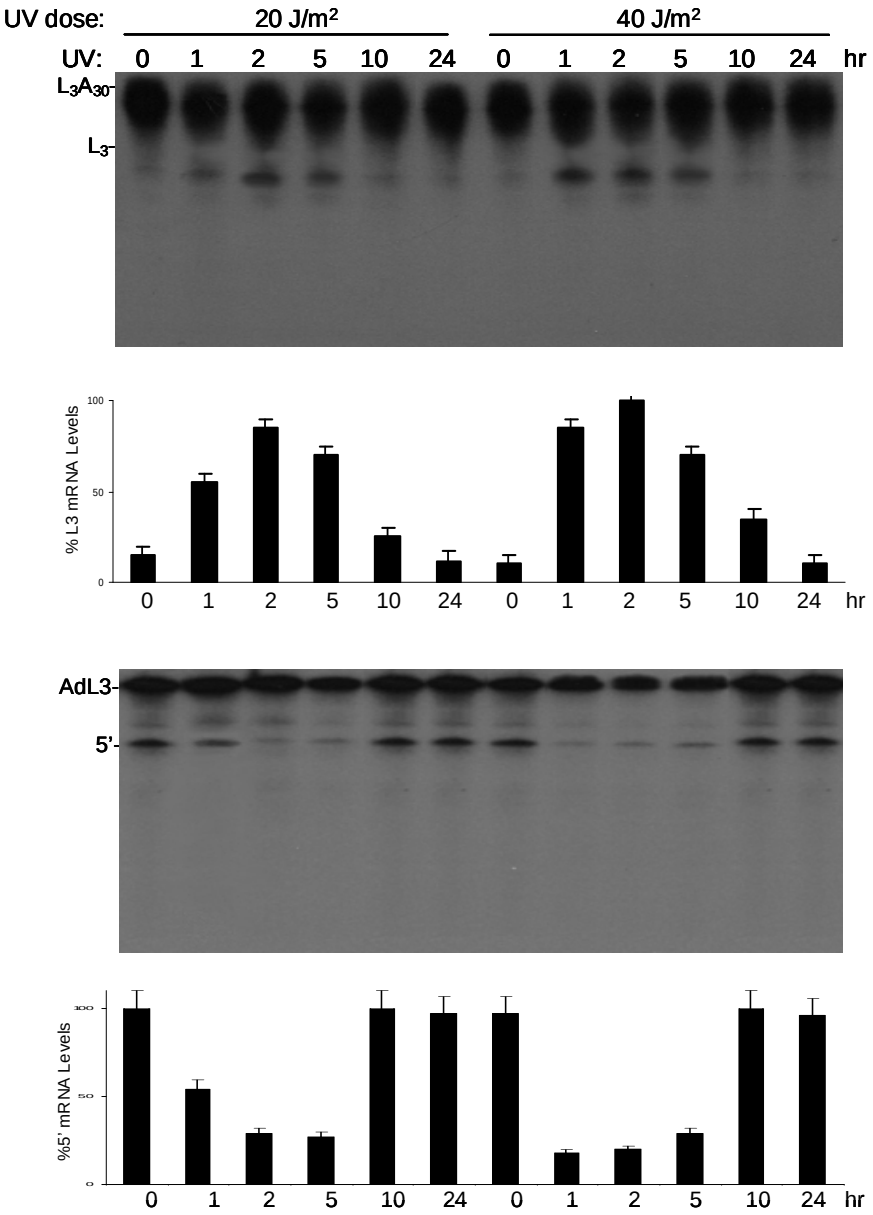

Figure 21: DNA damage induces nuclear PARN deadenylase activity. Effect of different UV doses on the activation of deadenylation and inhibition of 3' end cleavage. NEs from cells treated with UV irradiation (20 and 40 Jm^{-2}) and allowed to recover for the times indicated in the figure were analyzed for deadenylation and 3' end cleavage as described in 11B. The levels of 5' cleavage product and L_3 deadenylated product were quantified as in Figure 9B.

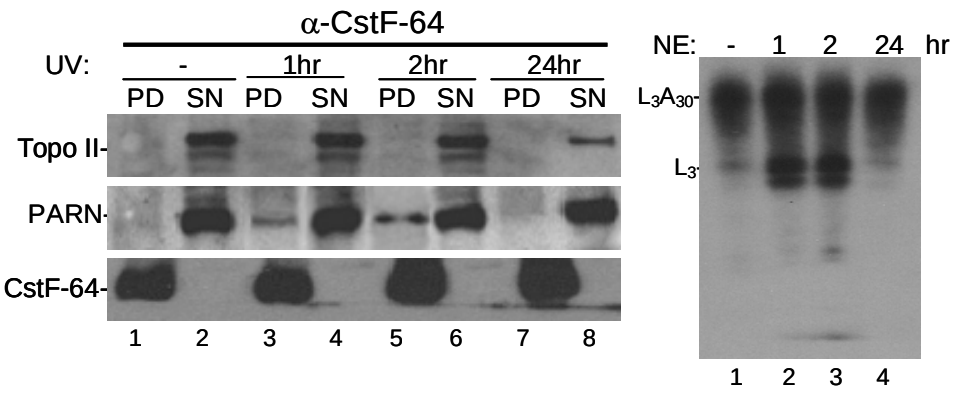

Figure 22: DNA damage induces nuclear PARN deadenylase activity. Increase of CstF and PARN coimmunoprecipitation from NE of HeLa cells treated with UV irradiation and allowed to recover for 1, 2 and 24 hours. NEs were immunoprecipitated with anti-CstF-64 antibodies. Samples were analyzed as in Figure 6. The NEs used in the immunoprecipitation assays were also tested in deadenylation assays as in Figure 11B.

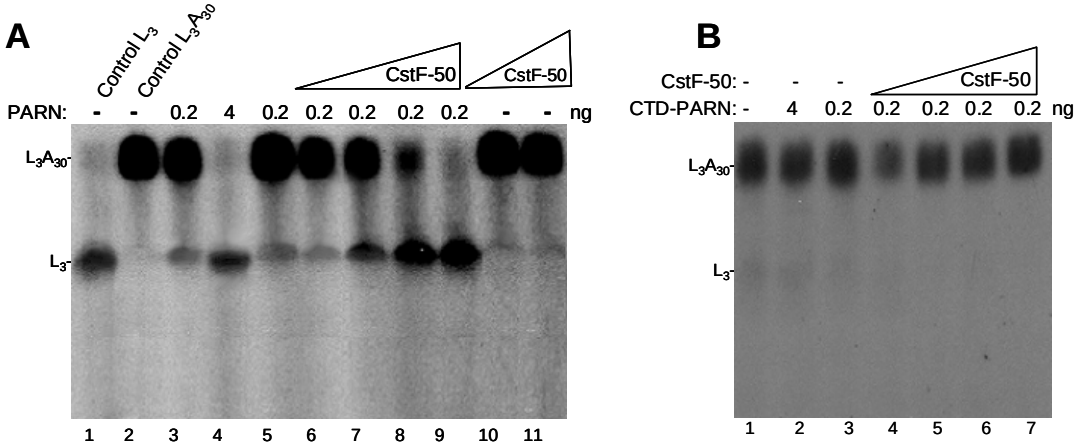

Figure 23: CstF50 can activate PARN deadenylase activity in the absence of any other factors. A) CstF-50 activates PARN deadenylase activity *in vitro*. The deadenylation assays with different concentrations of His-PARN were carried out in the presence of capped L_3A_{30} RNA substrate radioactively labeled. Increasing amounts of recombinant GST-CstF-50 (5, 10, 25, and 50 ng) were added to the reaction. The reactions were analyzed by electrophoresis in 10% polyacrylamide/7M urea gels. Positions of polyadenylated mRNA $L_3(A_{30})$ and the L_3 deadenylated product are indicated. **B)** CstF-50 was not able to activate the C-terminal derivative of PARN (His-CTD-PARN), which is inactive in deadenylation. Deadenylation reactions were analyzed as in (A). Deadenylated L_3 substrate is shown in lane 1 as control.

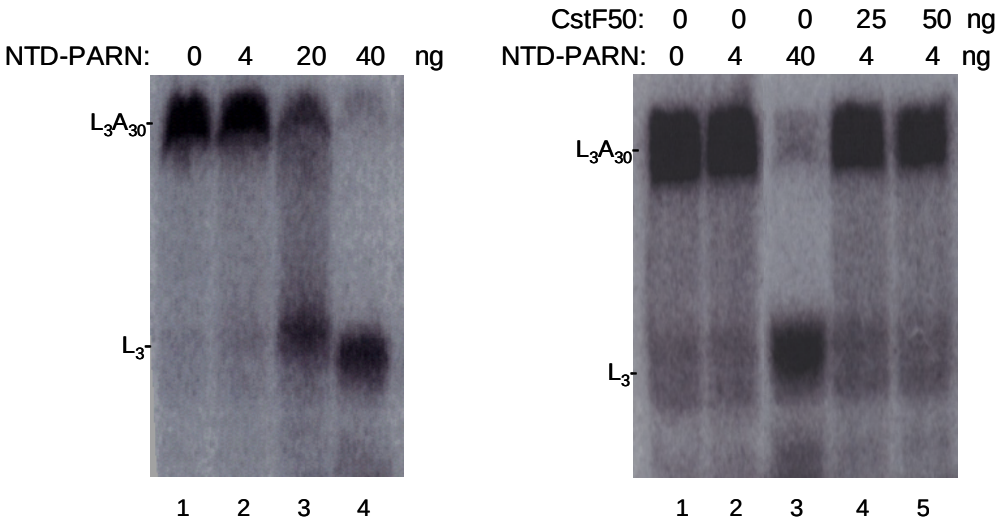

Figure 24: CstF-50 can activate PARN deadenylase activity in the absence of any other factors. The C-terminal domain of PARN is required for CstF-50 activation. Catalytically active His-NTD-PARN was analyzed for deadenylase activity as in Figure 14A.

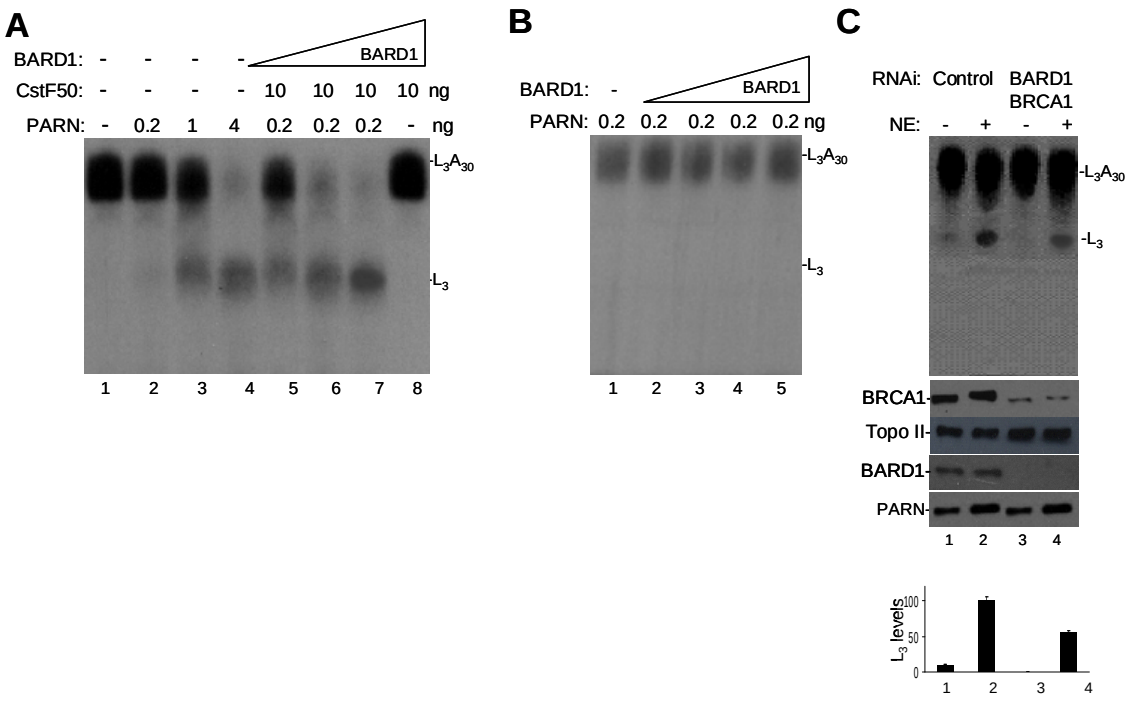

Figure 25. BARD1 increases CstF-50 and DNA induced activation of PARN deadenylase. A) CstF-50/BARD1 strongly activates PARN deadenylase activity *in vitro*. The deadenylation assays with different concentrations of His-PARN were carried out in the presence of capped $L_3(A_{30})$ RNA substrate radioactively labeled. Recombinant GST-CstF-50 and increasing amounts of GST-BARD1 (5, 25, 50 and 100 ng) were added to the reaction containing limiting amounts of PARN. The reactions were analyzed by electrophoresis in 10% polyacrylamide/7M urea gels. Positions of polyadenylated mRNA $L_3(A_{30})$ and the L_3 deadenylated product are indicated. **B)** Limiting amount of PARN and increasing amounts of BARD1 was not able to activate the full-length PARN deadenylase activity in the absence of CstF-50. **C)** siRNA knockdown of both BARD1/BRCA1 expression decreases the UV-induced activation of deadenylation. NEs from cells treated with control/BARD1-BRCA1 siRNA and allowed to recover for 2 hours after UV irradiation were analyzed for deadenylation. Protein levels of BRCA1, Topo II, BARD1 and PARN were analyzed by Western blot.

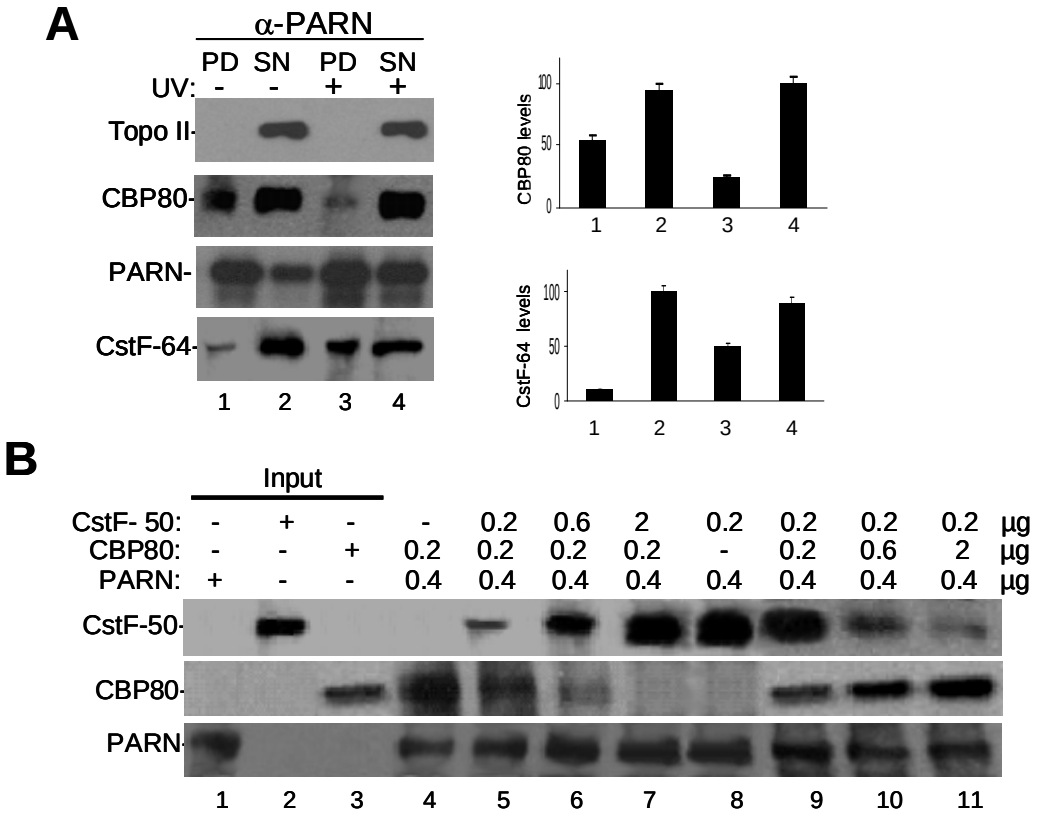

Figure 26. CstF-50 and CBP80 compete for binding to PARN. A) While CBP80 and PARN complex formation decreases in NE of cells treated with UV irradiation, the CstF/PARN complex formation is induced in NE of those cells. NEs were immunoprecipitated with anti-PARN antibodies. Samples were analyzed as in Figure 1C. Proteins were detected by immunoblotting with antibodies against CBP80, PARN and CstF-64. The amount of CBP80 coprecipitated with PARN was quantified. The relative density of each band was determined by Image J program. Data shown are the mean ± SEM from three independent experiments. **B)** CstF-50 and CBP80 bind to the same region of PARN. Immobilized His-PARN on nickel beads was incubated with either CBP80 and increasing amounts of CstF-50 (lanes 5-7) or CstF-50 and increasing amounts of CBP80 (lanes 9-11). Samples were analyzed as in Figure 8.

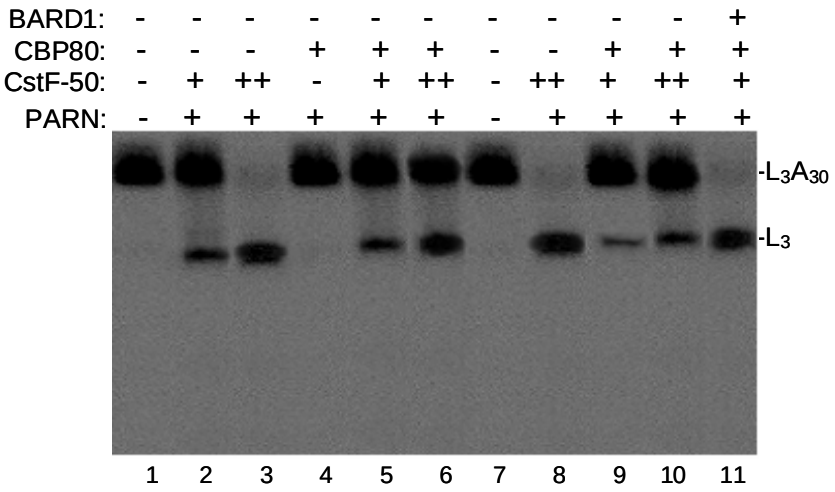

Figure 27. The CstF-50/BARD1 complex can rescue PARN deadenylase activity from the CBP80 induced inhibition. CstF-50 can revert the CBP80-induced inhibition of PARN deadenylase activity and BARD1-associated CstF-50 contributes to this CstF-50 function in deadenylation. GST-CBP80 (25 ng) and increasing amounts of GST-CstF-50 (+: 5 ng and ++: 25 ng) and BARD1 (25 ng) were used in the reactions. Deadenylase assays were performed as in Figure 11B.

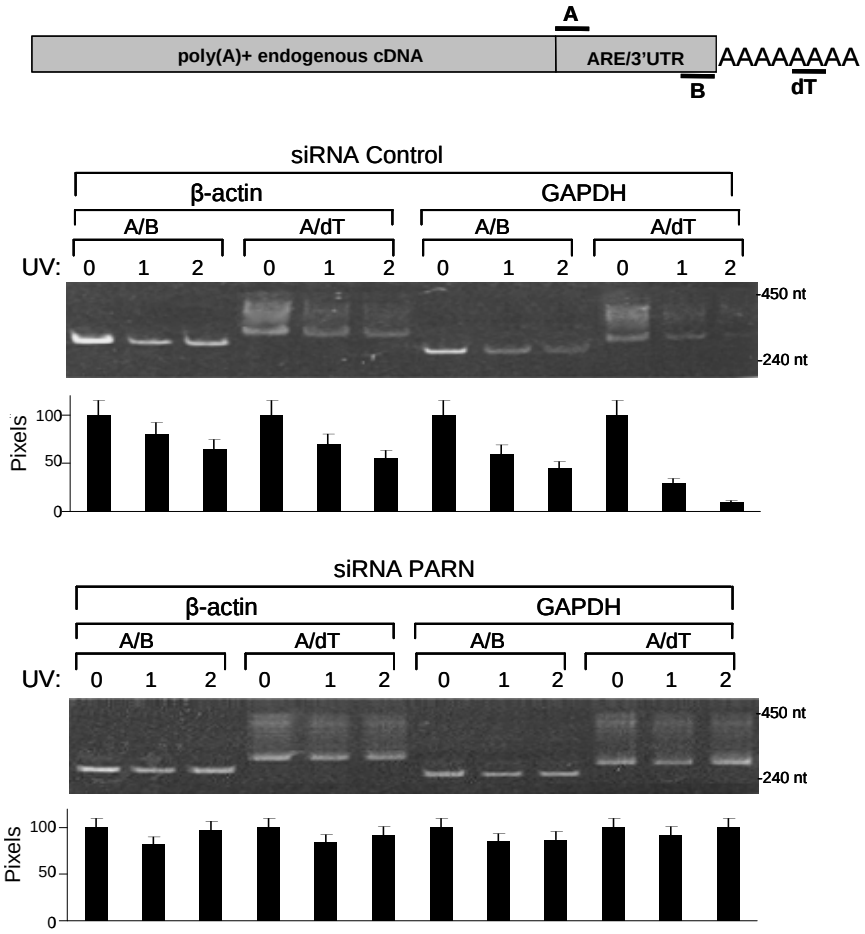

Figure 28. Analysis of the effect of PARN expression on endogenous gene expression after UV treatment via PAT assay. A) Upper panel, diagram of the RT-PCR analysis of endogenous housekeeping and ARE-containing genes. Lower panel**,** effect of PARN on the expression levels of housekeeping genes, GAPDH and β-actin, after UV treatment. RNA samples obtained from HeLa cells treated with control/PARN siRNAs and UV irradiation were analyzed by RT-PCR and PAT. Equivalent amounts of RNA were used in the reactions. Equal volumes of the PCR reactions were analyzed by 8% polyacrylamide gel electrophoresis. Molecular size markers are indicated.

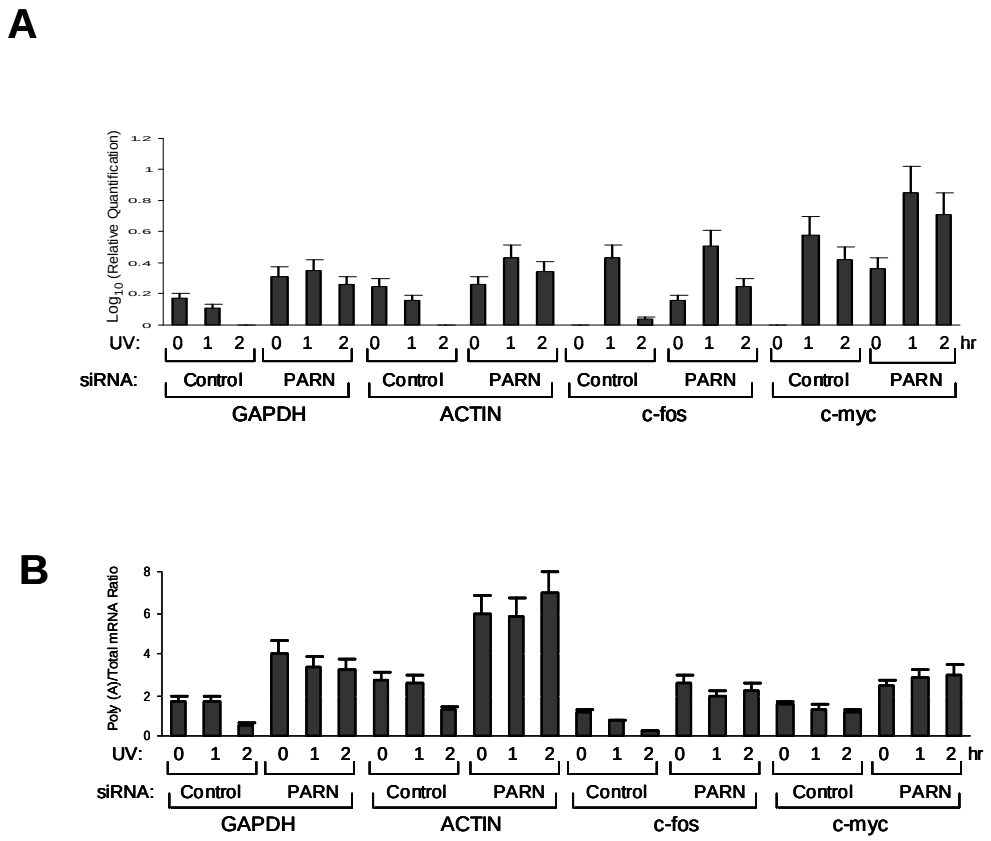

Figure 29. Analysis of the effect of PARN expression on endogenous gene expression after UV treatment via real-time PCR. A) Real-time PCR analysis of GAPDH, β-actin, c-fos and c-myc expression in RNA samples from cells treated with control/PARN siRNAs and UV irradiation. The RT products of GAPDH from cells treated with control siRNA and not treated with UV were used as endogenous control. Data shown are the mean ± SEM from three independent experiments. **B**) Real-time PCR analysis of GAPDH, β-actin, c-fos and c-myc mRNAs polyadenylation. Total and poly(A)$^+$ RNA were prepared from HeLa cells treated with control/PARN siRNA and UV irradiation. Equal mass of either preparation was used as a template in the RT-PCR reactions with primers specific for GAPDH, β-actin, c-fos and c-myc mRNAs. The results shown are the average of four PCRs from two different RNA extractions.

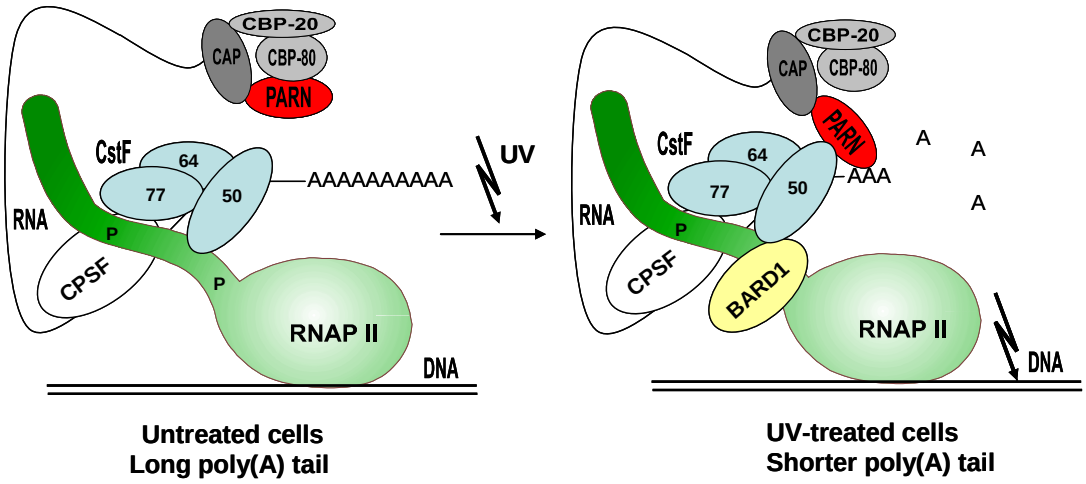

Figure 30: Model of poly(A) tail dynamics after DNA damage. In the absence of DNA damage treatment, CBP80 binds to nuclear PARN, inhibiting its deadenylase activity. In those conditions, CBC also enhances the polyadenylation of pre-mRNAs by increasing the stability of the RNA/CstF complex. As a result of these functional interactions, polyadenylation takes place and normal levels of total mRNA are observed. After exposure to UV treatment, the elongating RNAP II-CstF holoenzyme complex stalls at sites of damage. A BRCA1/BARD1-containing complex is recruited to sites of repair, inhibiting RNAP II and the associated polyadenylation machinery by ubiquitination followed by degradation of the RNAP IIO. In those conditions, CBP80 protein dissociates from PARN, allowing the binding of PARN to the CstF-50/BARD1 complex. As a result of these functional interactions, polyadenylation is inhibited and a deadenylation/dependent decay pathway is activated, generating the observed decrease in the levels of total mRNA. Given that CstF-50 can functionally interact with all the elements of this model, we propose an important role for this protein in the transcription-coupled DNA damage response.

CHAPTER VII

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